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FERTILITY PRESERVATION

# Impact of freezing and thawing of human ovarian tissue on follicular growth after long-term xenotransplantation

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**Abstract**
*Purpose* To assess follicular growth after xenografting in order to understand how freezing and/or grafting may affect follicular development.
*Methods* Human ovarian biopsies were used for fresh and frozen-thawed xenografting to SCID mice. After xenotransplantation, follicular morphology and proportion, oocyte and follicle diameter, and quantitative and qualitative parameters of antral follicles were analyzed.
*Results* The proportion of growing follicles was significantly higher in grafted than non-grafted ovarian tissue. Follicular growth to the antral stage was observed and there was no significant difference in oocyte or follicle diameter in fresh or frozen-thawed grafts. Although no significant difference was observed in antral area or zona pellucida thickness, the theca layer in antral follicles from frozen-thawed grafted tissue was found to be significantly thinner than in fresh grafts.
*Conclusion* Antral follicles obtained after grafting of frozen-thawed human ovarian tissue showed a thinner theca cell layer compared to those from fresh grafts, which could affect follicular development and function. Further studies are nevertheless warranted to confirm the identity of theca cells and assess if they retain the ability to respond to luteinizing hormone and produce androgens.

**Keywords** Ovarian tissue · Follicles · Xenotransplantation · Cryopreservation · Theca cell layer

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*Capsule* The theca layer in antral follicles from cryopreserved grafted human ovarian tissue is thinner than in fresh grafts.

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## Introduction

In recent years, advanced chemo/radiotherapeutic treatments have led to high survival rates in cancer patients, giving rise to new issues for cancer survivors. Indeed, one major concern is future fertility in these women, since they may face premature ovarian failure [1]. Cryopreservation of ovarian tissue before gonadotoxic treatments and its transplantation after disease remission has therefore emerged as an option for these patients, resulting in restoration of endocrine function [2] and 13 live births to date [3].

Cryopreservation has been shown to successfully safeguard the pool of primordial follicles [4, 5]. Moreover, after grafting of frozen-thawed ovarian fragments, these follicles are able to grow and develop [6]. However, some of them do not develop properly, as demonstrated by the low recovery rates of oocytes aspirated from antral follicles in women with transplanted cryopreserved ovarian tissue [2, 7] and the significant number of empty follicles observed [8]. This indicates that freezing and/or grafting may affect follicular development to some extent.

Studies using xenografting models have also shown successful follicular development, ovulation and corpus luteum formation after long-term transplantation of human cryopreserved ovarian tissue to mice [5, 6, 9, 10]. In

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addition, using the intraperitoneal pocket as the transplantation site might provide a direct clinical correlation, since live births obtained following ovarian transplantation in humans have utilized this technique [2, 11, 12]. Hence, this model was applied in this study to understand the possible impact of freezing and/or grafting on the population of human preantral follicles.

The development of human ovarian follicles up to the antral stage was analyzed after grafting of fresh and cryopreserved human ovarian tissue to an intraperitoneal site in SCID mice. The following parameters were assessed to compare differences between fresh and frozen grafts: (i) follicular morphology and proportion, (ii) oocyte and follicle diameter, and (iii) quantitative and qualitative evaluation of antral follicles. As no differences in follicle populations were recorded between fresh and frozen-thawed ovarian tissue after shorter xenotransplantation periods [13–16], the grafts were analyzed after 28 weeks.

## Materials and methods

### Collection of ovarian tissue

Use of human ovarian tissue for this study was approved by the Institutional Review Board of the Université Catholique de Louvain. After obtaining written informed consent, ovarian biopsies were taken from eight women between 20 and 30 years of age undergoing laparoscopic surgery for benign gynecological conditions. The ovarian tissue was transported to the laboratory in Leibovitz medium (L-15, Gibco, Carlsbad, CA, USA) at 4°C and then cut into small fragments (5.0 × 1.5 × 1.0 mm). One fragment of each biopsy was directly fixed in formalin or Bouin for histological analysis (fresh control) and the others were grafted (fresh grafts) or frozen for subsequent fixation (frozen-thawed control) or grafting (frozen grafts).

### Ovarian tissue freezing and thawing

Ovarian tissue fragments were slow frozen and thawed using 10% dimethylsulfoxide (DMSO, Sigma-Aldrich, Bornem, Belgium) as a cryoprotectant according to the protocol from Gosden et al [17], slightly modified [18]. After thawing one fragment was fixed in formalin for histological analysis (frozen-thawed control).

### Transplantation to SCID mice

Thirteen six-week-old female SCID mice (Charles River, L'Arbresle, France) were used for the study. The mice were housed and bred as previously described [19]. They were anesthetized by intraperitoneal injection of ketamine (75 mg/kg; Anesketin, Eurovet, Heusden-Zolder, Belgium) and medetomidine (1 mg/kg; Domitor, Pfizer, Cambridge, MA, USA), and buprenorphine (0.1 mg/kg; Temgesic, Schering Plough, Kenilworth, NJ, USA) was administered for analgesia. A ventral midline skin incision was made and the abdominal wall opened. Two ovarian grafts were stitched to the anterior wall of the peritoneum at the level of the bladder. A fresh ovarian fragment from one patient was placed on the right side and a frozen-thawed ovarian fragment from the previous patient on the left using non-absorbable sutures (7/0 Prolene; Ethicon, Johnson & Johnson International, Belgium). The abdominal wall and skin were then closed with absorbable 7/0 Vicryl (Ethicon). After surgery, anesthesia was reversed by injection of atipamezole (1 mg/kg; Antisedan, Pfizer).

### Gonadotropin stimulation

The mice were given intraperitoneal injections of follicle-stimulating hormone (FSH: 7.5 IU) (Menopur, Ferring, Aalst, Belgium) starting from 26 weeks after transplantation. Human chorionic gonadotropin (hCG: 20 IU) (Pregnyl, Organon, Oss, the Netherlands) was administered 36 h before euthanasia according to Dolmans et al. [19]. After 28 weeks, the animals were euthanized by $CO_2$ asphyxiation, and the grafts were recovered and fixed in Bouin's fixative.

### Follicle classification and morphology

Histological analysis was performed on fresh and frozen-thawed ovarian tissue before and after grafting. After fixation, to evaluate follicular morphology, the ovarian fragments were dehydrated, embedded in paraffin and serially sectioned (5-μm-thick sections). Every sixth slide was stained with hematoxylin-eosin (Merck, Darmstadt, Germany).

Follicles were counted in the entire fragment and classified according to stage into primordial, primary, secondary or antral follicles. Primordial follicles were characterized by one layer of flattened granulosa cells around the oocyte, primary follicles by one layer of cuboidal granulosa cells, secondary follicles by two or more layers of granulosa cells, and antral follicles by the presence of an antral cavity [20].

Antral follicles were also analyzed according to several parameters described by Gook et al. [21], such as degradation of theca and granulosa cells (separation, swelling or lysis), presence of cell remnants, granulosa cells and blood in the antral cavity, and separation of the corona cells.

### Follicle measurements

Follicle and oocyte diameters were calculated according to Griffin et al. [22]; using ImageJ software (National

Institutes of Health, USA) after calibration with a stage micrometer. In addition, a second measurement was taken at a right angle from the midpoint of the first measurement. The two measurements were then averaged and expressed as the diameter of the structure. Follicle diameters were calculated from the outer layer of the granulosa cells, while oocyte measurements included the zona pellucida (ZP), when present.

In total, 10 follicles from each developmental stage from each patient in each group were measured. When fewer than 10 follicles were present, all the follicles were evaluated. For antral follicles, theca layer and ZP thickness, as well as antral area, were also measured.

To determine theca layer thickness, higher-magnification (20X) pictures were taken using the Mirax microscope (Zeiss Systems, Germany) and the thickness was then measured using the Mirax Viewer program. The theca layer extended from the basement membrane of the follicle to the end of the visible difference between theca and stromal cells.

Antral area measurement was also carried out using the Mirax Viewer program. The total area was first marked with the freehand tool and then measured.

For ZP thickness, higher-magnification (100X) pictures of the oocytes were taken using the Mirax microscope (Zeiss Systems, Germany) and the thickness was measured using the Mirax Viewer program.

### Periodic acid-Schiff (PAS) and Masson's trichrome staining

In order to validate measurements of theca cell thickness, some slides containing antral follicles from fresh and frozen-thawed grafted tissue were PAS stained. This enabled us to examine morphological differences between theca layer and stromal cell populations and evidence the basement membrane that separates granulosa from theca cells. Oxidation of carbohydrate molecules (glycoproteins, proteoglycans and glycosaminoglycans) present in the basement membrane with periodic acid creates aldehydes that react with Schiff, a reagent for the carbonyl groups, mainly those of aldehydes, generating a magenta-violet color. In addition, some components of the extracellular matrix (i.e. proteoglycans and collagen) are also oxidized by periodic acid, resulting in an insoluble product colored pink-red with Schiff's reagent [23].

For PAS staining, sections were stained with 0.5% periodic acid for 30 min and then with Schiff's reagent for 20 min. After washing in running tap water for 5 min, nuclei were counterstained with hematoxylin for 3 min [24].

In addition, some slides were routinely colored with Masson's trichrome to differentiate the theca layer from stromal tissue through collagen staining, present in larger amounts in the latter.

### Statistical analysis

All data were analyzed using SAS 9.2. Descriptive analysis was performed to report results as mean, standard deviation (SD), median, range, minimum and maximum for continuous variables, and as frequency for categorical variables. Comparisons of means between groups were made using the Wilcoxon test for independent groups because distribution of variables was not normal. Frequencies were compared using $\chi^2$ analysis. If an expected frequency in at least one cell of the table in the $\chi^2$ test was less than 5, Fisher's exact test was used instead. A p-value $\leq 0.05$ was considered statistically significant.

## Results

### Graft recovery rate and macroscopic aspect

After 28 weeks of xenotransplantation, all 26 ovarian xenografts were easily recovered from the intraperitoneal cavity of the 13 mice. Six antral follicles (three in fresh and three in frozen-thawed tissue) and two corpora lutea measuring 4 mm in diameter were observed on the surface of the grafts, which indicates that they responded to the stimulation protocols eventually leading to ovulation.

### Histological evaluation of ovarian follicles

#### *Follicle proportions*

Altogether, 4087 follicles were identified and analyzed: 429 from fresh fragments, 759 after grafting of fresh ovarian tissue, 333 from frozen-thawed tissue, and 2566 after grafting of frozen-thawed ovarian tissue. Follicle distribution was uneven among the 26 grafts, with some grafts showing fewer follicles, but others exhibiting high follicle numbers. The mean number (± SEM) of follicles found per fragment was 61±69 in fresh fragments, 66±79 after grafting of fresh ovarian tissue, 184±82 from frozen-thawed tissue, and 510±369 after grafting of frozen-thawed ovarian tissue.

Similar follicular development was observed after grafting in both fresh and frozen-thawed groups. The proportion of growing (primary, secondary and antral) follicles was significantly higher ($p<0.05$), but the proportion of primordial follicles significantly lower ($p<0.01$), in both fresh and frozen-thawed ovarian xenografts than in ungrafted ovarian tissue (Fig. 1). When fresh and frozen-thawed grafts were compared, no statistical difference was observed in the number of primordial follicles or in the populations of growing follicles. It is important to note that, although the number of antral follicles was similar in fresh ($n=6$) and frozen-thawed grafted ovarian tissue ($n=10$), the proportion

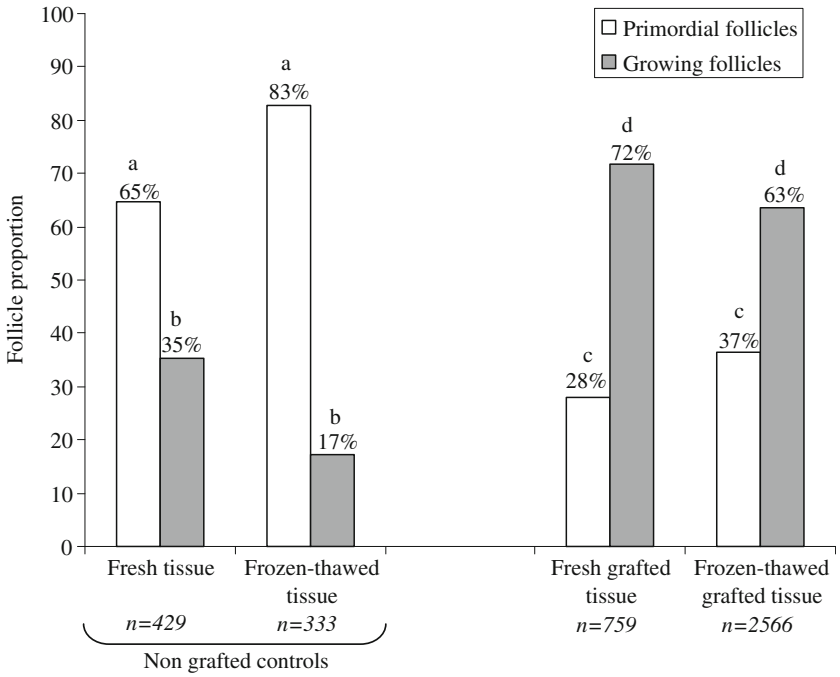



**Fig. 1** Proportion of primordial and growing (primary, secondary and antral) follicles before and after xenografting in fresh and frozen-thawed ovarian fragments. **a** significantly different from **c** ($p<0.01$). **b** significantly different from **d** ($p<0.05$)

of follicles forming an antral cavity tended to be higher in fresh grafts (0.79%) than in frozen grafts (0.39%).

*Follicle morphology*

Preantral follicles present in both fresh and frozen-thawed xenografts exhibited similar structural integrity to follicles from fresh ovarian tissue: they showed a healthy, round oocyte surrounded by varying numbers of granulosa cells in close contact with each other, enclosed by a visible basement membrane (Fig. 2). In secondary follicles, a distinct ZP could be identified around the oocyte, as well as layers of theca cells surrounding the basement membrane.

Antral follicles from both fresh and frozen-thawed xenografted ovarian tissue also looked healthy (Fig. 3). The oocyte was encapsulated within a thin ZP and surrounded by granulosa cells, which were sometimes detached from the oocyte. Occasionally, Call-Exner bodies

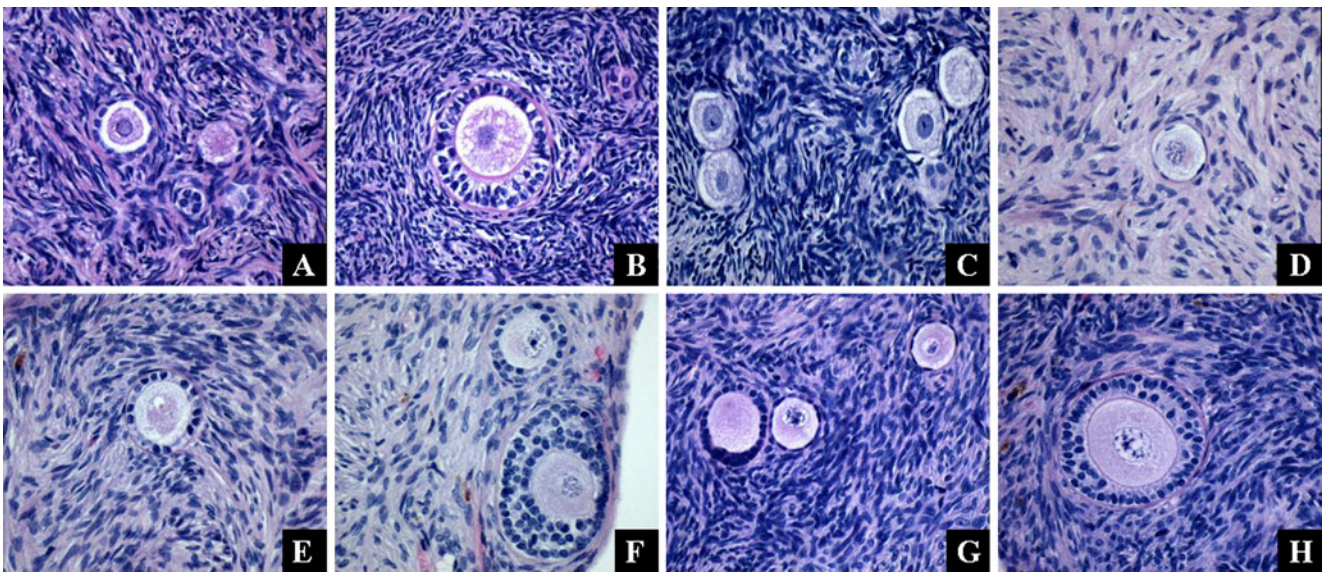



**Fig. 2** Preantral follicles stained with hematoxylin-eosin from fresh and frozen-thawed fragments of ovarian tissue before and after grafting. Primary (**a**) and secondary follicles from fresh non-grafted tissue (**b**); primordial follicles from frozen-thawed non-grafted tissue (**c**); primordial (**d**), primary (**e**) and secondary follicles from fresh grafted tissue (**f**); primordial, primary (**g**) and secondary follicles from frozen-thawed grafted tissue (**h**). Original magnification 20X

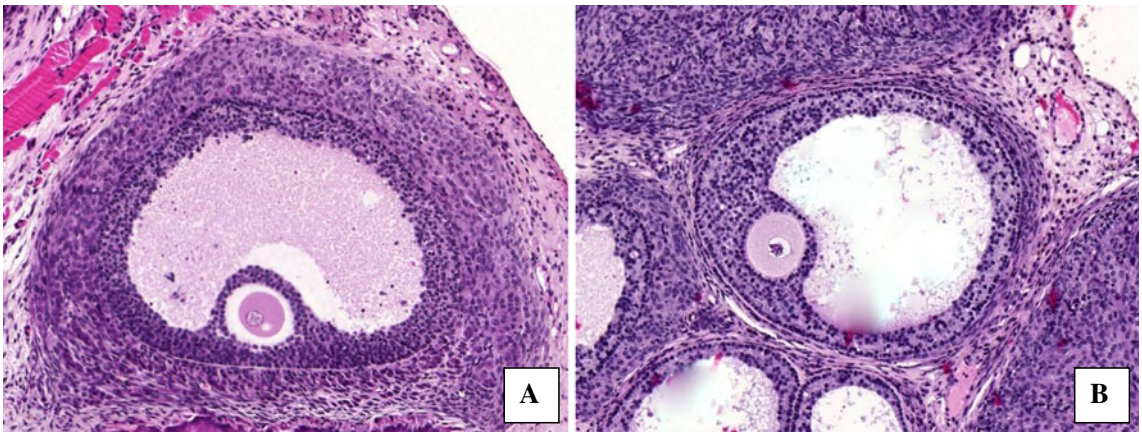



**Fig. 3** Representative photomicrograph of hematoxylin-eosin-stained antral follicles from fresh (**a**) (original magnification 10X) and frozen-thawed grafted ovarian tissue (**b**) (original magnification 5X)

and pyknotic cells were observed among the granulosa cells. A few pyknotic cells were also found in the antral cavity. All antral follicles from fresh grafted tissue exhibited distinct layers of theca interna and theca externa cells. By contrast, most follicles ($n$=7) from frozen-thawed ovarian tissue had a thin theca layer, with no clear discernible distinction between the two types of theca cells. Apart from theca layer thickness, no major morphological differences were observed between antral follicles from fresh or frozen-thawed xenografts.

*Follicle measurements*

In both fresh and frozen-thawed grafted tissue, oocyte and follicle diameter increased according to developmental stage. No significant difference was observed in oocyte or follicle diameters (Fig. 4) of primordial, primary, secondary or antral follicles from fresh or frozen-thawed grafts.

The theca layer in antral follicles from frozen-thawed grafted tissue (49.9±24.28 μm) was significantly thinner ($p$<0.05) than in fresh grafts (86.5±29.9 μm), while no significant difference was observed in antral area (fresh grafted—29,248.5±37,354.7 μm²; frozen-thawed grafted—29,285.4±27,028.6 μm²) or ZP thickness (fresh grafted—1.5±0.5 μm; frozen-thawed grafted—1.6±0.4 μm).

PAS and Masson's trichrome staining

As illustrated in Fig. 5, PAS and trichrome staining were observed at lesser and greater intensity among theca cells and in stromal tissue respectively. Theca layer thickness was again measured from the basement membrane stained with PAS and trichrome up to the border of the theca and stromal cells. The results obtained with such measurements corresponded to those observed on hematoxylin-eosin-stained slides.

## Discussion

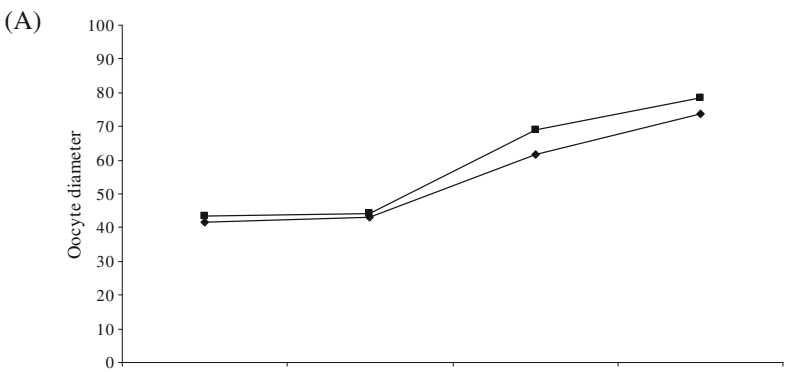
The present study evaluated the impact of freezing and/or grafting on the morphology and growth of human ovarian follicles. It also demonstrated, for the first time, that primordial follicles can grow to the antral stage in ovarian grafts transplanted to an intraperitoneal site in immunodeficient mice. After analysis of more than 4000 follicles, we observed that the morphology and development of preantral follicles do not appear to be altered after freezing or grafting procedures. However, antral follicles from frozen-thawed ovarian tissue exhibited a thinner layer of theca cells than follicles from fresh grafted tissue.

Preantral follicle population

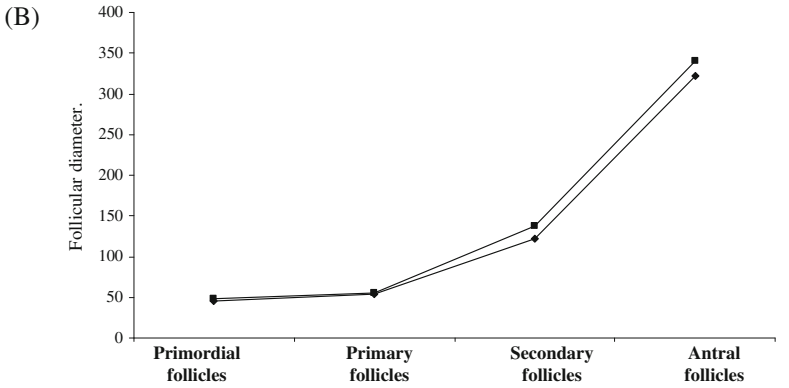
Based on our findings and data from the literature, one can assume that freezing and thawing procedures have little or no impact on initial follicular development, since preantral follicles appear to grow in a similar manner to fresh follicles.

It is also interesting to note that the proportion of frozen-thawed primordial follicles (36%) found after 28 weeks of grafting is comparable to that (41%) found after 3 weeks of grafting [16]. This shows that after initial massive recruitment, the primordial follicle population tends to stabilize, possibly due to neovascularization in the graft and production of inhibitory factors from growing follicles. As suggested in our previous studies [18], such activation of a high number of primordial follicles may be due to ischemic stress that the ovarian tissue is subjected to before its revascularization. Moreover, the absence of inhibitory factors, such as anti-Müllerian hormone involved in the maintenance of the pool of quiescent follicles, may also promote activation of primordial follicles [25].

After 28 weeks, the proportion of primordial follicles was found to have decreased in both fresh and frozen-thawed




| | Primordial follicles | Primary follicles | Secondary follicles | Antral follicles |
|---|---|---|---|---|
| **Fresh grafted** | 41.5 ± 5.2 | 43.1 ± 5.4 | 61.8 ± 21.4 | 73.84 ± 15.3 |
| **Frozen-thawed grafted** | 43.3 ± 5.1 | 44.3 ± 6.5 | 69.1 ± 16.1 | 78.5 ± 14.7 |




| | Primordial follicles | Primary follicles | Secondary follicles | Antral follicles |
|---|---|---|---|---|
| **Fresh grafted** | 45.9 ± 5.54 | 53.9 ± 7.05 | 121.5 ± 53.1 | 321.9 ± 94.0 |
| **Frozen-thawed grafted** | 47.9 ± 5.2 | 54.7 ± 7.0 | 137.7 ± 55.7 | 340.9 ± 103.3 |

**Fig. 4** (**a**) Oocyte diameter of preantral and antral follicles from fresh and frozen-thawed grafted ovarian tissue. (♦) Fresh grafted and (■) frozen-thawed grafted ovarian tissue. Values shown as mean±SD. (**b**) Follicular diameter of preantral and antral follicles from fresh and frozen-thawed grafted ovarian tissue. (♦) Fresh grafted and (■) frozen-thawed grafted ovarian tissue. Values shown as mean±SD

grafts, while the percentage of primary and secondary follicles increased. Similar results were previously published by our group after short-term xenografting [13, 16], and by others after long-term xenografting [5, 15]. These studies also reported that the percentage of preantral follicles (primordial, primary and secondary) did not differ between fresh and frozen-thawed grafts.

A similar increase in oocyte and follicle diameters was observed after grafting from both fresh and frozen-thawed groups. In addition, it was noted that the morphology of grafted preantral follicles from frozen-thawed tissue was similar to that in fresh grafted and non-grafted tissue, as reported after 3 weeks of xenografting [13, 16]. However, further ultrastructural studies need to be performed to further compare fresh and frozen thawed grafts [4].

## Antral follicle population

The number of antral follicles found after xenografting of fresh and frozen-thawed ovarian tissue was similar to that in previous reports in the literature [6, 14, 26–30], except in studies by Gook et al. [9, 21] and Soleimani et al. [10], who obtained a higher number of antral follicles. It is important to note that we did not observe any influence of the animal on the number of antral follicles identified in fresh and frozen-thawed ovarian fragments.

The proportion of follicles showing an antral cavity tended to be higher in fresh grafts (6/759) than in frozen-thawed grafts (10/2566). However, due to the low proportion of follicles reaching the antral stage, further studies are needed to draw conclusions on antral follicle proportions. A number of studies [5, 14, 15] have compared human

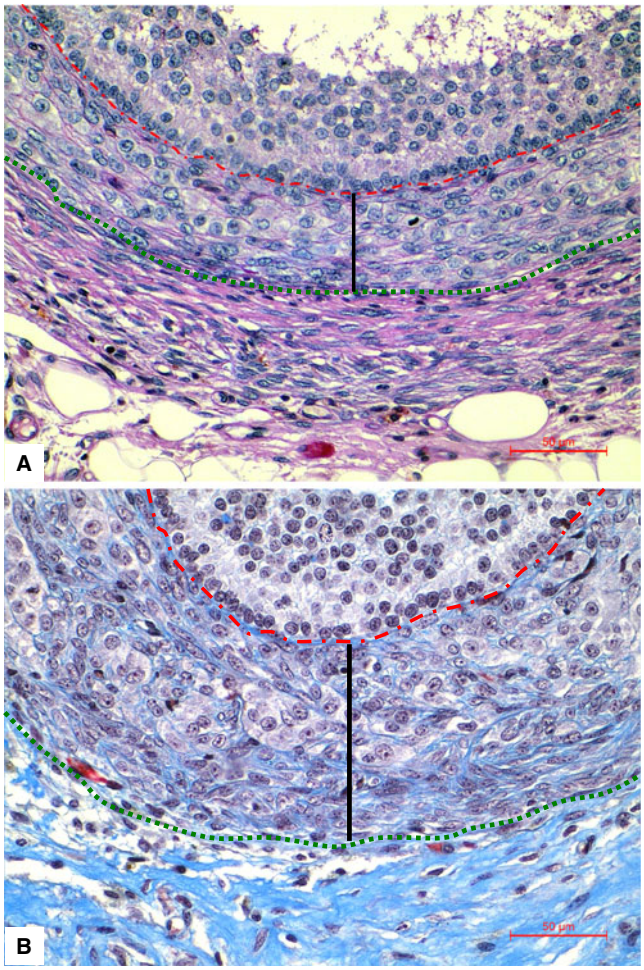



**Fig. 5** (**a**) Periodic acid-Schiff (PAS) staining on antral follicles from fresh grafted ovarian tissue. Theca cells (TCs) were differentiated from granulosa cells (GCs) due to basement membrane staining (▬ ▪ ▬) by PAS, which also stained collagen among the stromal cells (SCs). Since collagen fibers are present in higher proportions in stromal tissue than in the theca layer, the delimitation between these two cell types was clearly identified (▪▪▪▪▪). The theca layer was measured between the basement membrane and the boundary between the theca and stromal cells (▬▬). (**b**) Masson's trichrome (TRI) staining on antral follicles from fresh grafted ovarian tissue. Theca cells (TCs) were differentiated from stromal tissue by TRI, which strongly stained blue the collagen fibers present in higher numbers among the stromal cells (SCs)

follicles from fresh and frozen-thawed ovarian tissue after long-term xenografting, but only Gook et al. [14] obtained antral follicles from both groups. They reported that the number of antral follicles found after grafting was similar in fresh and frozen-thawed fragments.

Overall, antral follicles observed in this study looked healthy. Unlike Gook et al. [21], who reported variations in patterns of structural characteristics, we did not observe any major differences in morphological features of antral follicles between fresh and frozen grafts, apart from the thickness of the theca cell layer. Antral follicles from frozen-thawed grafted tissue indeed exhibited a thinner layer of theca cells than follicles from fresh grafted tissue. However, since the current findings are based on histological identification of the theca layer, with delineation of boundaries by PAS and Masson's trichrome colorimetric staining, further studies using theca-specific markers are needed to confirm theca cell identity. In particular, it would be interesting to evaluate whether theca cells from antral follicles originating from fresh and frozen-thawed xenografts express enzymes involved in androgen production, such as acute regulatory protein (StAR) and 3-beta hydroxysteroid dehydrogenase, as well luteinizing hormone receptors. The occurrence of corpora lutea in both graft types indicates that, in some grafts at least, theca cells retain the ability to luteinize.

An ultrastructural study carried out by our group [31] also showed a lack of concentric arrangement of perifollicular tissue forming the theca layers in secondary follicles after grafting of cryopreserved ovarian tissue. Such findings could be due to the deleterious effect of cryopreservation on the ovarian stroma [5, 31–35] and granulosa cells [32, 36, 37], since these cell populations are directly involved in the formation of the theca layer. During follicular development, granulosa cells stimulate the differentiation of stromal cells into theca cells [38, 39] through expression of different factors, such as kit ligand [40] and insulin-like growth factor (IGF)-I and/or -II [41]. Although the immature oocyte appears to be well maintained after cryopreservation [32, 36], different studies have reported that the freezing procedure negatively affects stromal cells [31–35] and granulosa cells [32, 36, 37]. It might therefore be expected that death of the theca cell precursor population and granulosa cells that express factors involved in the differentiation process could have a detrimental effect on the formation of the theca cell layer.

In conclusion, this study shows that although freezing and thawing procedures do not have an impact on initial follicular development, they affect the morphology of antral follicles, specifically the thickness of the theca cell layer. Since theca cells are implicated in follicular development, providing structural support for follicles and synthesizing androgens [41], a change in their population (number and/or function) could lead to failure of follicular development and function.

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