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Preservation of goat preantral follicles in saline or coconut water solution

Conservação de folículos pré-antrais caprinos em solução salina ou à base de água de coco

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SUMMARY

The present study investigated the efficiency of saline solution and coconut water solution in the preservation of goat preantral follicles enclosed in ovarian tissue, at different temperatures and for different incubation periods. At the slaughterhouse, the ovarian pair was divided into 19 fragments; one ovarian fragment was immediately fixed for histology (control-time zero). The other 18 ovarian fragments were preserved in both solutions at 4ºC, 20ºC or 39ºC for 4 h, 12 h or 24 h. The histological analysis showed that the storage of ovarian fragments in both solutions at 4ºC for up to 24 h kept the percentage of normal preantral follicles similar to the control values. In contrast, preservation at 20°C or 39ºC, in either solution, reduced significantly the percentage of normal preantral follicles compared to the control values, except in saline solution at 20ºC for 4 h or in coconut water solution at 20ºC for 4 h and 12 h. In conclusion, this study shows that both solutions can be used with the same efficiency to preserve goat preantral follicles at 4°C, irrespective of the incubation time. However, to preserve goat preantral follicles at higher temperatures, coconut water solution is recommended.

KEY-WORDS: Preservation. Goat. Preantral follicles. Coconut water. Saline solution.

INTRODUCTION

At birth, the mammalian ovary contains many thousands of oocytes enclosed in preantral follicles. However, only a tiny part (0,01%) will be used during the reproductive lifespan of the female, i.e., the other follicles become atretic during their growth and maturation (7). The biotechnology of manipulation of oocytes enclosed in preantral follicles (MOEPF) has been developed to rescue these follicles from the ovaries before they become atretic and culture these follicles in vitro up to maturation stages by preventing follicular atresia.

The biotechnology of MOEPF has already been successfully applied in laboratory species, and mouse offspring from preantral follicles isolated and cultured in vitro have been obtained (8, 10, 11, 20). However, in domestic species, a limiting factor for the success of this biotecnology is the quality of prantral follicles after removal and transportation of the ovaries, since the ovarian donor is commonly encountered far away from reproduction laboratories. Thus the preservation of oocytes enclosed in preantral follicles during transportation to laboratories is very important when using these kind of follicles in cryopreservation and/or in vitro culture protocols. Several studies have successfully used saline solution in the transport of ovaries of some species (1, 19, 23, 25). Coconut water solution is another efficient medium used to preserve goat (22), pig (26, 27) and sheep semen (13), as well as during murine oocyte maturation (3) and embryo culture (4). However, the effect of those solutions on the preservation of goat oocytes enclosed in preantral follicles is unknown.

The present study was carried out to evaluate the effect of saline and coconut water solutions on the preservation of goat oocyte enclosed in preantral follicles, using different incubation temperatures and times.

MATERIAL AND METHOD

Ovary Source

Ovaries (n=8) from 4 adult mixed breed goats were obtained at a local slaughterhouse. The ovaries were dissected from surrounding fatty connective tissues, washed in 70% alcohol for approximately 10 seconds and washed twice in saline solution and processed as described below.

Media

The efficiency of two different media on the

preservation of goat preantral follicles enclosed in ovarian tissue was evaluated in this study. The media tested were: 1) saline solution, and 2) coconut water solution**.** Coconut water in natura has an osmolarity of 500mOsm/L and a pH of 4.5 to 5.0. To correct these parameters, it is necessary to add purified water to reduce the osmolarity and 5% sodium citrate to increase the pH. Thus, the coconut water solution is composed of 50 ml of filtered coconut water, 25 ml of purified water and 25 ml of 5% sodium citrate (final osmolarity: 300mOsm/L and pH: 6.8).

Experimental protocol

The ovarian pair from the same animal was divided into 19 fragments at the slaughterhouse. One ovarian fragment was taken randomly and immediately fixed for classical histology (Control - Treatment 1 - Time zero). The other 18 ovarian fragments were randomly distributed in tubes containing 2mL of saline solution or coconut water at 4ºC, 20ºC or 39ºC for 4 h, 12 h or 24 h (Treatments 2 to 19) as shown in Figure 1. The temperatures were maintained using thermosflasks filled with water at 4ºC, 20ºC or 39ºC. For each treatment, parameters such as temperature and pH of the solution were monitored at the beginning and at the end of the treatments. Each treatment was repeated 4 times.

Qualitative Analysis of Goat Preantral Follicles Enclosed in Ovarian Tissue

The quality of the goat preantral follicles enclosed in small fragments of ovarian tissue was evaluated at the end of the treatments tested by processing the ovarian fragments as follows. The ovarian fragment from each treatment, including the control, was fixed individually in Carnoy for 12 h. They were then dehydrated in a graded series of ethanol, clarified with xylol and embedded in paraffin wax. The tissue was sectioned serially at a thickness of 7 mm. Every 5th section was stained PAS-Hematoxilin; each of these sections was deparaffinized with xylol and rehydrated in graded alcohol. Sections were examined by light microscopy (Leica).

The morphology of preantral follicles was evaluated based on the integrity of the basement membrane, density of the granulosa cells, presence of pycnotic bodies and integrity of the oocyte, using the nucleus as a marker. The proportion of atretic follicles in the control sample was considered as a baseline value for that seen in the stored sample, changes seen due to storage. Based on these parameters, preantral follicles were classified as morphologically normal (containing a healthy oocyte and well organized granulosa cells without pycnotic nuclei; Fig. 2A), degenerated Type 1 (containing an oocyte with pyknotic nucleus and sometimes retracted oocytes, but normal granulosa cells; Fig. 2B) and degenerated Type 2 (containing an shrunken oocytes with pycnotic nucleus and disorganized granulosa cells; Fig. 2C). These three classifications were assigned on the basis of atresia observed in the control and combined with changes that occurred as result of storage. Criteria for granulosa cell and oocyte degeneration were identified using ovaries fixed at time zero (Control).

Statistical Analysis

For each treatment, data of normal and degenerated preantral follicles from four ovarian fragments were pooled. The effects of the preservation solution (saline solution or coconut water), temperatures and incubation times on the percentage of normal and degenerated follicles were analyzed by Chi-square test (Instat for Macintosh). The mean pH values between the control and other treatments were compared using ANOVA and Fisher PSLD test (Stat View for Macintosh). Values were considered statistically significant when $P<0.05$.

RESULTS

Storage of Goat Preantral Follicles in Saline Solution or Coconut Water Solution

A total of 3,243 preantral follicles were examined. The Figure 3 shows the effect of temperature and storage time on the percentage of morphologically normal preantral follicles stored in saline solution or coconut water solution. It was observed that the storage of ovarian fragments in saline solution at 4ºC for up to 24 h and 20ºC for 4 h did not affect significantly the percentage of normal preantral follicles compared to the control ($P>0.05$). However, in the fragments preserved in saline solution at 20ºC for 12 h and 24 h or at 39ºC, in all incubation times tested, the percentage of normal follicles decreased significantly when compared with control values ($P<0.05$). Similar results were obtained with the coconut water solution, except for the ovarian fragments stored at 20ºC for 12 h in which the percentage of normal preantral follicles was similar to those obtained in the control ($P>0.05$).

Comparison between saline solution and coconut water solution at the same temperature and incubation time showed a significantly higher percentage of the normal preantral follicles only after storage in coconut water at 20ºC for 12 h and at 39ºC for all incubation times.

In saline solution and coconut water at 4ºC, the increase in incubation time from 4 h to 12 h and 24 h did not affect significantly the percentage of normal preantral follicles. In contrast, in the fragments stored in both solutions at 20ºC and 39ºC, there was a significant decrease in the percentage of normal preantral follicles with the increase in incubation time from 4 h to 12 h or 24 h and in coconut water at 20ºC in which the increase in incubation time from 12 h to 24 h decreased the percentage of normal preantral follicles ($P<0.05$).

Regarding to the effect of temperature at the same incubation time, the results showed that in saline solution, for all incubation times tested, there was a progressive

reduction of the percentage of normal preantral follicles with the increase in temperature from 4ºC to 20ºC and 39ºC ($P<0.05$), except for the storage time of 4 h, at which the temperature of 20ºC did not decrease the percentage of normal follicles when compared with the storage at 4ºC (Fig. 3). For the coconut water, the results showed that the percentage of normal preantral follicles did not decrease with the increase in temperature from 4ºC to 20ºC, except when fragments were preserved at 20ºC for 24 h. However, in saline solution a significant reduction in the percentage of normal preantral follicles was observed with the increase in temperature from 20ºC to 39ºC, at all incubation times tested. On the other hand, when the fragments were stored in coconut water, the increase in incubation temperature from 20ºC to 39ºC decreased the percentage of normal preantral follicles when preserved for 24 h.

Distribution of Follicular Degeneration Type in the Control and After Storage

Fig. 4 shows the distribution of degenerated Type 1 and Type 2 preantral follicles, in the control and after storage in the different treatments, in saline solution (Fig. 4A) and in coconut water (Fig. 4B). There were no significant differences in the percentage of degenerated type 1 follicles among the control and the different treatments when the fragments were stored in both solutions at 4ºC for all incubation times, as well as in saline solution at 20ºC for 4 h and in coconut water at 20ºC for 4 h and 12 h. Similar results were observed for the percentage of degenerated Type 2 follicles. However, fragments stored in saline solution at 20ºC for 12 h and 24 h or in coconut water solution at 20ºC for 24 h presented a significant increase in the percentage of degenerated type 1 follicles compared with the control. There was a significant predominance of degenerated type 2 follicles compared to the control in the fragments stored in saline solution at 20ºC for 12 h and 24 h and in coconut water at 20ºC for 24 h, as well as in both solutions at 39ºC for all incubation times tested.

With regard to the distribution of degenerated Type 1 and Type 2 preantral follicles, in each treatment, there was a significant predominance of degenerated Type 1 in the control and in fragments stored in saline solution at 4ºC and at 20ºC for all incubation times tested as well as in fragments stored in coconut water at 20ºC for 12 h. In contrast, when the fragments were stored in saline solution at 39ºC for all incubation times and in coconut water at 39ºC for 12 h and 24 h there was a predominance of degenerated type 2 follicles.

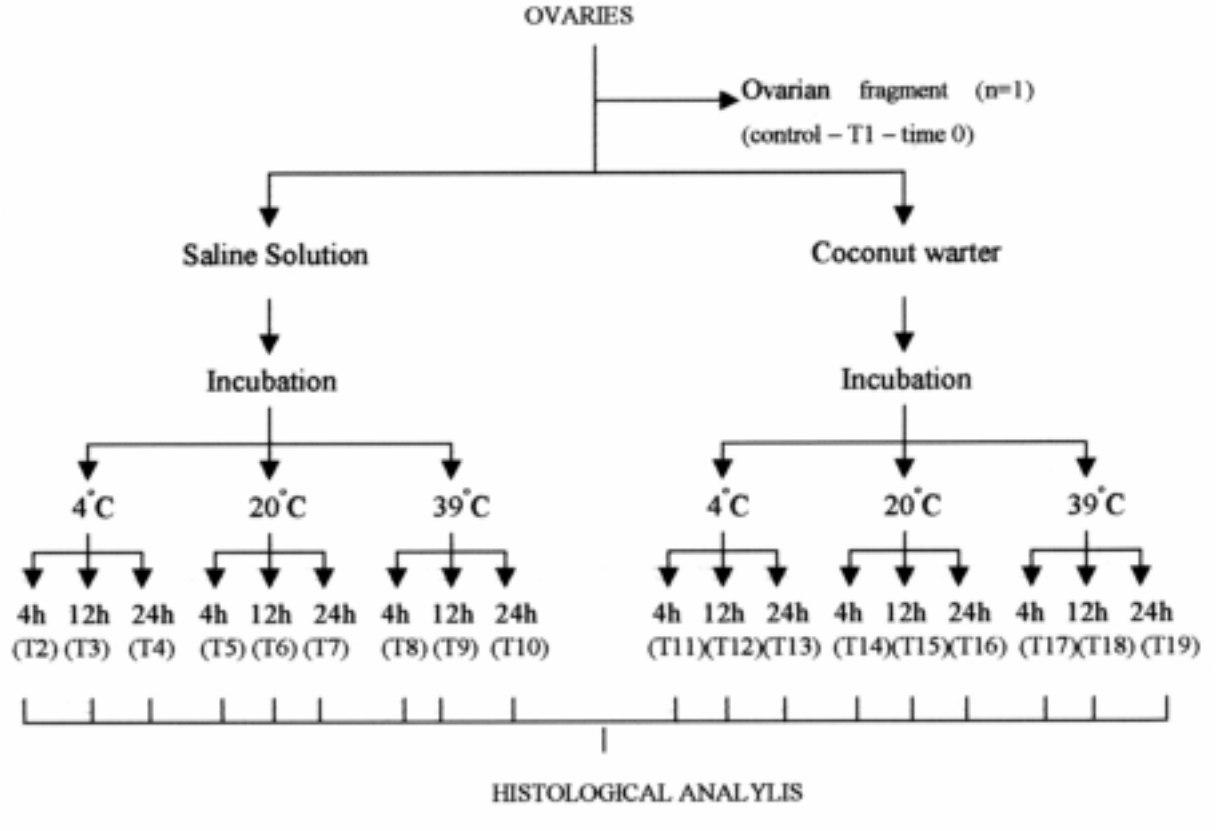

Figure 1

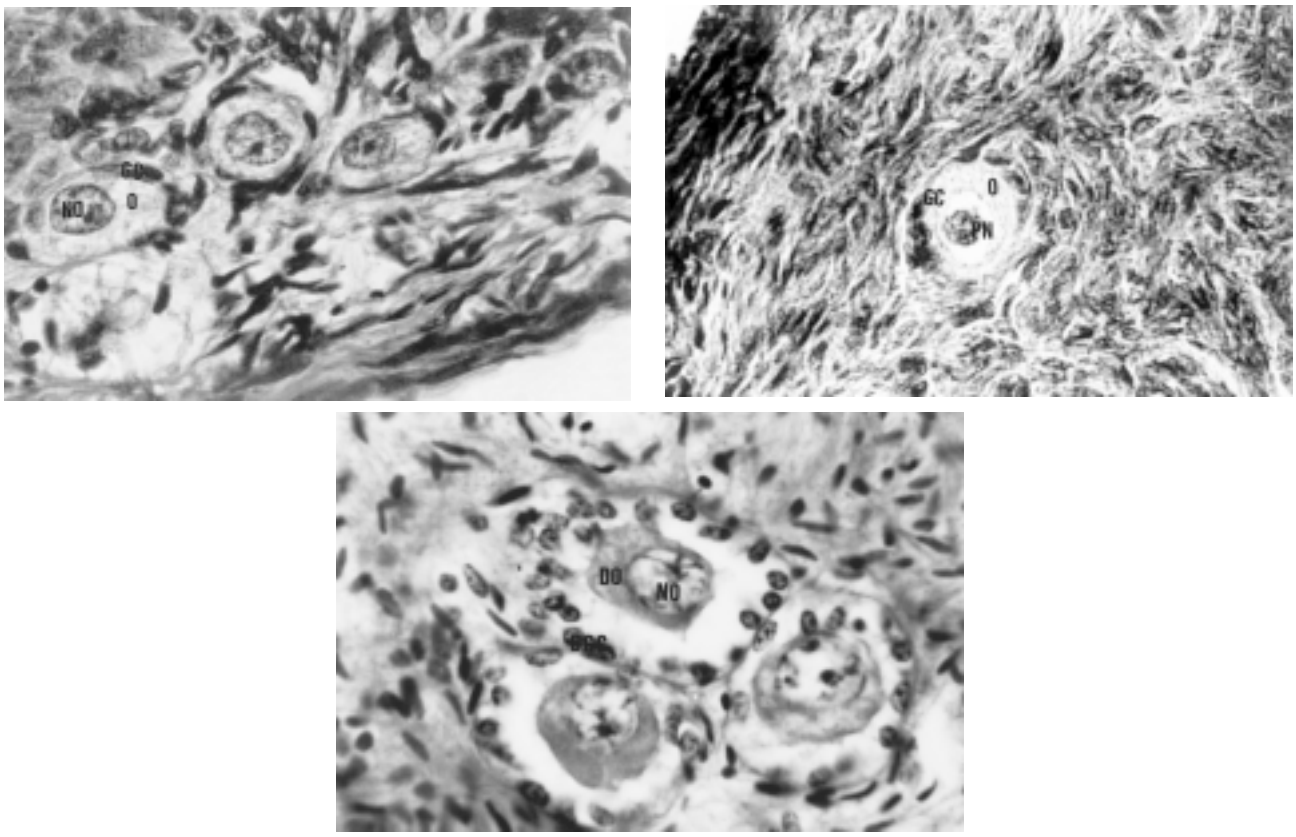

Figure 2

Histological section of ovarian fragment after staining with PAS-hematoxilin, showing A) normal preantral follicle (100x), B) degenerated Type 1 follicles (100x) and C) degenerated Type 2 follicle (100x). O: oocyte, NO: nucleus of oocyte, GC: granulosa cells, DO: degenerated oocyte, DGC: degenerated granulosa cells and PN: pycnotic nucleus.

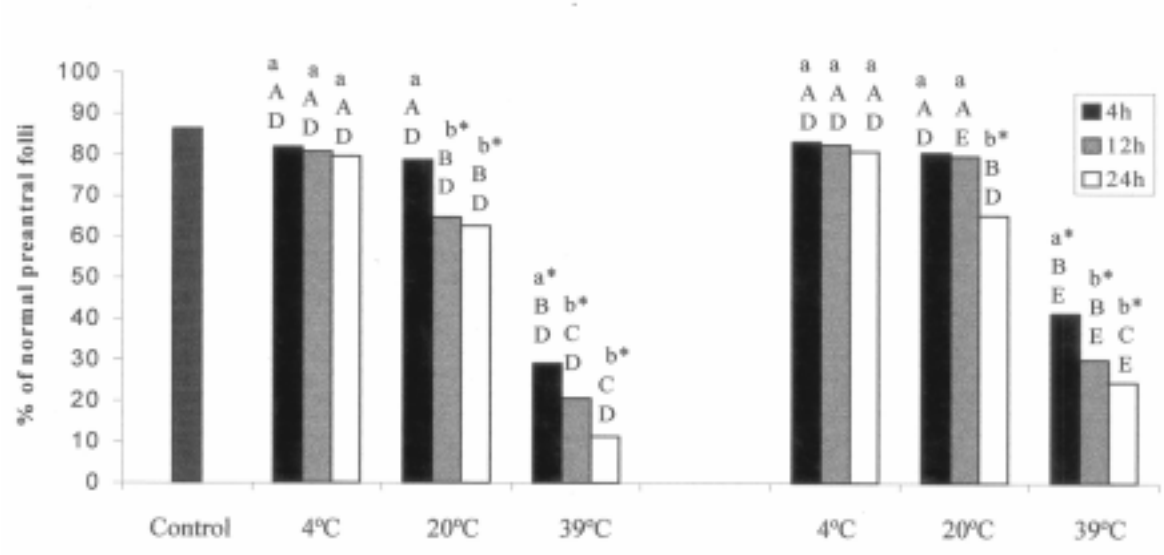

Figure 3

Effect of temperature and storage time on the percentage of morphologically normal preantral follicles preserved in saline solution or cocconut water solution.

*Differ significantly from the control .a, b Different letters at the same preservation temperature differ significantly. A, B, C Different letters in the same preservation time differ significantly

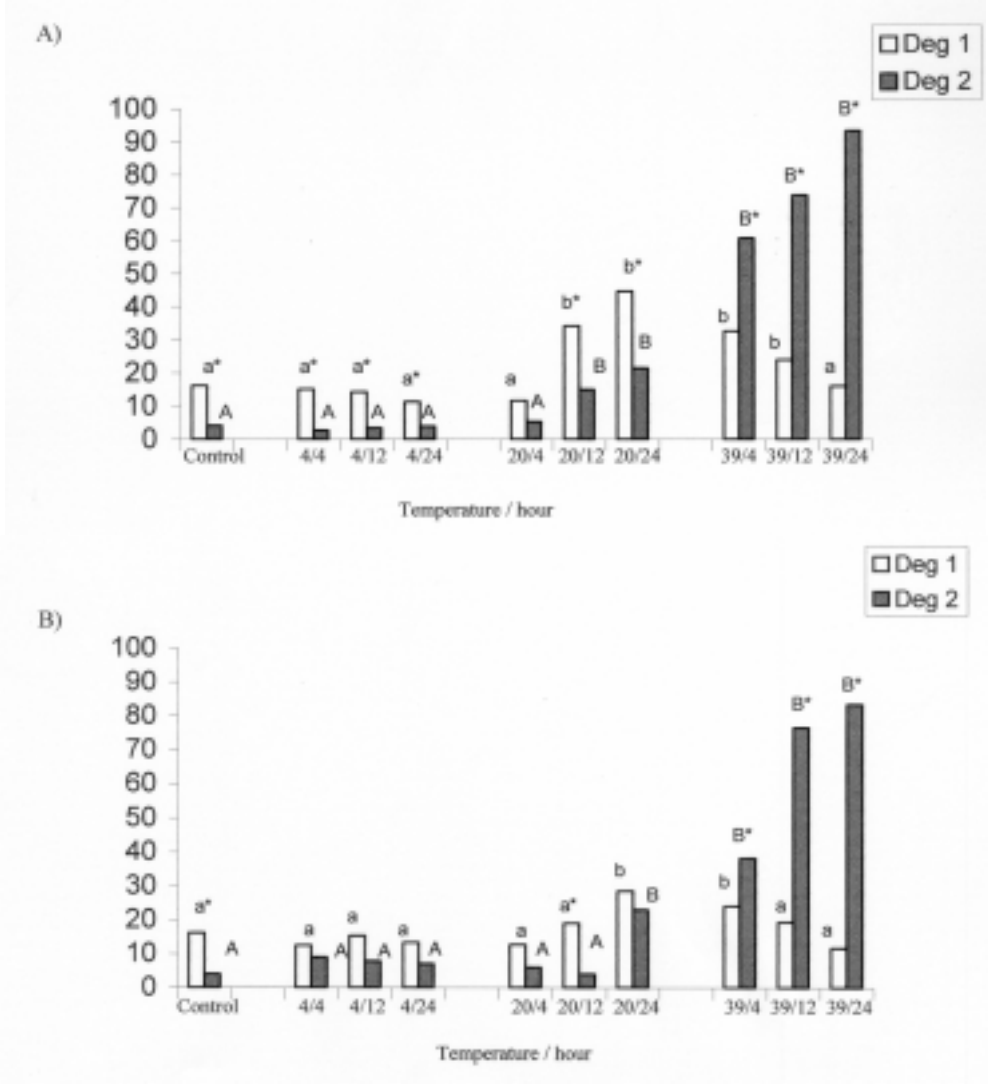

Figure 4

Percentage distribution of Type 1 and 2 degenerated preantral follicles, in the control and after storage in the different treatments, in saline solution (figure 4A) and in coconut water solution (figure 4B).* Denotes a significant difference of degeneration types within each treatment a,b Different letters shows significant difference between the percentage of degenerated Type 1 follicles found in the different treatments and control. A, B Different letters shows significant difference between the percentage of degenerated Type 2 degenerated follicles found in the different treatments and control

DISCUSSION

This study shows that it is possible to preserve goat preantral follicles enclosed in ovarian tissue successfully in saline solution or in coconut water solution at low temperatures. However, the effectiveness of each preservation solution is dependent on the temperature and incubation time.

The histological analysis of preantral follicles present in the control as well as in fragments stored at 4ºC or 20ºC showed that oocyte changes (type 1 degeneration) are the first sign of degeneration. Similar results were also observed after analysis of fresh (caprine - 19 and ovine - 18) or cold-stored preantral follicles at 4ºC (feline – 28). Hirshfield[14] also reported that in secondary follicles, between 75 µm and 150 µm in diameter, oocyte degeneration was usually the only sign of atresia. It was reported [28] that the first index of degeneration in preantral follicles is oocyte degeneration, whereas the first indicator in antral follicles is usually related to the granulosa cells. Braw-Tal and Yossefi[5] , after culture of bovine preantral follicles, reported that the oocytes degenerated or completely disappeared while the granulosa cells appeared healthy in the first days of culture. In contrast, in treatments where the preantral follicles enclosed in ovarian tissue were preserved at 39ºC, the most common type of degeneration was type 2, indicating that oocyte and

granulosa cells were similarly affected, i.e. susceptible to degeneration when preantral follicles were stored at 39ºC. It is important to note that in this study no pycnotic bodies in granulosa cells were observed. According to Cahil *et al*.[6] and Jorio *et al*[18]. while the pycnosis of granulosa cells occurs in antral follicles it is almost absent in preantral follicles. Although the duration of follicular atresia is unknown [16], this current studty shows that atresia signs are earlier in oocytes than in granulosa cells.

The storage of ovarian fragments at 4ºC in both solutions, as well as in saline solution or coconut water solution a 20ºC for 4 h and for up to 12 h, respectively, maintained the percentage of normal preantral follicles similar to that found in the ovary, immediately after animal death (control - time zero). These results may be due to the hypothermia provoked by low temperatures, that might have reduced cellular metabolism, consequently minimizing the metabolic need and increasing the resistance of follicles to the reduction of nutrients and oxygen during preservation in vitro. Roy and Treacy, observed that a lower metabolic rate at low temperatures may be beneficial in maintaining viable human preantral follicles after isolation and in vitro culture. The temperature of 4ºC has been successfully used in the preservation of domestic cat ovaries for 48 h [28] and bovine oocytes from antral follicles for 24 h [25].

The increase in incubation temperature from 4ºC to 20ºC or 39ºC, as well as the increase in the incubation time from 4 h to 12 and 24 h after preservation at 20ºC or 39ºC, in both solutions, decreased significantly the percentage of normal preantral follicles. The normal (39ºC) or subnormal (20ºC) metabolism associated with low oxygen tension in vitro could result in a higher rate of follicular degeneration in the treatments where the ovarian fragments were stored at 20ºC and 39ºC. Jennings *et al.* [17], suggested that changes in the cellular membrane permeability induced by lack of oxygen, caused changes at a level of intracellular Na^+, K^+ and Cl^-, that associated with changes in the distribution of Ca^{++} and increase in intracellular water, may lead to increased cellular volume and consequently cellular degeneration.

Comparing the media tested in this work, the coconut water solution was more effective than saline solution in preserving goat preantral follicles at 20ºC for 12 h and 39ºC for all incubation times. The good results obtained with coconut water solution are probably due to the nutrient composition of this medium, which is rich in proteins, salts, sugars, vitamins, growth factors and plant hormones[21]. Coconut water solution has been successfully used for semen preservation in goats [22], sheep [13] and pigs [26], as well as during murine oocyte maturation [3] and embryo culture [4]. Combarnous and Nunes isolated a component named Indole-3-acetic acid or IAA from coconut water. The IAA is the main representative of a group of plant hormones called auxins. In plants, the auxins increase cell wall plasticity, alter cell permeability, respiratory patterns and nucleic acid metabolism [12]. Regarding the effect of IAA in animals, it was related [27] that the percentage of living spermatozoa with undamaged acrosomes after 13 days of storage at 15°C was significantly higher in the presence of IAA compared to the control. Nunes[21] observed that IAA allows an increase in the motility and percentage of living spermatozoa and also increases the fertility rate. However, the mechanisms of IAA action in animal cells is not clear.

In conclusion, this work shows that saline solution and coconut water solution can be used with the same effectiveness for the preservation of goat preantral follicles at 4°C, irrespective of the incubation time. However, coconut water solution is recommended to preserve goat preantral follicles at higher temperatures.

RESUMO

O presente estudo investigou a eficiência da solução salina e solução à base de água de coco na preservação de folículos pré-antrais inclusos em tecido ovariano, em diferentes temperaturas e diferentes tempos de incubação. No abatedouro, o par ovariano foi dividido em 19 fragmentos; um fragmento ovariano foi imediatamente fixado para histologia clássica (controle-tempo zero). Os outros 18 fragmentos ovarianos foram conservados em ambas as soluções a 4ºC, 20ºC ou 39ºC por 4 h, 12 h ou 24 h. A análise histológica mostrou que a conservação de fragmentos ovarianos em ambas as soluções a 4ºC por até 24 h mantém a percentagem de folículos pré-antrais normais similar aos valores do controle. Ao contrário, a conservação a 20°C ou 39ºC, em ambas as soluções, reduziu significativamente a percentagem de folículos pré-antrais normais comparado aos valores do controle, exceto em solução salina a 20ºC por 4 h ou em solução à base de água de coco a 20ºC por 4 h e 12 h. Em conclusão, esse estudo mostrou que ambas as soluções podem ser usadas com igual eficiência para conservar folículos pré-antrais caprinos a 4°C, independente do tempo de incubação. No entanto, para conservar folículos pré-antrais caprinos a altas temperaturas, a solução à base de água de coco é recomendada.

PALAVRAS-CHAVE: Conservação. Cabra. Folículos pré-antrais. Água de coco. Solução salina.

REFERENCES

1. AMORIM, C. A. et al. . Mechanical method for the isolation of preantral follicles from adult ovine ovaries. In: XIII Reunião Anual da Sociedade Brasileira de Transferência de Embriões, Atibaia. pp. 215, 1998.

2. AMORIM, C. A. et al. Quantitative e qualitative analysis of the effectiveness of a mechanical method for the isolation of preantral follicles from from ovine ovaries. **Theriogenology**. v. 53, p. 1251-1262, 2000.

3. BLUME, H. et al. Avaliação da água de coco na maturação de oócitos bovinos. **Rev. Bras. Reprod. Anim**. v. 21, p. 72-75, 1997a.

4. BLUME, H. et al. Uso da Água de Coco no Cultivo de Embriões Bovinos. **Rev. Bras. Reprod**. v. 21, p. 79-81, 1997b.

5. BRAW-TAL R. and YOSSEFI, S. Studies in vivo and in vitro on the initiation of follicle growth in the bovine ovary**. J. Reprod. Fert**. v. 109, p. 165-171, 1997.

6. CAHILL, L. P. et al. Total follicular populations in ewes of high and low ovulation rates. **J. Reprod. Fert**. v. 55, p. 27-36, 1979.

7. CARROL, J. et al. . Extra Ovarian Production of Mature Viable Mouse Oocytes from Frozen Primary Follicles**. J. Reprod. Fert.** v. 90, 321-327, 1990.

8. CARROL, J. et al.. Effect of gonadotrophin environment on growth and development of isolated mouse primary ovarian follicles. **J. Reprod. Fert.** pp. 71-79, 1991.

9. COMBARNOUS Y. and FERREIRA-NUNES J. Sperm extender including indole derivative. **International patent** n° 2725342; PCT/FR95/01322 (FR 94-12122)

10. EPPIG, J. J. and SCHROEDER, A. C.. Capacity of Mouse Oocyte from Preantral Follicles Undergo Embryogenesis and Development to Live Young After Growth, Maturation and Fertilization in Vitro. **Biology of Reproduction**. 41, 268-276, 1989.

11. EPPIG, J. J. and O'BRIEN, M. J.. Development in vitro of mouse oocytes from primordial follicles. **Biology of Reproduction**. 54, 197-207, 1996.

12. GALSTON A.W. and PURVES W. K. Mechanism of action of auxin. **Am. J. Bot.** 41, 373-80, 1954.

13. GUERRA, F. F. A. and NUNES, J. F. Fertilidade in vivo e avaliação in vitro do sêmen ovino resfriado e conservado em água de coco por 72 horas. **Rev. Bras. Reprod. Anim.** 23, 287-289, 1999.

14. HIRSHFIELD, A. N. Compensatory ovarian hypertrophy in the long term hemicastrate rat: size distribution of growing and atretic follicles. **Biology of Reproduction**. 28, 271-278, 1983.

15. HULSHOF, S. C. J. et al. Isolation and characterization of preantral follicles from foetal bovine ovaries. **The Veterinary Quartely**. 18, 78-80, 1994.

16. INGRAM, D. L. Atresia. (In: Zuckerman, S. *The ovary*). pp. 247-273 (Academic Press, New York), 1962.

17. JENINGS, R. B., GANOTE, C. E. & REIMER, K. R. Ischemic tissue injury. **Am. J. Pathol**. 81, 179-197, 1975.

18. JORIO, A. et al. Development of the population of ovarian follicles during the prepubertal period in D'man and Timahdite sheep. **Anim. Reprod. Sci**. 26, 239-250, 1991

19. LUCCI, C. M. et al. Study of preantral follicle population in situ and after mechanical isolation from caprine ovaries at different reproductive stages. **Anim. Reprod. Sci**. 56, 223-236, 1999.

20. NAYUDU, P.L. & OSBORN, S.M. Factors Influencing the Rate os Preantral and Antral Growth of Mouse Ovarian Follicles in Vitro. **J. Reprod. Fert**. 95, 349-362, 1992.

21. NUNES, J. F. Utilização da água de coco como diluidor do sêmen de animais domésticos e do homem. **Rev. Bras. Reprod. Anim**. 22, 109-112, 1998.

22. NUNES, J. F. and SALGUEIRO, C. C. M. Utilização da água de coco como diluidor do sêmen de caprinos e ovinos. **Rev. Bras. Reprod. Anim**. 1, 17-26, 1999.

23. RODRIGUES, A. P. R. et al. Comparação Entre o Número de Folículos Pré-Antrais Isolados nos Ovários Direito e Esquerdo das Cabras Nativas da Região Nordeste: Resultados Preliminares. (In: *24º Congresso Brasileiro de Medicina Veterinária*, Goiânia) pp. 97, 1996.

24. ROY, S. K.and TREACY, B. J. Isolation and Long-Term Culture of Human Preantral Follicles. **Fertility and Sterility**. 59, 783-790, 1993.

25. SOLANO, R.et al. Short Term Preservation of Intrafollicular Oocytes At 4ºC. **Theriogenology** n.41, p. 299, 1994.

26. TONIOLLI, R. Conservação do Sêmen Suíno em Água de Coco. (In: *VIII Congresso Brasileiro de Reprodução Animal*, Belo Horizonte) pp. 138-142, 1989.

27. TONIOLLI, R. et al. Effect of indole-3-acetic-acid (plant auxin) on the preservation at 15°C of boar semen for atificial insemination. **Reprod. Nutr. Dev**. 36, 503-511, 1996.

28. WOOD, T. C. et al. Follicle-oocyte atresia and temporal taphonomy in cold-stored domestic cat ovaries. **Mol. Reprod. Dev**. 46, 190-200, 1997.

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