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Tissue Thickness May Influence the Outcome of Vitrification of Goat Ovarian Cortex

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ABSTRACT

Background: The main advantage of the cryopreservation of ovarian fragments is a thinner tissue, which facilitates the penetration of cryoprotective agents, but the size of tissue may not be a limiting factor in achieving a successful cryopreservation of the ovarian tissue. This information is highly significant considering that the cryopreservation of hemi-ovary or whole ovary may preserve the entire or major part of the contingent of primordial follicles of ovarian fragments. Therefore, the aim of this study was to evaluate the vitrification of different dimensions goat ovarian tissue on the follicular morphology, viability, diameter, and the stromal cell density.

Materials, Methods & Results: The ovarian tissue was vitrified as fragment, hemi-ovary, or whole ovary, and after warming, the preantral follicles were examined by trypan blue dye exclusion test and histological analysis. Preantral follicles incubated with trypan blue were considered viable if the oocyte and granulosa cells remained unstained. Preantral follicles were classified as morphologically normal only when they contained intact oocyte and granulosa cells. The follicular diameter was measured considering the major and minor axes of each follicle; the average of these 2 measurements was used to determine the diameter of each follicle. Ovarian stroma cells density was evaluated by calculating the number of stromal cell in an area of 100 × 100 µm. There was no difference in the percentage of morphologically normal and viable follicles after vitrification compared to the control (fresh tissue), regardless of the dimension of the vitrified ovarian tissue (P > 0.05). In addition, there were no differences in the follicular diameter after ovarian tissue vitrification, independent of the dimension (P > 0.05). However, after vitrification, a decrease in the ovarian stromal cells density was observed (P < 0.05). This reduction was more intense after the vitrification of the hemi-ovary and whole ovary, compared to the ovarian fragment vitrification (P < 0.05).

Discussion: No differences were observed in the percentages of morphologically normal and viable follicles from fresh or vitrified ovarian tissue (fragment, hemi-ovary, and whole ovary). These results are in agreement with other reports which no showed morphological changes after cryopreservation of the whole ovary, and the ovarian fragments. With respect to follicular diameter, only the diameter of the preantral follicles in ovarian tissue vitrified as hemi-ovary was similar to that observed in the fresh control, in the present study. The results demonstrate that fragments and whole ovary vitrification had greater cell dehydration (exposure to VS) and/or less cell rehydration (VS removal), showing that minor adjustments are needed in the protocols of cryoprotectants addition or removal from the fragments and the whole ovary. However, this reduction in follicular diameter did not appear to have affected the follicular architecture or cellular viability, which were maintained in all dimensions of ovarian tissue undergoing vitrification. A reduction in the stromal cell density was observed, especially in the hemi-ovary and whole ovary as compared to the ovarian fragment. Previous reports have shown that ovarian stromal cells are responsible for the production of essential substances for follicle development and these substances are fundamental for follicles development and these cells tend to be more sensitive to cryopreservation procedure than ovarian follicles. In conclusion, the maintenance of follicular morphology and viability demonstrated that vitrification of goat ovarian tissue under the conditions applied in this study can be performed in any dimension of ovarian tissue (fragment, hemi-ovary, and whole ovary).

Keywords: cryopreservation, follicular morphology, ovarian tissue thickness, stromal cell density.
INTRODUCTION

The cryopreservation of ovarian tissue has as its main objective to safeguard female fertility by preserving thousands of preantral follicles enclosed in the ovarian cortex [4]. Among the livestock species, goats are a commercially interesting species [20] and, therefore, efforts have been made to develop germplasm banks for the genetic preservation of animals possessing higher value [5]. Moreover, because the ovary dimensions from goats and sheep are similar to those from human [21], these species are considered as viable alternatives to achieve cryopreservation protocols in humans.

The ovarian tissue of ruminants has been cryopreserved in fragments [11] or even as hemi-ovary [2] or whole ovary [10]. The main advantage of the cryopreservation of ovarian fragments is to have a thinner tissue, which facilitates the penetration of cryoprotective agents [17], but the size of tissue may not be a limiting factor in achieving a successful cryopreservation of the ovarian tissue [16]. This information is highly significant considering that the cryopreservation of hemi-ovary or whole ovary may preserve the entire or major part of the contingent of primordial follicles that, when transplanted, can increase the chances of pregnancy [14].

The success of sheep ovarian tissue cryopreservation, for example, has been demonstrated by birth of healthy offspring after transplantation of different dimensions (ovarian fragments [9]; hemi-ovary [2]; whole ovary [10]). However, in goats, only the cryopreservation of ovarian tissue fragments has been reported [19].

The aim of this study was to evaluate the influence of different dimension of ovarian tissue (fragment, hemi-ovary, or whole ovary) on the ovarian stroma cells density, preantral follicles morphological changes, and preantral follicles viability enclosed in goat ovarian tissue vitrified.

MATERIALS AND METHODS

Collection and vitrification of goat ovarian tissue

Ovaries were collected at a local abattoir from adult crossbred goats (n = 5). Immediately postmortem, ovaries were washed in 70% alcohol, followed by two washes in HEPES-buffered minimum essential medium (MEM) supplemented with antibiotics (100 µg/mL penicillin and 100 µg/mL streptomycin). This medium was also used to transport ovaries (at 20°C) to the laboratory, within 1 h after they were recovered. In the laboratory, ovaries (average size 1.4×0.9×0.5 cm) were divided into 2 halves, in which a hemi-ovary was cut into small fragments (3×3×1 mm), parts of which were either immediately used a fresh control or subjected to the vitrification procedure. The remaining hemi-ovary, and the other whole ovary were also cryopreserved, resulting in 3 dimensions for ovarian tissue vitrification: fragments, hemi-ovary, and whole ovary. The vitrification solution 1 (VS1) was composed of MEM supplemented with 10% ethylene glycol (EG), 10% dimethylsulfoxide (DMSO), 0.25M sucrose, and 10 mg/mL bovine serum albumin (BSA). The vitrification solution 2 (VS2) had the same composition of VS1, except that the concentration of cryoprotectants was increased (20% EG and 20% DMSO). The ovarian fragments were exposed to VS1 for 4 min, and VS2 for 1 min. To vitrify the hemi-ovaries and whole ovaries, both were exposed to VS1 and VS2 for 8 and 2 min, respectively. The solutions were perfused through hilum into whole ovaries. All the tissue dimensions were vitrified using a cryodevice according to the logistics of the solid surface vitrification. This device is a cylinder made by stainless steel, a good conductor of heat, which enables rapid temperature reduction. Moreover, this device has a lid to be closed, avoiding tissue contact with liquid nitrogen during vitrification. After cryostorage (up to 1 week), the vitrified material was removed from the liquid nitrogen and warmed by maintaining the device at room temperature (~25°C; 1 min for ovarian fragments and hemi-ovaries, and 2 min for whole ovaries) followed by immersion in water bath at 37°C (ovarian fragments: 30 s; hemi-ovary: 1 min, and whole ovary: 2 min). After warming, the cortical tissue from hemi-ovary and whole ovary were cut into small fragments (3×3×1 mm). For each treatment (fresh control, fragment, hemi-ovary and whole ovary) two samples (i.e. fragments) of ovarian tissues were used for histological and viability analysis. The VS removal was performed by in a three-step equilibrium (5 min each) in (i) MEM + 3 mg/mL BSA + 0.5M sucrose, (ii) MEM + 3mg/mL BSA + 0.25 M sucrose, and finally (iii) MEM + 3 mg/mL BSA.

Histological analysis

The ovarian fragments were fixed in Carnoy’s solution (4 h), embedded in paraffin, serially sectioned...
(7 µm) and were stained with Hematoxylin-Eosin. Only preantral follicles with visible nuclei were counted. Preantral follicles (one or more layers of granulosa cells and no antral cavity, i.e., primordial, primary, and secondary follicles) were classified as morphologically normal only when they contained intact oocyte and granulosa cells (Figure 1A). The follicular diameter was measured using Nikon NIS Elements software, considering the major and minor axes of each follicle; the average of these 2 measurements was used to determine the diameter of each follicle. Thirty follicles were analyzed per treatment. Ovarian stroma cells density was evaluated by calculating the number of stromal cell in an area of 100 × 100 µm (Figure 1B). For each treatment, ten fields per slide were assessed and the mean number of stromal cell per field was calculated [8].

**Viability analysis**

The preantral follicles were isolated mechanically from all treatment using the method of Lucci [12]. Briefly, samples were cut into small pieces with a tissue chopper adjusted to a sectioning interval of 75 µm. Samples were placed in MEM supplemented with 3 mg/mL 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 obtained material was then passed through a 200 µm nylon mesh filter. This procedure was performed within 10 min at room temperature (RT; approximately 25°C). The viability of preantral follicles was assessed by the trypan blue dye exclusion test. Briefly, 5 µL of 0.4% trypan blue was added to 100 µL of isolated and suspended preantral follicles, which were incubated for 1 min at RT [4]. Afterwards, follicles were examined with an inverted microscope and classified as viable if the oocyte and granulosa cells remained unstained (Figure 1C).

**Statistical analysis**

For all data, Kolmogorov-Smirnov and Bartlett tests were used to confirm normal distribution and homogeneity of variances, respectively. The percentages of morphologically normal, stromal cell density and follicular diameters were submitted to ANOVA followed Student-Newman-Keuls test. The percentages of viable follicles were compared by Chi-Square test. For all the statistical analyses, *P* < 0.05 was considered significant, and the results were expressed as mean ± SD.

**Figure 1.** Morphological characteristics and viability of preantral follicles enclosed in different dimensions of goat ovarian tissue. (A1-A2) Photomicrograph of morphologically normal goat preantral follicles in the fresh control (A1) and atretic preantral follicle after the cryopreservation of the whole ovary (A2). Note the measurement of follicular diameter in A1, obtained by performing by 2 transverse measurements. In A2, the follicular degeneration is evidenced by a retraction of the ooplasm (black arrow) and nuclear pyknosis (*) [Bar = 15 µm]. (B1-B2) Photomicrographs of ovarian stroma with normal (fresh control; B1) and reduced (whole ovary; B2) cell density. Observe the marking areas randomly selected for evaluation [Bar = 50 µm]. (C1-C2) Images of preantral follicles unstained, viable follicle (fresh control; C1) and stained, non-viable follicle (hemi-ovary; C2), with trypan blue [Bar = 20 µm].
RESULTS

Follicular morphology displayed no significant variation among morphologically normal preantral follicles obtained from fresh control (68.80%) and preantral follicles from vitrified tissue in fragment (61.40%), hemi-ovary (53.80%), and whole ovary (56.00%, \( P > 0.05 \)) [Table 1]. Similar results were observed for follicular viability under these 3 vitrification conditions, i.e., fragment (90.00%), hemi-ovary (93.33%), and whole ovary (93.33%).

The stromal cell density was reduced after vitrification, irrespective of the tissue thickness, compared to the fresh control (355.98 cells; \( P < 0.05 \)). Moreover, this reduction post vitrification was significantly higher in hemi-ovary (239.24 cells) and whole ovary (216.08 cells) as compared to the reduction observed after the vitrification of ovarian fragments (299.66 cells; \( P < 0.05 \)).

The measurement of the follicular diameter before (fresh control) and after cryopreservation in the different dimensions of ovarian tissue showed that only the vitrification in hemi-ovary (31.33 µm) maintained follicular diameter similar to the fresh control (33.08 µm; \( P > 0.05 \)). However, no significant reduction in the follicular diameter (Table 1) was observed among the different dimensions of vitrified ovarian tissue, i.e., fragment (30.07 µm), hemi-ovary (31.33 µm), and whole ovary (29.49 µm).

Table 1. Morphology, viability, follicular diameter, and stromal cell density after ovarian tissue vitrification at different dimensions (fragment, hemi-ovary, and whole ovary).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Morphologically normal preantral follicles (%) (mean ± SD)</th>
<th>Viable preantral follicles (%) (mean ± SD)</th>
<th>Follicular diameter (µm) (mean ± SD)</th>
<th>Stromal cells density (cells/100 x 100 µm) (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh control</td>
<td>68.80 ± 6.50A</td>
<td>93.33A</td>
<td>33.08 ± 6.18A</td>
<td>355.98 ± 40.15A</td>
</tr>
<tr>
<td>Fragment</td>
<td>61.40 ± 7.02A</td>
<td>90.00A</td>
<td>30.07 ± 4.28B</td>
<td>299.66 ± 33.49B</td>
</tr>
<tr>
<td>Hemi-ovary</td>
<td>53.80 ± 10.66A</td>
<td>93.33A</td>
<td>31.33 ± 4.58AB</td>
<td>239.24 ± 43.97C</td>
</tr>
<tr>
<td>Whole ovary</td>
<td>56.00 ± 9.46A</td>
<td>93.33A</td>
<td>29.49 ± 3.01B</td>
<td>216.08 ± 17.12C</td>
</tr>
</tbody>
</table>

*Five animals were used. †For each treatment, two samples: one sample to histological analysis and another one to viability analysis were used for each animal. Ovarian tissues samples were obtained from hemi-ovary and whole ovary after vitrification. A,B,C Different superscripts letters indicate statistically significant differences (\( P < 0.05 \)).

DISCUSSION

The results of this study showed that vitrification of ovarian fragments as either hemi-ovary or whole ovary can be used for the cryopreservation of goat ovarian tissue. This observation is supported by the fact that no differences were observed in the percentages of morphologically normal and viable follicles from fresh or vitrified ovarian tissue (fragment, hemi-ovary, and whole ovary). These results are in agreement with other reports which showed that morphological changes observed after cryopreservation of the whole ovary [1], and the ovarian fragments [11] were similar to those obtained from fresh tissue.

With respect to follicular diameter, only the diameter of the preantral follicles in ovarian tissue vitrified as hemi-ovary was similar to that observed in the fresh control, in the present study. The results demonstrate that fragments and whole ovary vitrification had greater cell dehydration (exposure to VS) and/or less cell rehydration (VS removal), showing that minor adjustments are needed in the protocols of cryoprotectants addition or removal from the fragments and the whole ovary. However, this reduction in follicular diameter did not appear to have affected the follicular architecture and cellular viability, which were maintained in all dimensions of vitrified ovarian tissue undergoing vitrification. The trypan blue is a vital dye which is only able to enter cells with compromised membranes and color them blue [6]. The histological assessment of viability by trypan blue proves to be a reliable method [13,18], and it is noteworthy that the trypan blue dye exclusion test has been successfully used to evaluate viability of preantral follicles after cryopreservation and in vitro culture in mice [3], goats [20] and women [7].

In the present study, a reduction in the stromal cell density was also observed, especially in the hemi-ovary and whole ovary as compared to the ovarian fragment. We reiterated prior hypothesis that lesser
thickness in the fragments might have favored both ovarian perfusion of cryoprotectants as well as a uniform reduction of temperature throughout the entire length of the ovarian tissue, which in turn may have minimized the damage to the ovarian stromal cells. Additionally, previous reports have shown that ovarian stromal cells are responsible for the production of essential substances for follicular development, i.e., growth factors and peptides [15]. These substances are fundamental for follicles development in vivo or in vitro after cryopreservation and these cells tend to be more sensitive to cryopreservation procedure than ovarian follicles [8]. Thus, an evaluation of ovarian stromal cells can be a valuable tool in establishing cryopreservation protocols owing to the greater sensitivity of these cells, which may show cryoinjuries in the ovarian tissue not easily observed in the follicular analysis.

In conclusion, the maintenance of follicular morphology and viability demonstrated that vitrification of goat ovarian tissue under the conditions applied in this study can be performed in any dimension of ovarian tissue (fragment, hemi-ovary, and whole ovary). A more accurate analysis of ovarian tissue should be addressed in order to reveal changes in the hemi-ovary and whole ovary, which were not detect by histological analysis, as ultrastructural or functional changes that could be observed by transmission electron microscopy and in vitro culture/transplantation, respectively.

SOURCES AND MANUFACTURERS
1Sigma Chemical Co., St. Louis, MO, USA.
2Dinâmica Química, Diadema, SP, Brazil.
3The Mickle Laboratory Engineering Co., Gomshal, Surrey, UK.
4Nikon, Tokyo, Japan.

Ethical approval. This experiment was approved and performed under the guidelines of Ethics Committee for Animal Use of State University of Ceará.

Declaration of interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

REFERENCES


