Effect of Braun-Collins and Saline solutions at different temperatures and incubation times on the quality of goat preantral follicles preserved in situ

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Abstract

The present work has investigated the efficiency of Braun-Collins and Saline (0.9%) solutions in the conservation of goat preantral follicles in situ, at different temperatures and incubation times. For each animal the ovarian pair was divided into 19 fragments. One ovarian fragment was taken randomly and immediately fixed (control). The other 18 ovarian fragments were randomly distributed in tubes containing Braun-Collins or Saline (0.9%) solutions at 4, 20 or 39°C for 4, 12 or 24 h. A total of 3385, 372 and 191 primordial, primary and secondary follicles were examined, respectively. The quality of preantral follicles was evaluated by histology and transmission electron microscopy. The storage of ovarian fragments in Saline (0.9%) or Braun-Collins solutions at 4°C did not reduce significantly the percentage of morphologically normal follicles when compared with the control. The histological analysis revealed a morphological integrity of goat preantral follicles stored at 4°C for up to 24 h in both solutions, but these results were not confirmed by ultrastructural analysis. The transmission electron microscopy revealed that only preantral follicles stored at 4°C for a maximum of 12 h in both solutions were ultrastructurally normal. In conclusion, this study shows for the first time that goat preantral follicles can be stored in situ successfully at 4°C in Saline (0.9%) or Braun-Collins solution for up to 12 h.

Keywords: Preantral follicle; Goat; Conservation; Histology; Ultrastructure

1. Introduction

The use of oocytes from preantral follicles in reproductive techniques may offer significant new ways for the propagation of valuable animal stocks. A specific mechanical method for the isolation of a great number of intact preantral follicles per ovary was recently developed in the goat (Lucci et al., 1999a). The development of systems which support oocyte growth in vitro is valuable for the conservation of the female genetic pool. Alternatively, the genetic potential of preantral oocytes could also be conserved by freezing for later transplantation of the preantral follicles into ovaries of another animal (Jewgenow and Stolte, 1996).

The success of cryopreservation and/or culture of preantral follicles depends on the quality of the oocytes enclosed in these follicles. However, the ovarian donor of preantral follicles for in vitro studies is commonly encountered far away from the reproduction

laboratories. In this respect, the preservation of ovaries during transportation to such laboratories becomes very important. However, there are currently no studies regarding the preservation of caprine preantral follicles in situ. Saline solution (0.9%) is commonly used as a conservation media for ovaries during short periods of time (Solano et al., 1994, Schernthaner et al., 1997 and Azambuja et al., 1998), although this solution has not been tested as a medium for preantral follicle storage for longer periods. Braun-Collins solution is another medium that has been successfully used in the preservation of different organs, such as lungs (Fukuse et al., 1996), liver (Adam et al., 1996), kidney (Savioz et al., 1996) and heart (Demmy et al., 1997). However, the effect of this latter solution on the conservation of preantral follicles in situ is unknown.

The aims of the present study were to evaluate the effect of Braun-Collins solution and Saline solution (0.9%) on the conservation of goat preantral follicles in situ, at different temperatures and times of conservation, and to investigate the quality of the conserved/stored preantral follicles in situ by histological and ultrastructural analysis.

2. Materials and methods

2.1. Ovaries

Ovaries (n=10) from five adult (1–3 years) mixed breed goats were obtained at a local slaughterhouse. The ovaries were washed in 70% alcohol for approximately 10 s, then twice in Saline solution and processed as described below.

2.2. Experimental protocol

For each animal, the ovarian pair was divided into to 19 fragments, so that these 19 fragments constituted the complete ovarian cortex of the two ovaries. Then, one ovarian fragment was taken randomly and immediately fixed for histology (control — treatment 1 — time 0). The other 18 ovarian fragments were randomly distributed in tubes containing 2 ml of Braun-Collins solution (Braun-Collins — G 3.57 s/Mg, Laboratórios B. Braun S.A., Rio de Janerio, Brazil) or Saline solution (0.9%) at 4, 20 or 39°C for 4, 12 or 24 h (treatments 2–19) as shown in Fig. 1. The temperatures were maintained using thermoflasks filled with water at 4, 20 and 39°C. For each treatment, variables such as temperature, osmolarity and pH of the solutions

were controlled at the beginning and at the end of the treatments. Each treatment was repeated eight times.

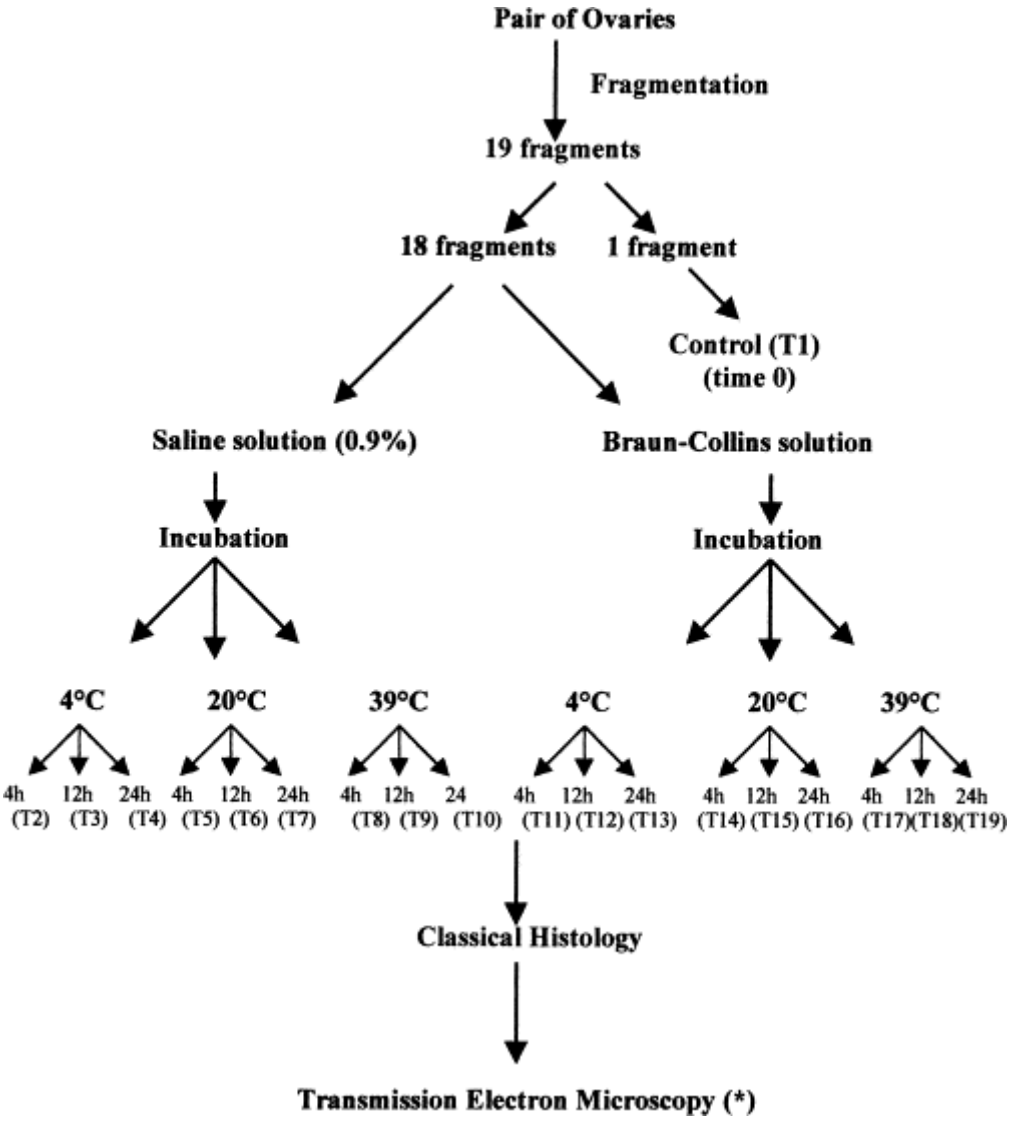

Fig. 1. General experimental protocol for conservation of caprine preantral follicles in situ. Asterisk (∗): only in the best treatments.

2.3. Qualitative analysis of caprine preantral follicles in situ

In order to evaluate the quality of the caprine preantral follicles, at the end of the treatments the ovarian fragments were processed as follows. The ovarian fragments from each treatment, including the control, were fixed individually in Bouin's solution for 12 h. Subsequently, the ovarian fragments were dehydrated in a graded series of ethanol, clarified with xilol and embedded in paraffin wax. The tissue was sectioned serially at a thickness of 7 μm, and the sections were stained with hematoxilyn and eosin and observed in a Zeiss Axiophot light microscopy. In the histological sections, preantral follicles were classified according to the stage of development as primordial (one layer of flattened or flattened-

cuboidal granulosa cells around the oocyte), primary (a single layer of cuboidal granulosa cells around the oocyte), or secondary (oocyte surrounded by more than one complete layer of cuboidal granulosa cells — Hulshof et al., 1994). Primordial, primary and secondary follicles were counted and evaluated in the section where the nucleus of the oocyte was visible. Criteria for follicular cell and oocyte degeneration were identified using ovaries fixed at time 0. Follicular quality was evaluated based on the integrity of the basement membrane, cellular density, presence or absence of pycnotic bodies and integrity of the oocyte. Based on these variables, preantral follicles were classified as morphologically normal, degenerated Type 1 follicles (only the oocyte was degenerated) and degenerated Type 2 follicles (when degeneration occurred at both levels, i.e. oocyte and granulosa cells). These three classifications were assigned on a basis of atresia observed in the control and/or combined with changes that occurred as a result of storage.

To better evaluate follicular quality, ultrastructural analysis was performed using preantral follicles from the control treatment as well as from the treatment that did not differ from controls. It is important to note that only preantral follicles classified as morphologically normal in semi-thin sections were evaluated. Briefly, small pieces of ovarian cortex were fixed in a solution containing 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2. After fixation, the specimens were rinsed in buffer and postfixed in 1% osmiun tetroxide, 0.8% potassium ferricyanide and 5 mM $CaCl_2$ in 0.1 M sodium cacodylate buffer. Subsequently the samples were dehydrated in acetone and embedded in Spurr. Semi-thin sections (3 μm) were stained with toluidine blue. Thin sections (70 nm) were contrasted with uranyl acetate and lead citrate, and examined using a Jeol 100 C and a Zeiss 912 transmission electron microscopes.

2.4. Statistical analysis

The effect of conservation solution (Braun-Collins solution or Saline solution), temperatures and incubation times on the percentage of morphologically normal follicles were analyzed by Chi-square-test using data from five replicates. Data from the other three replicates were used to compare the percentages of normal primordial, primary and secondary follicles in each treatment using a Chi-square-test. The values of osmolarity and pH were compared using a Fisher's PLSD-test (Stat View for Macintosh). Values were considered to be significantly different when $P<0.05$.

3. Results

3.1. Morphological aspects of goat preantral follicles in the control and after storage in situ

In the different treatments tested, including the control, morphologically normal preantral follicles contained an intact oocyte and well-organized granulosa cells without a pycnotic nucleus (Fig. 2a). Degenerated Type 1 follicles contained an oocyte that was sometimes retracted, with a pycnotic nucleus and strongly eosinophilic cytoplasm (Fig. 2b). In degenerated Type 2 follicles, degeneration was found in both the oocyte and granulosa cells. With this kind of degeneration the follicles, besides showing pycnosis at the oocyte level also showed disorganized granulosa cells and low cellular density (Fig. 2c). It is important to note that no pycnotic bodies in granulosa cells in follicles were observed from control or treatments stored in situ. The distribution of degenerated Type 1 and Type 2 follicles to primordial, primary and secondary follicles was 41.76 and 58.24%, 29.95 and 70.05% and 2.43 and 97.57%, respectively.

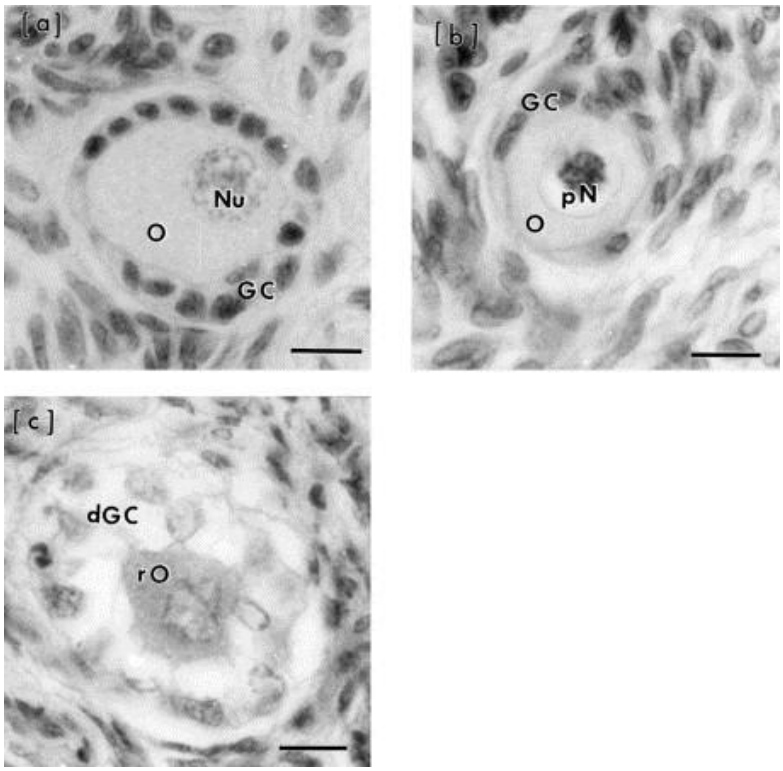

Fig. 2. Histological sections of a morphologically normal follicle (a), a degenerated Type 1 follicle (b) and a degenerated Type 2 follicle (c) after storage in situ. O: normal oocyte; Nu: nucleus of normal oocyte;

CG: granulosa cells; rO: retraced oocyte; pN: pycnotic nucleus; dCG: disorganized granulosa cells. Bars=10 µm.

3.2. Storage of goat preantral follicles in situ in Braun-Collins and Saline solutions

Fig. 3 depicts the effect of temperature and time of storage on the percentage of morphologically normal preantral follicles stored in Braun-Collins solution and in Saline solution evaluated by histology, independently of follicular classes. It was observed that storage of preantral follicles in Braun-Collins solution at 20 and 39°C, at all incubation times tested, decreased ($P<0.05$) the follicular viability when compared with controls. In contrast, the viability of stored follicles in this solution at 4°C for up to 24 h was not significantly affected in relation to control. Similar results were obtained with the use of Saline solution (0.9%), except for the treatment in which the ovarian fragment was stored at 20°C for 4 h; this treatment produced percentages of morphologically normal follicles similar to that obtained in the control.

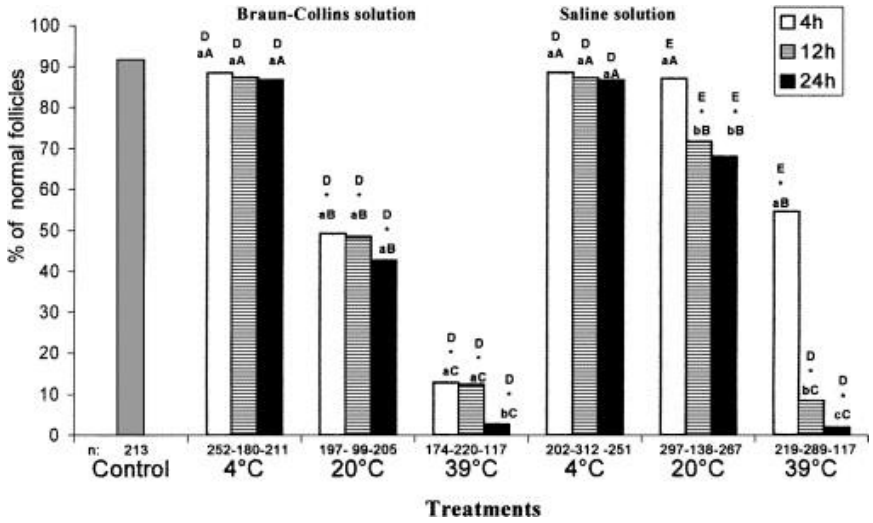

Fig. 3. Effect of temperature and storage time on the percentage of morphologically normal preantral follicles preserved in Braun-Collins solution and in Saline solution. Variable n: number of preantral follicles analyzed per treatment; asterisk (∗): differs significantly from control; a, b, c: different letters at the same conservation temperature show significant difference ($P<0.05$); A, B, C: different letters at the same conservation time show significant difference ($P<0.05$); D, E: different letters show significant difference between solutions for the same temperature and incubation time ($P<0.05$).

There was no effect of incubation time on the percentage of morphologically normal follicles on the conservation of follicles in situ at 4°C, in both solutions. Similar results were obtained with the fragments conserved in Braun-Collins solution at 20°C. However, in the fragments stored at 39°C in Braun-Collins solution there was a decrease ($P<0.05$) of the

percentage of normal follicles when stored for 24 h. In relation to the Saline solution at 20°C, there was a decrease ($P<0.05$) in the percentage of morphologically normal follicles after 12 h, however, at 39°C there was a decrease ($P<0.05$) of these percentages with the increase of incubation time.

With regard to the effect of temperature at the same incubation time, the results showed that for the Braun-Collins solution there was a effect ($P<0.05$) of temperature on the percentage of morphologically normal follicles at all times tested, with a progressive reduction of the percentage of morphologically normal follicles with the increase of temperature from 4 to 39°C being observed. Similar results were obtained for the Saline solution except for the storage time of 4 h, at which the temperature of 20°C did not decrease follicular viability when compared with storage at 4°C.

Comparisons were made between Braun-Collins solution and Saline solution at the same temperature and incubation time. Although, at 4°C no significant difference in the percentage of morphologically normal follicles between these solutions was detected, a greater ($P<0.05$) percentage of morphologically normal follicles was observed in Saline solution for all incubation times tested at 20°C, and at 39°C for 4 h.

In order to evaluate the sensitivity of follicular classes to the preservation conditions, a total of 711, 793 and 569 primordial, primary and secondary follicles were examined, respectively. The results showed that after preservation in saline solution at 39°C for 4 h, a greater percentage of normal primordial follicles was observed when compared with primary and secondary follicles ($P<0.05$). After preservation in Braun-Collins solution at 20°C for 24 h and at 39°C for 12 h, a greater percentage of normal primordial and primary follicles was observed when compared with secondary follicles ($P<0.05$), except after preservation at 20°C for 24 h which showed a similar percentage of normal primary and secondary follicles. Similar percentages of normal primordial, primary and secondary follicles were observed in the other treatments (Table 1).

Table 1
Percentage of morphologically normal primordial, primary and secondary follicles stored in Saline solution (SS)and Braun-Collins solution (BCS) in different temperatures and incubation timesa

Treatments	Primordial follicles (%)	Primary follicles (%)	Secondary follicles (%)
Control	94.5 a (34/36)	87.5 a (28/32)	91.7 a (33/39)
SS 4°C, 4 h	92.9 a (26/28)	86.9 a (20/23)	82.6 a (19/23)
SS 4°C, 12 h	89.2 a (33/37)	73.7 a (28/38)	90.6 a (29/32)
SS 4°C, 24 h	91.3 a (21/23)	80 a (28/35)	76.2 a (16/21)
SS 20°C, 4 h	89.7 a (26/29)	85 a (17/20)	85 a (17/20)
SS 20°C, 12 h	73.5 a (25/34)	63.2 a (24/38)	64.1 a (25/39)
SS 20°C, 24 h	75.5 a (37/49)	58.3 a (28/48)	67.5 a (29/43)
SS 39°C, 4 h	55.3 a (26/47)	30.8 b (20/65)	24.3 b (8/33)
SS 39°C, 12 h	9.1 a (5/55)	16.4 a (12/73)	13.9 a (5/36)
SS 39°C, 24 h	0 a (0/41)	3.1 a (2/65)	6.7 a (2/30)
BCS 4°C, 4 h	83.3 a (30/36)	83.0 a (39/47)	82.8 a (24/29)
BCS 4°C, 12 h	86.7 a (39/45)	90.9 a (50/55)	88.0 a (22/25)
BCS 4°C, 24 h	86.2 a (25/29)	86.4 a (19/22)	73.9 a (17/23)
BCS 20°C, 4 h	50 a (18/36)	60.7 a (17/28)	65.2 a (15/23)
BCS 20°C, 12 h	55.5 a (15/27)	55.0 a (11/20)	65.2 a (15/23)
BCS 20°C, 24 h	46.0 a (17/37)	34.6 ab (18/52)	16.7 b (4/24)
BCS 39°C, 4 h	16.7 a (5/30)	35.3 a (12/34)	13.3 a (4/30)
BCS 39°C, 12 h	13.7 a (7/51)	26.4 a (14/53)	0 b (0/48)
BCS 39°C, 24 h	2.4 a (1/41)	6.7 a (3/45)	6.5 a (2/31)

3.3. Distribution of follicular degeneration types in the control and other treatments

Fig. 4 depicts the distribution of degenerated Type 1 and 2 preantral follicles, in the control and after storage in the different treatments, in Braun-Collins solution (Fig. 4a) and in Saline solution (Fig. 4b). There was a predominance ($P<0.05$) of Type 1 degeneration in the fragments stored in both solutions at 4 or 20°C, at all incubation times tested. In contrast, at 39°C a predominance ($P<0.05$) of degenerated Type 2 follicles was observed, except in the fragments stored in saline solution for 4 h. Compared with the controls, a greater ($P<0.05$) percentage of degenerated Type 1 follicles was observed at 20°C in Braun-Collins solution at all times of incubation, and in Saline solution for 12 and 24 h. A greater ($P<0.05$) percentage of degenerated Type 2 follicles compared with controls was observed in follicles kept in Braun-Collins solution at 20 and 39°C at all incubation times, and in saline solution at 20°C, for 12 and 24 h, and 39°C at all incubation times.

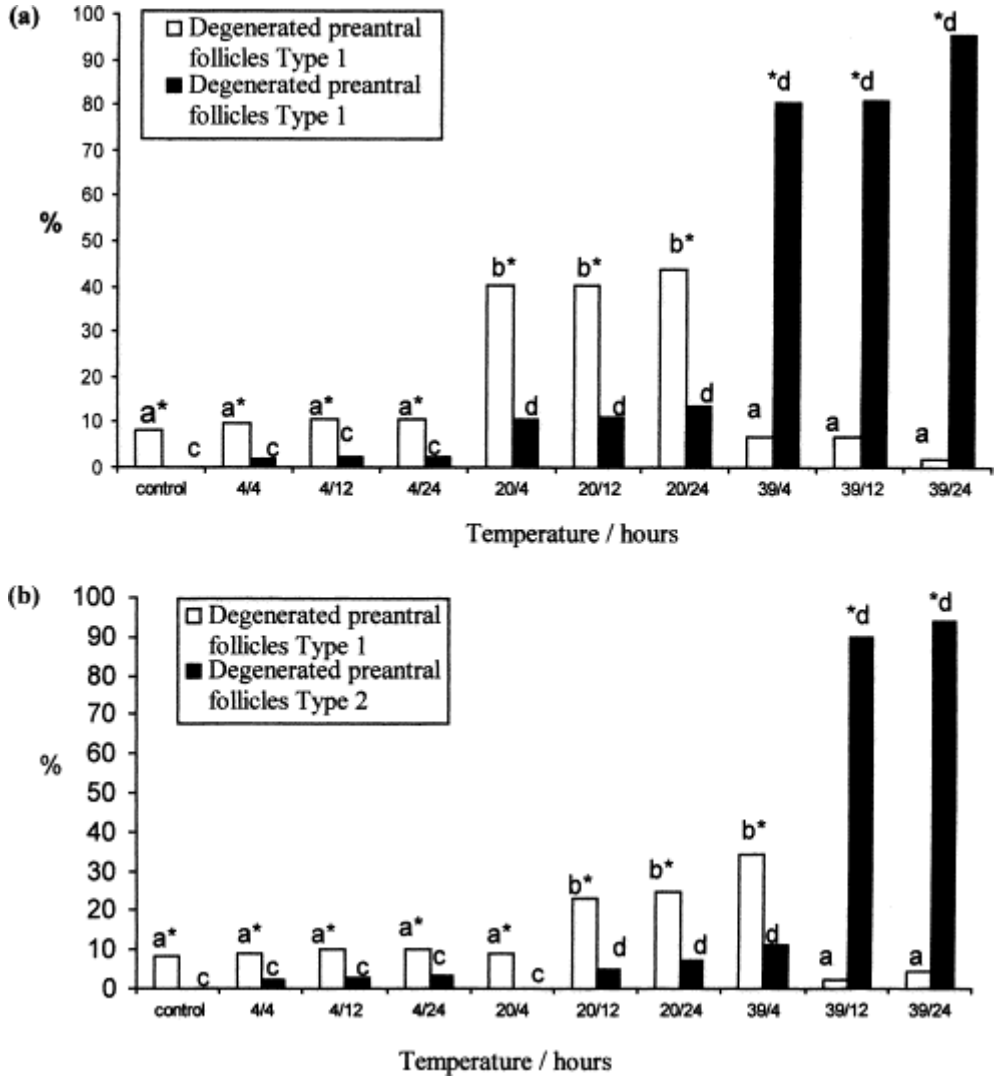

Fig. 4. Percentage distribution of the degenerated preantral follicles Type 1 and 2, from control and after conservation in different treatments, in Braun-Collins solution (a) and saline solution (b), respectively. Asterisk (∗): shows difference on degeneration types within each treatment (P<0.05); a, b: different letters show significant difference between the percentage of degenerated follicles; Type 1 found on different treatments and in control (P<00.05); c, d: different letters show significant difference between the percentage of degenerated follicle; Type 2 found on different treatments and in control (P<0.05).

3.4. Values of pH and osmolarity of Braun-Collins solution and Saline solution with conservation time

The mean values of pH and osmolarity in the fresh media (before the addition of the fragment), respective to Braun-Collins solution and Saline solution were 7.0±0.12 and 367±4.24 mOsm/l and 6.8±0.21 and 280±5.53 mOsm/l. The storage of ovarian fragments in situ at 4 or 20°C did not result in significant changes of pH and osmolarity of both solutions (Fig. 5a and b). On the contrary, there was a decrease (P<0.05) of pH and increase of osmolarity compared with the fresh media at 39°C in both solutions (Fig. 5a and b).

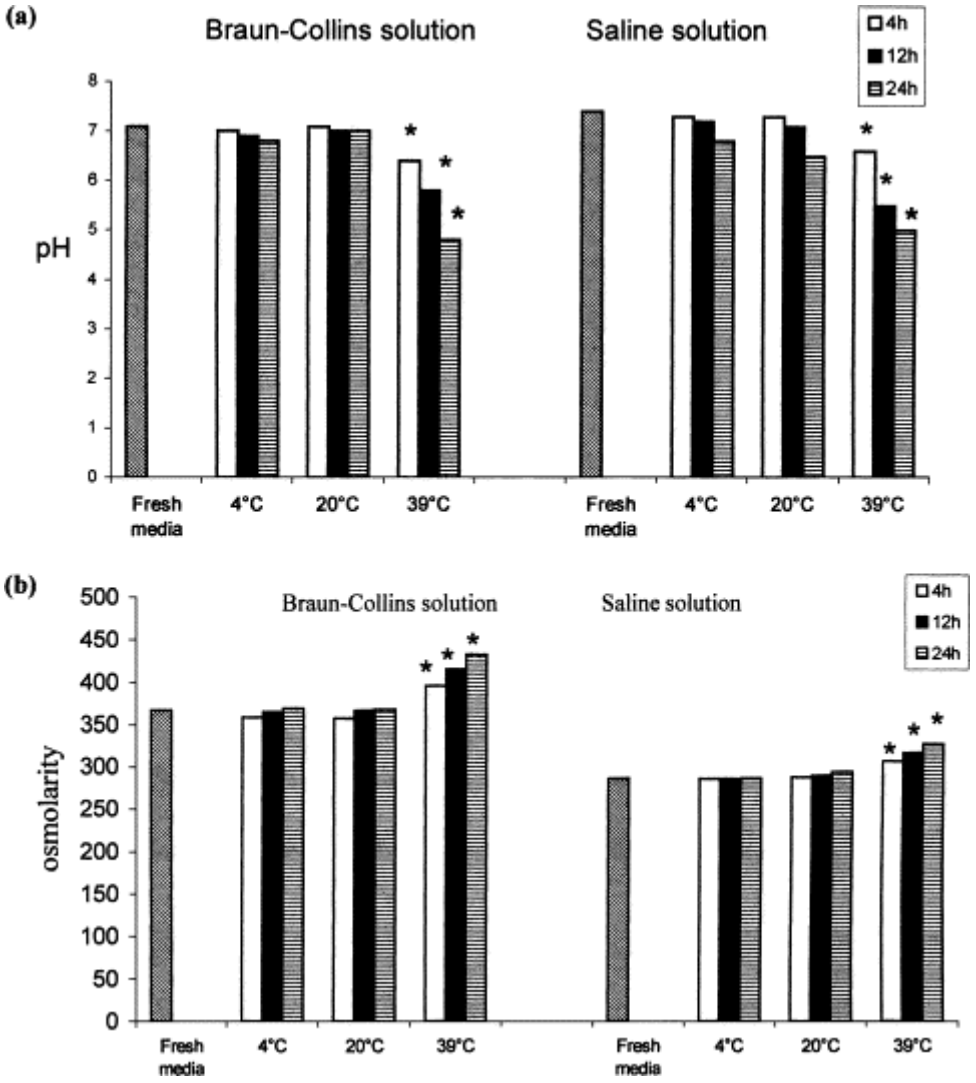

Fig. 5. pH (a) and osmolarity (b) of Braun-Collins solution and Saline solution along conservation times. Asterisk (∗) shows significant difference in relation to the flesh media ($P<0.05$).

3.5. Ultrastructural analysis of caprine preantral follicles preserved in situ

To better evaluate follicular quality, ultrastructural analysis was performed using morphologically normal preantral follicles (classified in semi-thin sections) from the control treatment as well as from the treatment in which the ovarian fragments were maintained in both solutions at 4°C (the longer preservation time). For both solutions, at 4°C for 24 h, follicles considered morphologically normal, after staining with toluidine blue, showed a high number of vacuoles on the oocyte ooplasm at the ultrastrutural level (Fig. 6a), suggesting an initial process of oocyte degeneration. A low cytoplasm density was also observed in the granulosa cells (Fig. 6a), indicating a morpho-functional compromise of the preantral follicles. Moreover, the nucleus of the oocyte was of an irregular shape (not shown), in contrast to controls, which had a round nuclear (Fig. 6b). However, ultrastructural analysis of follicles cold-stored in both

solutions at 4°C for 12 h confirmed the integrity of the oocyte, the granulosa cells and the basement membrane (Fig. 6c and d).

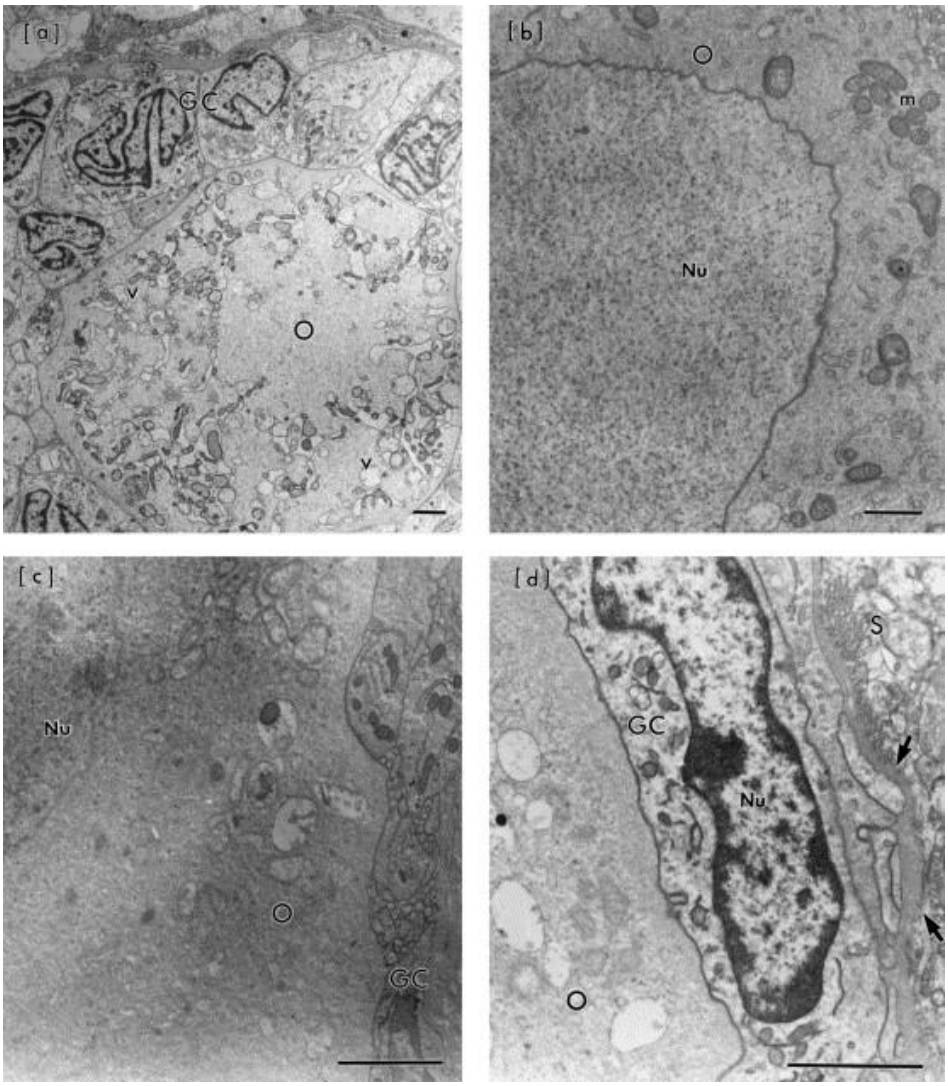

Fig. 6. Electron micrographs of an initial degeneration of an apparently normal follicles stored at 4°C for 24 h in Saline solution (a), showing a vacuolated oocyte cytoplasm and granulosa cells with low cytoplasm density, and morphologically normal follicles from control (time 0) (b) and stored at 4°C for 12 h in Braun-Collins solution (c) and Saline solution (d). O: oocyte; GC: granulosa cell.; Nu: nucleus; v: vacuoles; m: mitochondria, S: ovarian stroma; basement membrane (arrows). Bars=2 µm.

4. Discussion

The present study shows for the first time that goat preantral follicles can be stored successfully in situ for a long time in Saline solution or Braun-Collins solution at low temperature.

Qualitative analyses of goat preantral follicles in situ (Bezerra et al., 1998 and Lucci et al., 1999b) or after isolation (Lucci et al., 1999a) were performed using classical histology and transmission electron microscopy. However, these analyses were performed using only fresh preantral follicles. This is the first study to quantify the levels of follicular degeneration in situ

after storage of goat ovarian fragments at different temperatures and for different duration of time. The histological analysis of preantral follicles present in the controls as well as in fragments stored at 4 or 20°C showed that changes at the oocyte level constitute the first observed degenerative sign. Similar results were also observed with fresh ovine (Jorio et al., 1991) and caprine (Lucci et al., 1999b) preantral follicles, cold stored at 4°C feline preantral follicles (Wood et al., 1997) and cultured bovine preantral follicles in vitro (Figueiredo et al., 1994 and Braw-Tal and Yossefi, 1997). These results show that the oocyte is much more sensitive to degeneration than granulosa cells. In contrast, in the treatments where the ovarian fragments were stored at 39°C, the degeneration of the granulosa cells was frequently observed. The granulosa cells of follicles within these treatments ware disorganized with low cellular density, probably because they were enlarged in volume. It is important to note that in this study pycnotic bodies were not observed in granulosa cells. This result may be due to the short time of incubation to which the ovarian fragments were submitted. Moreover, according to Ingram (1962) the atresia is a degenerative process of unknown duration. Cahill et al. (1979) and Jorio et al. (1991) have described that while the pycnosis of granulosa cells occurs in antral follicles, it is almost absent in preantral follicles.

The storage of ovarian fragments in saline solution or Braun-Collins solution at 4°C did not significantly reduce the percentage of morphologically normal follicles when compared with controls. The desirable results obtained after storage of the preantral follicles at 4°C may be due to the fact that this temperature provided lower rates of cellular metabolism, consequently minimizing the metabolic need and, thus, increasing the resistance of follicles to the absence of nutrients and oxygen. Studies involving goat preantral follicle storage in vitro are not currently available. However, the conservation of cumulus oocyte complexes in situ or in vitro after puncture has been described. The temperature of 4°C has been successfully used in the preservation of the domestic cat ovaries for 48 h (Wood et al., 1997) and cow ovaries for up to 24 h from assessment of the blastocyst production in vitro (Solano et al., 1994). In contrast, Yang et al. (1990) have reported that the storage of cattle ovaries at 4°C for 24 h resulted in a very low cleavage rate and percentage of blastocysts. This may be due to the specific culture systems used by these authors.

This study shows that increasing temperatures from 4–20 and to 39°C progressively reduces the percentage of morphologically normal preantral follicles during the conservation times. The increase of conservation temperature and consequently the increase of cellular metabolism and oxygen consumption could result in the grater rate of follicular degeneration found with the treatments where the ovarian fragments were stored at 20 and 39°C. It is important to note that ovarian fragments were stored in closed tubes, i.e. under anaerobic

conditions, besides that both solutions were deficient in nutrients. Jennings et al. (1975) suggested that changes in the cellular membrane permeability induced by lack of oxygen, caused changes at a level of intracellular Na+, K+ and Cl2−, that were associated with changes in the distribution of Ca2+ and increased intracellular water, which may lead to increased cellular volume and consequently cellular degeneration. Boland et al. (1994) in a study in vitro demonstrated that preantral follicles cultured in the absence of oxygen grew at a significantly slower rate over the first 2 days of the culture and exhibited an abnormal morphology which was characterized by a shrunken oocyte and appearance of dark degenerative patches in the granulosa cells. It is important to note that in the treatment where the ovarian fragments were stored at 39°C the follicular degeneration was followed by pH and osmolarity alterations, that could mean cellular necrosis. Necrotic cells can exhibit rupture of cellular membranes, which release acidic contents within the dead cells (Farber, 1982), provoking changes in pH and osmolarity of used solutions.

In spite of the fact that the Braun-Collins solution exhibited a comparable efficiency for the storage of goat preantral follicles at 4°C, at 20°C (all incubation times) and at 39°C for 4 h, the percentage of morphologically normal follicles was significantly greater in the Saline solution. Braun-Collins solution has been successfully used in preservation at 4°C of lung (Fukuse et al., 1996), liver (Adam et al., 1996), kidneys (Savioz et al., 1996) and heart (Demmy et al., 1997) tissues. At greater temperatures, our results were similar to those obtained by Savioz et al. (1996) with kidney preservation at 14°C. These authors showed the low efficiency of Braun-Collins solutions when its use was not associated with the hypothermic principle. The Braun-Collins solution has as its principle hypothermic preservation in hyperosmotic solution, followed by a low cellular dehydration, avoiding consequent cell swelling. However, the increase of the temperature from 4 to 20 and 39°C results in an increase of cellular metabolism that, associated with a low oxygenation and cellular dehydration, could have caused high rates of follicular degeneration found in the treatments where the fragments were stored at 20 and 39°C.

In general, primordial, primary and secondary follicles can be equally preserved in vitro, except in saline solution at 39°C for 4 h and in Braun-Collins solution at 20°C for 24 and at 39°C for 12 h. Under these conditions a lower percentage of normal secondary follicles was observed when compared with primordial follicles, however, the underlying cause remains undefined.

The histological analysis of follicles in both solutions revealed a morphological integrity of goat preantral follicles stored at 4°C for 24 h, but these results were not confirmed by ultrastructural analysis. However, the transmission electron microscopy analysis of the

preantral follicles stored for 12 h revealed the ultrastructural integrity of these follicles. Hay et al. (1976) studied structural changes occurring during atresia in sheep ovarian follicles and emphasized that most follicles described as non-atretic after staining with toluidine blue contained degenerated cells, characterized by the presence of a pycnotic nucleus and vacuolated cytoplasm. These vacuoles represent endoplasmic reticulum swelling. The presence of vacuoles was also observed in equine oocytes matured in vitro (Willis et al., 1994) and bovine oocytes after exposure to vitrification solution (Fuku et al., 1995).

In conclusion, the present study shows for the first time that goat preantral follicles can be stored in situ successfully at 4°C in Saline solution or Braun-Collins solution for up to 12 h. The conservation of ovaries during transportation in these conditions might be very useful in the future to optimize the use of oocytes enclosed in preantral follicles.

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