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Development of caprine preantral follicles after orthotopic autotransplantation of ovarian tissue

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Abstract

Objective: The aim of this study was to evaluate the follicle morphology, density, development and hormone production after orthotopic autransplantation of fresh or vitrified goat ovarian tissue. Methods: Fresh and vitrified ovarian cortex was orthotopically autotransplanted for six months in two and three adults bilaterally ovariectomized goats, respectively. The animals were monitored during 196 days and blood samples collected. Results: It was observed that the percentage of morphologically normal preantral follicles (MNPF) after grafting of fresh ovarian tissue was similar to control. The follicular density in the *fresh graft* reduced significantly when compared to *fresh control.* unfortunately, after transplantation of vitrified tissue it was not possible to identified any follicles after recovery. Furthermore, the proportion of developing follicles was higher ($P < 0.05$) in the fresh auto-grafts than in control fragments. Moreover, progesterone plasma levels increased significantly from day 179 to day 195 of transplantation. Conclusion: In conclusion, orthotopic transplantation of fresh ovarian tissue was able to keep healthy the preantral follicles, as well as the restoration of goat endocrine function.

Keywords: vitrification; ovarian transplantation; preantral follicles; endocrine function.

Introduction

The mammalian ovarian cortex contains the preantral follicles reserve which comprises of approximately 80-90% of primordial follicles and only 10-20% constitute the primary and secondary follicles.[1] However, the absence of inhibitory signal or even the presence of toxic environment such as chemotherapy can negatively affect the oocytes in the ovarian reserve, causing death or abnormal activation leading to atresia, all of which results in depletion of ovarian follicles and hormonal imbalance.[2] Therefore, ovary tissue cryopreservation followed by transplantation has been extensively investigated with the aim of restoring the reproductive function in different species. In large mammals, for instance, transplantation of ovarian tissue has been shown a sign of a realistic alternative to obtain complete follicular development in goat.[3] Moreover, it has been shown live-births after autotransplantation of vitrified ovine ovary tissue.[4] This technique has also shown promising results in human, especially if tissue is cryopreserved applying conventional freezing instead of vitrification.[5] Currently, 86 successful births have been reported from transplantation of cryopreserved ovarian tissue.[6]

Conflicts of interest: The authors have declared that no competing interests exist. All authors gave their informed consent prior to their inclusion in the study.
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The study was carried out at Laboratory of Manipulation of Oocytes and Preantral Follicles (LAMOFOPA), State University of Ceará, Fortaleza, CE, Brazil and at Laboratory of Molecular Biology and development, University of Fortaleza (UNIFOR), Fortaleza, CE, Brazil.

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However, despite the good results obtained after transplantation of cryopreserved ovary, this technique has still been considered as experimental.[7]

Due to ethical concerns and scarce availability of human ovarian tissue, as well as the need to develop alternatives to preserve endangered animal breeds and species,[8] the number of investigations using different surgical approaches, transplantation sites, and several animal models is increasing.[9] Nevertheless, goat ovary is a candidate for such research based on structural and functional similarities with women ovaries.[10] According to the literature, to date, the only study performed with ovarian goat tissue transplantation was described by Santos et al.[3] These authors showed that, the percentage of normal preantral follicles in fresh or frozen-thawed ovarian tissue after one year of transplantation was lower than fresh or non-cryopreserved (control) ovarian tissue. In addition, these authors also reported that only primordial follicles survived after transplantation of frozen-thawed ovarian tissue. Then, the aim of this study was to verify if transplantation of fresh and vitrified goat ovarian and short time transplantation (6 months instead 1 year) of the ovarian tissue is better to preserve the morphology, density and follicular development. Additionally, the endocrine function after 6 months of ovary autotransplantation was also analyzed.

Materials and methods

Sexually mature, non-pregnant and normal cycling goats (n = 5) were hemi (n = 3) or bilateral (n = 2) ovariectomized after a ventral midline skin incision and anesthesia. Immediately after ovariectomy, one fragment of each 5 animals was fixed in paraformaldehyde 4% (PAF) for classical histology and served as *fresh control*. Afterward, the animals submitted to bilateral ovariectomy received 22-23 ovarian cortical fragments (3×3×1 mm) which were sutured together and grafted under the curvature minor region of the left uterine horn side as described by Santos et al.[3] and considered as *fresh graft*. Furthermore, the ovary of the animals submitted to hemilateral ovariectomy was vitrified according to Carvalho et al.[11] for 14 days and after warming, one fragment of each animal was fixed and served as *vitrified control.* The remaining fragments (10-12) were autotransplanted and considered as *vitrified graft* following a complete ovariectomy. After transplantation, all the animals were monitored during 196 days (~ 6 months). Blood samples were collected on days 48, 60, 178 and after this last day it was done four times per week during 14 days before recovery of the fragments to assess progesterone plasma levels. Concentrations of progesterone were determined using the ARCHITECT® platform (Abbott Diagnostics, Abbott Park, IL, USA). Additionally, two non-sterilized healthy goats were used as reference for comparative plasma progesterone analysis. After 184 days of transplantation, the animals were treated with progesterone through intravaginal implant to regulate their estrus cycle, since no estrous behavior was observed naturally after this period. Finally, after 196 of transplantation, the goats were euthanized and the fresh and vitrified ovary fragments recovered. Fresh or vitrified controls fragments as well as the grafts (fresh or vitrified) were processed histologically and follicles were classified according to development stage (*primordial*, *transition*, *primary* or *secondary*) and morphology (*normal* or *degenerated*) as described by Carvalho et al.[11] The follicular density was also evaluated according by Santos et al.[12] The percentages of normal early stage follicles were compared by ANOVA and Tukey's test. Mean values of follicular density per square millimeter were compared by Student's t-test and ANOVA. The follicle was the experimental unit.

Results and discussion

In the current experiment, it was observed three antral follicles in fresh graft ovarian fragments. Moreover, 71% and 60% of fresh and vitrified grafts were recovered respectively after 196 days of transplantation. It is observed in Figure 1A that the percentage of MNPF in the *vitrified control* (30%) or even *fresh graft* (66%) was not significant different from *fresh control* (64%). In contrast to what was observed in the present study, Santos et al.[3] using the same species, observed a significant decrease of MNPF in fresh graft in comparison with fresh control. This difference of results could be due to the fact that in the current study, the duration of transplantation was lower (6 months) while the time used by those authors was one year. Unfortunately, in this work it was not possible to find any follicles after transplantation of vitrified ovarian fragments. This result cohoborate those reported by Donnez et al.[13] as they observed no follicles in three patients after transplantation of frozen-thawed ovarian tissue. We believe that, the absence of follicles in *vitrified graft* could be due to the ovary quality before the transplantation, because the fresh control already had a high percentage of degenerate follicles in comparison of what was previously established for the caprine species. According to Silva et al.,[14] the acceptable percentage of degeneration in the fresh ovary is 12%, however, in the present study this percentage was three times higher (36%). Moreover, cryopreservation makes follicles more susceptible to cell death by increasing osmotic stress[15] caused by high concentration of cryoprotectants used for vitrification. This reinforces the high percentage of degenerated follicles associated with the absence of follicles in the *vitrified graft* observed in our study. In addition, several others factors can affect ovarian graft longevity, including: ovarian reserve (follicular density, which is age dependent); graft size and method of ovarian tissue preparation (freezing-thawing techniques); inhomogeneous

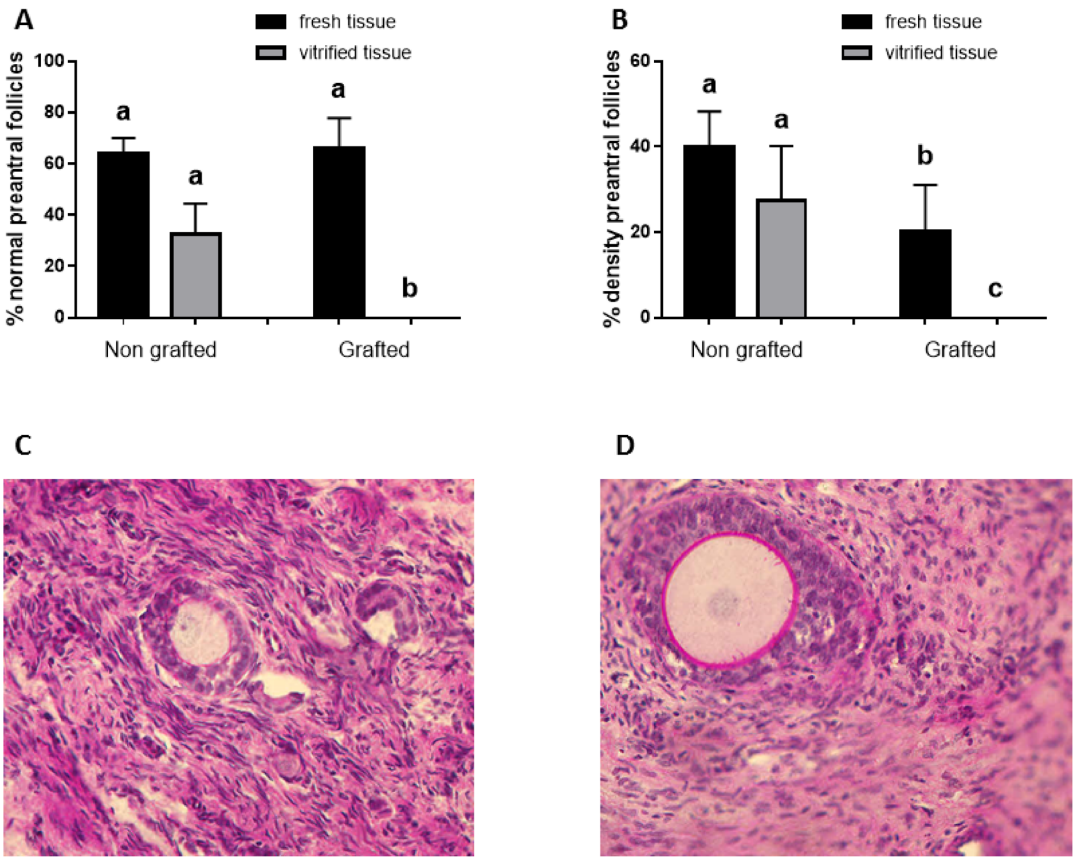

Figure 1. Proportion of normal preantral follicles (A) and follicle density (B) in fresh and vitrified ovary fragments before and after transplantation; (C) and (D) histological normal secondary follicles before and after grafting, respectively. a, b differs within the experimental group. Scale bar= 50μm, Original magnification 40X.

distribution of follicles in grafted cortical pieces; and angiogenic potential of the graft site influencing the degree of ischemia after transplantation.[13]

The follicular density, in the *fresh graft* reduced significantly when compared to *fresh control* (Figure 1B). Experimental studies have indicated that the fall in number of primordial follicles in grafted tissue is due to hypoxia and the delay before reimplanted cortical tissue becomes revascularised.[16] This could explain the decrease of follicular density observed after ovary transplantation. Moreover, the proportion of developing (transition, primary and secondary) follicles was higher in the *fresh grafts* than in *fresh control* (Figure 2A). Such an increase in the rates of developing follicles together with a decreased density of normal follicles can be indicative of massive follicular activation. Usually, graft revascularization takes places in few days[3] and, before this, ovarian tissue will be challenged with hypoxia and consequent oxidative stress, which plays an important role in massive follicular activation and burn out of the follicular reserve.[17]

An increase in progesterone plasma levels was observed from day 179 to day 195. Considering that progesterone was administered day 184 only, the initial increased levels indicate that grafts were allowing the animals to recover their reproductive/endocrine function (Figure 2B). The orthotopic transplantation of ovarian tissue was previously proved to be efficient for restoration of endocrine activity as evidenced by circulating steroid levels 70 days after grafting and it was maintained up to one year without hormonal stimulation.[3] In the present study, we obtained restoration of ovarian function just after six months from bilaterally ovariectomized animals with low percentage of normal follicles. In conclusion, orthotopic transplantation of fresh ovarian tissue was able to maintain a similar percentage of morphological normal preantral follicles with fresh control as well as the restoration of goat endocrine function. Moreover, to transplant cryopreserved ovary tissue, it is recommended to analyze previously the percentage of morphological normal follicles and this percentage should be high than 30%. Moreover, vitrification protocol should be improved to minimize follicles loss.

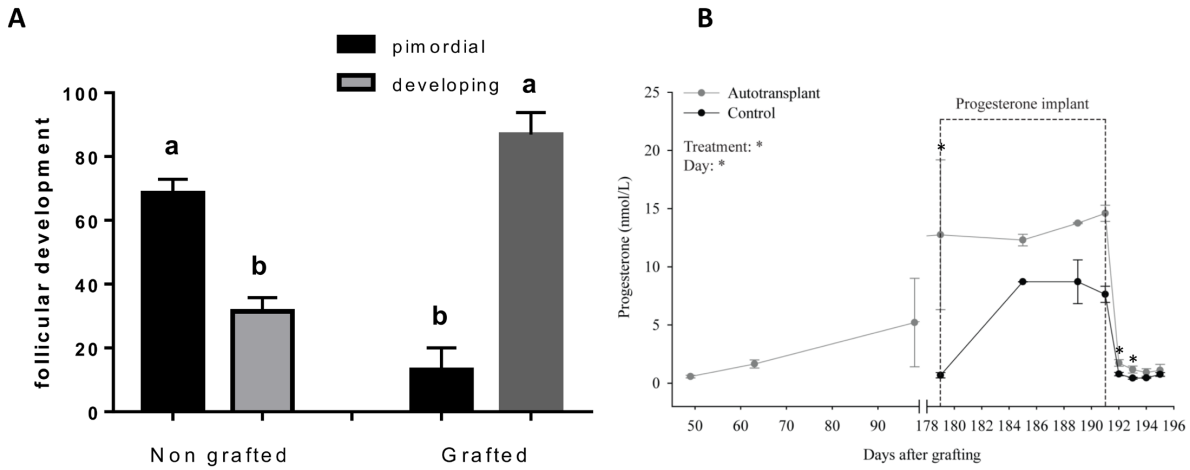

Figure 2. Proportion of follicular development in fresh control fragments and fresh graft (A) and Mean progesterone concentration (± SEM) produced by animals from fresh transplanted group during the experimental period of 195 days (B). The progesterone production from control group animals was evaluated since the insertion of hormonal implant (179 days). a, b differs within the experimental group. *Statistical difference among treatments in the same day ($P < 0.05$).

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Statement of animal's rights

The Ethics Commission for the Use of Animal Institutional Care and Use Committee of the State University of Ceará approved this study (number: 2917497/2015).

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Authors contribution

NJD performed the laboratory work and write the first draft of the manuscript. KAA coordinated and help in the execution of the experiment. BGA performed the statistical analysis. LTM, CEMC, SGN, LHA performed the surgery and transplantation. MB provided the animals and technical support. JRF provided the technical support and revised the manuscript. RRS and JS revised the manuscript. SFSD performed the hormonal assay. APRR designed the experiment, provided the funding and helped with the drafting of the manuscript. All authors read the final draft and agreed to its submission.