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FERTILITY PRESERVATION

Can frozen-thawed human ovary withstand refreezing-rethawing in the form of cortical strips?

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Abstract

Purpose The aim of this study was to elucidate whether ovarian tissue is able to withstand a double freezing-thawing procedure.

Methods Human ovarian cortical biopsies from 4 thawed whole ovaries were divided into 4 experimental subgroups: (a) frozen-thawed non-grafted group, (b) frozen-thawed xenografted group, (c) refrozen-rethawed non-grafted group, and (d) refrozen-rethawed xenografted group. Xenografting was performed using 8 severe combined immunodeficient mice for a total duration of 21 days. The following analyses were conducted: classic hematoxylin and eosin staining, Ki67 immunolabeling, transmission electron microscopy, Masson's green trichrome, and double CD34 immunostaining.

Results Morphologically normal preantral follicles were detected in all groups. We observed a dramatic decline of more than 65% in early preantral follicle survival rates after grafting of both frozen-thawed ($p < 0.0001$) and refrozen-rethawed ($p < 0.0001$) ovarian tissue. However, mean follicle densities remained comparable between the frozen-thawed and refrozen-rethawed non-grafted groups, as well as both grafted groups. Equivalent proportions of proliferating early preantral follicles were identified in frozen-thawed and refrozen-rethawed samples, whether the tissue was grafted or not. Furthermore, we did not observe any significant difference in atretic follicle rates between any of the four groups, and the ultrastructural quality of follicles appeared unaffected by the refreezing procedure. Similar proportions of fibrosis were noted in the frozen-thawed and refrozen-rethawed groups, irrespective of grafting. Finally, no significant differences were witnessed in terms of vascularization.

Conclusion We were able to demonstrate, for the first time, that refrozen-rethawed ovarian tissue has the same functional characteristics as frozen-thawed ovarian tissue.

Keywords Refreezing · Ovarian tissue cryopreservation · Fertility preservation · Xenografting · Electron microscopy

Introduction

Over the past two decades, major advances in cancer diagnosis and therapy have resulted in an overall decline in cancer mortality rates of 27% [1], significantly extending the life expectancy of cancer survivors. Unfortunately, as cancer treatments like chemotherapy and/or radiotherapy are highly toxic to the gonads, they threaten ovarian function in both girls and women, subsequently leading to infertility [2–4]. There are now a number of fertility preservation options that can be proposed to patients, with embryo, oocyte, and ovarian tissue cryopreservation topping the list as the most widely established techniques [4, 5].

Ovarian tissue cryopreservation is the only available option for prepubertal girls or when life-saving therapy cannot be postponed [4–7]. It is also the only alternative that allows both restoration of fertility and resumption of ovarian endocrine function, avoiding the morbidity and mortality associated with premature menopause [8]. Ovarian tissue cryopreservation and transplantation in the form of cortical strips has recently been endorsed by the American Society for Reproductive

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Medicine (ASRM) [9]. Moreover, researchers all over the world are continually working on improving the technique in order to boost its efficiency [4, 10, 11], extend the application to subgroups of patients (e.g., Turner patients) [12, 13], and ensure the safety of the procedure particularly in cancer patients [14, 15].

Nevertheless, it was not so long ago that cryopreservation and transplantation of ovarian cortical fragments had not yet proved its worth, with the first live birth taking place in 2004 (Belgium) [16]. Back then, researchers were investigating different techniques to preserve ovarian tissue, including whole ovary cryopreservation and grafting, which resulted in successful vascular transplantation in sheep [17–23], yielding healthy offspring [18, 22, 23]. In humans, several studies demonstrated the efficacy of cryopreservation protocols for whole ovary preservation [24–26], but only fresh whole ovaries were actually transplanted [27–30], yielding one live birth [30]. Today, patients who had their whole ovaries frozen back in time are returning to the oncofertility unit to use their frozen organs. However, researchers are still struggling with the risk of vascular clotting when grafting frozen-thawed whole ovaries, which may lead to loss of the entire organ [22, 31].

In this study, we propose an original way of managing these patients who had a whole ovary cryopreserved a decade ago, in order to allow them to safely use their frozen tissue by thawing the whole ovary and dissecting it into cortical strips. One third of these cortical strips could then be transplanted back to the patient, avoiding the risk of losing the whole organ upon vascular transplantation, while the remaining cortical strips could be refrozen in order to allow successive transplantations and hence increase the chances of motherhood. In addition, the fact that human ovarian tissue can be refrozen may potentially open up new research perspectives in the field of fertility preservation. The present study was therefore initiated to elucidate whether human ovarian tissue is able to actually sustain whole ovary freezing-thawing, followed by refreezing-rethawing of cortical strips.

Materials and methods

Experimental design

In order to study the viability of human ovarian tissue after refreezing, ovarian biopsies from 4 thawed whole ovaries were divided into 4 experimental subgroups: (a) frozen-thawed non-grafted group, (b) frozen-thawed xenografted group, (c) refrozen-rethawed non-grafted group, and (d) refrozen-rethawed xenografted group (Fig. 1). The tissue was xenografted to severe combined immunodeficient (SCID) mice for a total duration of 21 days, as described below. The following analyses were conducted in the four groups: (a) follicle density and survival rates by classic hematoxylin and eosin staining, (b) Ki67 immunolabeling to assess follicle proliferation, (c) ultrastructural analysis using transmission electron microscopy (TEM), (d) Masson's green trichrome to evaluate the proportion of fibrotic areas, and (e) double CD34 immunostaining to investigate vessels of both murine and human origin.

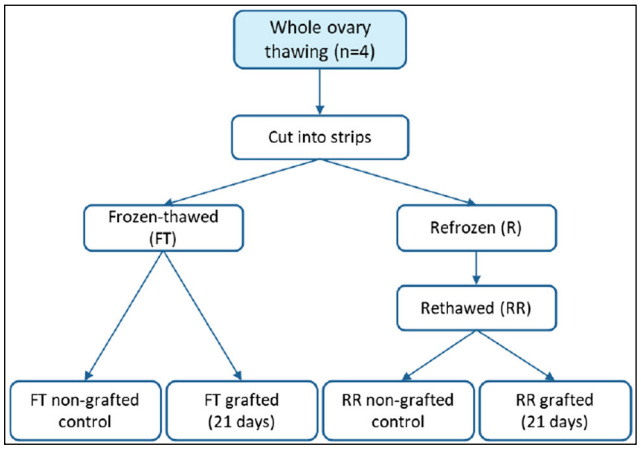

Fig. 1 Experimental design

Ethical approval

The Institutional Review Board of the Universite Catholique de Louvain (reference 2012/23MAR/125) approved the use of human ovarian tissue for this study after obtaining written informed consent from all subjects. Animal welfare guidelines were followed, and the protocol was approved by the Committee on Animal Research of the Universite Catholique de Louvain (reference 2018/UCL/MD/40).

Patients

Between 2004 and 2007, 4 patients aged 25–34 years underwent cryopreservation of their whole ovaries, with careful dissection of the vascular pedicle in each case [32]. At the time, cryopreservation of ovarian tissue in the form of a whole ovary was considered a possible way of preserving fertility, since cortical strips had not yet proved their superiority in terms of efficacy [18, 26, 33]. Patients gave their informed consent for whole ovary cryopreservation, subsequently donating their unused tissue to research. All 4 women were suffering from non-ovarian cancers and wished to safeguard their fertility before commencing cancer treatment. The freezing procedure applied at the time was the one described by Martinez-Madrid et al., which was shown to be effective [24, 25]. Briefly, the ovary was first flushed through its pedicle with a heparinized solution and then transported to the laboratory. There, it was perfused and immersed in a bath containing a cryoprotective solution composed of Leibovitz-15 medium (Gibco, Scotland), supplemented with 10%

dimethyl sulfoxide (DMSO) (Sigma, MO) and 0.4% human serum albumin (Red Cross, Belgium) for 5 min at 4 °C. Ovarian perfusion was performed using a precalibrated pump (Terumo Corporation, Japan) maintaining a flow rate of 2.5 ml/min. After pre-equilibration of the whole ovary in its cryovial containing the cryoprotective solution for 10 min at 4 °C, the cryovial was placed in a 5100 Cryo 1 °C freezing container (Nalgene, VWR, Belgium) precooled at 4 °C and deposited in a − 80 °C freezer. This confers a theoretical cooling rate of − 1 °C/min. After 24 h at − 80 °C, the cryovial containing the ovary was transferred to liquid nitrogen for storage.

Thawing of whole ovaries

The frozen whole ovaries were thawed according to our previously established protocol [24, 25]. In short, each cryovial was directly transferred from liquid nitrogen to a 60 °C water bath until the ice surrounding the ovary melted (taking approximately 8 min), allowing us to retrieve the frozen whole ovary from its cryovial. To flush out the cryoprotectant, the ovary was bathed and perfused for 30 min using three consecutive thawing media containing decreased sucrose concentrations to avoid osmotic injury. Ovarian perfusion was performed as explained above. Once thawed, the whole ovary was dissected in order to separate the cortex and cut it into strips of approximately 5 × 7 × 1 mm. The cortical strips were distributed among the experimental subgroups, with 2 strips undergoing immediate fixation (frozen-thawed non-grafted group) either in 4% formaldehyde for histological analysis or 2.5% glutaraldehyde for ultrastructural assessment. Two additional strips were xenografted to SCID mice (frozen-thawed grafted group), and the remaining fragments were refrozen. The time required for whole ovary thawing and cortical strip refreezing was less than 1 h in total.

Refreezing and rethawing of cortical strips

Refreezing and rethawing of cortical strips was done according to our routine slow-freezing and fast-thawing procedure [34]. Briefly, the fragments were placed inside cryovials filled with freezing medium composed of 1.5 M DMSO (Sigma, MO). The cryovials were slow frozen in a programmable freezer (Kryo 10, Series III, Planer, UK), before being transferred to liquid nitrogen for storage. For thawing, the cryovials were first maintained at room temperature for 2 min, then immersed in a water bath at 37 °C for another 2 min, and finally washed three times in fresh HEPES-MEM medium (Gibco, Scotland). One cryovial per patient was rethawed, 2 fragments were xenografted (refrozen-rethawed grafted group), and the remaining tissue was immediately fixed in either 4% formaldehyde or 2.5% glutaraldehyde (refrozen-rethawed non-grafted group).

Xenografting

Eight female SCID mice (Charles River Laboratories, France) were used for the transplantation experiments and were bred as described elsewhere [35]. A median incision was made in the abdominal wall of the animal, and human ovarian implants were attached to the inner side of the peritoneum with 1–2 stitches of 6/0 Prolene (Ethicon, Johnson & Johnson International, USA). The abdominal wall was then closed using 6-0 Prolene (Ethicon, Johnson & Johnson International, USA). The mice were kept in sterile conditions and euthanized by cervical dislocation after 21 days. All ovarian grafts were recovered and fixed for further histological and ultrastructural analysis.

Histology

Samples for histology were embedded in paraffin and serially sectioned at a thickness of 5 μm. Every fifth section was stained with hematoxylin and eosin (Merck, Germany). Slides were digitized using the Pannoramic P250 Flash III scanner (3DHISTECH, Hungary) and analyzed with CaseViewer (3DHISTECH, Hungary). Only morphologically normal follicles with a visible oocyte were taken into consideration for further analysis [36].

Follicle outcomes

Follicle density

Follicle density was established as the total number of follicles per mm^3 by counting ovarian follicles in twelve sections per sample spaced 50 μm apart, thereby avoiding counting preantral follicles twice [37]. Follicles were further classified according to stage into primordial, intermediate, primary, or secondary, based on specific criteria [38]. Primordial and intermediate follicles were defined as early preantral follicles.

Follicle survival rate

We evaluated early preantral follicle survival rates by calculating remaining follicle density (early preantral follicles/mm^3) after grafting and normalizing it to early preantral follicle density in non-grafted controls (considered as 100%).

Follicle atresia

Follicle atresia was assessed using specific morphological criteria, namely, ooplasm eosinophilia, irregularly shaped follicles and/or oocytes, granulosa cell pyknosis, cytoplasmic contraction, and/or the presence of vacuoles [38].

Follicle proliferation

Nine sections per sample spaced 50 μm apart were subjected to anti-human Ki67 immunohistochemical staining, as previously described [39]. Antibodies used were mouse anti-human Ki67 (1/100, clone MIB, mAb ref. M7240, Dako, Belgium), followed by EnVision goat anti-mouse immunoglobulin G (IgG; 1:2 dilution, Dako, Belgium). Negative controls were incubated solely with the secondary antibody**.** Only early preantral follicles were considered for analysis and subsequently classified into 2 categories: resting, when all granulosa cells were negative for Ki67, or growing, with at least 1 granulosa cell positive for Ki67.

Transmission electron microscopy

In order to conduct ultrastructural analyses in all subgroups, tissue samples were fixed in 2.5% glutaraldehyde in phosphate buffer. After fixation, samples were processed as detailed elsewhere [40]. They were further visualized and photographed using a Zeiss EM10 electron microscope operating at 80 kV (Zeiss, Germany). Analysis was based on the morphological characteristics of granulosa cells and oocytes, along with evaluation of their organelles, basal and plasmatic membranes, and the nuclear envelope. Characteristics of chromatin, such as shape, morphology, distribution, and electron density, were also investigated.

Fibrosis

At least 2 slides per sample spaced 200 μm apart were stained with Masson's green trichrome to evaluate proportions of fibrotic areas in the ovarian tissue using Visiopharm® software (version 2017.2). Visiopharm® software is a semi-automated histomorphometric computer-based image analysis system. To quantify proportions of tissue fibrosis, we developed an algorithm allowing recognition of cells and the extracellular matrix (ECM) using Author™ in Visiopharm®. Areas positive for Masson's green trichrome (expressed as ECM) were segmented by watershed transformation using cells as segmentation points. A threshold method then identified faintly or strongly stained ECM areas. This original approach resulted in identifying fibrotic areas with strongly stained ECM content but low cell density. Manual corrections were included when necessary. Results are expressed as the proportion of fibrotic surface area out of total section area.

Vascularization

Vessels of both murine and human origin were assessed by double CD34 immunostaining, which stains endothelial cell membranes, as described in an earlier publication [41]. Primary antibodies used were rat anti-mouse CD34 (1:100, clone MEC 14.7, Hycult Biotech, the Netherlands) and mouse anti-human CD34 (1:500, clone QBend/10, Biocare Medical, the Netherlands), followed by rabbit anti-rat biotinylated IgG (1:100, BA 4001, Vector Laboratories, UK) and alkaline phosphatase-conjugated goat anti-mouse IgG (1:300, Jackson Immunoresearch, UK), respectively. Negative controls were incubated with the secondary antibody only. One section per sample was assessed, and vessels were considered positive when at least one endothelial cell showed staining. Results are expressed in terms of vessel density.

Statistical analysis

GraphPad Prism (GraphPad software, version 8.1.2, USA) was used for statistical analysis. For follicle proliferation and atresia analyses, only patients with follicles in all four groups were taken into account in order to reduce bias from extreme values. Sidak's test was carried out after one-way repeated measures analysis of variance (ANOVA) in all investigations (parametric statistics), except for follicle density analysis, where Friedman's test followed by Dunn's correction were applied (non-parametric statistics). Results are expressed as mean ± SEM. A p value < 0.05 was considered statistically significant.

Results

Follicle outcomes

Follicle density

Morphologically normal preantral follicles were detected in all four groups. Mean follicle density showed a decline after grafting in the frozen-thawed group, but it was not significant ($p = 0.684$) (Table 1). The same was evidenced in the refrozen-rethawed group, but again it did not reach statistical significance ($p = 0.055$). Frozen-thawed and refrozen-rethawed non-grafted groups showed comparable mean follicle densities ($p > 0.999$), and there was no significant difference between the two grafted groups ($p > 0.999$). Furthermore, we witnessed high variability in follicle density between patients.

Follicle survival rate

We observed a significant decline of more than 65% in the early preantral follicle survival rate after grafting of frozen-thawed ($p < 0.0001$) and refrozen-rethawed ($p < 0.0001$) ovarian tissue (Table 1).

Table 1 Results

	Frozen-thawed non-grafted	Frozen-thawed grafted	Refrozen-rethawed non-grafted	Refrozen-rethawed grafted	*p* value
Follicle density (#/mm^3)	273.5 ± 197.7	208.1 ± 143.1	261.8 ± 133.4	84.9 ± 48.4	Ns
Follicle survival rate	100%a	34.75% ± 14.3%b	100%c	20.75% ± 1.75%d	a ≠ b, $p < 0.0001$ c ≠ d, $p < 0.0001$
Follicle atresia	13.2% ± 4.9%	5.7% ± 3.3%	11.4% ± 5.5%	11.4% ± 2.1%	Ns
Follicle proliferation	5.5% ± 0.9%a	68.2% ± 2.5%b	5.1% ± 0.9%c	62.7% ± 4.2%d	a ≠ b, $p = 0.01$ c ≠ d, $p = 0.02$
Fibrosis	3% ± 1.3%a	51% ± 6.7%b	3.8% ± 0.9%c	66.2% ± 9.4%d	a ≠ b, $p = 0.01$ c ≠ d, $p = 0.02$
Total vascular density (#/mm^2)	156.4 ± 37.9	131 ± 18.1	107.7 ± 15.8	176.6 ± 56	Ns
Human vascular density (#/mm^2)	/	102.7 ± 12.3	/	149 ± 51	Ns
Murine vascular density (#/mm^2)	/	28.4 ± 7.7	/	27.6 ± 9.2	Ns

Data are expressed as mean ± SEM. All statistically significant differences are highlighted by different letters in the *p* value column (a ≠ b and c ≠ d). *Ns* not significant

Follicle atresia

There were no significant differences in proportions of atretic follicles within the frozen-thawed ($p = 0.872$) and refrozen-rethawed ($p > 0.999$) groups individually (Table 1). Likewise, similar atretic follicle rates were found between frozen-thawed and refrozen-rethawed non-grafted ($p = 0.720$) and grafted ($p = 0.664$) tissues, respectively.

Follicle proliferation

Although significantly higher proliferation rates were detected after grafting in both frozen-thawed ($p = 0.012$) and refrozen-rethawed ($p = 0.023$) tissues, proportions remained equivalent when comparing the frozen-thawed and refrozen-rethawed non-grafted ($p > 0.999$) and grafted ($p = 0.884$) groups, respectively (Table 1).

Ultrastructural assessment

A total of 17 follicles were analyzed by LM and TEM in the frozen-thawed ($n = 4$) and refrozen-rethawed ($n = 13$) groups. After grafting, no follicles were detected, probably due to the small amount of tissue fixed for TEM. All analyzed follicles were at primordial/primary stages. Seventy-five percent (3/4) of follicles in the frozen-thawed group were classified as well preserved, compared with 76.9% (10/13) in the refrozen-rethawed group.

Well-preserved follicles

By LM, well-preserved follicles appeared regularly rounded in shape, with a diameter ranging between 40 and 80 μm. They all contained an oocyte completely surrounded by a single layer of flattened or cuboidal follicular cells (Fig. 2a, b, inset). By TEM, the oocytes in these healthy-looking follicles showed a large vesicular nucleus, with 1 or 2 nucleoli and finely dispersed chromatin (Fig. 2a, c). Cytoplasmic organelles were aggregated around the nucleus (Balbiani body) and were mainly identified by small mitochondria with a pale matrix and a few transverse or arch-like cristae (Fig. 2c, d). Mitochondria were often clustered in clouds, and some were in close contact with vesicles or forming rosettes around an electron-dense amorphous substance (Fig. 2d, e). Free ribosomes, smooth (SER) and rough endoplasmic reticulum (RER) cisternae, the Golgi apparatus, multivesicular bodies, and compound aggregates were also observed inside the oocyte cytoplasm (Fig. 2e). At the oocyte-follicular cell interface, close interdigitations were detected between oocyte microvilli and follicular cell projections (Fig. 2f). Follicular cells had a high nucleus/cytoplasm ratio, and the nucleus was large and irregularly shaped, with low levels of condensed chromatin along its membrane (Fig. 2g, h). In some follicles, the quantity and quality of condensed chromatin varied between cells in the same follicle. Scattered follicular cells exhibited more condensed chromatin and a darker cytoplasmic matrix (Fig. 2h), while others showed more distended chromatin, less patchy along the nuclear membrane, and a clearer cytoplasm. Cytoplasmic organelles had intact membranes, and mitochondria were elongated with

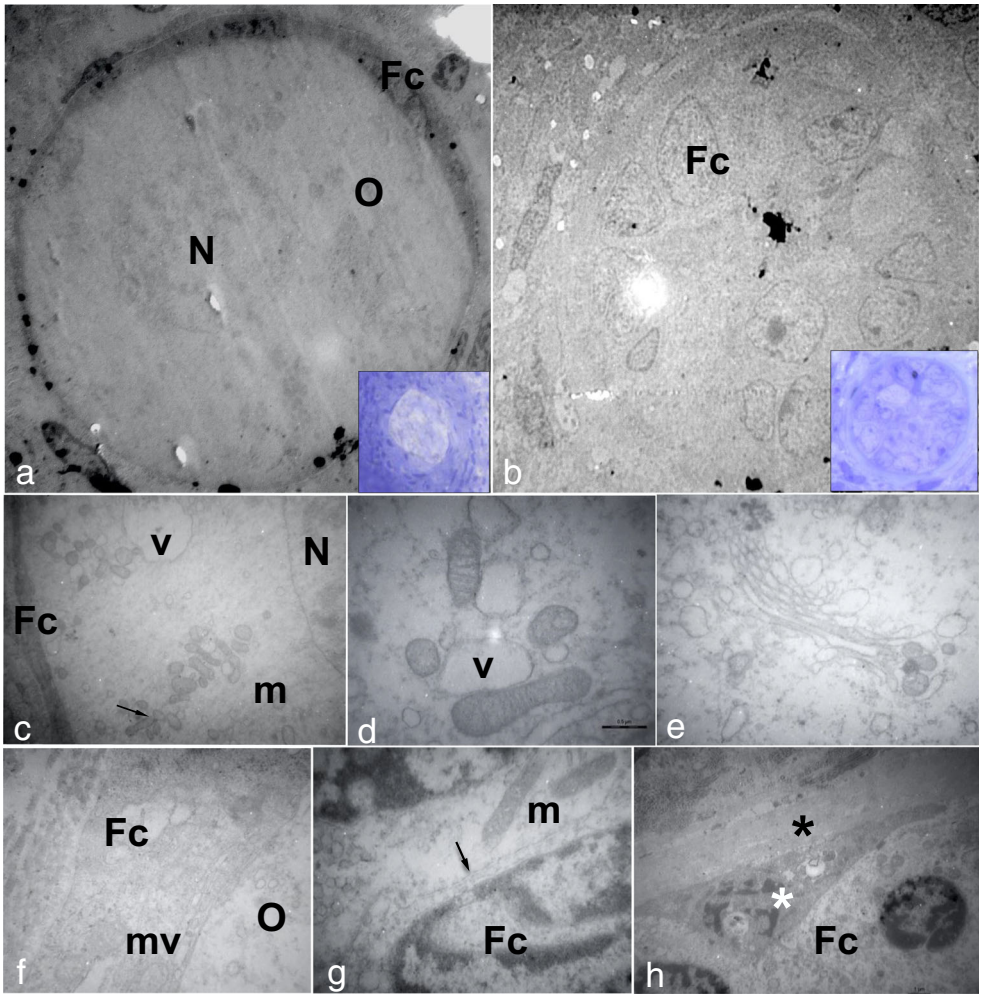

Fig. 2 Transmission electron microscopy illustrations of healthy-looking follicles. (**a**) Primordial follicle and (**b**) intermediate follicle, with their respective semithin sections illustrated in the insets. (**c**) Oocyte cytoplasm containing numerous mitochondria clustered in clouds. Note some mitochondria arranged in rosettes around a dense substance (arrow). (**d**) Mitochondria with a few transverse cristae in close contact with vesicles. (**e**) The Golgi apparatus in the oocyte cytoplasm. (**f**) Oocyte microvilli in contact with follicular cell projections. (**g**) Two follicular cells in close contact (arrow). Indented nuclei show condensed chromatin along the nuclear membrane. In the cytoplasm, elongated mitochondria are visible with transverse cristae. (**h**) Follicular cells lie on a continuous basal membrane (black asterisk). One follicular cell shows a darker cytoplasm and more condensed chromatin (white asterisk). Fc, follicular cell; O, oocyte; N, nucleus; m, mitochondria; v, vesicle; mv, microvilli. Original magnification: LM, × 20 (**a**, inset), × 40 (**b**, inset); TEM, × 700 (**a**, **b**), × 3000 (**c**, **h**), × 7000 (**e**), × 12,000 (**d**, **f**, **g**)

transverse cristae (Fig. 2g). Follicular cells lay on a continuous basal membrane, separating follicles from the surrounding stromal compartment.

Altered follicles

By LM and TEM, one follicle from the frozen-thawed non-grafted group (25%) and three follicles from the refrozen-rethawed non-grafted group (23.1%) showed abnormal structural and ultrastructural features typical of atretic follicles. Follicular cells displayed an irregular shape (Fig. 3a, b, inset), large intercellular spaces, oocyte and follicular cell detachment (Fig. 3a, c), excess lipids in the cytoplasm (Fig. 3d), shrinkage, and nuclear and cytoplasmic condensation (Fig. 3b, e).

Fibrosis

Grafting induced significantly higher rates of fibrosis within frozen-thawed ($p = 0.012$) and refrozen-rethawed ($p = 0.022$) ovarian tissues individually (Table 1). However, proportions of fibrosis remained similar between the frozen-thawed and refrozen-rethawed non-grafted ($p = 0.765$) and grafted ($p = 0.127$) groups, respectively.

Vascularization

In terms of total vessel density, no significant difference was observed after grafting of frozen-thawed ($p = 0.953$) or refrozen-rethawed ($p = 0.689$) tissues (Table 1). Similar results were also obtained in the frozen-thawed and refrozen-

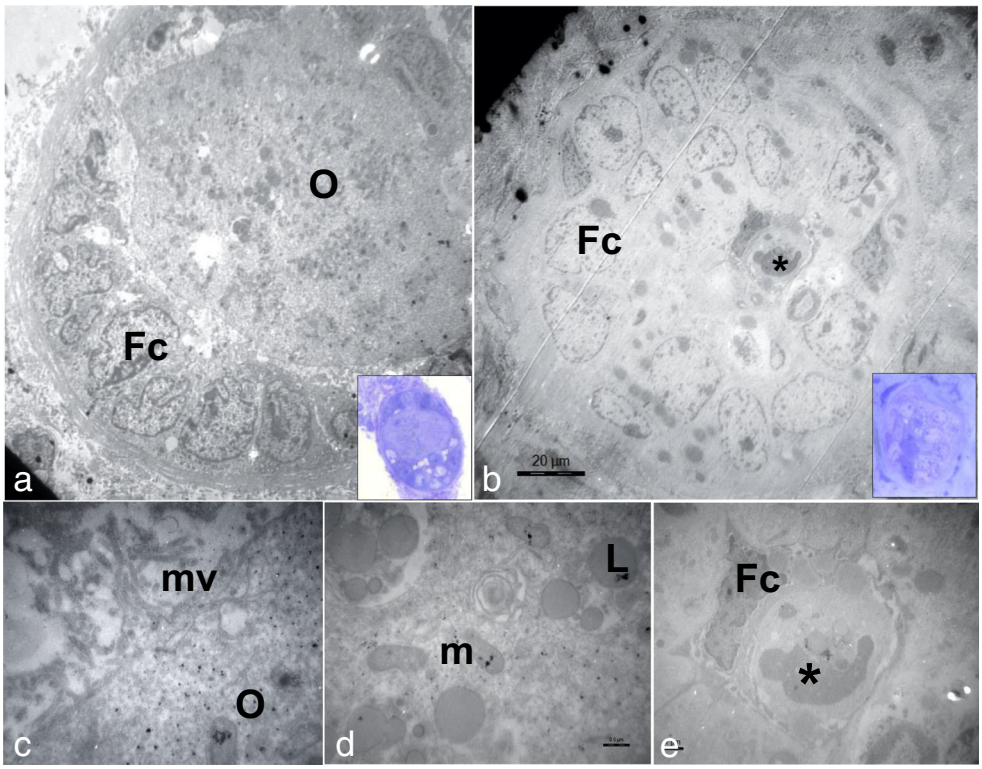

Fig. 3 Transmission electron microscopy illustrations of altered-looking follicles. (**a**) Intermediate follicle and (**b**) primary follicle, with their respective semithin sections illustrated in the insets. (**c**) Detachment between oocyte microvilli and follicular cell projections. (**d**) Increased number of lipid droplets in the oocyte cytoplasm. (**e**) Pyknosis and shrinkage of follicular cells (asterisk). Fc, follicular cell; O, oocyte; mv, microvilli; L, lipid droplet; m, mitochondria. Original magnification: LM, × 20 (**a**, inset), × 40 (**b**, inset); TEM, × 700 (**a**, **b**), × 3000 (**e**), × 7000 (**c**, **d**)

rethawed non-grafted ($p = 0.806$) and grafted ($p = 0.954$) groups, respectively. In addition, no significant differences were evidenced in either of the grafted groups with regard to human ($p = 0.939$) or murine ($p = 0.916$) vessel density.

Discussion

The aim of this study was to assess the viability of human ovarian tissue subjected to whole ovary freezing-thawing, followed by refreezing-rethawing of cortical strips and subsequent xenografting. The protocol used to freeze whole ovaries back in time was the one established by Martinez-Madrid et al. [24, 25]. This protocol proved its efficacy, with the authors describing high survival rates of follicles, small vessels and stromal cells, and a normal histological structure in all ovarian components after thawing compared with fresh biopsies taken before freezing [24]. In a follow-up study, the same team demonstrated that their protocol was not associated with any signs of apoptosis or ultrastructural alterations in any cell types after thawing [25].

Follicle outcomes

Morphologically normal preantral follicles were identified in the tissue of all patients after refreezing-rethawing and subsequent xenografting. In the non-grafted groups, mean follicle density was maintained, and the early preantral follicle pool showed a similar proliferation rate after refreezing to that observed after a single freezing procedure. This suggests that refreezing does not affect follicle density nor follicle growth. In the grafted groups, no significant difference was evidenced when comparing their mean follicle densities with their respective non-grafted controls. This lack of significance may be explained by the heterogeneous distribution of follicles in human ovarian cortex and large variations in follicle density from patient to patient [42–44]. Nevertheless, the process of follicle burnout, which is widely acknowledged after transplantation of frozen-thawed ovarian tissue [45–48], occurred at similar rates in the refrozen-rethawed grafted group. Indeed, in both the frozen-thawed and refrozen-rethawed groups, we observed a dramatic drop in the early preantral follicle survival rate, associated with a significant increase in the proportion of proliferating early preantral follicles after grafting. All in all, this demonstrates that the refreezing procedure does not affect the follicle's ability to grow nor does it impair or accelerate follicle burnout more than the once-only freezing protocol.

Several studies have also shown serious but similar levels of follicle loss (> 65%) after grafting of fresh and frozen-thawed human ovarian fragments [35, 49], indicating that follicle loss is due to warm ischemia following avascular transplantation of cortical strips rather than cryopreservation itself. These assumptions were further confirmed by Van Eyck et al. [50], who identified a hypoxic period until day 5 of transplantation, followed by gradual reoxygenation. In our study,

follicle loss evidenced after xenografting of human ovarian tissue from frozen-thawed whole ovaries (> 65%) is in line with that documented in the literature after xenografting of frozen-thawed human cortical strips (50–90%) [35, 47, 48]. This suggests that the follicle loss is more likely the result of warm ischemia after avascular transplantation than any hypothetical supplementary damage that may have been caused by whole ovary freezing.

Atresia and ultrastructural assessment

Histological evaluation of atresia showed no difference in atretic follicle rates between refrozen-rethawed tissues and their respective frozen-thawed controls, even after grafting. This proves that refreezing does not induce higher rates of follicle atresia. On the other hand, TEM provides essential information on cell integrity and cellular interactions, crucial for normal folliculogenesis, and represents a valuable tool in the field of reproductive medicine [51]. Our findings reveal that the refreezing procedure does not markedly impact the ultrastructural quality of follicles, with similar rates of follicles exhibiting equally well-preserved features after refreezing as after single freezing. Interestingly, ultrastructural analysis of non-grafted tissue showed the same atretic features after both thawing and rethawing as those typically described in atretic follicles physiologically found in fresh ovarian tissue [40, 52, 53]. Moreover, the proportion of atretic follicles detected after rethawing was similar to that encountered after thawing of whole ovaries, which is consistent with the atretic follicle rate reported in the literature after thawing of cortical strips [40]. Taken together, this demonstrates that refreezing does not harm follicle ultrastructure.

Fibrosis

Stromal compartment analysis in non-grafted tissue showed a very similar proportion of fibrotic surface area after refreezing to that observed after once-only freezing. This indicates that refreezing does not induce any fibrotic remodeling of the tissue when performed in good conditions (< 1 h between whole ovary thawing and cortical strip refreezing). Although very little literature is available on tissue refreezing using liquid nitrogen, a former publication revealed that refrozen canine aortic valve tissue showed comparable histological features in conjunctive tissue to those after single freezing [54], which is in line with our observations. After grafting, we detected a significant increase in the rate of fibrosis in the refrozen-rethawed group but at a similar proportion to the frozen-thawed group. Indeed, fibrosis has already been described repeatedly after transplantation of frozen-thawed ovarian tissue [35, 46, 47, 55], demonstrating that the major cause in this case is grafting and not refreezing.

Vascularization

Since cortical strip transplantation is achieved by avascular grafting, revascularization of ovarian implants is a key process to ensure and sustain tissue survival [50, 56–60]. Interestingly, no significant difference was found in terms of vessel density between the two non-grafted groups, suggesting that refreezing does not affect the vascular compartment. Moreover, no significant difference was evidenced in vascular patterns when comparing frozen-thawed and refrozen-rethawed grafted tissues, proving that revascularization of ovarian implants 21 days after grafting is not altered by refreezing.

Furthermore, in recent years, we have stressed the importance of host vessel invasion (murine vessels) in the initiation of vessel establishment in ovarian grafts [57–59]. Host angiogenesis is mainly induced by proangiogenic factors secreted by ovarian implants in response to hypoxic conditions triggered by the avascular grafting procedure [10, 57]. Our results show similar rates of host vessel density after grafting of both frozen-thawed and refrozen-rethawed ovarian tissue. It therefore appears that refrozen-rethawed implants are able to synthesize proangiogenic factors required for revascularization.

Potential indications

First, we describe an original way of managing patients who have had a whole ovary cryopreserved, without risking loss of the entire organ following vascular transplantation. We propose dissecting the thawed whole ovary into cortical strips and refreezing the remaining non-grafted fragments, thereby enabling further transplantations and increasing the chances of motherhood and repeated pregnancies.

Second, as ovarian tissue cryobanks do not usually have enough patients suffering from the same disease to conduct powerful studies, and amounts of human ovarian tissue available for research purposes are very limited, refreezing could allow more sparing use of this precious human tissue and promote collaborations between centers. Indeed, collaborations are crucial to accomplishing large-scale and conclusive studies in the field, especially when the aim is to extend the indications or ensure the safety of ovarian tissue cryopreservation and transplantation in subgroups of patients suffering from low-prevalence diseases (e.g., Turner or leukemia patients). To this end, one cryovial could be thawed in one institution, with several tests performed on a piece of frozen-thawed tissue, and the remaining tissue could then be refrozen and transported in liquid nitrogen to a second institution to allow further testing. In this way, collaborative studies could be done using only one cryovial per patient instead of two. Refreezing-rethawing of ovarian tissue could also be undertaken in order to allow successive studies in the same center, since one cryovial often contains a relatively large amount of

ovarian cortical tissue, which may even be considered excessive for a single study.

Conclusion

Our outcomes in terms of follicle survival, fibrosis, and vascularization were similar to what was described in the literature after grafting of frozen-thawed cortical strips [35, 46–48, 55]. To our knowledge, this is the first study to provide proof of concept that refrozen-rethawed ovarian tissue has the same functional characteristics after grafting as once-only frozen-thawed tissue. We also outline an original and safe way of managing patients who have previously had a whole ovary cryopreserved by refreezing their ovarian tissue in the form of cortical strips. Furthermore, the ability of ovarian tissue to withstand a double freezing procedure may well open up new research perspectives in the field of fertility preservation. It could promote collaborations between centers, which are essential for the development of more powerful studies in the field, and enable more judicious use of this precious human tissue, an important consideration given the low availability of the latter for research purposes. A limitation of this study could be that it was performed using frozen-thawed whole ovaries, so doubts may persist regarding the tolerance of frozen-thawed cortical strips to refreezing. However, cryopreserving an entire ovary is far more challenging than freezing cortical strips, so we hypothesize that if ovarian tissue from a frozen-thawed whole ovary is able to withstand refreezing, frozen-thawed cortical strips should at least be as tolerant to refreezing.

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Authors' contributions C.H.: Conception and design of the study, experimental procedures, analysis of results, statistical analysis, and article preparation. A.C.: Experimental procedures, analysis of results, and discussion contribution. L.C.: Experimental procedures and analysis of results and discussion contribution. T.Y.T.N.: Experimental procedures. R.M.: Experimental procedures and analysis of results. J.D.: Data evaluation, discussion contribution, and article revision. M.M.D.: Conception of the study, data evaluation, discussion contribution, and article revision.

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Data availability Not applicable.

Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

Ethics approval The Institutional Review Board of the Université Catholique de Louvain (reference 2012/23MAR/125) approved the use of human ovarian tissue for this study after obtaining written informed consent from all subjects. Animal welfare guidelines were followed, and the protocol was approved by the Committee on Animal Research of the Université Catholique de Louvain (reference 2018/UCL/MD/40).

Consent to participate Not applicable.

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