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Morphologic, viability and ultrastructural analysis of vitrified sheep preantral follicles enclosed in ovarian tissue

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Abstract

The main objective was to compare the efficiency of vitrification techniques and solutions on the preservation of morphology, ultrastructure and viability of sheep preantral follicles enclosed in ovarian tissue fragments. The fragments were cryopreserved by using macrotube vitrification (MTV), solid-surface vitrification (SSV) or conventional vitrification (CV). These techniques were combined with one of the six solutions containing 6 M ethylene glycol (EG) and with or without sucrose (SUC) (0.25 or 0.50 M) and with or without fetal calf serum (FCS) (10%). After one week, samples were warmed and histological analysis was performed, showing that the percentage of normal follicles after CV (66.20 ± 8.87%) using a solution containing 6 M EG, 0.25 M SUC and 10% FCS (vitrification solution 4 – VS4) was similar to fresh control (79.40 ± 7.83%), MTV (53.40 ± 10.60%) and SSV (56.75 ± 15.33%), all of them with the same vitrification solution ($P < 0.05$). For follicular viability evaluation, ovarian fragments were vitrified as described above. After warming, follicles were assessed by trypan blue dye. Controversially, the highest percentage of viable follicles was observed in MTV (97.06%) and was similar to fresh control (92.62%) ($P < 0.05$), but was significantly different from SSV (81.08%) and CV (83.81%) ($P < 0.05$). These results were validated by transmission electron microscopy that showed normal follicles observed in MTV and in fresh control. In addition, to verify the MTV with VS4 (a combination of the best technique plus the best solution), follicle viability was evaluated after 48 h *in vitro* culture. The viability assay was performed by fluorescence microscopy (calcein-AM and ethidium homodimer-1) analysis as follows: follicles isolated from fresh tissue were forthwith analyzed or underwent 48 h *in vitro* culture before analysis, whereas others fragments were vitrified/warmed and immediately analyzed or underwent 48 h *in vitro* culture before analysis. These results showed that, although follicular viability after MTV/VS4 (65%) was reduced when compared to the non-vitrified follicles at day 0 (100%), follicular viability after MTV/VS4 at day 2 (36.5%) was similar to follicles vitrified at day 0 (65%) and similar to non-vitrified follicles at day 2 (62.5%) ($P > 0.05$). As the decrease of viability in non-vitrified follicles at day 2 was similar to the decrease of MTV/VS4 in the same time, follicle viability at day 2 is not affected by MTV/VS4. In conclusion, using the

experimental conditions of the present study, an efficient solution (VS4: 6 M EG, 0.25 M SUC and 10% FCS) and technique (MTV) were successfully used to vitrify ovine ovarian tissue.

Keywords
Short-term culture; Ovarian fragments; Cryopreservation; Cryoprotectant; Ovary; Ewes

1. Introduction

Advances in cryopreservation techniques and protocols for germinal tissue over the past decades have contributed greatly to the establishment of germplasm banks. These genetic banks are crucial for the preservation of genetic material with potentially high economic value or for use with endangered species populations (Liu et al., 2008 and Santos et al., 2010). In addition, the association between cryotechnology and assisted reproduction techniques (ART) has important clinical relevance, as it permits the development of alternative strategies for restoring fertility in women at risk of premature ovarian failure, especially those undergoing cancer therapies. Admittedly, high dose chemotherapy and radiotherapy destroy a significant portion of ovarian follicular population, often times leading to permanent infertility in women (Meirow and Nugent, 2001 and Chemaitilly et al., 2006).

The main alternatives for fertility preservation in routine clinical use are limited to the protection of the ovaries (oophoropexy) against radiation, or emergency in vitro fertilization (IVF) (Sonmezer and Oktay, 2004). Although oophoropexy may offer some protection to germ cells, this technique can greatly reduce the success of future pregnancies (Wallace et al., 2005). There are also serious limitations in the emergency use of IVF in patients with cancer, as hormonal stimulation is required to obtain mature oocytes. The possibility of utilizing these hormones in patients with hormone-sensitive cancers, as well as in prepubertal patients (Sonmezer and Oktay, 2004), is immensely restricted. Currently, cryopreservation of ovarian tissue is a possible fertility preservation alternative for patients in need of treatment for malignant diseases and is recommended by the American Society of Clinical Oncology (ASCO) (Lee et al., 2006).

In veterinarian medicine, embryo cryobiology has been emphasized when regarding conservation of endangered species or pets. However, this practice is not feasible in cases of accidental or sudden loss of valuable females and, therefore, cryopreservation of ovarian tissue is indicated as a better alternative in these situations (Takahashi et al., 2001). With regard to the ovarian tissue cryopreservation of livestock animals, such as sheep, several studies have reported the feasibility of applying both slow freezing (Gosden et al., 1994, Salle

et al., 2002, Salle et al., 2003 and Imhof et al., 2006) and vitrification (Bordes et al., 2005 and Lornage et al., 2006) methods through the birth of healthy offspring after transplantation of ovarian tissue.

Itrification is a fairly recent alternative method of cryopreservation and, when compared to slow freezing, is quicker and cheaper. However, the vitrification method is characterized by using high concentrations of cryoprotectants (Vajta et al., 1998), which can increase the toxic effect caused by these substances on preantral follicles. Moreover, it is known that factors such as high concentrations of cryoprotectant agents, osmotic stress and the techniques used for vitrification loading may contribute to the reduction of normal preantral follicles after warming (Huang et al., 2008).

In the last decade, studies have been completed using ethylene glycol (EG) with vitrified ovarian tissue or isolated preantral follicles in rat (Sugimoto et al., 2000), mouse (Kagabu and Umezu, 2000 and Kim et al., 2010), goat (Santos et al., 2007 and Carvalho et al., 2011), cow (Gandolfi et al., 2006 and Kagawa et al., 2009), pig (Moniruzzaman et al., 2009) and human (Isachenko et al., 2003 and Silber et al., 2010). However, very few investigators have tested EG in vitrification solution with ovine preantral follicles enclosed in fragments of ovarian tissue (Amorim et al., 2003 and Melo et al., 2011). Developments in sheep ovarian vitrification may have relevance as ewe ovaries are similar to the human ovary in its anatomy and physiology (Gosden et al., 1994, Oktay et al., 2000 and Salle et al., 2002). While positive results have recently been obtained with the vitrification of mouse ovaries (Wang et al., 2011), these methods cannot be easily transferred to human tissue. This is, in part, due to the vast morphological and physiological differences between mouse and human ovaries. Despite having larger ovaries, neither bovine nor porcine can be considered a relevant model for human tissue vitrification (Gandolfi et al., 2006). In addition, researchers have published promising results regarding ovarian tissue cryopreservation in the presence of an extra-cellular cryoprotectant, like sucrose (SUC) (Santos et al., 2006a) or fetal calf serum (FCS) (Chen et al., 2006). Information detailing whether the addition of sucrose at concentrations of 0.25 or 0.5 M with or without 10% FCS may be essential for ovarian tissue vitrification, despite being important, is limited in sheep.

The current study aimed (1) to compare different vitrification techniques in ovine ovarian tissue and (2) to test the effects of varying concentrations of SUC, FCS or both combined with 6 M EG as a vitrification solution (VS). Morphology, by classical histology and transmission electron microscopy, and viability, by trypan blue stain and fluorescent markers, were assessed in fresh ovarian fragments, vitrified/thawed fragments, and vitrified/thawed samples after in vitro culture (IVC).

2. Materials and methods

2.1. Source and preparation of ovarian tissue

Ovaries (n = 30) were collected at a local abattoir from 15 adult non-pregnant mixed-breed ewes. Immediately after postmortem, under aseptic conditions, the ovaries were washed in 70% alcohol for 10 s, followed by two washes in HEPES buffered minimum essential medium (MEM) (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 100 g/mL penicillin and 100 g/mL streptomycin. The ovaries were transported into tubes containing 20 mL of MEM within thermos flasks maintained at 20 °C to the laboratory within 1 h after they were recovered.

2.2. Experiment I: morphology, viability and ultrastructure of preantral follicles in vitrified ovarian cortex

2.2.1. Ovarian tissue vitrification: solution composition and technique

At the laboratory, ovarian pairs (n = 5) were stripped of adhering tissue and fat, and cut with a scalpel into approximately 3 mm × 3 mm × 1 mm (9 mm3) or 1 mm × 1 mm × 1 mm (1 mm3) fragments, according to the vitrification technique used, macrotube vitrification (MTV), solid-surface vitrification (SSV) or conventional vitrification (CV) and were randomly assigned to each treatment. One fragment (9 mm3) from each pair of ovaries was immediately fixed in Carnoy's solution for 12 h for histological analysis (fresh control). Twelve 9 mm3 fragments (for MTV or for SSV) and six 1 mm3 fragments (for CV) were exposed to one of the six vitrification solutions (VS): (VS1–6, description to follow) for 5 min at 20 °C. After this duration, the fragments underwent MTV, SSV or CV. The base medium (BM), composed of 6 M ethylene glycol (EG) in MEM, was supplemented with SUC with or without 10% FCS to produce the six VS and are referred to as: VS1: (BM); VS2: (BM + FCS); VS3: (BM + 0.25 M SUC); VS4: (BM + 0.25 M SUC + FCS); VS5: (BM + 0.50 M SUC) and VS6: (BM + 0.50 M SUC + FCS). For clarification, refer to Table 1.

Table 1.
Composition of six vitrification solutions and their arrangement with vitrification techniques.

	Composition of vitrification solution	Macrotube vitrification (MTV)	Solid-surface vitrification (SSV)	Conventional vitrification (CV)
VS1	BM	MTV/VS1	SSV/VS1	CV/VS1
VS2	BM + 10% FCS	MTV/VS2	SSV/VS2	CV/VS2
VS3	BM + SUC 0.25 M	MTV/VS3	SSV/VS3	CV/VS3
VS4	BM + SUC 0.25 M + 10% FCS	MTV/VS4	SSV/VS4	CV/VS4
VS5	BM + SUC 0.5 M	MTV/VS5	SSV/VS5	CV/VS5
VS6	BM + SUC 0.5 M + 10% FCS	MTV/VS6	SSV/VS6	CV/VS6

BM, base medium; FCS, fetal calf serum; SUC, sucrose; VS, vitrification solution; MTV, macrotube vitrification; SSV, solid-surface vitrification; CV, conventional vitrification.

For the MTV, each fragment was inserted into a macrotube (Minitub do Brasil Ltda., Porto Alegre, RS, Brazil) containing 1.8 mL of VS (VS1–6) (20 °C), which was then immersed in liquid nitrogen (LN2) after 5 min (Carvalho et al., 2011). The SSV procedure was performed as previously reported by Santos et al. (2007) with slight modifications. A total of six fragments were exposed to 1.8 mL VS (VS1–6) (20 °C) for 5 min, removed from the solutions, and dried (using an absorbent paper). These samples were then individually placed on the surface of a metal cube floating in LN2. After this, vitrified fragments were transferred (with LN2 cooled forceps) into cryovials for storage in LN2. The CV procedure was conducted according to the methodology previously described by Chen et al. (2006). Briefly, six fragments were loaded individually into 0.5 mL French straws that were partially filled with a column of VS (VS1–6) (∼2 cm) (20 °C), followed by an air space (∼1 cm), and another VS column. After insertion of a tissue sample, another air space, and a final column of VS were drawn into the straws. The straws were then heat-sealed and after 5 min, plunged into LN2.

After one week of cryostorage, all treatment fragments were removed from the LN2, kept at room temperature (RT) (∼25 °C) for 1 min, and then immersed in a water bath at 37 °C until the VS was completely melted (∼1–2 min). The cryoprotectant was removed from ovarian cortex fragments in three step washes containing MEM supplemented with 10% FCS and decreasing concentrations of SUC (0.50 M, 0.25 M, and no SUC, respectively) for 5 min each. The efficiency of these VS for the preservation of preantral follicles was evaluated histologically.

2.2.2. Histological analysis

After 12 h of fixation in Carnoy's fluid, fresh and vitrified ovarian tissue fragments were dehydrated in a graded series of ethanol, clarified with xylene and embedded in paraffin wax. Serial sections 7 µm thick were cut and every fifth section was mounted on glass slides and stained with periodic acid Schiff (PAS)–hematoxylin, and evaluated using a light microscope

(Nikon, Tokyo, Japan) at 400× magnification. In each treatment, a total of 150 preantral follicles were examined and were defined according to Hulshof et al. (1994) as follows: (1) primordial follicle: had an oocyte surrounded by one layer of flattened pre-granulosa cells; (2) primary follicle: had an oocyte surrounded by a single layer of cuboidal granulosa cells; or (3) secondary follicle: had an oocyte surrounded by two or more layers of cuboidal granulosa cells without antrum formation. Antral follicles were not counted in this study. Normal morphology was defined as a follicle containing a spherical oocyte with uniform cytoplasm and well-organized granulosa cell layers. Degenerated follicles were those containing a retracted oocyte with or without a pyknotic nucleus or degeneration of either oocyte or granulosa cells. To avoid evaluating and counting the same follicle more than once, preantral follicles were analyzed only in the sections in which an oocyte nucleus was observed.

2.2.3. Follicular isolation, trypan blue staining and assessment of follicular viability

To evaluate the effect of vitrification on preantral follicle viability in ovarian tissue, samples underwent the three tested techniques: MTV, SSV and CV. The only VS utilized was VS4, as it yielded the highest percentage of morphologically normal follicles shown by histological analysis from the MTV/VS4, SSV/VS4 and CV/VS4 treatments.

Four fragments were retrieved from five pairs of ovaries (n = 5), with one fragment immediately undergoing follicle isolation (fresh control) and viability testing. The other three fragments were first vitrified in MTV/VS4, SSV/VS4 and CS/VS4 and stored for one week. After this time, fragments were warmed and follicle isolation was completed. Preantral follicles were isolated from ovarian tissue by using a mechanical procedure as described by Amorim et al. (2000). Briefly, samples were cut into small pieces with a tissue chopper (The Mickle Laboratory Engineering Co., Gomshal, Surrey, UK) adjusted to a sectioning interval of 87.5 µm. Samples were then placed in 2 mL of MEM supplemented with 3 mg/mL bovine serum albumin (BSA), and suspended 100 times with a large Pasteur pipette (inner diameter ∼ 1600 µm), followed by 100 times with a smaller Pasteur pipette (inner diameter ∼ 600 µm) to dissociate preantral follicles from stroma. The suspension was successively filtered through 500 and 100 µm nylon-mesh filters. This procedure was performed within approximately 10 min at RT.

After follicular isolation, preantral follicle viability was assessed by trypan blue dye. Briefly, 300 µL of the suspension plus 15 µL of 0.4% trypan blue (Sigma Chemical Co., St. Louis, MO, USA) were incubated for 1 min at RT (Celestino et al., 2008). Afterwards, follicles were viewed and scored with an inverted microscope (Nikon, Tokyo, Japan) and classified as either viable if

unstained or non-viable if stained with trypan blue. An average of 93 preantral follicles was analyzed in each treatment (total of 372 follicles) and the percentages of viable follicles were calculated.

2.2.4. Ultrastructure evaluation

The procedures for ultrastructure evaluation were conducted according to Santos et al. (2006b). Briefly, ultrastructural studies were carried out using fresh control fragments and from fragments that underwent MTV/VS4, SSV/VS4 and CV/VS4 treatments. For these, tissue fragments with a maximum dimension of 1 mm3 were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 4 h. After fixation and five washes, 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. Subsequently, the samples were dehydrated through a gradient of acetone solutions (31–100%) and the tissues were embedded in Spurr. Semi-thin sections (3 µm) were stained with Toluidine blue. The ultra-thin sections (60–70 nm) were contrasted with uranyl acetate and lead citrate, and examined under a Jeol JEM 1011 transmission electron microscope (Jeol, Tokyo, Japan).

2.3. Experiment II: in vitro culture of preantral follicles after vitrification

Ewe ovarian tissue was cultured in vitro after vitrification with the MTV/VS4 treatment, which yielded the highest percentage of viable preantral follicles after being dyed with trypan blue in experiment I.

In each replicate (n = 5), four cortex fragments were prepared; one was immediately analyzed as a fresh control for viability using fluorescence microscopy. Of the remaining three fragments, two were vitrified in MTV/VS4 and the remaining one was cultured in vitro for 48 h. One week after vitrification, both fragments were warmed. One of the fragments was then assessed for viability using fluorescence markers, whereas the other one was further cultured in vitro for 48 h. After culture, fragments were processed for viability assessment, as described below.

Preantral follicles were first isolated and incubated at 37 °C for 15 min in 4 µM calcein-AM and 2 µM ethidium homodimer-1 (Molecular Probes, Invitrogen, Karlsruhe, Germany) (Santos et al., 2007). Afterwards, follicles were examined using an epifluorescence microscope (Nikon, Tokyo, Japan) at magnification 400×. The emitted fluorescent signals of calcein-AM and ethidium homodimer-1 were collected at 488 and 568 nm, respectively. The first probe

detected intracellular esterase activity of viable cells, whereas the latter labeled nucleic acids of non-viable cells with plasma membrane disruption. 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). While trypan blue only detects membrane intactness, fluorescent markers give information regarding DNA integrity. Examples of follicular viability using these probes have been published by our team (Carvalho et al., 2011).

The culture medium comprised α-MEM (pH 7.2–7.4) supplemented with ITS (10 μg/mL insulin, 5.5 μg/mL transferrin, 5 ng/mL selenium), 2 mM glutamine, 2 mM hypoxanthine, 3 mg/mL bovine serum albumin, 50 μg/mL ascorbic acid, 50 μg/mL recombinant follicle stimulating hormone (rFSH), and 100 μg/mL penicillin–streptomycin. Fresh and vitrified/warmed ovarian tissues were transferred to 24-well culture dishes containing 1 mL of culture media per well and were cultured at 39 °C in 5% CO_2 in a humidified incubator for 48 h.

An average of twenty preantral follicles was analyzed in each treatment (fresh control, in vitro culture with or without prior vitrification), which resulted in a total of 60 examined preantral follicles.

2.4. Statistical analysis

For follicle morphology data, Kolmogorov–Smirnov and Bartlett tests were used to confirm normal distribution and homogeneity of variances, respectively. Two-way ANOVA was then carried out using PROC GLM procedure of SAS (SAS Institute Inc., Cary, NC, USA) according to a 3 × 6 factorial arrangement of treatments with vitrification technique (MTV, SSV and CV) and VS (EG with or without SUC and with or without FCS – VS1–6) as the main effects. Ovarian fragments were considered as the experimental unit and the following model was used:

$$Y_{ij} = \mu + VTi + VSj + (VTi \times VSj) + e_{ij}$$

where Y_{ij}, dependent variable (percentage of morphologically normal preantral follicles); VT_i, vitrification technique; VS_j, vitrification solution; $VT_i \times VS_j$, interaction between vitrification technique and vitrification solution; and e_{ij}, residual error.

When main effects or the interaction was significant, means were compared by Student–Newman–Keuls (SNK) test, whereas Dunnett's test was applied for comparisons of each treatment to fresh control group. Percentages of viable follicles assessed by trypan blue or fluorescent markers were compared by Chi-square test or by Fisher's exact test (when the number of replicates was smaller than 30 units). For all statistical analyses, $P < 0.05$ was considered significant, and results were expressed as mean ± SD.

3. Results

3.1. Percentages of morphologically normal sheep preantral follicles

A total of 2850 preantral follicles (150 follicles each treatment) were examined by classic histology. They were evaluated according to oocyte, granulosa cell and membrane integrity. Morphologically normal (Fig. 1A and B) and atretic preantral follicles (Fig. 1C and D) were found after all treatments as well as in fresh control. The predominant degenerative characteristics were shrunken oocytes, pyknosis of oocyte nucleus, oocyte cytoplasm vacuolization and disorganized granulosa cells.

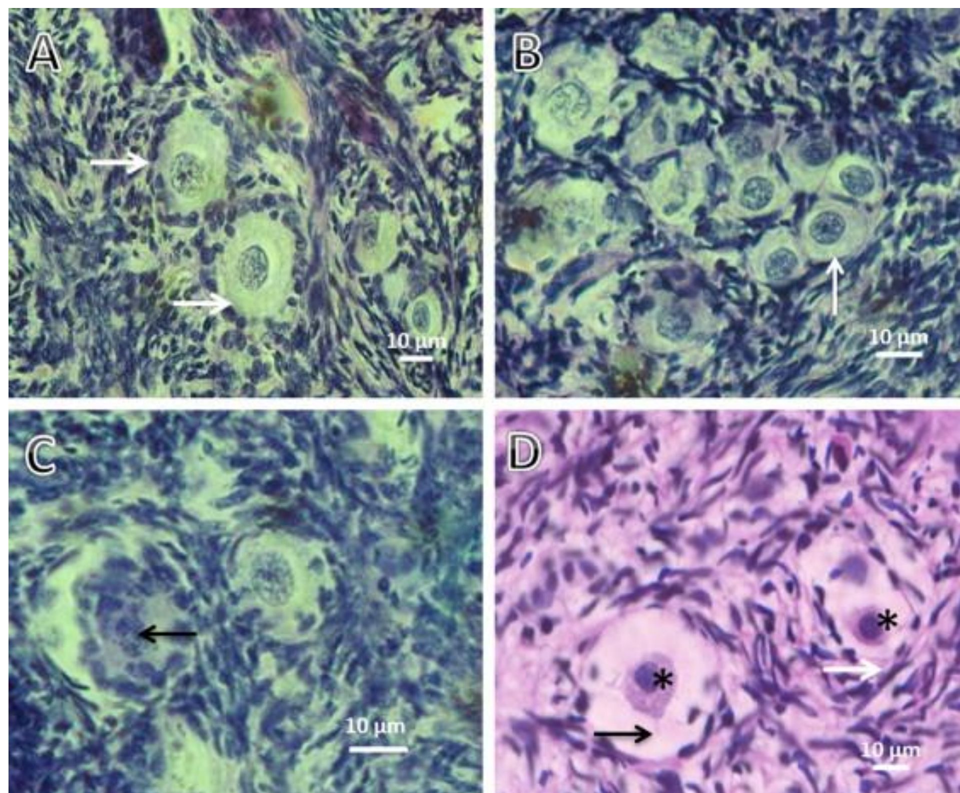

Fig. 1. Photomicrographs of ovarian cortical histological sections showing preantral follicles before (A) and after vitrification (B–D). (A) Normal follicles with one to two layers of cuboidal granulosa cells (white arrows). Normal follicles (white arrow) after vitrification through MTV/VS4 (B). (C) Nucleus of degenerated follicle (black arrow) after vitrification through SSV/VS4. (D) Degenerated follicles displaying oocyte nuclear pyknosis (asterisks), slight cytoplasm retraction (black arrow) and disorganization of granulosa cells layers (white arrow) after vitrification through CV/VS4.

The percentage of morphologically normal preantral follicles in ovarian fragments from fresh control (79.40 ± 7.83%) and after all vitrification treatments is shown in Table 2. All treatments had significantly reduced percentages of morphologically normal follicles when compared to fresh control ($P < 0.05$), except after CV/VS4 treatment (66.20 ± 8.87%) ($P > 0.05$).

Vitrification techniques only presented significant differences, between them, when using VS6, with a higher percentage of morphological normal follicles with SSV (59.00 ± 13.09). Concerning the vitrification solution, no differences were observed when performing MTV but the CV using the VS4 allowed attaining the higher percentage of morphological normal follicles. When using SSV, both VS6 and VS4 allowed the observation of a higher percentage of morphological normal follicles.

Table 2.
Percentages of morphologically normal ovine preantral follicles in fresh (control) and in vitrified ovarian tissue by using macrotube vitrification (MTV), solid-surface vitrification (SSV) or conventional vitrification (CV) and six solutions containing 6 M ethylene glycol (EG) and with or without sucrose (SUC) (0.25 or 0.50 M) and with or without fetal calf serum (FCS) (10%).

Control	79.40 ± 7.83		
Vitrification solution	Vitrification technique		
	MTV	SSV	CV
VS1: BM	$46.60 \pm 12.52^{*,Aa}$	$39.40 \pm 9.32^{*,Ab}$	$45.60 \pm 15.65^{*,Aab}$
VS2: BM + 10% FCS	$50.60 \pm 13.81^{*,Aa}$	$44.80 \pm 10.50^{*,Aab}$	$47.00 \pm 11.40^{*,Aab}$
VS3: BM + SUC 0.25 M	$50.00 \pm 9.70^{*,Aa}$	$52.60 \pm 11.97^{*,Aab}$	$49.20 \pm 11.10^{*,Aab}$
VS4: BM + SUC 0.25 M + 10% FCS	$53.40 \pm 10.60^{*,Aa}$	$56.75 \pm 15.33^{*,Aa}$	66.20 ± 8.87^{Aa}
VS5: BM + SUC 0.5 M	$47.20 \pm 11.39^{*,Aa}$	$44.80 \pm 5.02^{*,Aab}$	$48.60 \pm 10.48^{*,Aab}$
VS6: BM + SUC 0.5 M + 10% FCS	$42.00 \pm 3.08^{*,Ba}$	$59.00 \pm 13.09^{*,Aa}$	$33.20 \pm 14.18^{*,Bb}$

Data are displayed as the mean ± SD. A,BDiffering capital letters within a row illustrate significantly differing percentages among vitrification techniques (P < 0.05). a,bDiffering lowercase letters within a column illustrate significantly differing percentages among vitrification solutions (P < 0.05).
MTV, macrotube vitrification; SSV, solid-surface vitrification; CV, conventional vitrification; VS, vitrification solution; BM, base medium; FCS, fetal calf serum; SUC, sucrose.
*Percentage significantly different (P < 0.05) from the non-vitrified ovarian cortex (control).

The percentage of normal follicles was significantly greater in VS4 compared to VS1 and VS6 (when using the SSV or CV techniques). Therefore, VS4 was selected for assessment of viability and ultrastructure.

3.2. Preantral follicle viability using trypan blue after vitrification of ovine ovarian tissue

Viable and nonviable follicles obtained from vitrified ovarian cortex fragments are shown in Fig. 2. The percentage of viable preantral follicles was similar to that in the fresh control (92.62%) only after MTV/VS4 (97.06%) (P < 0.05). Furthermore, MTV/VS4 preserved the follicular viability better than SSV/VS4 (81.08%) or CV/VS4 (83.81%) (P < 0.05). These results were decisive for choosing MTV as the technique to be used in experiment 2.

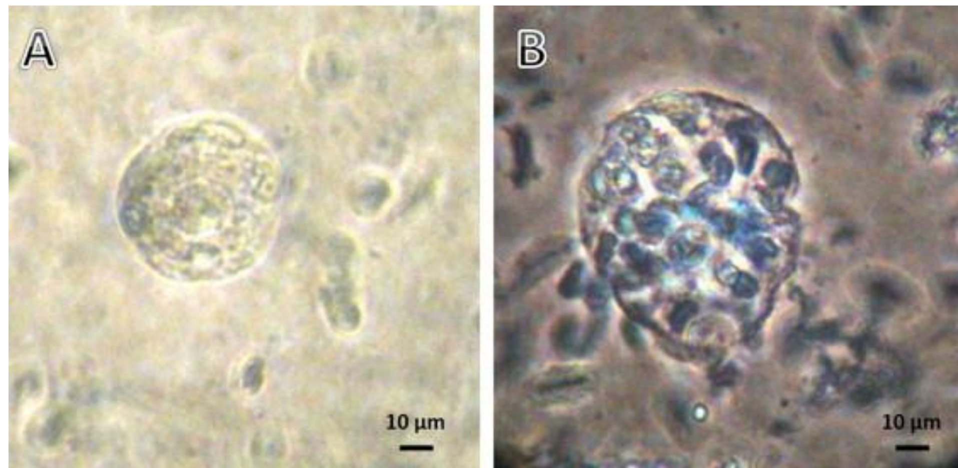

Fig. 2. Photomicrographs of trypan blue dye-treated preantral follicles that were mechanically isolated from vitrified ovarian tissue showing (A) viable follicle (not stained) and a (B) nonviable follicle (stained). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.3. Ultrastructural analysis of vitrified preantral follicles

The ultrastructural analysis showed that follicles from the fresh control group (Fig. 3A) and follicles from MTV/VS4 (Fig. 3B) treatment were similar, presenting oocytes with a large central nucleus well-defined by a nuclear envelope. Organelles were uniformly distributed throughout the homogeneous cytoplasm, with mitochondria being the most evident organelle, and granulosa cells were normal in appearance. In the fresh control group (Fig. 3A), it is possible to see some vesicles. Although MTV/VS4 (Fig. 3B) follicular ultrastructure was similar to fresh control follicles, some discreet changes could be observed, like a slight shrinkage in the nuclear envelope.

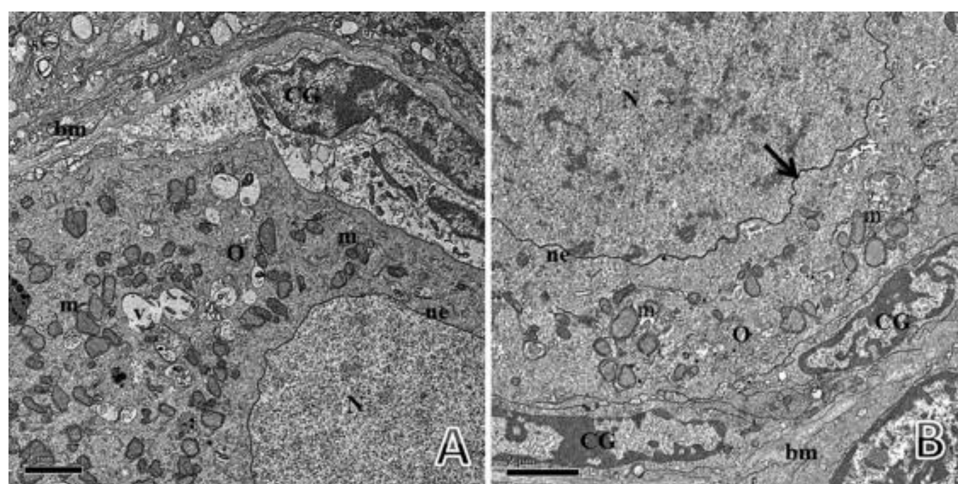

Fig. 3. Electron micrography of a normal sheep preantral follicle in ovarian tissue before (A) and after (B) MTV/VS4 showing an oocyte (O) with intact nucleus (N), both presented a homogeneous appearance, nuclear envelope (ne), granulosa cells (CG), basement membrane (bm) and mitochondria (m). Note vesicles (v) in (A) and in (B) a slight shrinkage (black arrow), but intact, nuclear envelope.

Conversely, more drastic alterations could be observed during ultrastructure analysis of SSV/VS4 (Fig. 4A) and CV/VS4 (Fig. 4B–D) follicles. For example, oocytes exhibited cytoplasm vacuolization with a granulated appearance and contained empty spaces. In the SSV/VS4 follicles, granulosa cells had a loss of cytoplasmic content. CV/VS4-treated follicles resulted in the greatest ultrastructural abnormalities with vacuolated follicles and the majority of organelles lost in the oocyte cytoplasm (Fig. 4B), which presumably left empty spaces in the oocyte cytoplasm content. The ovarian stromal area also had signs of degeneration, with largely vacated areas (Fig. 4C).

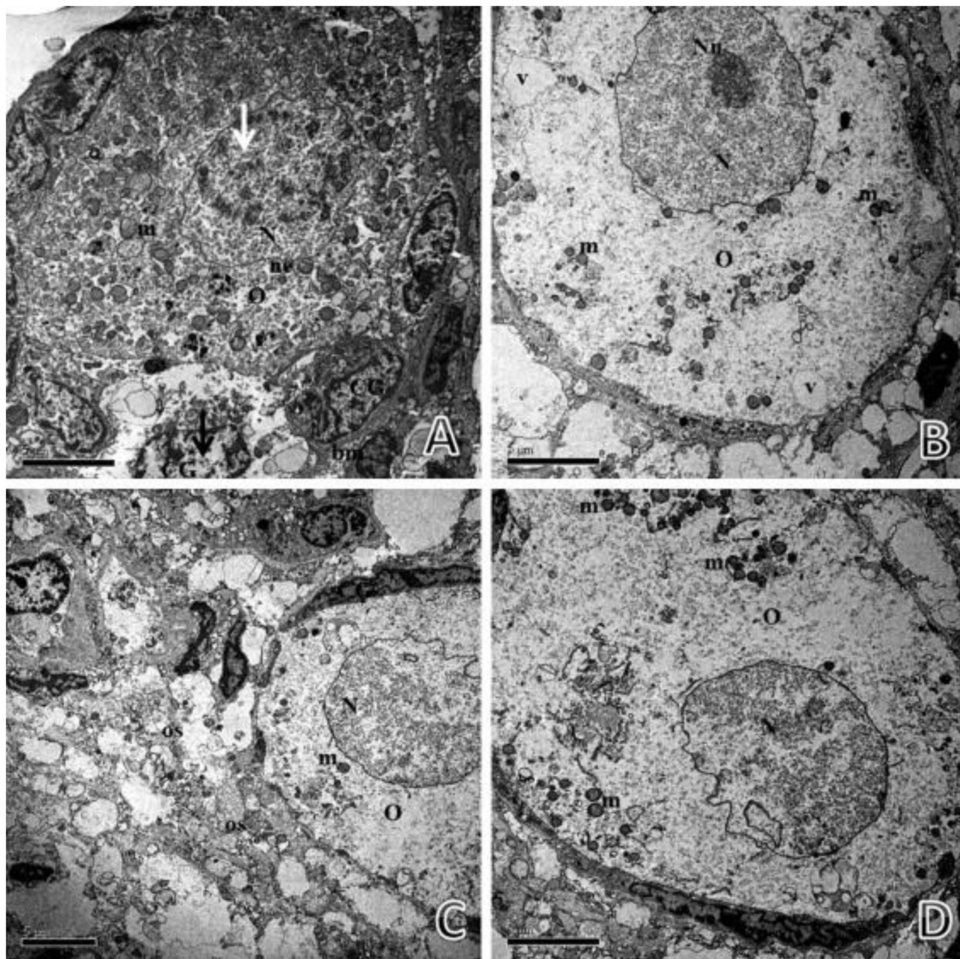

Fig. 4. Electron micrography of sheep preantral follicles in ovarian tissue vitrified in SSV/VS4 (A), CV/VS4 (B–D) showing some signs of degeneration. Despite (A) shows normal features such as basement membrane (bm), mitochondria (m) and an intact nuclear envelope (ne) with areas of condensation of chromatin (white arrow), this nuclear envelope membrane has slight shrinkage, a widespread cytoplasmic vacuolization, a granulosa cell (CG) with a loss of cytoplasmic content (black arrow). (B) Shows a widespread vacated area in the oocyte (O) cytoplasm content and nucleus (N) of oocyte (O) with nucleolus (Nu). (C) Shows the ovarian stroma (os) area extremely vacuolated. (D) Shows an empty space in the oocyte cytoplasm content.

3.4. In vitro culture of preantral follicles after vitrification

Follicular viability, based on fluorescent markers (Fig. 5), immediately after MTV/VS4 (65%) was reduced when compared to the non-vitrified follicles at day 0 (100%) ($P < 0.05$) (Table 3). However follicular viability after MTV/VS4 at day 2 (36.5%) was similar to follicles vitrified at day 0 (65%) and similar to non-vitrified follicles at day 2 (62.5) ($P > 0.05$). As the decrease of viability in non-vitrified follicles at day 2 was similar to the decrease of MTV/VS4 in the same time, follicle viability at day 2 is not affected by MTV/VS4.

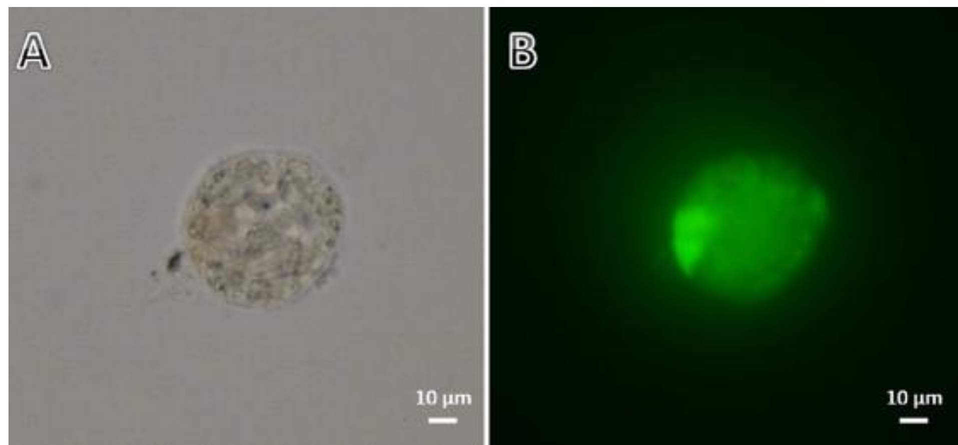

Fig. 5. View of viability evaluation of isolated preantral follicles. (A) Direct observation of isolated ovarian follicles. (B) Fluorescent staining with calcein-AM and ethidium homodimer-1. No dead follicle is shown in this view.

Table 3.
Percentages of viable ovine preantral follicles non-vitrified and vitrified at day 0 and day 2 of in vitro culture.

	Day 0	Day 2
Non-vitrified	$100\%^{Aa}$	$62.5\%^{Bb}$
Vitrified	$65\%^{Ab}$	$36.5\%^{Ab}$

A,B Differing capital letters within a row illustrate significantly differing percentages between follicles before (at day 0) and after (at day 2) 48 h in vitro culture ($P < 0.05$). a,bDiffering lowercase letters within a column illustrate significantly differing percentages between follicles before (non-vitrified) and after MTV/VS4 ($P < 0.05$)

4. Discussion

In this study were evaluated the effects of three different techniques for vitrification of sheep ovarian tissue: the vitrification in macrotubes (MTV), solid-surface vitrification (SSV) and conventional vitrification (CV), combined with six different vitrification solutions, on the follicular morphology and viability preservation. Based on our previous experimental data (Carvalho et al., 2011), the solutions tested were composed of minimum essential medium (MEM) supplemented with 6.0 M ethylene glycol (EG) and supplemented or not with 10% fetal

calf serum (FCS) and with or without sucrose (SUC) in two different concentrations (0.25 or 0.50 M).

In the first experiment, we found higher percentage of morphologically normal preantral follicles cryopreserved when used the VS4 (EG + SUC 0.25 M + 10% FCS) in all the vitrification techniques. In addition, this solution was the only that presented percentage of normal follicles similar to fresh control (CV/VS4). This result is probably due to the optimized combination of low SUC concentration (0.25 M) with FCS (10%). The absence of SUC in VS probably reduced its dehydration potential, allowing higher intracellular water and, consequently, promoting more intracellular water crystallization (Bao et al., 2010), thereby demonstrating that a delicate balance between SUC and FCS is essential for preserving normal preantral follicle morphology after cryopreservation process. On the other hand, VS with higher concentration of SUC could have promoted deleterious dehydration to the tissue, causing evaluable damage. Both, the SUC and the FCS, acted synergistically permitting to influx and efflux of substances through the plasma membranes of preantral follicles (Jain and Paulson, 2006). This effect results in a favorable transport of EG and water in this cellular type. Moreover, the combination between 0.25 M of SUC and FCS in the basic medium as observed above was beneficial for the preservation of follicular morphology in all vitrification techniques (CV/VS4, MTV/VS4 and SSV/VS4). And at the same time, high SUC concentration (0.5 M) associated with FCS (10%) also permitted the preservation of percentage of morphologically normal follicles in SSV/VS6. Probably, the fragments were better preserved in this association because in SSV the thawing procedure occurred without contact with the vitrification solution, whereas the thawing procedure in CV and MTV keeps the fragments in contact with the vitrification solution, causing damage due to an additional exposure to this solution (Jain and Paulson, 2006).

The results were similar to those previously reported by Bao et al. (2010) that in vitrified bovine follicles in a solution containing, among others, EG and FCS, the addition of SUC was essential to maintain the follicle morphology and the potential for further development to advanced stages. One study performed by Santos et al. (2007) demonstrated that the combination of EG and SUC was the most suitable for vitrification of goat ovarian tissue, obtaining higher rates of morphologically viable follicles. Despite the use of a slow freezing technique, recently, Santos et al. (2011) also showed that sheep preantral follicles are best preserved (morphology and viability) when SUC was associated with EG in the freezing solution.

The cryoprotectant EG, a basic component of vitrification solution in this study, was chosen because it is considered an intracellular cryoprotectant with low molecular weight

(Massip, 2001 and Amorim et al., 2003) and low toxicity (Zhang et al., 2010). The cryoprotectants reduce cell damage caused by cryopreservation, since they act by partially replacing the water inside the cell and binding to hydrogen molecules in the intracellular water, increasing the viscosity of the cryopreservation solution and thus reducing the freezing point (Fuller and Paynter, 2004).

Unlike intracellular cryoprotectants, the extracellular cryoprotectants, as SUC, remain outside the cell and interact with the water-free present in the solution, influencing indirectly the processes of osmotic cellular dehydration. The SUC acts as a buffer against osmotic stress during the addition and the removal of intracellular cryoprotectant and has the potential to stabilize the cell membrane, minimizing cell damage (Fabbri et al., 2001). Despite the process of cellular dehydration prevents intracellular ice formation (IIF), dehydration can also cause excessive osmotic stress (Vajta et al., 1998). Therefore, we believed this is the reason why the low SUC concentration used in this experiment (0.25 M) had supported better results.

The FCS, which composition is not defined, or in some cases semi-defined, also participates in the controlling of the water flow through cell membranes. Its action occurs through proteins and large macromolecules, which bind to the lipid membranes, protecting these structures (Wang et al., 1997). In our study we found that the FCS is related to the preservation of the morphology of sheep preantral follicles after vitrification, similarly as shown in goat ovarian tissue by Carvalho et al. (2011). We believe that this substance promotes the exchange of fluids between the vitrification solution and cellular environment, an effect that apparently was enhanced by the association with low SUC concentration (0.25 M). In addition to the osmotic equilibrium maintenance, the FCS can act as a source of nutrients (protein, carbohydrates, lipids and vitamins), possibly aiding in the recovery of cellular metabolism after the cryopreservation procedures (Wang et al., 1997).

Unlike the observed in the morphological analysis, whose best results were obtained with the CV technique, when concerning follicular viability it was observed that follicles were better preserved using MTV. Furthermore, this same treatment MTV/VS4 was the only that showed follicles with ultrastructural characteristics similar to those from fresh control. This can be explained by the fact that histological studies identify only a few signs of follicular atresia, such as nuclear pyknosis, detachment of the granulosa cells of the oocyte and the basement membrane abnormalities.

Thus, the visual observation of follicular morphology alone is insufficient to assess the efficacy of the cryopreservation process by itself (Martinez-Madrid et al., 2004).

In this regard, the viability and ultrastructure assessments determined that MTV was the best loading device for sheep ovarian fragments. MTV is a technique that permits the

vitrification of larger fragments than CV technique, because the French straws are narrow and do not allow proper handling of 9 mm3 fragments. Similar to MTV, the SSV technique, described by Begin et al. (2003) and still used by many researchers (Santos et al., 2007 and Carvalho et al., 2011), permits the vitrification of larger fragments. However, to perform this technique the tissue must to be in contact with a cooled aluminum metal partly immersed into liquid nitrogen (Begin et al., 2003). Such tissue exposure to liquid nitrogen is discouraged, since there is the possibility of cellular contamination by nitrogen contact (Criado et al., 2011 and Isachenko et al., 2009). While some authors indicate techniques such as ultraviolet sterilization or filtration of liquid nitrogen, these procedures would not prevent cross-contamination between cells of different individuals in the same cryogenic tank (Parmegiani et al., 2010).

Studies have shown that short-term culture can be used as a valuable tool to verify the developmental ability of cryopreserved preantral follicles (Choi et al., 2008). Based on these previous studies, we conducted a second experiment including the short-term in vitro culture as an additional tool to precisely evaluate the follicular viability after MTV/VS4. Also, it is known that this type of culture has no intention of promoting follicular growth, but allows the tissue to return to its normal metabolic conditions, recovering from possible cryoinjuries, or even allowing the cells to express the molecular damage that may have occurred during cryopreservation, undetectable at the post-thaw (Paynter et al., 1999).

To assess the percentages of viable non-vitrified and vitrified ovine preantral follicles at day 0 and day 2 of in vitro culture, we used the fluorescent markers ethidium homodimer-1, which enters into cells with disrupted plasmatic membrane and binds to DNA, and calcein-AM, which assesses esterase activity in viable cells. This method is more sensitive than histology for the assessment of follicular quality and more accurate than the trypan blue previously used in the first experiment of this study, by evaluating both, morphological and functional structures simultaneously. With these markers, it was found that follicular viability immediately after MTV/VS4 (65%) was reduced when compared to the non-vitrified follicles at day 0 (100%) ($P < 0.05$). Similarly, other studies (Gonçalves et al., unpublished data; Oskam et al., 2011) did not obtain satisfactory results using similar conditions of in vitro culture even after 2 and 4 h after freezing sheep ovarian tissue compared to non-vitrified follicles at day 0. However, in the present study the significant reduction in viability from day 0 to day 2 was observed only in follicles from non-vitrified tissue. Thus, follicular viability after MTV/VS4 at day 2 (36.5%) was similar to follicles vitrified at day 0 (65%) and similar to non-vitrified follicles at day 2 (62.5) ($P > 0.05$). Due to the decrease of viability in non-vitrified follicles at day 2, the viability at day 2 was similar between non vitrified and vitrified follicles (MTV/VS4). Thus, the use of the

MTV/VS4 is suitable for the preantral follicles, because they are able to survive after short-term in vitro culture as successfully as fresh follicles were.

In conclusion, when using the experimental conditions of the present study, an efficient solution (VS4: 6 M EG, 0.25 M SUC and 10% FCS) and technique (MTV) were successfully used for the vitrification of ovine ovarian tissue.

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