DOI: 10.4322/1984-3143-AR772

Anim. Reprod., v.13, n.1, p.28-35, Jan./Mar. 2016

Influence of the ovarian fragmentation before storage at 4°C on the apoptosis rates and *in vitro* development of ovine preantral follicles

R.S. Barberino, R.J.S. Gonçalves, V.G. Menezes, V.R.P. Barros, T.L.B.G. Lins, B.B. Gouveia, T.J.S. Macedo, L.P. Santos, M.H.T. Matos[1]

Nucleus of Biotechnology Applied to Ovarian Follicle Development, Federal University of San Francisco Valley, Petrolina, PE, Brazil.

Abstract

The aim of this study was to evaluate the effect of ovarian tissue fragmentation before preservation at 4°C in MEM on the morphology, apoptosis, and growth of ovine preantral follicles. After collection, the ovaries were divided into two halves, being one divided into two fragments (1/4 of the ovary). One fragment was subdivided into two fragments (1/8), being one fixed for histology (fresh control). The remaining whole ovary, 1/2, 1/4 and 1/8 of the ovary were preserved in MEM at 4°C for 6, 12 or 24 h. The tissue was further destined to histology. *In vitro* culture and TUNEL technique were performed in treatments that showed the best results of follicular survival after preservation. Storage of 1/8 of the ovary increased the normal follicles compared with half or whole ovary. After preservation in 1/8 of the ovary and culture for 7 days, the percentage of apoptotic cells was similar to the fresh control and non-cultured fragments. The percentage of growing follicles increased after preservation of 1/8 of the ovary compared with 1/4. In conclusion, ovine preantral follicles can be preserved in fragments of 1/8 of the ovary in MEM at 4°C for 24 h, without affecting apoptosis and their ability to grow *in vitro*.

Keywords: culture, oocyte, preservation, sheep, survival.

Introduction

Many reproductive biotechnologies have been employed to allow the acceleration of *in vitro* production of mammalian embryos (Gupta *et al*., 2007; Garcia-Alvarez *et al*., 2011). The *in vitro* development, maturation and fertilization of thousands of oocytes from preantral follicles present in the ovaries may optimize the oocyte potential from valuable animals and contribute to the genetic improvement. However, the successful *in vitro* embryo production depends on the maintenance of oocyte viability during transportation of the ovaries, because generally the females are far from specialized laboratories (Evecen *et al*., 2010; García-Alvarez *et al*., 2011). In this context, the preservation media, temperature and conservation period of the ovaries are extremely important to ensure the subsequent follicular survival, development and complete maturation (Matos *et al*., 2004; Chaves *et al*., 2008; Tellado *et al*., 2014).

Studies in ovine and caprine species, which are present on all continents and commercially seen as highly attractive livestock, have shown that chilling ovarian fragments in different media at 4°C during transport is more efficient for maintaining follicular morphology than higher temperatures such as 20 or 35°C (Andrade *et al*., 2001; Matos *et al*., 2004; Costa *et al*., 2005). In addition, some authors have shown that the transportation of small caprine ovarian fragments (3 x 3 x 1 mm) in Minimal Essential Medium (MEM) at 4°C for 4 h maintained the percentages of morphologically normal follicles similar to those observed in control tissues and also kept follicular viability after *in vitro* culture (Chaves *et al*., 2008). However, the transportation of the whole ovary has been more practicable than fragmented ovarian tissue (Rosseto *et al.,* 2009; Esmaielzadeh *et al*., 2013). A previous study stored different sizes of sheep ovarian tissue (whole, half and 1/8 of the ovary) at 4°C for 24 h in 0.9% saline solution. After preservation, the percentage of histologically normal follicles was similar among the different ovarian fragments (Matos *et al*., 2002). However, they did not evaluate whether this storage may affect the apoptosis rate or the ability of preantral follicles to grow *in vitro*. Moreover, the MEM, which has usually been used as a basic culture medium for preantral follicles, was not evaluated as a storage medium for ovine ovaries at 4°C, and a richer solution might be able to improve follicular viability in this case. Therefore, it is not known whether the size of the ovarian tissue (whole or half of the ovarian tissue, or smaller fragments) would affect morphology, apoptosis rates and further *in vitro* development of ovine preantral follicles stored in MEM at 4°C.

Thus, the aim of this study was to evaluate the effect of ovarian tissue fragmentation before preservation at 4°C in MEM on the morphology and apoptosis of ovine preantral follicles, and to test the ability of these follicles to survive and grow *in vitro* after being stored.

Materials and Methods

This experiment was approved and performed under the guidelines of the Committee of Ethics and

[1]Corresponding author: helena.matos@univasf.edu.br
Phone: +55(87)2101-4839
Received: August 15, 2015
Accepted: January 28, 2016

Deontology Studies and Research at the Federal University of San Francisco Valley (0006/261011). Unless otherwise mentioned, culture media, supplements and chemicals used in the present study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Source, collection and in vitro *preservation of ovarian tissue*

Ovarian cortical tissues (n = 30 ovaries) were collected at a local slaughterhouse from 15 adult (1-3 years old) mixed-breed sheep. The animals used in this study were non-pregnant and presumed to be undergoing normal oestrous cycles as judged by the presence of normal corpora lutea at slaughter. Immediately after death, the ovaries were washed once in 70% alcohol (Dinâmica, São Paulo, Brazil) for 10 seconds and then twice in Minimum Essential Medium containing HEPES (MEM-HEPES) and antibiotics (100 µg/ml penicillin and 100 µg/ml streptomycin).

Still in the slaughterhouse, for each animal, one of the ovaries was divided into two halves (1/2 of the ovary). Subsequently, one half was divided into two fragments (1/4 of the ovary). Then, one of these fragments was subdivided into two fragments of 1/8 of the ovary, being one of them immediately fixed for histological analysis (fresh control). The remaining whole ovary and the other fragments (1/2, 1/4 and 1/8 of the ovary) were individually preserved in tubes containing 5 ml of MEM-HEPES supplemented with antibiotics (100 µg/ml penicillin and 100 µg/ml streptomycin) at 4°C for 6, 12 or 24 h (simulating transport; Fig. 1). After preservation periods, ovarian tissue fragments were fixed and destined to histological analysis. Each treatment was repeated 5 times in each preservation period (five ovarian pairs for each tested incubation time).

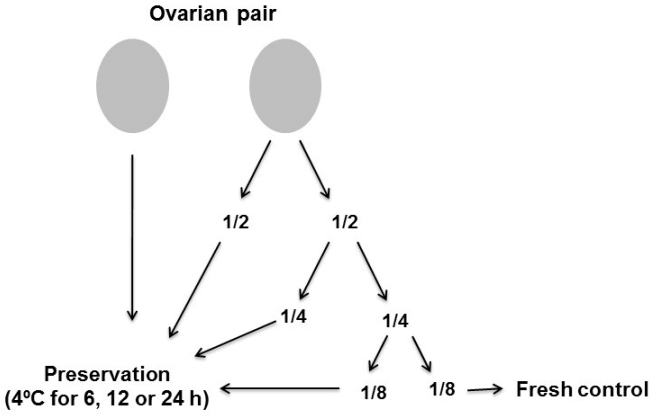

Figure 1. General experimental protocol for preservation of ovine ovarian tissue.

In vitro *culture of preantral follicles after preservation*

After histological analysis, for better evaluation of follicular quality and growth, *in vitro* culture was performed on treatment groups that maintained follicular morphology for a longer period after preservation. Additional pairs of sheep ovaries (n = 10 ovaries) were collected and divided into fragments of 1/4 or 1/8 of the ovary. For each animal, one fragment of tissue was randomly selected and immediately fixed for histological analysis (fresh control). The remaining fragments were preserved in MEM at 4ºC for 24 h. After this period, the fragments were fixed for morphological and TUNEL analysis (non-cultured fragments) or destined to *in vitro* culture (cultured fragments). To this end, ovarian cortex samples from each ovarian fragment (1/4 or 1/8 of the ovary) were cut into slices approximately 3 mm x 3 mm (1 mm thick) in size using a needle and scalpel under sterile conditions. The slices of ovarian cortex were cultured individually in 1 ml of culture medium in 24-well culture dishes for 7 days; the culture conditions were 39°C in an atmosphere of 5% CO_2 in air. The culture medium consisted of α-MEM (pH 7.2-7.4; Gibco, Invitrogen, Karlsruhe, Germany) supplemented with ITS (insulin 10 µg/ml, transferrin 5.5 µg/ml and sodium selenite 5.0 ng/ml), 2 mM glutamine, 2 mM hypoxanthine, 1.25 mg/ml bovine serum albumin, 50 µg/ml ascorbic acid, 50 ng/ml recombinant follicle-stimulating hormone (rFSH; Nanocore, São Paulo, Brazil), 100 µg/ml penicillin and 100 µg/ml streptomycin. The culture medium was stabilized at 39°C for 2 h prior to use and replenished every second day. Each treatment was repeated 5 times, thus using the ovaries of five different animals. After preservation and/or *in vitro* culture for 7 days, ovarian fragments were fixed and destined to histological analysis.

Morphological analysis of ovarian preantral follicles

Tissues from all treatments (fresh control, preserved or those preserved and then cultured) were

fixed in 10% buffered formaldehyde (Dinâmica) for 18 h and then dehydrated in increasing concentrations of ethanol (Dinâmica). After paraffin embedding (Dinâmica), the ovine tissue pieces were cut into 7 µm sections, and every section was mounted on glass slides and stained by Periodic Acid Schiff and hematoxylin (Dinâmica). Follicle stage and survival were examined by microscopy (Nikon, Tokyo, Japan) at 400X magnification.

The developmental stages of preantral follicles have been defined previously as follows (Silva *et al*., 2004): primordial (one layer of flattened granulosa cells around the oocyte) or growing follicles (intermediate: one layer of flattened to cuboidal granulosa cells; primary: one layer of cuboidal granulosa cells, and secondary: two or more layers of cuboidal granulosa cells around the oocyte and no sign of antrum formation). Additionally, these follicles were classified individually as histologically normal when an intact oocyte was present and surrounded by granulosa cells that were well organized in one or more layers and have no pyknotic nuclei. Atretic follicles were defined as those with a retracted oocyte, pyknotic nucleus, and/or disorganized granulosa cells detached from the basement membrane. Overall, 150 follicles were evaluated for each treatment (30 follicles per treatment-replicate x 5 replicates = 150 follicles).

Assessment of in vitro *follicular activation and growth after culture*

To evaluate follicular activation (transition from primordial to growing follicles, when surrounding squamous pregranulosa cells become cuboidal and begin to proliferate) and growth, only morphologically normal follicles with a visible oocyte nucleus were recorded, and the proportion of primordial and growing follicles was calculated on day 0 (fresh control), after 24 h of preservation (non-cultured) and after 7 days of culture (preserved and then cultured). In addition, from the basement membrane, major and minor axes of each oocyte and follicle were measured using Image-Pro Plus® software. The average of these two measurements was used to determine the diameters of both the oocyte and the follicle.

Detection of apoptotic cells by TUNEL assay

Terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick-end labeling (TUNEL) assay was used for a more in-depth evaluation of ovine preantral follicle quality before (fresh control), after preservation in 1/4 or 1/8 of the ovary for 24 h (non-cultured), and after preservation and *in vitro* culture for 7 days. TUNEL was performed using a commercial kit (In Situ Cell Death Detection Kit, Roche Diagnostics Ltd., Indianapolis, USA) following the manufacturer's protocol, with some modifications. Briefly, sections (5 µm) mounted on glass slides were deparaffinized and rehydrated through graded alcohols, then rinsed in PBS (pH 7.2). Antigen retrieval by microwave treatment was performed in a sodium citrate buffer (pH 6.0; Dinâmica) for 6 min. Endogenous peroxidase activity was blocked by 3% H_2O_2 (Dinâmica) in methanol (Dinâmica) at room temperature for 10 min. After rinsing in Tris buffer (Dinâmica), the sections were incubated with a TUNEL reaction mixture at 37°C for 1 h. Then, the specimens were incubated with Converter-POD in a humidified chamber at 37°C for 30 min. The DNA fragmentation was revealed by incubation of the tissues with diaminobenzidine (DAB; 0.05% DAB in Tris buffer, pH 7.6, 0.03% H_2O_2) during 1 min. Finally, sections were counterstained with Harry's haematoxylin in a dark chamber at room temperature for 1 min, dehydrated in ethanol, cleared in xylene, and mounted with balsam (Dinâmica). For negative controls (reaction controls), slides were incubated with label solution (without terminal deoxynucleotidyl transferase enzyme) instead of TUNEL reaction mixture.

The apoptosis in preantral follicles was evaluated as described previously (Santos *et al*., 2014). Only follicles that contained an oocyte nucleus were analyzed for apoptotic assay. The number of brown TUNEL positive cells (oocyte and granulosa cells) was counted in ten randomly fields per treatment using Image-Pro Plus® software. The percentage of TUNEL positive or apoptotic cells was calculated as the number of apoptotic cells out of the total number of cells (x 100).

Statistical analysis

Percentages of morphologically normal, primordial and developing follicles were submitted to ANOVA and Tukey´s test was applied for comparison among treatments. The values of apoptotic cells were submitted to Qui-square and differences were considered to be statistically significant when $P < 0.05$. The results of follicular survival and growth were expressed as the mean ± SD and the results of follicular activation were expressed as the mean ± SEM.

Results

Effect of storage conditions on follicular morphology

Among the preantral follicles analyzed after preservation, 1,756 were primordial, 384 were intermediate, 78 were primary and 32 were secondary follicles. Fig. 2 shows the percentage of morphologically normal preantral follicles before (fresh control) and after preservation in MEM for 6, 12 or 24 h. The percentage of normal follicles in the fresh control (~70%) was similar ($P > 0.05$) among groups of five ovarian pairs from each tested preservation period. The percentage of morphologically normal preantral follicles

decreased significantly in all treatments when compared with the fresh control. After comparing the different treatments, the percentage of morphologically normal follicles preserved in 1/8 of the ovarian tissue was similar (P > 0.05) to that observed in 1/4 of the ovary. However, the storage of smaller ovarian fragments (1/8) increased significantly the percentage of normal follicles compared with the preservation of half or whole ovarian tissue irrespective of preservation periods. In addition, more normal follicles were observed after preservation of 1/4 of the ovary for 24 h than in half or whole ovarian tissue (P < 0.05). When the same treatment was compared throughout the preservation periods, storage of 1/4 or half of the ovarian tissue decreased (P < 0.05) the percentage of normal follicles from 6 to 24 h of storage.

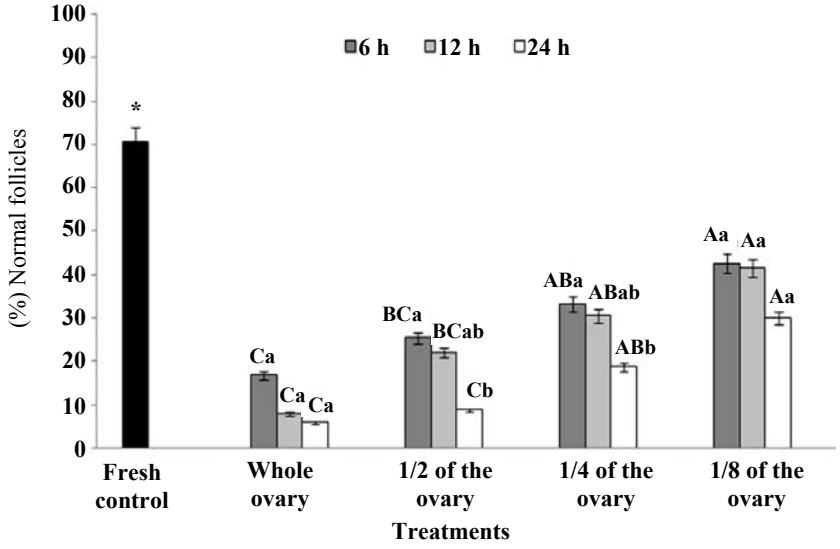

Figure 2. Percentages of morphologically normal ovine preantral follicles in the fresh control and after preservation of whole ovary, half, 1/4 or 1/8 of the ovary in MEM for 6, 12 or 24 h. *Differs significantly from fresh control (P < 0.05). [A,B,C]Different letters denote significant differences between preservation forms in the same period (P < 0.05). [a,b]Different letters denote significant differences between preservation periods in the same treatment (preservation forms; P < 0.05).

Effect of storage conditions on follicular morphology after in vitro *culture*

Among the 1,050 preantral follicles analyzed after *in vitro* preservation and culture of ovine ovarian tissue, 657 were primordial, 369 were intermediate, 13 were primary and 11 were secondary follicles. Fig. 3A shows the percentage of morphologically normal preantral follicles before (fresh control), after preservation in 1/4 or 1/8 of the ovary for 24 h (non-cultured), and after preservation and further *in vitro* culture for 7 days. There was a significant reduction in the percentage of morphologically normal preantral follicles in all treatments after preservation (non-cultured) and after preservation followed by culture, when compared with the fresh control. In addition, after 7 days of culture, all the treatments significantly reduced the percentage of normal follicles compared with non-cultured treatments (only preserved). However, there were no differences (P > 0.05) in the percentage of normal follicles among preservation forms (1/4 or 1/8 of the ovary) in the same condition (only preserved or preserved and cultured).

Apoptotic cell detection before and after in vitro *culture*

Fig. 3B shows the percentage of TUNEL positive cells in ovine preantral follicles before (fresh control) and after preservation in 1/4 or 1/8 of the ovary for 24 h (non-cultured) and further culture *in vitro* for 7 days. After 7 days of culture, the percentage of TUNEL positive cells of preantral follicles preserved in 1/8 of the ovary was similar (P > 0.05) to that observed in the fresh control and after preservation of ovarian tissue (non-cultured fragments). Nevertheless, preservation of follicles in 1/4 of the ovary and further culture for 7 days significantly increased the percentage of TUNEL positive cells when compared to fresh control and non-cultured tissue. However, there were no differences (P > 0.05) in the percentage of apoptotic cells among preservation forms (1/4 or 1/8 of the ovary) in the same condition (only preserved or preserved and cultured).

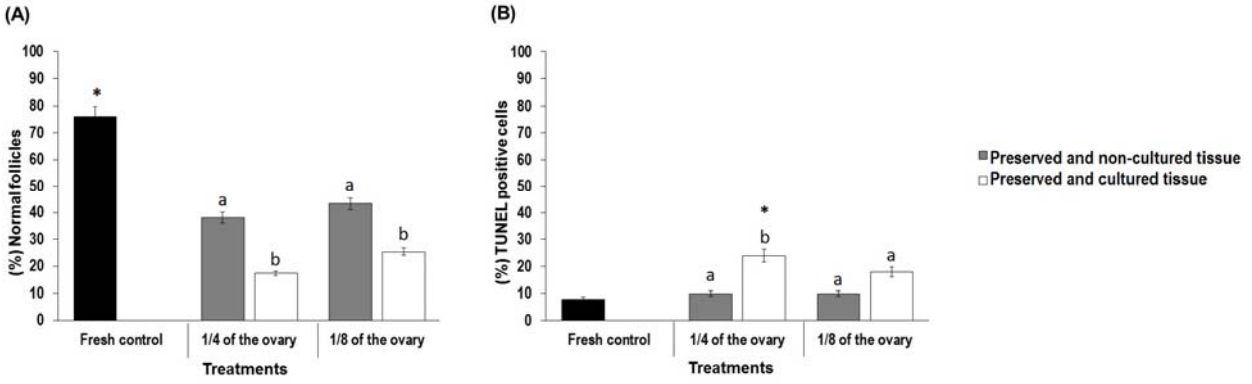

Figure 3. Percentages of morphologically normal ovine preantral follicles (A) and percentages of TUNEL positive cells (B) before (fresh control) and after preservation in 1/4 or 1/8 of the ovary for 24 h without (preserved and non-cultured tissue) or with subsequent *in vitro* culture for 7 days (preserved and cultured tissue). *Differs significantly from fresh control ($P < 0.05$). [a,b]Different letters denote significant differences between preserved and non-cultured tissue versus preserved and cultured tissue ($P < 0.05$).

Figure 4 shows histological sections after TUNEL analysis. Ovarian follicles preserved in 1/8 of the ovary and further culture did not show or showed less apoptotic cells (Fig. 4A). Nevertheless, oocyte apoptosis was commonly found after preservation in 1/4 of the ovary and further culture, which was highlighted by the brown staining (Fig. 4B). Negative control did not show staining for TUNEL analysis (Fig. 4C).

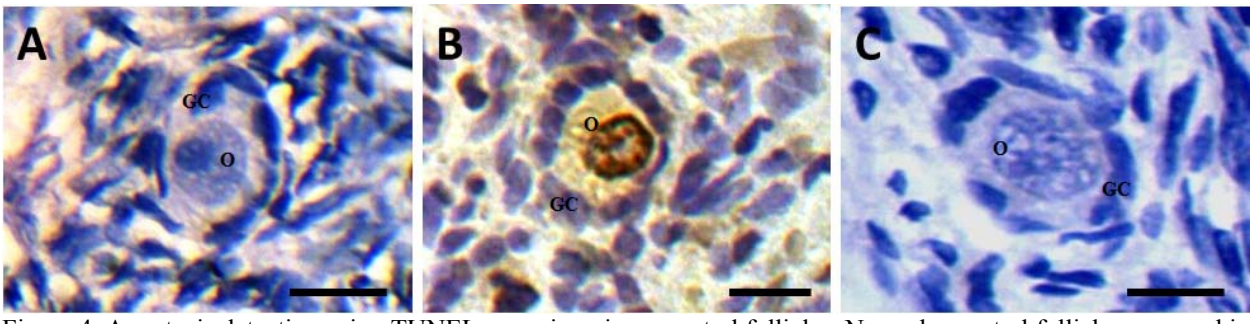

Figure 4. Apoptosis detection using TUNEL assay in ovine preantral follicles. Normal preantral follicle preserved in 1/8 of the ovary and further culture (A), apoptotic follicle after preservation in 1/4 of the ovary and further culture, (B) and negative control (C). In Fig. B, note the apoptotic oocyte. O: oocyte; GC: granulosa cells. Scale bar: 25 µm (400X).

Follicular activation and growth during in vitro *preservation and culture*

The percentage of primordial and growing follicles (intermediate, primary and secondary) in fresh tissue (control) or in tissues preserved in 1/4 or 1/8 of the ovary for 24 h (non-cultured) and further culture *in vitro* for 7 days is shown in Fig. 5. Fresh ovarian tissues predominantly contained primordial follicles (83.3%). No change in the percentage of primordial or growing follicles was observed between fresh and preserved tissues (non-cultured) in both sizes (1/4 or 1/8) of ovarian fragments ($P > 0.05$). Nevertheless, after 7 days of culture, a significant reduction in the percentage of primordial follicles was observed concomitant with an increase ($P < 0.05$) in the percentage of growing follicles compared with the fresh control and non-cultured tissues. Moreover, after culture, the percentage of growing follicles increased ($P < 0.05$) after preservation of 1/8 of the ovary compared with 1/4 of the ovary.

Follicle and oocyte diameters are shown in Table 1. After 7 days of culture, there was a significant increase in follicular and oocyte diameters in all treatments compared with the fresh control, but there is no significant influence ($P > 0.05$) of ovarian fragment size.

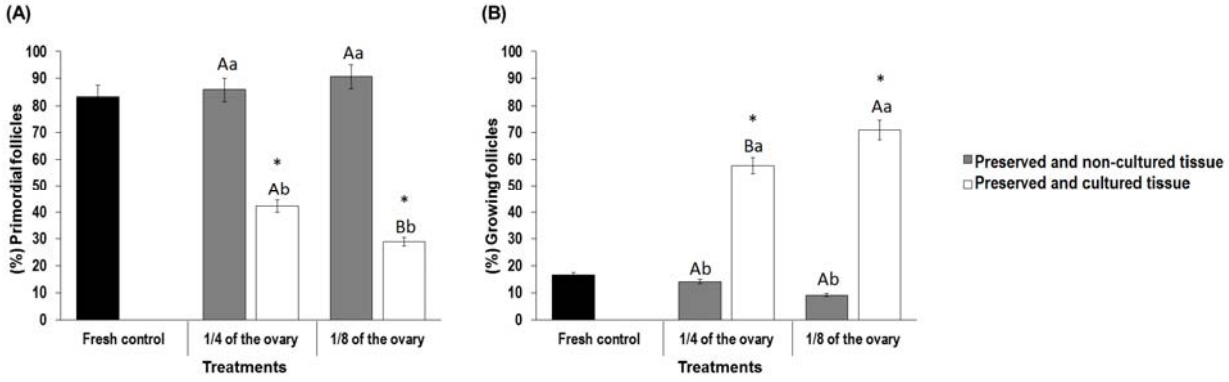

Figure 5. Percentages (mean ± SEM) of primordial (A) and growing (B) follicles in the fresh control, after preservation of 1/4 or 1/8 of the ovary for 24 h without (preserved and non-cultured tissue) or with subsequent *in vitro* culture for 7 days (preserved and cultured tissue). *Differs significantly from fresh control ($P < 0.05$). ^{A,B}Different letters denote significant differences between treatments in the same condition (only preserved or preserved followed by *in vitro* culture; $P < 0.05$). ^{a,b}Different letters denote significant differences between preserved and non-cultured tissue versus preserved and cultured tissue ($P < 0.05$).

Table 1 - Mean oocyte and follicular diameter (mean ± SD) in the fresh control, after preservation in 1/4 or 1/8 of the ovary for 24 h (non-cultured) and further culture *in vitro* for 7 days.

	Oocyte diameter (µm)		Follicle diameter (µm)	
Treatments	1/4 of the ovary	1/8 of the ovary	1/4 of the ovary	1/8 of the ovary
Fresh control	40.42 ± 6.93		53.43 ± 9.21	
Preserved and non-cultured tissue	41.40 ± 7.81	41.07 ± 8.72	53.99 ± 9.01	53.78 ± 10.7
Preserved and cultured tissue	44.06 ± 11.3*	44.04 ± 12.26*	58.13 ± 12.1*	59.05 ± 12.56*

* Differs significantly from fresh control ($P < 0.05$).

Discussion

The preservation of the ovine ovaries during the transportation from the farms or slaughterhouses to the specialized laboratories is of great importance for the maintenance of follicular integrity, ensuring good quality oocytes for cryopreservation and/or *in vitro* culture techniques. Although some authors have shown that it is possible to preserve caprine ovarian fragments (3 x 3 x 1 mm) at 4°C for up to 4 h before *in vitro* culture of preantral follicles (Chaves *et al*., 2008), the transportation of the whole ovary has been more practicable (Esmaielzadeh *et al*., 2013). In fact, the transportation of follicles in larger sizes of ovarian fragments or even into the whole ovary would make the collect easier and faster. Therefore, the present study evaluated whether the size of the ovine ovarian fragment, which was preserved at 4°C, had any influence on the morphology, apoptosis and *in vitro* development of preantral follicles.

After storage, we showed that the ovarian fragment size directly influences the percentage of morphologically normal preantral follicles, since as the size decreases, the follicular survival increases. However, no differences in the percentage of normal follicles were observed after preservation of 1/4 or 1/8 of the ovary in the different periods. Based on cryopreservation studies (Onions *et al*., 2013; Torre *et al*., 2013), the fragmentation may facilitate the permeation of the medium into the ovarian tissue, enhancing nutrient absorption by the preantral follicles preserved *in vitro*. Some authors prefer the use of small ovarian cortex fragments instead of the whole organ because the smaller size and thickness of the tissue facilitates the adequate perfusion and diffusion of the cryoprotectant through the ovarian stroma and the population of primordial and growing follicle (Onions *et al*., 2013; Torre *et al*., 2013). A study has demonstrated a significant decrease in the proportion of normal follicle structures after cryopreservation when the bovine ovarian tissue fragments are cut with all their dimensions larger than 2 mm compared to those cut with at least one of their dimensions measuring ≤2 mm, allowing an optimum penetration of the cryoprotectant into the ovarian tissue fragment (Ferreira *et al*., 2010). Nevertheless, Silva *et al*. (1999) demonstrated that the size of the ovarian tissue (whole ovary or fragment) did not influence the percentage of normal caprine preantral follicles preserved in coconut water solution at 4, 20 or 39°C for 24, 12 and 4 h, respectively. It is likely that coconut water solution has some nutrients that are important for the maintenance of follicular morphology

in the earlier stages of development, such as amino acids, protein, growth factors and energetic substrates. Therefore, the low rates of normal follicles observed in our study after preservation of larger ovarian fragments could be explained by the absence of an additional supplementation (for example, energetic substrates and hormones) in the preservation medium (MEM-HEPES).

In vitro culture may be used as a reliable evaluation of follicles and/or oocyte viability (Abd-Allah, 2010) after preservation of ovarian tissue. In the present study, after preservation for 24 h and further 7 days of culture, all the treatments reduced the percentage of normal follicles compared with non-cultured treatments. Nevertheless, the percentage of normal follicles observed after preservation of 1/8 fragment for 24 h and further 7 days of culture (25.33%) could be considered satisfactory since the culture of ovine and caprine ovarian tissue in α-MEM, without preservation, resulted in an average rate of 44% normal follicles (Bruno *et al*., 2009; Rosseto *et al*., 2009; Lima *et al*., 2013; Santos *et al*., 2014). Although preantral follicles are small and have a low metabolic rate, they are also sensitive to adverse conditions *in vitro*. The removal of the ovaries from the animal, their transportation for long periods and further culture lead to tissue hypoxia and necrosis, due to the occlusion of blood flow and reduction of the supply of oxygen and energy to the organ (Wongsrikeao *et al*., 2005; Meirow and Wallace, 2009).

Terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick-end labeling (TUNEL) happens to be a very sensitive method for the in situ visualization of apoptosis at the cellular level (Sreejalekshmi *et al*., 2011). Our results demonstrated that TUNEL positive cells increased in preantral follicles preserved in 1/4 of the ovary after 7 days of culture when compared to fresh control and non-cultured tissue. One factor that could have influenced this apoptosis rate is that larger ovarian tissues have higher cell population (stroma and follicles), thus increasing the nutritional requirements of follicles. Maffei *et al*. (2014) have demonstrated that conventional cryopreservation of whole ovaries increased the percentage of apoptotic cells after 7 days of culture, compared to the fresh control.

After 7 days, follicular and oocyte diameters increased in all treatments compared with fresh control, as well as follicular activation was higher in cultured tissues than in fresh control or non-cultured tissues. This can be due to the fact that α-MEM is an enriched medium, containing non-essential amino acids, carbohydrates, sodium pyruvate, lipoic acid, biotin, vitamins, and DNA precursors, all of which promote cell division (Faustino *et al*., 2013), besides being supplemented with rFSH, insulin, and ascorbic acid, which were considered important for ovarian follicle development *in vitro* (Adhikari and Liu, 2009; Rossetto *et al*., 2009). However, preservation of 1/8 of the ovarian tissue maintained preantral follicle ability to grow *in vitro* since this storage significantly increased the percentage of growing follicles after 7 days of culture compared with 1/4 of the ovary. In addition, the lower apoptotic rate in ovarian follicles preserved in this condition (1/8 of the ovary) is an indicator of good oocyte quality, in terms of a greater capacity to be fertilized and to produce embryos.

In conclusion, ovine preantral follicles can be successfully preserved in fragments of 1/8 of the ovary in MEM at 4°C, for up to 24 h, without affecting apoptosis in the ovarian follicles and their ability to grow and activate *in vitro*. Moreover, storage of follicles in 1/8 of the ovary, when compared to 9 mm^3 fragments, is more practicable, making the collection of the organ easier and faster. The present data represent one more contribution to the available information on the factors that affect the success of the ovarian tissue storage during transportation, an important step to ensure the oocyte cryopreservation and production of quality embryos. For future studies, we suggest that supplementation of the preservation medium would improve the normal follicle rates after storage of ovary fragments for longer transportation periods.

Acknowledgments

R.S. Barberino receives a scholarship from the CNPq/Univasf. M.H.T. Matos is supported by a grant from CNPq.

References

Abd-Allah SM. 2010. Effects of storage conditions of dromedary camel ovaries on the morphology, viability and development of antral follicular oocytes. *Anim Reprod*, 7:65-69.

Adhikari D, Liu K. 2009. Molecular mechanisms underlying the activation of mammalian primordial follicles. *Endocr Rev*, 30:438-464.

Andrade ER, Rodrigues APR, Amorim CA, Carvalho FCA, Dode MAN, Figueiredo JR. 2001. Short term maintenance of sheep preantral follicles in situ in 0.9% saline and Braun-Collins solution. *Small Rumin Res*, 41:141-149.

Bruno JB, Celestino JJH, Lima-Verde IB, Lima LF, Matos MHT, Araújo VR, Saraiva MVA, Martins FS, Name KPO, Campello CC, Báo SN, Silva JRV, Figueiredo JR. 2009. Expression of vascular endothelial growth factor (VEGF) receptor in goat ovaries and improvement of in vitro caprine preantral follicle survival and growth with VEGF. *Reprod Fertil Dev*, 21:679-687.

Chaves RN, Martins FS, Saraiva MVA, Celestino JJH, Lopes CAP, Correia JC, Lima-Verde IB, Matos MHT, Bao SN, Name KPO, Campello CC, Silva JRV, Figueiredo JR. 2008. Chilling ovarian fragments during transportation improve viability and growth of goat preantral follicles cultured in vitro. *Reprod Fertil*

Dev, 20:640-647.

Costa SHF, Andrade ER, Silva JRV, Rodrigues APR, Amorim CA, Lôbo RNB, Ohashi OM, Figueiredo JR. 2005. Preservation of goat preantral follicles enclosed in ovarian tissue in saline or TCM 199 solutions. *Small Rumin Res*, 58:189-193.

Esmaielzadeh F, Hosseini SM, Nasiri Z, Hajian M, Chamani M, Gourabi H, Shahverdi AH, Vosough AD, Nasr-Esfahani MH. 2013. Kit ligand and glial-derived neurotrophic factor as alternative supplements for activation and development of ovine preantral follicles in vitro. *Mol Reprod Dev*, 80:35-47.

Evecen M, Cirit U, Demir K, Ozdas OB, Tas M, Birler S, Pabuccuiglu S. 2010. Effects of estrous cycle stage and transport temperature of ovaries on in vitro maturation of canine oocytes. *Anim Reprod Sci*, 117:160-165.

Faustino LR, Lima IMT, Carvalho AA, Silva CMG, Castro SV, Lobo CH, Lucci CM, Campello CC, Figueiredo JR, Rodrigues APR. 2013. Interaction between keratinocyte growth factor-1 and kitligand on the goat preantral follicles cultured in vitro. *Small Rumin Res*, 114:112-119.

Ferreira M, Bos-Mikich A, Frantz N, Rodrigues JL, Brunetto AL, Schwartsmann G. 2010. The effects of sample size on the outcome of ovarian tissue cryopreservation. *Reprod Domest Anim*, 45:99-102.

García-Álvarez O, Maroto-Morales A, Berlinguer F, Fernández-Santos MR, Esteso MC, Mermillod P, Ortiz JA, Ramon M, Pérez-Guzmán MD, Garde JJ, Soler AJ. 2011. Effect of storage temperature during transport of ovaries on in vitro embryo production in Iberian red deer (*Cervus elaphus hispanicus*). *Theriogenology*, 75:65-72.

Gupta PSP, Rameshb HS, Nandi S, Ravindra JP. 2007. Recovery of largepreantral follicles from buffalo ovary: effect of season and corpus luteum. *Anim Reprod Sci*, 101:145-152.

Lima LF, Rocha RM, Alves AMCV, Saraiva VA, Araújo VR, Lima IMT, Lopes CAP, Baó SN, Campello CC, Rodrigues APR, Figueiredo JR. 2013. Dynamized follicle-stimulating hormone affects the development of ovine preantral follicles cultured in vitro. *Homeopathy*, 102:41-48.

Maffei S, Pennarossa G, Brevini TAL, Arav A, Gandolfi F. 2014. Beneficial effect of directional freezing on in vitro viability of cryopreserved sheep whole ovaries and ovarian cortical slices. *Hum Reprod*, 29:114-124.

Matos MHT, Machado VP, Brasil AF, Rodrigues APR, Santos RR, Andrade ER, Amorim CA, Figueiredo JR. 2002. Efeito da fragmentação ovariana sobre a conservação de folículos pré-antrais ovinos em solução salina 0,9%. *Ciênc Anim*, 12:47-55.

Matos MHT, Andrade ER, Lucci CM, Báo SN, Silva JRV, Santos RR, Ferreira MAL, Costa SHF, Celestino JJH, Figueiredo JR. 2004. Morphological and ultrastructural analysis of sheep primordial follicles preserved in 0.9% saline solution and TCM 199. *Theriogenology*, 62:65-80.

Meirow D, Wallace WH. 2009. Preservation of fertility in patients with cancer. *N Engl J Med*, 360:2682-2683.

Onions VJ, Webb R, Pincott-Aleen C, Picton HM, Campbell BK. 2013. The effects of whole ovarian perfusion and cryopreservation on endothelial cell-related gene expression in the ovarian medulla and pedicle. *Mol Hum Reprod*, 19:205-215.

Rossetto R, Lima-Verde IB, Matos MHT, Saraiva MVA, Martins FS, Faustino LR, Araújo VR, Silva CMG, Name KPO, Báo SN. 2009. Interaction between ascorbic acid and follicle-stimulating hormone maintains follicular viability after long-term in vitro culture of caprine preantral follicles. *Domest Anim Endocrinol*, 37:112-123.

Santos JMS, Menezes VG, Barberino RS, Macedo TJS, Lins TLB, Gouveia BB, Barros VRP, Santos LP, Gonçalves RJS, Matos MHT. 2014. Immunohistochemical localization of fibroblast growth factor-2 in the sheep ovary and its effects on pre-antral follicle apoptosis and development in vitro. *Reprod Domest Anim*, 49:522-528.

Silva JRV, Carvalho FCA, Lucci CM, Santos RR, Figueiredo JR. 1999. Conservação de folículos pré-antrais caprinos in situ em solução à base de água de coco. *Ciênc Anim*, 9:27-36.

Silva JRV, Van denHurk R, Matos MHT, Santos RR, Pessoa C, Moraes MO, Figueiredo JR. 2004. Influences of FSH and EGF on primordial follicles during in vitro culture of caprine ovarian cortical tissue. *Theriogenology*, 61:1691-1704.

Sreejalekshmi P, Raghavendra BS, Subramani TS,Murthy VC, Jamuna KV, Prasad RV, Ravindra JP, Selvaraju S. 2011. Detection of follicular apoptosis in water buffalo (*Bubalus bubalis*) ovary by histology and nick end labelling technique. *Reprod Domest Anim*, 46:59-65.

Tellado MN, Alvarez GM, Dalvit GC, Cetica PD. 2014. The conditions of ovary storage affect the quality of porcine oocytes. *Adv Reprod Sci*, 2:57-67.

Torre A, Ben Brahim F, Popowski T, Boudjenah R, Salle B, Lornage J. 2013. Factors related to unstained areas in whole ewe ovaries perfused with a metabolic marker. *Hum Reprod*, 28:423-429.

Wongsrikeao P, Otoi T, Karja NWK, Agung B, Nii M, Nagai T. 2005. Effects of ovary time and temperature on DNA fragmentation and development of porcine oocytes. *J Reprod Dev*, 51:87-97.