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Original Article

Follicular survival, activation of primordial follicles and DNA fragmentation after storage of goat ovaries at 35ºC in supplemented Minimal Essential Medium

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A B S T R A C T

This study evaluated the effect of caprine ovarian tissue transportation conditions (medium supplementation and transportation duration) on the morphology, DNA fragmentation and development of cultured and non-cultured preantral follicles. After the fragmentation of ovaries, one fragment was fixed (fresh control) while the remaining slices were placed individually in two different conservation media (Minimal Essential Medium - MEM without supplementation or supplemented MEM, i.e. MEM$^+$) and stored at 35ºC for 6 or 12 h without (non-cultured) or with a subsequent 5-day *in vitro* culture in supplemented α-MEM. After transportation, followed or not by *in vitro* culture, the fragments were processed for histological and Terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick-end labeling (TUNEL) examination. For the preserved and non-cultured fragments, the percentages of normal follicles after the storage of ovarian tissue in MEM$^+$ for 6 h and the DNA fragmentation rates after preservation in MEM for 6 h and MEM$^+$ for 6 or 12 h were maintained similar to the fresh control. However, all cultured treatments reduced the proportion of normal follicles and increased the percentage of TUNEL-positive cells as compared to the fresh control and non-cultured treatments. On the contrary, all culture conditions (except after preservation in MEM for 6 h) promoted an increase in primordial follicle activation. In conclusion, the use of an enriched medium (MEM$^+$) during ovary transportation is preferable to maintain satisfactory rates of normal follicles after the preservation of caprine ovarian tissue at 35ºC for up to 6 h, without affecting the ability of the primordial follicle to grow *in vitro*.

INTRODUCTION

Based on studies which focused on assisted reproductive technologies, an area that still requires improvement is the maintenance of ovarian preantral follicle viability, after the removal and storage of ovaries during transportation to the laboratory. Thus, the components of the preservation medium, temperature and conservation period of the ovaries are important to ensure subsequent oocyte development and complete maturation (CHAVES et al., 2008; TELLADO et al., 2014).

In goats, the transportation of ovarian tissue in the Minimal Essential Medium (MEM, used as a base culture

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medium for preantral follicles), at 4°C for a short period (4 h), maintained the percentage of histologically normal follicles similar to the observed in fresh tissues and kept follicular viability after *in vitro* culture. On the other hand, conservation at 35°C in MEM for 2 or 4 h, affected the follicular morphology after 7 days of culture (CHAVES et al., 2008). Although preservation at low temperatures may be recommended, temperatures close to physiological values (30–37°C) have been successfully used to collect and transport ovaries to the laboratory for further *in vitro* culture of preantral follicles (ARUNAKUMARI; SHANMUGASUNDARAM; RAO, 2010; SUN; LI, 2013) or *in vitro* maturation studies (MUKHERJEE et al., 2014; SHIRAZI et al., 2009; WAN et al., 2009). Ovine ovarian tissue was successfully preserved in supplemented MEM⁺ at 35°C for up to 6 h without affecting DNA fragmentation in the preantral follicles and their ability to develop *in vitro* (GONÇALVES et al., 2015). In addition, cleavage rates were higher after transportation of sheep ovaries for 4 h at 30–35°C (47%) than for 12 h at 5–8°C (23%) (GARCÍA-ÁLVAREZ et al., 2011). The storage of porcine ovaries at 15°C impaired oocyte maturation, while a near-physiological temperature (35°C) maintained oocyte quality and allowed both meiotic and cytoplasmic developmental competence (TELLADO et al., 2014; WONGSRIKEAO et al., 2005). Additionally, oocyte maturation rates were high after the transportation of equine ovaries at 30-35°C for up to 15 h (49%; DEL CAMPO et al., 1995). Nevertheless, there are no efficient protocols for the transportation of goat ovaries at temperatures close to physiological values.

It is known that the addition of supplements (e.g. ITS, glutamine, hypoxanthine, and BSA) into MEM could decrease ovarian follicle apoptosis after *in vitro* conservation (GONÇALVES et al., 2015) or culture (SILVA et al., 2004) of ovarian tissue. It is believed that a richer medium could maintain follicle viability and ability to grow in goat ovaries stored at near-physiological temperatures for a duration more than 4 h. However, a supplemented MEM is yet to be evaluated for the transportation of goat ovaries. The importance of small ruminants as providers of essential food, meat and dairy products, has been well reported all over the world (SOUZA-FABJAN et al., 2014). In addition, besides acting as an important source of fertilizable oocytes for further *in vitro* embryo production (GUPTA et al., 2007), the use of culture systems for preantral follicles could help in improving our knowledge about the mechanisms involved in early folliculogenesis in mammals (SANTOS et al., 2014).

Therefore, the aim of the present study was to evaluate the effect of different media (non-supplemented or supplemented MEM) and duration (6 or 12 h) of storage at near-physiological temperature (35°C) on the survival and further *in vitro* growth of caprine preantral follicles enclosed in ovarian tissues.

MATERIALS AND METHODS

Ovarian tissue collection

Caprine ovaries (n = 10) were obtained from adult cross-bred goats (n = 5), at a local slaughterhouse. The ovaries were washed in 70% alcohol (Dinâmica) for 10 s and then twice in MEM containing HEPES (5.95 mg/mL) and antibiotics (100 µg/mL penicillin and 100 µg/mL streptomycin). All chemicals used were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless stated otherwise.

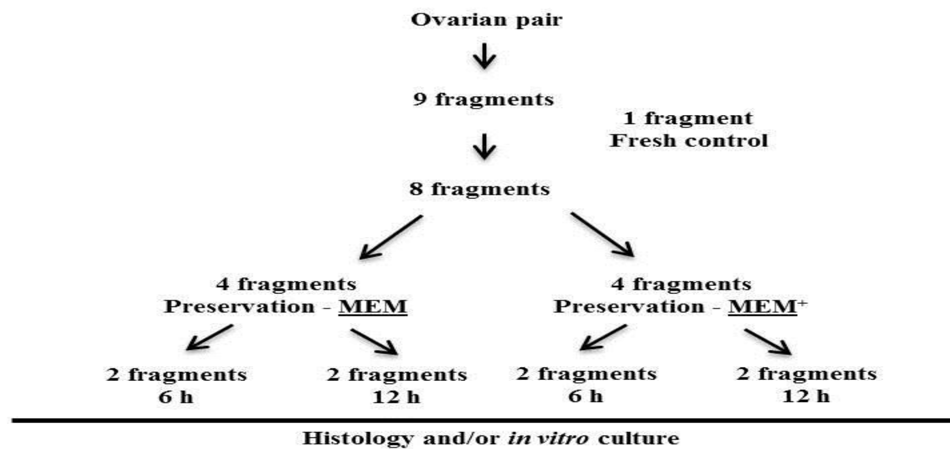
Conservation and culture of ovarian fragments

At the slaughterhouse, each ovarian pair was cut into approximately 9 mm^3. For each animal, one slice of tissue was randomly selected and immediately fixed for histological analysis (fresh control). The remaining slices of the ovarian cortex were placed individually in cryotubes containing 2 mL of conservation medium (MEM without supplementation or supplemented MEM [MEM⁺]- see composition below) and stored (simulating transport) at 35°C for 6 or 12 h, followed or not by a subsequent 5-day *in vitro* culture period (Figure 1). The fragments of ovaries were stored and transported to the laboratory using an oocyte transporter12-Compact® (Wtavet, São Paulo, Brazil). The preservation media were MEM without supplementation (the same used for washing the ovaries, containing 5.95 mg/mL HEPES, 100 µg/mL penicillin and 100 µg/mL streptomycin) or MEM supplemented with ITS (10 µg/mL insulin, 5,5 µg/mL transferrin and 5 ng/mL sodium selenite), 2 mM glutamine, 2 mM hypoxantine, 50 µg/mL ascorbic acid, 1.25 mg/mL BSA, 50 ng/mL recombinant FSH (Nanocore, São Paulo-Brazil), 100 µg/mL penicillin and 100 µg/mL streptomycin. The latter medium was named MEM⁺. The pH of the media was monitored before and after the preservation periods (pH meter - PG2000, Gehaka, São Paulo, Brazil).

Right after the transportation periods, four ovarian fragments were fixed for morphological and TUNEL analyses (non-cultured fragments) and four fragments were cultured *in vitro* (cultured fragments). The fragments were cultured individually for 5 days in 1 mL of medium in 24-well culture dishes at 39°C in an atmosphere of 5% CO_2 in the air. The base culture medium consisted of α-MEM (pH 7.2–7.4; GIBCO, New York, USA) supplemented with ITS (10 µg/mL insulin, 5,5 µg/mL transferrin and 5 ng/mL sodium selenite), 0.23 mM pyruvate, 2 mM glutamine, 2 mM hypoxantine, 1.25 mg/mL BSA, 50 µg/mL ascorbic acid, 50 ng/mL recombinant FSH (Nanocore, São Paulo, Brazil), 100 µg/mL penicillin and 100 µg/mL streptomycin. It is important to emphasize that α-MEM was used instead of MEM, although the supplements (i.e., ITS, pyruvate, glutamine, hypoxantine, BSA, ascorbic acid and FSH) and the concentrations used during the *in vitro* culture were

the same utilized in the preservation medium (MEM+). The culture medium was replenished every two days. Each treatment was repeated five times, thus the ovaries of five different animals were used.

Figure 1. General experimental protocol for the preservation of caprine ovarian tissue.



Classical histology

Non-preserved tissues (fresh control), those preserved for 6 or 12 h without subsequent *in vitro* culture and those preserved for 6 or 12 h followed by a 5-day culture period were fixed in 4% buffered formaldehyde (Dinâmica) for 18 h and used for histological processing. After paraffin embedding (Dinâmica), the ovarian tissue was cut into 5 µm sections, which were mounted on glass slides and stained with Periodic Acid-Schiff (PAS)-hematoxylin (Dinâmica).

The preantral follicles were examined by light microscopy (400 x magnification; Nikon, Tokyo, Japan) for morphological aspects (histologically normal or atretic) and developmental stages (primordial or growing follicles). Only follicles showing an intact oocyte with well organized granulosa cells and without signs of degeneration were classified as normal. Atresia was considered when retracted oocyte, disorganized granulosa cells and/or pyknotic nucleus were observed. The follicles were classified as primordial (oocyte surrounded by one layer of flattened granulosa cells) 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 surrounding the oocyte and no sign of antrum formation) (SILVA et al., 2004). A total of 150 follicles were evaluated in each treatment.

The percentage of primordial and growing follicles was calculated at day 0 (fresh control), after 6 or 12 h of preservation without (preserved and non-cultured group) or with (preserved and cultured group) subsequent *in vitro* culture. By using the Image-Pro Plus® software, the average of the major and minor axes of each oocyte and follicle was used to determine the diameters.

TUNEL

For a more in-depth evaluation of follicular quality in the treatments, the ovarian tissue was subjected to the terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick-end labeling (TUNEL) assay (SANTOS et al., 2014). Briefly, sections (5 µm) were mounted on glass slides and submitted to antigen retrieval with sodium citrate buffer (pH 6.0; Dinâmica) in a microwave for 6 min. Endogenous peroxidase activity was blocked by 3% H_2O_2 (Dinâmica) in methanol (Dinâmica) at room temperature for 10 min. Thereafter, the sections were incubated with a TUNEL reaction mixture at 37ºC for 1 h, followed by incubation with Converter-POD for 30 min. 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) for 1 min. Finally, the sections were counterstained with haematoxylin for 1 min. For negative controls (reaction controls), the slides were incubated with a labeled solution (without terminal deoxynucleotidyl transferase enzyme) instead of the TUNEL reaction mixture.

The DNA fragmentation in preantral follicles was evaluated as described previously (SANTOS et al., 2014). Only follicles containing an oocyte nucleus were analyzed. Brown stained cells (oocyte and granulosa cells) were considered as TUNEL positive cells. The percentage of DNA fragmentation was calculated as the number of TUNEL positive cells out of the total number of cells (x 100).

Statistical analysis

For comparison among treatments, the percentages of histologically normal, primordial and growing follicles were submitted for ANOVA test and Tukey´s test. The values of pH and TUNEL-positive cells were submitted

for Chi-Square test 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 activation were expressed as the mean ± SEM.

RESULTS

In the present study, a total of 1,350 preantral follicles were analyzed. For the ovarian fragments that were preserved for 6 or 12 h without subsequent *in vitro* culture, the percentages of histologically normal follicles after conservation in MEM⁺ for 6 h and the percentages of TUNEL-positive cells after conservation in MEM for 6 h and MEM⁺ for 6 or 12 h were similar ($P > 0.05$) to those observed in the fresh control (Table 1). After 5 days of culture, all treatments significantly reduced the percentage of normal follicles and increased the rates of TUNEL-positive cells as compared to the fresh control and non-cultured treatments. However, no differences were observed among different media and periods of preservation.

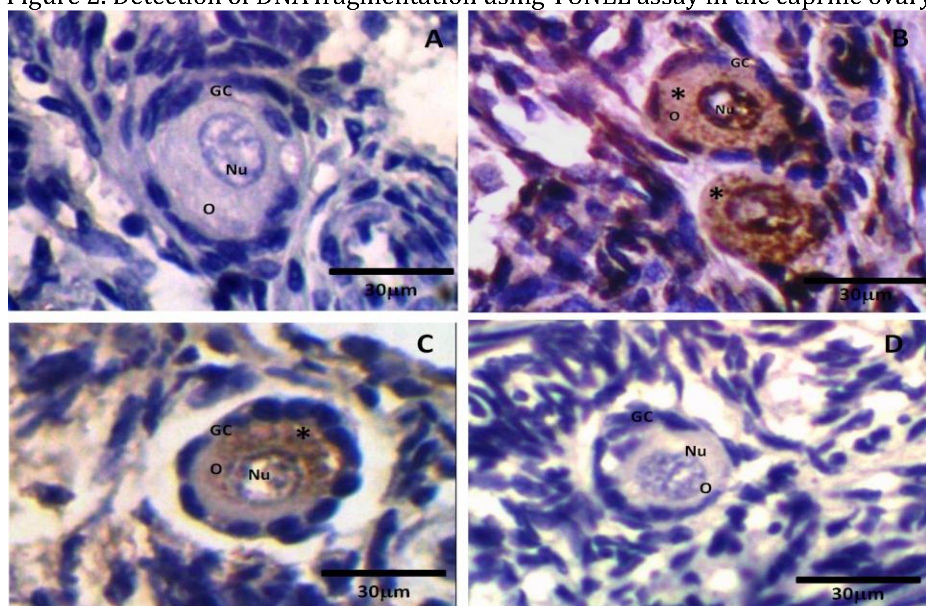
Table 1 – Percentages of morphologically normal goat preantral follicles and TUNEL-positive cells in the fresh control, after preservation for 6 or 12 h in different media without (non-cultured fragments) and with a subsequent 5-day culture period (cultured fragments).

Treatment group	Normal follicles (%)		TUNEL-positive cells (%)	
Fresh control	74.00 ± 10.50		21.08 ± 4.35	
Storage medium/duration	Non-cultured fragments	Cultured fragments	Non-cultured fragments	Cultured fragments
MEM/6 h	46.60 ± 10.25 *a	32.67 ± 13.32 *b	25.50 ± 6.67 b	43.20 ± 4.87 *a
MEM/12 h	46.00 ± 11.75 *a	29.33 ± 10.95 *b	30.89 ± 8.75 *b	53.85 ± 5.88 *a
MEM⁺/6 h	54.00 ± 15.70 a	28.67 ± 11.20 *b	23.00 ± 9.75 b	44.22 ± 3.56 *a
MEM⁺/12 h	51.40 ± 12.98 *a	32.00 ± 14.15 *b	26.92 ± 6.18 b	52.87 ± 4.45 *a

MEM: non-supplemented MEM; MEM⁺: supplemented MEM.
* Denote significant differences from fresh control ($P < 0.05$). ([a, b]) Different letters denote significant differences between non-cultured X cultured fragments in the same preservation medium and transportation period (6 or 12 h), $P < 0.05$.

Figure 2 shows a normal primordial follicle with no sign of DNA fragmentation in the fresh control (Figure 2A), TUNEL-positive cells in follicles preserved for 12 h in MEM (Figure 2B), for 6 h in MEM followed by 5 days of culture (Figure 2C), and the negative control of the TUNEL reaction (Figure 2D). The mean (± SD) pH values in the MEM or MEM⁺ after 6 h of preservation were 7.33 and 7.31, respectively. The storage of ovarian fragments for 12 h did not result in changes ($P > 0.05$) of pH in either solution (7.26 and 7.22 for MEM and MEM⁺, respectively).

Figure 2. Detection of DNA fragmentation using TUNEL assay in the caprine ovary.



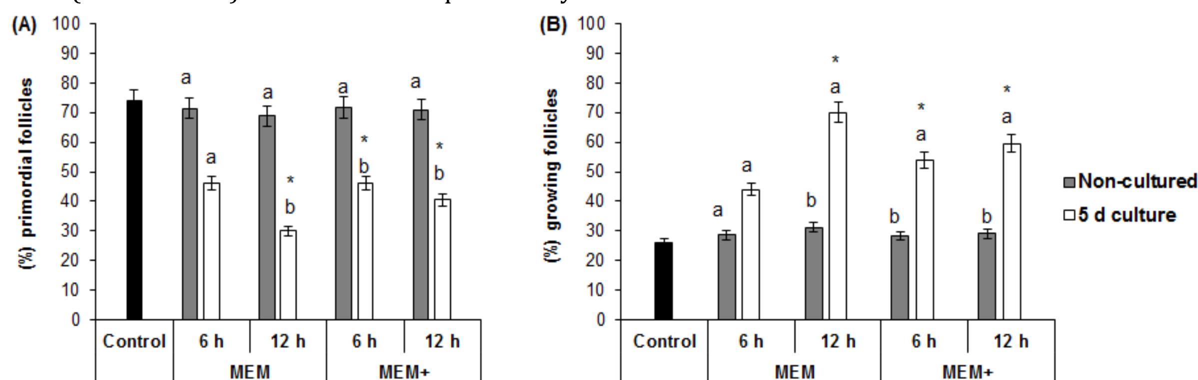
Normal follicle in the fresh control (A); TUNEL-positive follicles preserved for 12 h in MEM (B) or for 6 h in MEM followed by 5 days of culture (C); negative control (D). O = oocyte; Nu = oocyte nucleus; GC = granulosa cell (x 400).

There was no observed change in the percentage of primordial or growing follicles between fresh and preserved tissues (non-cultured) in both media ($P > 0.05$; Figure 3). Nevertheless, all culture conditions

(except after preservation in MEM for 6 h) promoted a significant reduction in the percentage of primordial follicles concomitant with an increase (P < 0.05) in the percentage of growing follicles as compared to the fresh control and non-cultured tissues.

The storage of follicles in both media and preservation periods, followed by the *in vitro* culture, increased (P < 0.05) follicular and oocyte diameters when compared to the fresh control and non-cultured treatments (Table 2).

Figure 3. Percentages (mean ± SEM) of primordial (A) and growing (B) follicles in the fresh control, after storage for 6 or 12 h without (non-cultured) and with a subsequent 5-day *in vitro* culture.



*Differs significantly from the fresh control, P < 0.05. [a, b] Different letters denote significant differences between treatments in the same preservation medium at the same period (non-cultured x cultured fragments), P < 0.05.

Table 2. Oocyte and follicular diameter (mean ± SD) in the fresh control, after preservation for 6 or 12 h in different media without (non-cultured fragments) and with a further *in vitro* culture (cultured fragments).

Treatment group	Oocyte diameter (µm)		Follicle diameter (µm)	
Fresh control	39.8 ± 4.8		51.2 ± 4.2	
Storage medium/duration	Non-cultured fragments	Cultured fragments	Non-cultured fragments	Cultured fragments
MEM/6 h	40.3 ± 3.2[b]	46.9 ± 2.2*[a]	51.6 ± 2.9[b]	59.2 ± 3.5*[a]
MEM/12 h	40.8 ± 3.8[b]	48.2 ± 3.1*[a]	51.3 ± 3.5[b]	61.0 ± 3.9*[a]
MEM+/6 h	39.7 ± 3.3[b]	48.7 ± 4.2*[a]	49.9 ± 2.8[b]	60.8 ± 3.2*[a]
MEM+/12 h	40.7 ± 4.3[b]	48.8 ± 3.9*[a]	51.9 ± 3.8[b]	60.8 ± 4.5*[a]

MEM: non-supplemented MEM; MEM+: supplemented MEM.
* Differs significantly from the fresh control (P < 0.05). [a, b] Different letters denote significant differences between non-cultured X cultured fragments in the same preservation medium and transportation period (6 or 12 h), P < 0.05.

DISCUSSION

An ideal protocol for the storage of ovaries during transportation is of great importance to the preservation of follicular integrity, allowing good quality preantral follicles for further cryopreservation and/or *in vitro* culture (ABD-ALLAH, 2010; CASTRO et al., 2014). In the present study, we evaluated the effect of preserving caprine ovarian cortex into an oocyte transporter, using a temperature of 35°C (close to the physiological temperature of goat) as well as different storage media and periods.

In the current study, the preservation (without subsequent *in vitro* culture) of the ovarian cortex in supplemented MEM+ for 6 h at 35°C, was the only treatment that resulted in similar percentages of normal follicles (follicular survival), as those found in the fresh control. On the contrary, some studies have demonstrated that the storage of caprine ovarian fragments at physiological temperatures (39°C) for 4 or 12 h, increased the rates of follicular atresia in about 60-100% (Coconut water and Braun-Collins solutions: SILVA et al., 2000; Saline solution: COSTA et al., 2005). It is known that the removal of ovaries from the animal result in the occlusion of blood flow and reduction of the oxygen and energy supply to the organ, thereby culminating in tissue hypoxia and necrosis (MEIROW; WALLACE, 2009; WONGSRIKEAO et al., 2005). Ovarian tissue necrosis could be potentialized by the long period of transport (12 h), which is associated with some of the morphological features observed in the preserved follicles (e.g. cell swelling) and consequently, the low percentage of normal follicles. In the present study, the maintenance of pH values and the use of the oocyte transporter, which kept the temperature constant (35°C) throughout the conservation period, may have contributed to the satisfactory rates of normal follicles after ovary transport in MEM+ for 6 h.

In addition, better results were obtained in the present study after storage at near-physiological temperature for

6 h (up to 54% of normal follicles). This may be explained by the preservation medium (MEM) used, which is richer in nutrients (e.g. amino acids, glucose and vitamins) than those used previously for storage of caprine tissues. Moreover, the supplementation of the medium (MEM$^+$) with antioxidants (ascorbic acid, transferrin, sodium selenite), energy substrates (glutamine, BSA) and hormones (FSH, insulin) might help in the maintenance of follicular survival after *in vitro* preservation. Among the antioxidants, transferrin and ascorbic acid prevent the formation of hydroxyl radical by preventing their participation in reactive oxygen species reactions. Selenium can participate in the decomposition of peroxides by converting them to nontoxic products (ZIMMERMAN et al., 2015; SURRAI; FISININ; KARADAS, 2016), and it also suppresses apoptosis in the granulosa cells of follicles cultured *in vitro* (TILLY; TILLY, 1995). Additionally, glutamine is an effective energy substrate for oocytes (SONGSASEN; SPINDLER; WILDT, 2007), while BSA improves caprine follicular development and provides meiotically competent oocytes after the *in vitro* culture of isolated preantral follicles (RODRIGUES et al., 2010). Regarding the hormones, recombinant FSH maintained the ultrastructural integrity of caprine preantral follicles, promoted primordial follicle activation and further growth of cultured follicles (MAGALHÃES et al., 2009). Finally, insulin acts synergistically with FSH to promote granulosa cell differentiation and proliferation. It also facilitates FSH-dependent steroid production and LH receptor induction in cultured granulosa cells (DULEBA et al., 1997; MAY et al., 1980). This combination of hormones also stimulated follicular development and maintained follicular survival, as well as increased estradiol secretion and promoted oocyte meiosis resumption in goat preantral follicles (CHAVES et al., 2012).

After the preservation of ovarian tissue, *in vitro* culture and TUNEL can be used as reliable methods for the evaluation of follicular viability and DNA fragmentation, respectively (ABD-ALLAH, 2010; SREEJALEKSHMI et al., 2011). In the current study, the percentage of histologically normal follicles reduced when ovaries were stored in both media and preservation periods and then cultured for 5 days. Other authors have also shown a decrease in the normal caprine follicles after the preservation of ovaries at 32ºC, followed by culturing for 5 days (MARTINS et al., 2005; SILVA et al., 2004). The DNA fragmentation rates also increased in all cultured treatments when compared to the fresh control and preserved treatments (non-cultured). The presence of other components in our culture medium (other hormones and/or growth factors) could have been another important factor in the maintenance of follicular viability. For example, the addition of Epidermal Growth Factor (EGF; CELESTINO et al., 2009) or Bone Morphogenetic Protein-15 (BMP-15; CELESTINO et al., 2011) to the medium maintained follicular survival similar to the fresh control after culturing caprine ovarian tissue for 7 days.

In the present study, cultured tissues showed greater follicle and oocyte diameters than the fresh control or only preserved tissues. Similarly, follicular activation increased in all cultured tissues, except for tissues preserved in MEM for 6 h. Regardless of the medium used, these results showed that the storage of ovarian tissue at near-physiological temperature did not affect caprine primordial follicle activation and further growth *in vitro*. However, it is possible to suggest that in the tested conditions, a richer and supplemented preservation medium (MEM$^+$) could accelerate the initiation of primordial follicle growth *in vitro* (6 h for MEM$^+$ versus 12 h for non supplemented MEM). This finding is important since some authors have shown that body temperature is more desirable for the transportation of goat viable cumulus-oocyte complexes and fresh embryos to the laboratory (WAN et al., 2009; QUAN et al., 2010).

CONCLUSION

The use of an enriched medium (MEM$^+$) during caprine ovarian tissue transportation at 35°C for 6 h is preferable to maintain the rates of normal follicles and DNA fragmentation similar to the fresh control. Moreover, these conditions did not affect the ability of the primordial follicle to become activated and grow *in vitro*. The preservation of ovaries during transportation in these conditions can be very useful in situations where laboratories are distant from the farms, allowing the recovery of good oocyte enclosed in preantral follicles, thereby ensuring the successful *in vitro* production of embryos in the future.

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