Successful vitrification and autografting of baboon (Papio anubis) ovarian tissue

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STUDY QUESTION: Can a vitrification protocol using an ethylene glycol/dimethyl sulphoxide-based solution and a cryopin successfully cryopreserve baboon ovarian tissue?

SUMMARY ANSWER: Our results show that baboon ovarian tissue can be successfully cryopreserved with our vitrification protocol.

WHAT IS KNOWN ALREADY: Non-human primates have already been used as an animal model to test vitrification protocols for human ovarian tissue cryopreservation.

STUDY DESIGN, SIZE, DURATION: Ovarian biopsies from five adult baboons were vitrified, warmed and autografted for 5 months.

PARTICIPANTS/MATERIALS, SETTING, METHODS: After grafting, follicle survival, growth and function and also the quality of stromal tissue were assessed histologically and by immunohistochemistry. The influence of the vitrification procedure on the cooling rate was evaluated by a computer model.

MAIN RESULTS: After vitrification, warming and long-term grafting, follicles were able to grow and maintain their function, as illustrated by Ki67, anti-Müllerian hormone (AMH) and growth differentiation factor-9 (GDF-9) immunostaining. Corpora lutea were also observed, evidencing successful ovulation in all the animals. Stromal tissue quality did not appear to be negatively affected by our cryopreservation procedure, as demonstrated by vascularization and proportions of fibrotic areas, which were similar to those found in fresh ungrafted ovarian tissue.

LIMITATIONS, REASONS FOR CAUTION: Despite our promising findings, before applying this technique in a clinical setting, we need to validate it by achieving pregnancies.

WIDER IMPLICATIONS OF THE FINDINGS: In addition to encouraging results obtained with our vitrification procedure for non-human ovarian tissue, this study also showed, for the first time, expression of AMH and GDF-9 in ovarian follicles.

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Key words: ovarian tissue / pre-antral follicles / baboon / vitrification / autografting

Introduction

Cryopreservation and transplantation of ovarian tissue has emerged as a promising alternative to safeguard and restore fertility in cancer patients at risk of infertility due to chemo/radiotherapy. It is particularly indicated for prepubertal girls or when cancer treatment cannot be delayed, and it has shown positive results, including restoration of endocrine function (Donnez...
sheep ovarian tissue (Wang et al., 2008) and >20 live births to date (Donnez et al., 2011; Revel et al., 2011).

Ovarian tissue cryopreservation is usually carried out using conventional freezing procedures (Donnez et al., 2011). Such methods rely on the formation of extracellular ice (Pölge et al., 1949); as this forms, the concentration of the extracellular solution increases and osmotic dehydration of cells occurs (Mazur, 1966). However, ice crystal formation can be harmful to cells, since it may destroy cell interrelations and even lead to cell death. Indeed, different studies have demonstrated the deleterious effect of freezing on ovarian tissue (Nottola et al., 2008; Schubert et al., 2008; Keros et al., 2009).

To avoid this problem, ovarian tissue fragments can be vitrified. The vitrification approach differs from freezing because there is no water precipitation and consequently no ice formation (Fahy, 1986). Instead, the water is quickly cooled, achieving a vitrified state thanks to a huge increase in viscosity during cooling (Fahy et al., 1984). Although vitrification is theoretically advantageous and there is mounting evidence for its use in human embryo and oocyte cryopreservation (Kuwayama et al., 2005; Vajta and Nagy, 2006; Gook and Edgar, 2007), studies of its application in the context of ovarian tissue are still very limited and show variable results (Gandolfi et al., 2007; Rahimi et al., 2009, 2010; Amorim et al., 2011, 2012; Oktem et al., 2011).

In our previous study (Amorim et al., 2012), we developed a new vitrification solution (VS) for human ovarian tissue, containing lower concentrations of penetrating cryoprotectants (CPAs), combined with non-penetrating CPAs. The goal was to achieve vitrification of the tissue without causing any significant damage to pre-antral follicles, notably oocytes, by high concentrations of CPAs. Our results showed that after vitrification and 1 week of xenografting to nude mice, follicles were able to survive and resume their development. Regarding the vitrification procedure itself, a novel carrier using acupuncture or insulin needles has been successfully applied to vitrify human, mouse and sheep ovarian tissue (Wang et al., 2008; Xiao et al., 2010; Fathi et al., 2011). Among the advantages of needle-immersed vitrification (Wang et al., 2008; Xiao et al., 2010) or cryopin use (Fathi et al., 2011), we can cite ease of manipulation of tissue fragments during exposure to VS and convenience. It has also been hypothesized that such carriers can increase the cooling rate (Wang et al., 2008; Xiao et al., 2010).

Based on such promising results, we decided to test our solution mimicking the clinical setting. The aim of this study was, therefore, to vitrify and autograft non-human primate ovarian tissue over the longer term. For this, we used baboons, a large primate commonly utilized as an animal model for studies of human reproduction (Stevens, 1997), as pre-antral follicle morphometry, menstrual cycles and hormone patterns during cycles are similar between humans and baboons (Stevens, 1997; Wandji et al., 1997).

Materials and Methods

Collection of ovarian tissue

Approval for this study was obtained from the Animal Care and Use Committee of the Institute of Primate Research (Nairobi, Kenya).

Five adult female baboons (mean weight 13 ± 1.9 kg) were studied at the Institute of Primate Research. All the animals were trapped in the wild and maintained in quarantine for 3 months. All tested negative for common pathogens (bacterial and viral infections as well as parasites) and were screened for tuberculosis, simian T-lymphotropic virus-I and simian immunodeficiency virus. After this period, the animals were housed in single cages and fed commercial monkey pellets (Gold Star Products, Kenya) and seasonal fruits and vegetables, with free access to water.

For the surgical procedure, the animals were anesthetized with a mixture of ketamine (Anesketin, 15 mg/kg; Eurovet NV/SA, Heusden-Zolder, Belgium) and xylazine (Bomazine 2%, 2 mg/kg; Bomac Laboratories Ltd, Auckland, New Zealand) administered intramuscularly for induction, and 1–2% halothane (Halothane; Nicholas Piramal India Ltd, Andhra Pradesh, India) with N2O/O2 (70%/30%) for maintenance.

Video-assisted laparoscopy was performed by qualified gynecologists (J. Donnez and M.M. Dolmans) by introducing a 10 mm scope (Karl Storz Company, Tuttlingen, Germany) into the umbilicus after CO2 pneumoperitoneum induction. Two trocars 5 mm in diameter were placed in the suprapubic area to facilitate pelvic exploration once the baboons were in the Trendelenburg position. One ovary was almost entirely removed and immediately transported to the laboratory in minimal essential medium + Glutamax™ (MEM, Gibco, Carlsbad, USA).

After surgery, the animals received antibiotics for 1 week (Clamoyxil LA; Pfizer, Paris, France), and pain was controlled with ibuprofen (Ketofen; Merial, Lyon, France) for 3 days.

Ovarian tissue vitrification and warming

The ovary was cut into halves and the medulla was removed. The remaining cortical area was then cut into strips (~ 8 × 3 × 1 mm) and one small fragment of ovarian tissue was immediately fixed in formalin (fresh control). A 25G (0.5 × 16 mm, BD Microlance™, BD, Drogheda, Ireland) or 29G (0.33 × 12.7 mm, BD Micro-Fine™, BD, Le Pont de Claix-Cedex, France) needle was inserted along the length of each tissue strip, according to Fathi et al. (2011), in order to facilitate handling of the ovarian strips and maximize the cooling rate.

For the vitrification procedure, we used a VS we developed previously (Amorim et al., 2012) containing 10% dimethyl sulfoxide (DMSO, Sigma, Bornem, Belgium) and 26% ethylene glycol (EG, Sigma) in minimal essential medium (MEM) + Glutamax™ (MEM, Gibco, Carlsbad, CA, USA) supplemented with 20 mg/ml human serum albumin (HSA, Sanquin, Amsterdam, the Netherlands), 2.5% polyvinylpyrolidone (PVP, MW 10 000, Sigma) and 1 M sucrose (Sigma).

The strips were first equilibrated in 25% VS/MEM + HSA (7 min), then 50% VS/MEM + HSA (4 min) and finally 100% VS (3 min). All VS baths were performed at room temperature. The strips were then placed on aseptic absorbent gauze to remove the remaining VS and directly plunged into LN2. The needles were inserted into precooled 5 ml cryotubes (Corning Inc., Corning, New York) and stored in the LN2 for 24 h.

Before autografting, the ovarian strips were immersed in warming solution 1 (WS1) containing 1 M sucrose in MEM supplemented with 20 mg/ml HSA at 37°C. The tissue remained in WS1 for <15 s, long enough to warm up. The samples were then transferred to WS2 (0.5 M sucrose), WS3 (0.25 M sucrose) and WS4 (0 M sucrose). All baths lasted for 5 min at 37°C. No tissue was collected immediately after vitrification and warming, because fixation so soon after cryopreservation would evidence neither possible changes nor injuries caused by the procedure (Hovatta et al., 1996; Gosden, 2000).

Temperature profiles in tissue cooled with and without a needle probe (cryopin)

Numerical simulations were performed using the FLUENTTM CFD software to assess the impact of inserting a chilled needle (cryopin carrier) to enhance the cooling rate of tissues immersed in LN2. The simulation Tested negative for common pathogens (bacterial and viral infections as well as parasites) and were screened for tuberculosis, simian T-lymphotropic virus-I and simian immunodeficiency virus. After this period, the animals were housed in single cages and fed commercial monkey pellets (Gold Star Products, Kenya) and seasonal fruits and vegetables, with free access to water.
domain was modeled in the CAD software and imported to FLUENT. The computational grid consisted of about 100,000 elements, and a combination of both hexahedral and tetrahedral elements was used where appropriate. For example, rectangular geometry was meshed with hexahedral elements, while a tetrahedral mesh was utilized for curvilinear features (i.e. with the needle). Temperature distribution in the whole domain was solved using the unsteady heat conduction equation

\[
\frac{dT}{dt} = \alpha \frac{d^2T}{dx^2}
\]

where \(T(x, y, z, t)\) is temperature and \(\alpha = k/\rho c_p\) the thermal diffusivity of the material. Boundary conditions were dependent on the conditions of the problem being studied. For example, constant boundary condition \(T(x, y, z, t) = C\) was applied when the whole domain was maintained at a uniform temperature, while convection boundary condition

\[
-k \frac{dT}{dx} = h(T - T_\infty)
\]

was used when the surface was exposed to air. In this case, \(T_\infty\) is the free stream of temperature of the environment while \(h\) corresponds to the convection heat transfer coefficient at 25°C.

Transplantation

One day after biopsy removal, vitrified-warmed strips were autotransplanted by vertical laparotomy. Before reimplantation of the tissue, the remaining ovary was completely decorticated. The ovarian strips were then placed onto the decorticated area, stitched using non-absorbable sutures (6/0 Prolene; Ethicon, Johnson & Johnson International, Diegem, Belgium) and covered with Interceed (Johnson & Johnson, Raritan, USA). At the time of transplantation, the intact ovary still present in the animals was removed.

After 5 months, the animals were anesthetized and euthanized using T61 (Hoechst GmbH, Munich, Germany), and the grafts were recovered and fixed in formalin.

Histological analysis

Histological analysis was performed on fresh and vitrified-warmed grafted tissue. After fixation, to evaluate follicular morphology, the ovarian fragments were dehydrated, embedded in paraffin and serially sectioned (5 μm-thick sections). Every tenth slide was stained with hematoxylin–eosin (Merck, Darmstadt, Germany) for histological evaluation; the other slides (Superfrost® Plus slides, Menzel-Glaser, Braunschweig, Germany) were kept for immunostaining.

Evaluation of the quality of follicles was based on the integrity of the basement membrane, cellular density, presence or absence of pyknotic bodies and integrity of the oocyte. Based on these criteria, pre-antral follicles were classified as morphologically normal or atretic. The percentage of each type of follicle was calculated in both groups.

Morphologically normal follicles (MNFs) were further classified according to stage into primordial or growing (primary, secondary and antral) follicles (Gougeon, 1986).

Follicle density

Follicle density was estimated by counting ovarian follicles in three random 1 mm² areas using at least three sections (extremities and middle) of each fresh and grafted ovarian tissue fragment from every animal. To this end, sections were scanned using Mirax Scan (Zeiss, Jena, Germany) and pictures were taken at ×100 magnification. Using the grid tool from ImageJ, a freely available image-processing and analysis program developed at the National Institutes of Health (http://rsb.info.nih.gov/ij/), 1 mm² squares were randomly distributed across the picture and the captured follicles were counted and classified as primordial, primary, secondary, antral or atretic.

Fibrosis

Relative areas of fibrosis were evaluated on 3–5 Masson’s trichrome-stained sections (Amorim et al., 2012), depending on the size of the graft: two sections from both extremities and 1–3 sections from the middle of the graft depending on its size. Fibrotic areas were characterized by poor cellularity, as evidenced by a reduced number of cell nuclei and collagen deposits as previously described (Dath et al., 2010). Masson’s trichrome staining turns tissue that has been replaced with collagenous connective tissue green, rendering fibrotic areas easily recognizable.

Sections were scanned by Mirax Scan (Zeiss), and measurement of fibrotic areas and total section areas was carried out using the Mirax Viewer program. Areas were delimited with the freehand tool and then measured (Amorim et al., 2012).

Immunohistochemistry

The following markers were selected to assess follicle growth and function, stromal cell apoptosis and graft vascularization: Ki67, anti-Müllerian hormone (AMH), growth differentiation factor-9 (GDF-9), caspase-3 and cluster of differentiation 31 (CD31). Paraffin sections were deparaffinized with Histocfree (Ysolvab SA, Beerse, Belgium) and rehydrated in alcohol series. After blocking endogenous peroxidase activity with 0.3% H₂O₂ diluted in demineralized water (for caspase-3) or 3% H₂O₂ diluted in methanol (for Ki67, AMH, GDF-9 and CD31), a demasking step was performed for 75 min at 98°C with citrate buffer and Triton X100 before the sections were subjected to an antigen retrieval step. Antigen retrieval steps, antibody dilutions and incubation conditions are summarized in Table I. Diaminobenzidine was used as a chromogen (SK 4100, Vector Laboratories, Peterborough, UK). The slides were then counterstained with hematoxylin and mounted with DPX neutral mounting medium (Prosan, Merelbeke, Belgium). Negative controls consisted of the dilution solution without any primary antibody.

Follicles containing at least one granulosa cell (GC) staining positive for Ki67 were classified as proliferative follicles. For quantitative analysis of AMH and GDF-9 expression, a minimum of 10 follicles from each follicular stage (primordial, primary, secondary and antral) were evaluated for each animal and treatment group. If fewer than 10 follicles were present, all the follicles were taken into account. Follicles were considered AMH-positive when at least one GC was immunostained, and GDF-9-positive when the oocyte cytoplasmic staining was immunostained. For caspase-3 and CD31 immunostaining, depending on the size of the graft, 3–5 slides were scanned by Mirax Scan (Zeiss) and visualized using the Mirax Viewer software. The area around each ovarian tissue graft was defined and all caspase-3-positive stromal cells and vessels present in the grafts were counted.

Statistical analysis

The R software (version 2.15.1, R Foundation for Statistical Computing, Vienna, Austria) combined with the JAGS software (Just Another Gibbs Sampler, Martyn Plummer, 2003) and the SPSS 14 program for Windows (SPSS, Inc., Chicago, IL, USA) were used for statistical analysis. Follicle proportions before and after vitrification and grafting were compared using either a binomial or multinomial model. Numbers of vessels per area were compared using a mean/variance estimation procedure in the log scale. Proportions of fibrotic areas were analyzed using a mean/variance estimation procedure in the log scale. Comparisons of proportions of follicles positive and negative for Ki67, AMH and GDF-9 were
Table I  Summary of immunohistochemical protocols applied to assess follicle growth and function and graft vascularization.

<table>
<thead>
<tr>
<th>Antigen (source of antibody)</th>
<th>Antibody type</th>
<th>Dilution</th>
<th>Incubation</th>
<th>Positive control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki67 (M7240; Dako, Glostrup, Denmark)</td>
<td>Monoclonal</td>
<td>1:100 in TBS + 1% NGS + 0.1% BSA</td>
<td>4°C O/N</td>
<td>Proliferative endometrium</td>
</tr>
<tr>
<td>AMH (MCA2246; Serotec, Gentaur, Brussels, Belgium)</td>
<td>Monoclonal</td>
<td>1:100 in TBS + 1% NGS + 0.1% BSA</td>
<td>4°C O/N</td>
<td>Human ovary with antral follicles</td>
</tr>
<tr>
<td>GDF-9 (sc-12244; Santa Cruz Biotechnology, Santa Cruz, USA)</td>
<td>Polyclonal (goat)</td>
<td>1:100 in TBS + 2.5% BSA + 2% milk</td>
<td>4°C O/N</td>
<td>Human ovary with growing follicles</td>
</tr>
<tr>
<td>Caspase-3 (G748A; Promega, Madison, USA)</td>
<td>Polyclonal (rabbit)</td>
<td>1:200 in TBS + 4% NGS + 0.4% BSA</td>
<td>1 h RT</td>
<td>Human tonsil</td>
</tr>
<tr>
<td>CD31 (2530-1; Epitomics, Burlingame, USA)</td>
<td>Monoclonal</td>
<td>1:500 in TBS + 1% NGS + 0.1% BSA</td>
<td>1 h RT</td>
<td>Human uterus</td>
</tr>
</tbody>
</table>

made using the chi-square test. Values of $P < 0.05$ were considered statistically significant.

Results

Temperature profiles in tissue cooled with and without a needle probe (cryopin)

Simulations were performed for two relevant scenarios of tissue freezing: (i) tissue vitrification in a liquid nitrogen (LN$_2$) suspension and (ii) tissue vitrification in an LN$_2$ suspension in the presence of a chilled needle. In the first case, the tissue was modeled as a rectangular prism ($L = 8$ mm, $H = 3$ mm and $w = 1$ mm) with a constant boundary condition of $T = -160^\circ$C (LN$_2$ temperature) applied to all its sides. The initial temperature of the prism (i.e., tissue) at time point ($t$) = 0 s was 27°C. Unsteady temperature distribution was solved from $t = 0$ s with step-size increments of $\Delta t = 0.1$ s. The tissue attained an equilibrium temperature of $-160^\circ$C after a period of 4 s.

In the second case, simulation was also carried out to analyze the vitrification time of the tissue modeled as a rectangular prism ($L = 8$ mm, $H = 3$ mm and $w = 1$ mm) with a constant boundary condition of $T = -160^\circ$C (LN$_2$ temperature) applied to all its sides, with insertion of a chilled needle modeled as a cylinder protruding out of the cuboid. This needle was exposed to air, and therefore, convective boundary conditions were also applied to its surface. It should be noted that the tissue was at a temperature of 27°C (room temperature) and there was temperature distribution across the needle due to convection caused by air surrounding its surface. Unsteady temperature distribution was solved from $t = 0$ s with step-size increments of $\Delta t = 0.1$ s. The tissue with a chilled needle inserted attained an equilibrium temperature of $-160^\circ$C after a period of $\sim 3$ s, i.e. 1 s earlier than that without. In other words, the time needed to cool a tissue section was significantly shorter, and the tissue cooling rate significantly higher, with the use of a cryopin, with no difference observed between needle diameters.

Graft recovery rate and macroscopic aspect

After 5 months of transplantation, all the grafted tissues were easily recovered from the five baboons. Some vessels connecting the grafts to surrounding tissue were observed. Grafts from all animals contained a visible corpus luteum on their surface. After harvesting, all the grafts were fixed with 4% formalin.

Histological evaluation of ovarian follicles

Follicle proportions

A total of 1960 follicles were counted and classified: 1136 from fresh ovarian tissue and 824 from vitrified-warmed grafted fragments. In fresh ovarian tissue, the proportions of primordial, primary, secondary, and atretic follicles were 57.4, 36.6, 4.9 and 1.1%, respectively. No antral follicles were found in hematoxylin–eosin sections from fresh ovarian fragments. After vitrification, warming and grafting, the proportion of primordial follicles significantly decreased (22.2%), while no difference was observed in primary (41.9%) or secondary populations (14.8%). On the other hand, the proportions of antral (5.4%) and atretic follicles (15.8%) were significantly higher than in fresh tissue. MNFs exhibited a round oocyte, surrounded by varying numbers of GCs in close contact with each other, enclosed by a visible basal membrane (Fig. 1).

Follicle density

MNF density varied according to follicular class. In vitrified-warmed grafted fragments, the density of primordial follicles was statistically lower than in fresh tissue (Table II), but this difference was not observed for primary follicles. In vitrified-warmed grafted tissue, the density of secondary and antral follicles significantly increased, as well as the population of atretic follicles. Overall, MNF density taking into account all stages of follicular development did not differ significantly between groups.

Immunohistochemical evaluation of ovarian follicles

Follicle survival and growth

For analysis of proliferation after Ki67 immunostaining, a total of 810 follicles were counted in fresh ($n = 616$) and vitrified-warmed grafted ($n = 194$) tissue. Table III shows the proportion of Ki67-positive follicles in each follicle category in fresh and vitrified-warmed grafted ovarian tissue. All follicle categories except primordial follicles
showed staining for Ki67, which demonstrates that after vitrification, warming and grafting, primordial follicles remain in a quiescent state, while growing follicles are able to continue their development. Only secondary follicles showed a statistical difference between groups: the proportion that expressed Ki67 in fresh tissue was higher than in fragments after vitrification, warming and grafting.

**Figure 1** Morphologically normal follicles from fresh (A, C and E) and vitrified-warmed autografted (B, D and F) tissue. Pre-antral follicles stained with hematoxylin-eosin from fresh (A) and vitrified-warmed autografted (B) tissue; scale bar = 200 μm (A) and 500 μm (B). Antral cavity (asterisk) in an antral follicle can be seen in the grafted group (B). Fibrotic areas were evidenced by Masson’s trichrome staining in fresh (C) and vitrified-warmed and autografted (D) tissue stained green due to collagen fibers present in higher numbers among stromal cells; scale bar = 500 μm. CD31 immunostaining (E and F): positive staining (brown) in fresh ovarian (E) and vitrified-warmed grafted (F) tissue; scale bar = 100 μm.
Autografting of vitrified baboon ovarian tissue

Table II Follicle density (mean ± SD) in fresh ungrafted and vitrified-warmed grafted tissue.

<table>
<thead>
<tr>
<th>Follicle density (n follicles /mm²)</th>
<th>Morphologically normal follicles</th>
<th>Atretic follicles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Primordial</td>
<td>Primary</td>
</tr>
<tr>
<td>Fresh control tissue</td>
<td>130 ± 102a</td>
<td>83 ± 21</td>
</tr>
<tr>
<td>Vitrified-warmed grafted tissue</td>
<td>36 ± 46b</td>
<td>68 ± 86</td>
</tr>
</tbody>
</table>

a,bValues with different superscripts differ significantly between rows in terms of relative proportions with respect to each other (multinomial model, significance level P < 0.05).

Table III Proportion of primordial, primary, secondary and antral follicles showing Ki67 immunostaining in fresh ungrafted and vitrified-warmed grafted tissue.

<table>
<thead>
<tr>
<th>Follicle stage</th>
<th>Proportion of Ki67-positive follicles (%) (n positive follicles/n follicles evaluated)</th>
<th>Fresh ungrafted control tissue</th>
<th>Vitrified-warmed grafted tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primordial</td>
<td>0% (0/339)</td>
<td>0.9% (1/106)</td>
<td></td>
</tr>
<tr>
<td>Primary</td>
<td>15% (28/187)</td>
<td>11.1% (3/27)</td>
<td></td>
</tr>
<tr>
<td>Secondary</td>
<td>79.2%a (61/77)</td>
<td>55.8%b (29/52)</td>
<td></td>
</tr>
<tr>
<td>Antral</td>
<td>100% (13/13)</td>
<td>100% (9/9)</td>
<td></td>
</tr>
</tbody>
</table>

a,bValues with different superscripts differ significantly between columns (P < 0.05).

Follicle function

Immunostaining for AMH was observed in GCs of primordial, primary, secondary and antral follicles, ranging from faint to strong in intensity (Fig. 2). Oocytes of follicles from fresh and vitrified-warmed grafted tissue showed weak, nonspecific brown staining, and no staining at all was observed in the theca layer. A total of 1047 follicles were counted in fresh (n = 877) and vitrified-warmed grafted (n = 170) tissue. No statistical difference was observed between primordial, primary and secondary follicles from the two groups. For antral follicles, no comparison could be made between fresh and vitrified-warmed grafted tissue due to the absence of follicles in fresh tissue (Fig. 3).

Strong GDF-9 staining was observed in the oocyte cytoplasm of primordial (n = 318), primary (n = 706) and secondary (n = 53) follicles from fresh and vitrified-warmed grafted tissue (Fig. 2). Primordial, primary and secondary follicles from both groups were GDF-9-positive and no statistical difference was observed between groups. Faint cytoplasmic staining in GCs was also observed at all follicle stages. No antral follicles were found in the control group, but antral follicles (n = 12) in vitrified-warmed grafted tissue stained positive for GDF-9.

Evaluation of ovarian tissue

In all the grafts, stromal tissue in hematoxylin-eosin sections looked healthy and did not differ from fresh ovarian tissue. Vessels were well distributed throughout the grafts, and stromal cells were not pyknotic and were in close contact with each other (Fig. 1).

Assessment of stromal cell apoptosis

An average of 4.2 ± 5.2 caspase-3-positive stromal cells/mm² were found in vitrified-warmed grafted tissue. This number was significantly higher than in fresh ovarian fragments, where no caspase-3-positive stromal cells were observed.

Quantification of stromal tissue fibrosis

Figure 1 shows ovarian fragments from fresh and vitrified-warmed grafted tissue stained with Masson’s trichrome to evidence fibrotic areas. Fibrosis was expressed by fibrotic area (fresh tissue: 423.17 ± 227.01 μm²; vitrified-warmed grafted tissue: 1440.27 ± 1934.36 μm²) over total area of grafted ovarian tissue (fresh tissue: 3497.09 ± 1030.93 μm²; vitrified-warmed grafted tissue: 13220.02 ± 7719.21 μm²). Fibrotic areas were similar between the two groups (12.1%—fresh tissue; 10.8%—vitrified-warmed grafted tissue).

Immunohistochemical evaluation of ovarian tissue

Vascularization

In general, stromal tissue proved to be well vascularized in vitrified-warmed ovarian tissue from all the animals after 5 months of grafting (Fig. 1), with vessels on the surface, at the periphery and in the center of grafts. Cells and tubular structures staining positive for CD31 were observed throughout the vitrified-warmed fragments. The mean number of vessel sections identified in grafts was 251.6 ± 95 (total area analyzed: 23994.7 ± 8596.1 mm²), which did not differ significantly from the number of vessels found in fresh tissue, namely 107 ± 49.6 (total area analyzed: 3077.3 ± 1131.6 mm²).

Stromal tissue survival and proliferation

After vitrification, warming and long-term grafting, stromal tissue was similar to fresh ovarian tissue, but stromal cell proliferation was significantly higher in the latter group: 0.6 ± 0.2 Ki67-positive cells/mm² in vitrified-warmed grafted tissue and 2.5 ± 1.7 Ki67-positive cells/mm² in fresh fragments.

Discussion

The results of the present study show that after vitrification, warming and long-term autografting of baboon ovarian fragments, follicles can survive and grow, as indicated by the presence of follicles from all
stages of development (primordial, primary, secondary and antral). Their healthy status was confirmed by proliferation of GCs identified by Ki67 immunostaining, and demonstration of GCs and oocyte function evidenced by AMH and GDF-9 immunostaining. Moreover, corpora lutea were observed in all the grafts, proving successful follicle growth and ovulation. Such promising results were possibly due to both vitrification-warming and transplantation procedures.

**Effect of vitrification procedure**

The cryopreservation protocol was previously tested on human ovarian tissue (Amorim et al., 2012) and developed by our team to permit the use of lower concentrations of penetrating CPAs. It is important to point out that of all solutions (VS) used for vitrification of non-human primate ovarian tissue in existing studies (Yeoman et al., 2005; Hashimoto et al., 2010; Ting et al., 2011; Suzuki et al., 2012), ours has one of the lowest concentrations of penetrating CPAs. In order to increase the viscosity of the solution and favor vitrification, non-penetrating CPAs were used. According to Anchordoguy et al. (1987), sugars like sucrose may decrease CPA toxicity, since they form hydrogen bonds with phospholipids from the membrane, contouring the outer membrane. Sugars also aid dehydration, reducing the risk of intracellular crystallization, and protect cells against osmotic shock during CPA removal (Courbiere et al., 2009). Polymers like PVP show increasingly high viscosity at lower temperatures, preventing water molecules from fusing in growing ice crystals (Sutton, 1991; Fuller, 2004). PVP also has the advantage of low toxicity levels.

To vitrify ovarian strips, we applied the cryopin (needle) procedure described by Fathi et al. (2011), as promising results were obtained for vitrification of sheep ovarian tissue. A similar vitrification procedure with acupuncture needles (needle-immersed vitrification) also yielded successful outcomes in mouse (Wang et al., 2008) and human ovarian tissue (Wang et al., 2008; Xiao et al., 2010). Wang et al. (2008) and Xiao et al. (2010) hypothesized that needle-
immersed vitrification may maximize cooling rates and consequently facilitate the vitrification process. This theory was confirmed by the present study, where our numerical simulations showed that the needle placed longitudinally in the tissue strips acted as a ‘fin’ and indeed enhanced the cooling rate. Moreover, with the cryopin carrier, the fragments could be easily handled during the entire procedure of dehydration, vitrification in LN₂ and insertion into the cryovials. It stopped the strips from curling, so the tissue did not accumulate unnecessary VS and vitrified homogeneously.

Effect of transplantation procedure

For transplantation of the ovarian fragments, we applied the procedure described by Donnez et al. (2008, 2010), which was successfully used to graft human ovarian tissue. In this protocol, tissue strips are sutured to the decorticated ovary, directly to the medulla. However, unlike in patients, we grafted the ovarian tissue 1 day after biopsy removal. Possibly due to the angiogenic potential of the ovary (Donnez et al., 2011) and rich vascular bed present in the medulla, together with the beneficial effect of the wound-healing process that started in previous day, the ovarian fragments were efficiently revascularized and follicles were able to resume their growth.

An advantage of our grafting procedure is that it can allow natural pregnancy (Donnez et al., 2011), which in turn renders the whole procedure very simple, as there is no need for oocyte pickup and in vitro maturation, embryo transfer (Ting et al., 2011) or follicle isolation and in vitro culture (Suzuki et al., 2012).

Follicle survival, growth and function after vitrification, warming and transplantation

Follicle survival and growth were evidenced by histological analysis and different immunohistochemical staining. Like us, Suzuki et al. (2012) also obtained antral follicles from vitrified-warmed autografted ovaries from cynomolgus monkeys, and Ting et al. (2011) from vitrified-warmed ovarian tissue and in vitro culture of isolated secondary follicles. However, it is hard to compare success rates between the studies, since Suzuki et al. (2012) hyperstimulated the grafts with gonadotrophins and Ting et al. (2011) used only secondary follicles from cryopreserved tissue.

After vitrification, warming and grafting, the population of primordial follicles significantly decreased compared with fresh tissue. Such a reduction could have been due to follicle loss caused by the vitrification procedure, but also to massive activation of primordial follicles caused by ischemia-reperfusion (Baird et al., 1999; Nisolle et al., 2000). Although it is difficult to precisely quantify follicle death after long-term grafting due to natural atresia that occurs during folliculogenesis, follicular recruitment and growth can be more easily calculated. After grafting, the number of secondary and antral follicles significantly increased, indicating that vitrification and warming did
not affect the ability of follicles to resume their development. This was confirmed by Ki67 staining in primary, secondary and antral follicles. Secondary follicles were the only type to show significantly higher expression of Ki67 in fresh ovarian tissue than in vitrified-warmed grafted tissue. Although we cannot explain the reason for such a variation, we can at least presume that it is a transitory feature, as the proportion of Ki67-positive antral follicles again became similar between groups. It is also important to point out that <1% of primordial follicles were Ki67-positive, demonstrating that despite follicle growth after vitrification, warming and grafting, a population of quiescent primordial follicles can still be found in ovarian tissue.

We confirmed follicle function using AMH and GDF-9, as they are expressed by GCs and oocytes, respectively and play an essential role throughout folliculogenesis. Moreover, we previously studied expression of these factors after cryopreservation and xenotransplantation of human ovarian tissue (David et al., 2011, 2012). To our knowledge, there are no studies in baboons showing AMH and GDF-9 expression in follicles.

AMH is a member of the transforming growth factor β (TGFβ) superfamily, known to have an inhibitory effect on primordial follicle activation (Visser et al., 2006). In rodents and most primates, AMH expression is observed in GCs from primary to small antral follicles (Weenen et al., 2004; Visser et al., 2006; Thomas et al., 2007), but not in primordial follicles. In baboon ovaries, we detected AMH expression in GCs from follicles at all stages of development, including primordial, a phenomenon previously seen in bonnet monkeys (Macaca radiata) (Modi et al., 2006). Our results showed that around 50% of primordial follicles were AMH-positive. David et al. (2012) also observed AMH expression in a small percentage of primordial follicles from xenografted human ovarian tissue, and Stubbs et al. (2004) in fresh human ovaries. It is important to point out that we used a very sensitive protocol for AMH immunostaining and this explains our findings in primordial follicles. Indeed, Modi et al. (2006) reported that variations in results could be due to the sensitivity of methods used for AMH detection, fixatives and staining protocols.

Four of 11 antral follicles found in the vitrified-warmed grafted group were negative for AMH. Coincidentally or not, they were the largest follicles (1.5–3.5 mm) found on the slides. According to Weenen et al. (2004), human antral follicles up to 4 mm in size have a strong AMH signal and then expression gradually decreases until they reach 8 mm in diameter. One can hypothesize that AMH expression in baboon ovarian follicles differs from that of humans and its signal starts declining in smaller antral follicles. Another explanation for our findings could be the size of baboon antral follicles. Indeed, according to our personal observations, pre-ovulatory follicles in baboon ovaries are smaller than in humans. Consequently, if their size at the final stage of development is half that of human follicles, AMH expression may well decrease sooner. Unspecific AMH staining observed in oocytes was also described in marmoset and human ovarian follicles (Weenen et al., 2004; Thomas et al., 2007).

GDF-9 is also a member of the TGFβ superfamily and plays an essential role in early folliculogenesis (Dong et al., 1996), regulating recruitment of primordial follicles and proliferation of GCs (Gilchrist et al., 2006; Peng et al., 2010). It is involved in GC differentiation and atresia of ovarian follicles, ovulation and fertilization (Orisaka et al., 2009). In the present study, GDF-9 expression was found in the cytoplasm of oocytes and GCs from primordial follicles onwards. Like Gougeon and Busso (2000), who described GDF-9 expression in oocytes from pre-ovulatory and antral follicles in cynomolgus monkey ovaries, we also observed an increase in staining in antral follicles. Moreover, in our study, GCs stained positive for GDF-9. Although Gougeon and Busso (2000) did not mention the presence of any GC staining, Duffy (2003) reported that GCs from antral follicles in rhesus monkey ovaries can be GDF-9-positive. We presume that such differences between our results and the literature are probably due to species-specific variations. It is important to bear in mind that all oocytes from fresh and vitrified-warmed grafted groups stained positive for GDF-9, showing that vitrification and warming procedures did not affect GDF-9 expression.

### Preservation of stromal tissue integrity after vitrification, warming and grafting

Despite increased numbers of caspase-3-positive stromal cells after vitrification, warming and transplantation compared with fresh ovarian tissue, values remain very low (4.2 ± 5.2 cells/mm²). We, therefore, assume that this does not negatively affect follicle development or tissue quality. Indeed, no increase in relative fibrotic area was observed, probably due to use of an efficient VS for vitrification of ovarian tissue, and the cryopipet that helped increase cooling rates and decrease the likelihood of ice crystal formation. Our percentages were lower than those obtained in xenografting of frozen-thawed human ovarian tissue, which yielded a relative fibrotic area between 19 and 69% (Nisolle et al., 2000; Dath et al., 2010), indicating that vitrification is indeed more efficient in preserving stromal tissue than conventional freezing (Keros et al., 2009).

Similar to fibrotic areas, no difference was observed in the number of vessel sections between groups, which also suggests that our vitrification procedure was successful in maintaining the viability of CD31 cells after warming. Nisolle et al. (2000) previously observed that the number of vessels was significantly lower in cryopreserved xenografted human ovarian tissue than in fresh xenografts, showing a negative impact of the conventional freezing procedure on neovascularization after transplantation.

After vitrification, warming and grafting, stromal cell proliferation decreased. However, this did not appear to greatly affect the quality of the stromal tissue and follicle survival and development, as confirmed by evaluation of fibrotic areas and follicle quality.

### Conclusion and perspectives

In conclusion, this study shows that non-human primate ovarian tissue can be successfully vitrified using a VS containing DMSO, EG, PVP, HSA and sucrose associated with the cryopipet procedure. Moreover, orthotopic transplantation performed 24 h after biopsy removal may also be a factor contributing to graft survival. Such vitrification and grafting allowed survival and development of ovarian follicles. Follicle function was confirmed by expression of growth and inhibitory factors in GCs and oocytes and formation of corpora lutea. However, the most reliable proof of the efficacy of our protocol would be pregnancy and birth, so we plan to extend our transplantation period and mate the animals. We will then be able to propose our protocol to patients.
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Authors’ roles

C.A.A. and M.M.D. designed the study; C.A.A., M.M.D. and J.D. conducted research; M.M.D. and J.D. performed surgery; C.A.A., S.J., R.V.D., J.V., V.L. and J.J. analyzed data; A.V.L. and J.D. reviewed the manuscript; C.A.A. wrote the manuscript.

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Conflict of interest

None declared.

References


Ting AY, Yeoman RR, Lawson MS, Zelinski MB. In vitro development of secondary follicles from cryopreserved rhesus macaque ovarian tissue after slow-rate freeze or vitrification. Hum Reprod 2011; 26:2461–2472.


