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FERTILITY PRESERVATION

In vitro survival of follicles in prepubertal ewe ovarian cortex cryopreserved by slow freezing or non-equilibrium vitrification

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Abstract

Purpose Vitrification is a well-accepted fertility preservation procedure for cryopreservation of oocytes and embryos but little is known regarding ovarian tissue, for which slow freezing is the current convention. The aim of the present study was to assess the efficiency of non-equilibrium vitrification compared to conventional slow freezing for ovarian cortex cryopreservation.

Methods Using prepubertal sheep ovaries, the capacity of the tissue to sustain folliculogenesis following cryopreservation and in vitro culture was evaluated. Ovarian cortex fragments were cultured in wells for 9 days, immediately or after cryopreservation by conventional slow freezing or non-equilibrium vitrification in straws. During culture, follicular populations within cortex were evaluated by histology and immunohistochemistry for PCNA and TUNEL. Steroidogenic activity of the tissue was monitored by assay for progesterone and estradiol in spent media.

Results No significant differences in follicle morphology, PCNA, or TUNEL labeling were observed between cryopreservation methods at the initiation of culture. Similar decreases in the proportion of primordial follicle population, and increases in the proportion of growing follicles, were observed following culture of fresh or cryopreserved ovarian tissue regardless of cryopreservation method. At the end of culture, PCNA and TUNEL-positive follicles were not statistically altered by slow freezing or vitrification in comparison to fresh cultured fragments.

Conclusions Overall, for both cryopreservation methods, the cryopreserved tissue showed equal capacity to fresh tissue for supporting basal folliculogenesis in vitro. Taken together, these data confirm that both non-equilibrium vitrification and slow-freezing methods are both efficient for the cryopreservation of sheep ovarian cortex fragments.

Keywords Fertility preservation · Ovarian cortex · Cryopreservation · Vitrification · In vitro folliculogenesis

Introduction

Advances in cancer diagnosis and treatment have improved the survival rates of cancer patients. While lifesaving, cancer treatments such as chemotherapy commonly lead to destruction of the oocytes within the ovary and ultimately infertility [1]. Ovarian tissue cryopreservation (OTC) and autotransplantation is an important fertility preservation option for many female cancer patients, particularly those requiring immediate gametotoxic therapy for aggressive malignancies and when there is insufficient time to undergo classical ART programs. Furthermore, the cryopreservation of ovarian tissue represents the only fertility preservation strategy for prepubertal girls or for women who have hormone-sensitive malignancies [2–4].

OTC methodologies were first successfully developed in sheep models using slow freezing of ovarian cortical strips preceding restauration of fertility by autografting [5]. This technique was subsequently attempted in humans, with the first successful transplantation of ovarian tissue performed in a patient with Hodgkin's lymphoma by Donnez et al. [6]. After more than two decades, the autologous transplantation of cryopreserved ovarian tissue has been performed many times throughout the world, and to date, at least 86 live births

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have been reported using this method [7, 8]. However, the reported pregnancy rates following cryopreservation and transplantation remain low as reduced follicle density and oocyte quality are generally observed [9]. Indeed, it is generally accepted that after reimplantation, half of the follicle population is rapidly lost primarily due to hypoxia-induced stress to the ovarian tissue awaiting neovascularization, even with vascular bed preparation [3]. During this period, precocious primordial follicle activation occurs within the tissue and contributes to the loss of graft quality [10]. Interestingly, using a bovine ovarian xenograft model, Gavish et al. [11] reported that reduction of the resting follicle pool and a "burn out" phenomenon (rapid loss of primordial follicles) were associated with reduction of ovarian cortex thickness and underscored the ability of stromal cells to contribute to normal regulation of folliculogenesis, through providing a more rigid environment within the cortex [11]. Moreover, these data underline the importance of preserving both the follicular population and the integrity of the stromal compartment when OTC is performed.

If slow freezing of the ovary currently represents the most characterized procedure, only a 20–30% pregnancy rate can be reasonably expected [12]. It is very likely that the improvement of tissue integrity following cryopreservation would represent an important step forward in subsequent fertility restoration strategies. In this aim, vitrification has been recently developed for ovarian cortex tissue and represents an interesting alternative to slow freezing [13]. The vitrification of mouse [14], sheep [15], and human [16] ovarian tissue has resulted in the birth of live offspring, providing evidence of the effectiveness of vitrification. Although relatively little is known about vitrification in comparison with extensive data on slow freezing, it has been proposed as a better option to both preserve preantral follicles and stroma integrity [17–19]. Furthermore, as vitrification protocols have continued to be developed [13, 16, 20, 21], the efficiencies between the two methods have been particularly difficult to address and few studies have proposed extensive direct comparisons [22]. Studies to date have often been limited to characterizing the ultrastructure and morphology of slow-frozen and vitrified tissue shortly after thawing and warming, respectively [17, 23, 24]. Beyond the morphological and cytotoxicity evaluation of OTC strategies, there is major interest in describing their possible consequences from a functional point of view, utilizing in vitro culture or xenotransplantation of the tissue [20, 25]. Furthermore, a lack of consensus for the use of several vitrification protocols has led to problematic interpretation of results and often conflicting outcomes [23, 24]. Accordingly, the impacts of vitrification protocols on ovarian tissue are still required to be characterized and the methodology optimized before fertility preservation applications [26].

Using prepubertal ovine ovarian tissue as it represents a good model for the study human folliculogenesis, the main objective of the present study was to compare the efficiency of a non-equilibrium vitrification protocol with clinically used slow-freezing method. A validated culture system, allowing a robust evaluation of basal folliculogenesis, has been developed to assess tissue functions after warming/thawing of cryopreserved cortical pieces.

Materials and methods

Experimental design

The experimental design is summarized in Fig. 1. An initial experiment was designed to establish and assess a culture system that permitted survival of cortex tissue and to monitor follicular activation and development. Two different culture systems (culture in well vs. culture on insert) were compared. After dissection, ovarian cortex fragments from six ewe lambs were cultured for 2 or 5 days. At the end of culture, fragments were fixed for histology/immunohistochemistry analysis to assess the kinetics of follicular activation and development in comparison with uncultured fragments (control). In a second experiment, dissected ovarian cortex fragments from 6 ewe lambs were cultured fresh in wells or exposed to cryoprotectant agents (CPA) for slow freezing or non-equilibrium vitrification then cultured after thawing or warming, respectively.

Source of ovaries

Ovine ovaries were collected in pairs from prepubertal females (90–120 days old) from a local slaughterhouse and rinsed in saline solution (0.9% *w*/*v* NaCl; Braun, Germany) supplemented with 80 μg/mL of gentamycin (Sigma, Saint-Quentin Fallavier, France). Ovaries were then transported at 15–20 °C in HEPES-buffered tissue culture medium-199 (H-TCM; Invitrogen, France) supplemented with 0.2% bovine serum albumin (HyClone; Utah, USA) and 80 μg/mL gentamycin. Beneath a laminar flow hood at room temperature, ovaries were rinsed with H-TCM and excess tissue was removed. In a Petri dish, the ovary was dissected, and the medulla removed from the cortical tissue. The cortex was then laid flat and cut into 1-mm-wide strips. The strips were then cut into 1-mm^3 pieces. Three pieces from each animal were immediately fixed in Bouin solution for histological analysis of follicle distribution on day 0 (non-cultured controls). The remaining pieces were randomly divided into treatment groups for culture or cryopreservation with three cortex pieces per treatment, per culture time, and per animal (6 females per treatment). The same process was repeated for the different culture experiments.

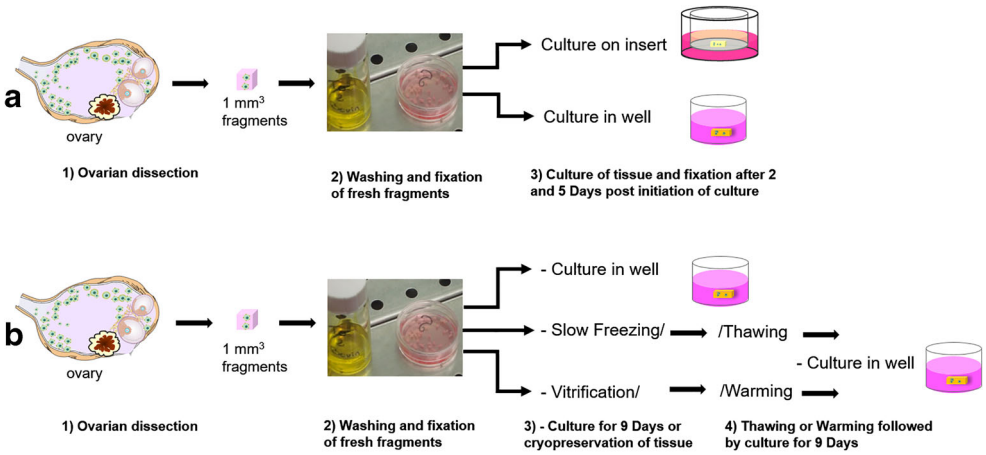

Fig. 1 Schematic illustration of experimental design evaluating well and insert culture systems (**a**) and cryopreservation techniques (**b**) for ovine ovarian cortex

Chemicals and culture media

All chemicals were purchased from Sigma-Aldrich (Sigma, Saint-Quentin Fallavier, France) unless otherwise stated. Pre-culture washing was conducted in H-TCM supplemented with 0.2% bovine serum albumin and 125 μg/mL gentamycin. The culture medium was Waymouth medium MB 752/1 (31220-023, Invitrogen®; Illkirch, France) supplemented with 25 mg/L sodium pyruvate, antibiotics (50 IU/mL penicillin, 50 μg/mL streptomycin; Invitrogen), and ITS+ solution (6.25 μg insulin, 6.25 μg of transferrin, 6.25 μg of selenium, 1.25 mg BSA, and 5.35 μg of linoleic acid per milliliter; Becton Dickinson Labware, Le Pont de Claix, France). Culture media was also supplemented with testosterone (30 ng/mL) and FSH (50 ng/mL). Purified ovine FSH was obtained from Dr. Yves Combarnous (Nouzilly, France; lot no. CY1771-11; FSH activity = 28 times the activity of NIH FSH S1). Slow-freezing solution was the culture medium described above supplemented with 10% DMSO, 10% FCS, and 0.1 M sucrose, and vitrification solution was culture medium supplemented with 20% DMSO, 20% ethylene glycol, 10% FCS, and 0.5 M sucrose).

Culture of ovarian cortex pieces

After dissection, cortical tissue was handled with sterile forceps and washed four times in H-TCM medium. A last wash was performed in cultured medium (1 mL/3 fragments).

In the first experiment, cortical tissue was cultured in 4-well plates (Nunc, Roskilde, Denmark) with 1 mL of culture medium or were cultured on insert supports (Millicell-CM, 0.4-μm pore size; Millipore Corporation, Bedford, MA) in six-well plates (Nunc) at 38.8 °C in humidified atmosphere of 5% CO_2 in air. Three cortical tissue were cultured per well or per culture insert. Cortical tissue was laid on cell culture insert then covered with 20 μl of collagen solution. In each case, culture medium was renewed every 48 h and fragments were fixed in Bouin's fixative at 2, 5, and 9 days after the initiation of culture. In the second experiment, the culture of fresh, slow frozen/thawed, or vitrified/warmed cortical tissue was performed in 4-well plates as described in experiment 1. This experiment was repeated twice (6 females per treatment).

Cryopreservation

Slow freezing

After dissection and preparation, ovarian tissue (27 fragments per animal) was exposed to slow-freezing solution (culture medium supplemented with 10% DMSO, 10% FCS, and 0.1 M sucrose) in four-well plates (3 fragments per well, 1 mL of slow-freezing solution) at 4 °C. After 15 min in solution, fragments were gently introduced in 0.5-mL straws with freezing solution using sterile forceps (6 fragments per straw) and straw was sealed with polyvinylic alcohol. A 30-min exposure with cryoprotectant at 4 °C was performed before the start of the slow-freezing program. Slow freezing was performed in a Freeze Control CL-8800 system (CryoLogic, Victoria, Australia) and Cryogenesis 5 (CryoLogic) software. Kinetics of slow freezing was adapted from Gosden et al. [5] and consisted of 15-min equilibration of the straws at 4 °C followed by a first cooling to reach − 9 °C (5 min, cooling rate of 2 °C/min). After 2 min at − 9 °C, seeding was then performed manually using cooled forceps (exposed to liquid nitrogen). After seeding, cooling to − 40 °C was performed at 0.3 °C/min. A final cooling rate of 6 °C/min was applied to allow temperature decrease from − 40 to − 70 °C. At the end of the slow-freezing program, straws were plunged into liquid nitrogen and stored at − 196 °C until thawing.

In order to assess the cytotoxicity of cryoprotectants, 3 fragments were exposed to freezing solution at 4 °C during 45 min. At the end of the equilibration period, ovarian cortex pieces were exposed to Thawing Solution 1 (culture medium supplemented with 10% FCS and 0.5 M sucrose) during 5 min

at room temperature then in Thawing Solution 2 (culture medium supplemented with 10% FCS and 0.125 M sucrose) during 5 min. Finally, ovarian cortex fragments were rinsed in culture medium supplemented with 10% FCS for 10 min before being transferred in culture medium and incubated at 38.8 °C in 5% CO_2 humidified atmosphere in air. After 2 h, fragments were fixed in Bouin solution and prepared for histology as described above.

Thawing was performed 1 week after freezing. Straws were removed from liquid nitrogen storage, exposed to air for 5 s and plunged into a water bath at 35 °C. Straws were then cut and ovarian cortex fragment were recovered in 35-mm Petri dish before exposure to Thawing Solutions 1 and 2, culture medium supplemented with 10% FCS and incubation in culture medium as described above. At the end of the incubation period, ovarian tissue was cultured in wells as described for fresh (non-exposed) counterpart fragments. Fragments were fixed in Bouin solution and prepared for histology at initiation of culture and after 2, 5, and 9 days of culture.

Vitrification

The vitrification protocol was a modification of the method described by Zhou et al. [21]. After dissection and preparation, ovarian tissue (27 fragments per animal) was exposed to vitrification solutions at room temperature. First exposure was performed for a period of 5 min in vitrification solution 1 (VS1: culture medium supplemented with 5% DMSO, 5% ethylene glycol, 10% FCS, and 0.125 M sucrose). Tissue was then exposed for 5 min in Vitrification Solution 2 (VS2: culture medium supplemented with 10% DMSO and ethylene glycol, 10% FCS, and 0.25 M sucrose) then another 2 min in vitrification solution 3 (VS3: culture medium supplemented with 20% DMSO, 20% ethylene glycol, 10% FCS, and 0.5 M sucrose). At the end of exposure to cryoprotectant, the tissue was gently aspirated with VS3 and inserted using forceps into 0.5-mL straws (CBS, Cryo Bio System, L'Aigle, France). Straws were sealed with polyvinyl alcohol and plunged directly into liquid nitrogen.

In order to assess the cytotoxicity of cryoprotectants, 3 fragments per animal were exposed to the vitrification protocol as described above, exception made for insertion in straws and liquid nitrogen exposure. Fragments were then exposed to VS2 for 5 min at room temperature then in VS1 during 5 min. Finally, ovarian cortex fragments were rinsed in culture medium supplemented with 10% FCS for 10 min before being transferred in culture medium and incubated at 38.8 °C in 5% CO_2 humidified atmosphere in air. After 2 h, fragments were fixed in Bouin solution and prepared for histology as described above.

Warming was performed 1 week after vitrification. Straws were removed from liquid nitrogen storage, plunged into a water bath at 20 °C. Straws were then cut and ovarian cortex fragments were recovered in 35-mm Petri dish before exposure to VS2 and VS1 (5 min each), culture medium supplemented with 10% FCS (10 min) and 5-min incubation in culture medium as described above. At the end of the incubation period, ovarian tissue was cultured in the well as described for fresh (non-exposed) tissue. Fragments were fixed in Bouin solution and prepared for histology at initiation of culture and after 2, 5, and 9 days of culture.

Histological assessment

Ovarian tissue was fixed in Bouin solution for 12–24 h followed by dehydration and embedding in paraffin. For experiment 1, pieces were fixed on days 0, 2, and 5. Tissue was sectioned from the middle of each piece at a thickness of 7 μm. A total of 40–60 sections were deposited per slide. Slides were deparaffinized and stained with hematoxylin and examined for follicle distribution. Both histological, TUNEL, and PCNA analyses were performed on adjacent sections (consecutives slides). Follicles were judged normal when no pyknosis was found in granulosa cells. However, the proportion of pyknotic follicles was negligible. Follicles were classified as primordial (one layer of flattened pre-granulosa cells around the oocyte), intermediate (at least one cuboidal granulosa cell), primary (oocytes with a single complete layer of cuboidal granulosa cells), secondary (follicles with two or more complete layers of granulosa cells around the oocyte), and antral (follicles where an antrum had formed). To avoid counting follicles more than once, only those follicles with a complete and visible oocyte nucleus were included for analysis.

Immunohistochemistry

PCNA immunohistochemistry

Tissue was deparaffinized in toluene and rehydrated and endogenous peroxidases were blocked by 1.2% hydrogen peroxide. Sections were preincubated with horse serum for 15 min at room temperature and washed three times in washing solution (PBS). The first antibody, a monoclonal mouse anti-PCNA (Millipore Merck), was diluted 1:200 in PBS, and 1% horse serum was added. The sections were incubated overnight in a humidified chamber at 4 °C. Sections were washed three times and then incubated with donkey anti-mouse peroxidase–conjugated secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA) diluted 1:800 in PBS/BSA 0.1% at room temperature for 4 h in a humidified chamber. Immunostaining was developed by incubating sections in 50 mM Tris-HCl (pH 7.8) containing 0.4 mg/mL 3,3′-diaminobenzidine tetrahydrochloride dehydrate (Sigma, Saint-Quentin Fallavier, France) and 0.0072% H_2O_2 for a maximum of 10 min at room temperature. Negative control sections were treated the same but the

primary antibody was replaced with sheep IgG. Follicles were considered positive for PCNA if the oocyte was labeled and/or more than 50% of the granulosa compartment was labeled. Those follicles positive for the PCNA antibody were considered healthy and growing. PCNA positively stained follicles were counted and categorized according to the total number of follicles.

TUNEL assay

Tissue was deparaffinized in toluene and rehydrated. Apoptotic oocytes and follicles in ovarian sections were stained for using TUNEL. The ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore, St Quentin en Yvelines, France) was used as per the manufacturer's instructions. All samples were treated the same to prevent inter-experimental staining. Images were captured with a computer-based program (Olympus DP Controller; Olympus Optical, Japan). Follicles were considered positive for TUNEL if the oocyte was labeled and/or more than 50% of the granulosa compartment was labeled. TdT positively stained follicles were counted and categorized according to labeling of either the oocyte or the granulosa in addition to follicle class. Those follicles with both the oocyte and more than 50% of the granulosa labeled were placed in the oocyte labeled group. Those follicles positive for the TdT enzyme were considered atretic while those remained unstained were considered healthy. The proportion of stroma cells positive for TUNEL was determined using public domain ImageJ software (https://imagej.nih.gov/ij/ v1.52a) and a cell counter plugin (Kurt De Vos, University of Sheffield, Academic Neurology). The evaluation of stroma was performed on tissue slides with ovarian follicles present. A total of 23285 stroma cells were evaluated over three treatment groups and culture times (days 0, 5, and 9).

Steroid assays

Spent medium was collected and stored at − 20 °C for subsequent steroid hormone assays. All samples were analyzed in a single assay to avoid inter-assay variations. As biotin in culture medium may interfere with assays, an extraction of steroids was performed on samples. Extraction was performed on 300-μL culture medium using 3 mL of 1:1 ethyl acetate/cyclohexane solution in 5-mL glass tubes. After mixing by vortex and incubation during 2 h at room temperature, tubes were centrifuged 15 min at 3000*g* and 4 °C. Tubes were snap frozen in liquid nitrogen and organic phase was recovered in a new glass tube. Samples were then evaporated using moderate heating and controlled air flow. Dry extracted samples were diluted in 300 μL of Tris 0.1 M-EDTA 1 mM solution, vortexed and incubated overnight at 4 °C.

The concentrations of progesterone (P4) in samples were measured by Laboratoire Phénotypage-Endocrinologie (INRA UMR 7247–PRC) using 10 μL of extracted samples by ELISA as previously described [27]. Standard curve concentrations ranged from 0.25 to 30 ng/mL P4 and standard reference of 2 ng/mL was employed to assess intra- and inter-assay coefficients of variation. The limit of detection of the assay was 0.25 ng/mL and the intra-assay coefficient of variation was 4%.

Estradiol (E2) concentrations were assayed in extracted samples with a commercial E2 immunoassay (ELISA) kit according to the manufacturer's instructions (DIAsource Immunoassays SA, Nivelles, Belgium). E2 standard references (0.39 to 200 pg/mL), control (25 pg/mL), and samples (150 μL volume in duplicates) were added to 96-well plates coated with rabbit Anti IgG. Horseradish peroxidase–labeled estradiol and the manufacturer anti-estradiol antibody were added in 50-μL volume to each well. Plates were incubated overnight at 4 °C using a microplate shaker. After incubation, plates were washed 5 times with 0.4 mL manufacturer washing solution. The staining was revealed by the incubation of 200 μL of tetramethylbenzidine-H_2O_2-acetate/citrate buffer solution for 30 min in the dark. The reaction was stopped by the addition of 50 μL of 1.8 M H_2SO_4 solution and the absorbance was measured at 450 nm (specific staining) and 650 nm (non-specific background). The limit of detection of the assay was 1.56 pg/mL and the intra-assay coefficient of variation was 6%.

Statistical analysis

All data are presented as means ± SEM. Development data were arcsine transformed ($y = 2 \arcsin \sqrt{(p)}$) [28]. Follicle distribution data according to culture system, time of culture, or treatment were subjected to two-way ANOVA and paired *t* test comparison. The TUNEL and PCNA data analyses were compared using a similar procedure. Steroid concentrations data were also analyzed using a two-way ANOVA, repeated measures ANOVA, and paired *t* test. However, because of the large amount of variation observed in the concentration of estradiol, log2 transformation was performed before analysis. Values were determined to be significant when $P < 0.05$. Analysis was carried out using Graphpad (v 5.01, Prism).

Results

Effect of insert vs. well culture systems on follicular activation

Histological analysis was performed on 4115 follicles from ovarian cortex fragments of 6 animals cultured in two independent experiments. The activation and development of preantral follicles following organotypic culture of fresh ovarian cortex fragments in well or in insert are represented in Fig. 2.

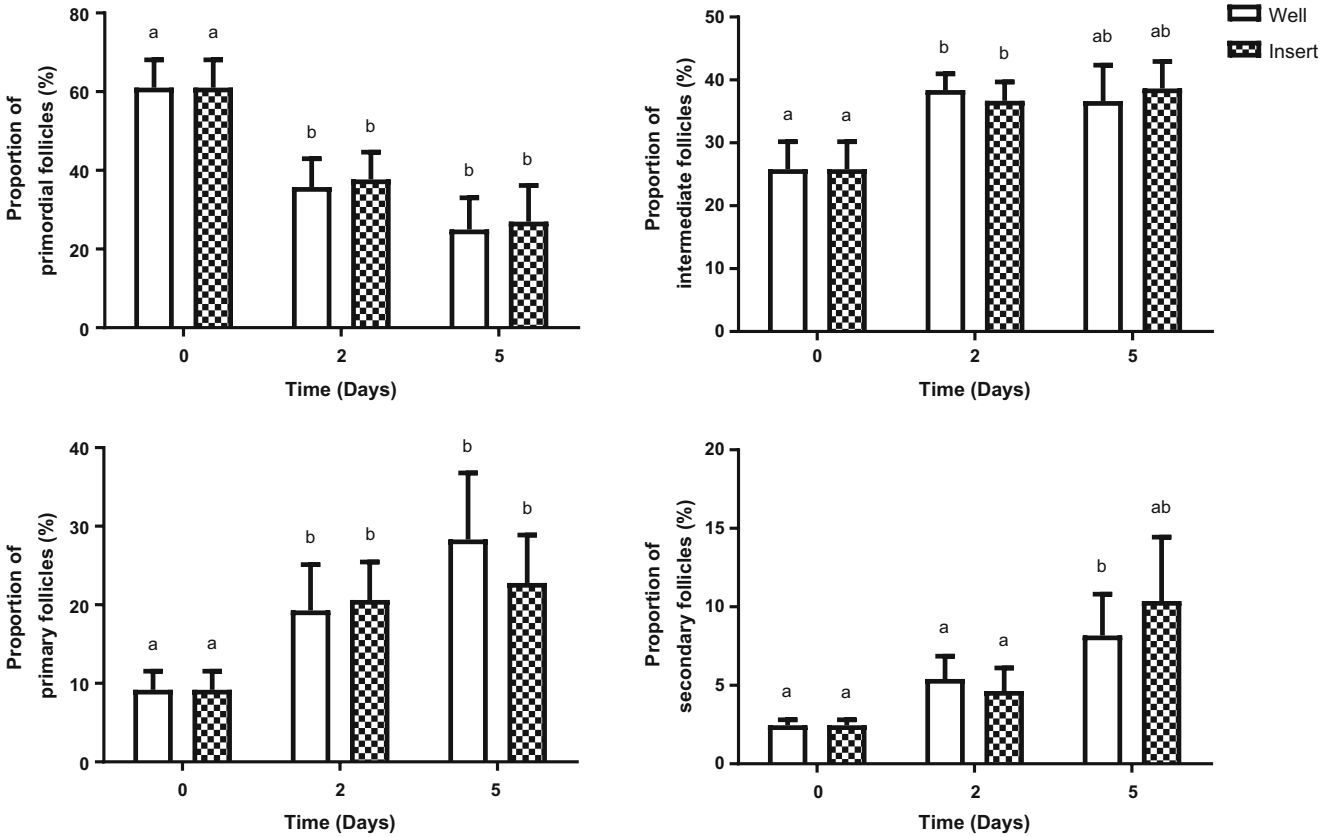

Fig. 2 Development of primordial, intermediate, primary, and secondary follicle populations after prepubertal sheep ovarian tissue cultured in wells or inserts. Data are presented as mean percentage ± SEM (given as indication). Within treatment, values with different superscript differ significantly, $P < 0.05$

A significant decrease in the proportion of primordial follicles was observed for both culture treatments as early as day 2 of culture when compared with day 0 ($P < 0.05$). The proportion of intermediate follicles was increased significantly after 2 days of culture ($P < 0.05$) but this increase was not sustained at day 5 in either culture system. The proportion of primary follicles increased significantly from day 0 to day 5 of culture in both culture systems ($P < 0.05$). However, the proportion of secondary follicles significantly increased only when the tissue was cultured in a well ($P < 0.05$), whereas a tendency for a larger proportion was observed when tissue was cultured in inserts ($P = 0.09$).

Effects of cryopreservation on follicular activation

Changes in the follicular populations of the ovarian cortex fragments after organotypic culture in wells for fresh, frozen/thawed, or vitrified/warmed conditions are represented in Fig. 3. A total population of 10,570 follicles from cortex fragments from 6 animals was assessed by histology (Fig. 4) in two different experiments.

Cryopreservation strategy did not significantly affect follicular population at the initiation of culture (day 0). As described in experiment 1, histological analysis of fresh cultured tissue showed significant differences in the proportions of follicles of different stages after 9 days of culture. This was characterized by a continuous significant decrease in the proportions of primordial follicles as early as day 2 (58.5 ± 6.5% vs. 45 ± 5.75%, mean ± SEM at day 0 vs. day 2, respectively, $P < 0.05$). A significant increase was observed for the transitory follicles on day 2 (34.5 ± 5.2% vs. 43.4 ± 5.2%, mean ± SEM at day 0 vs. day 2, respectively, $P < 0.05$), for the primary follicles (6.9 ± 2.3% vs. 26.6 ± 5.3%, mean ± SEM at day 0 vs. day 5, respectively, $P < 0.05$) and secondary follicles from day 5 (0.17 ± 0.11% vs. 5.1 ± 1.8%, day 0 vs. day 5, respectively, $P < 0.05$). These proportions were generally not statistically affected by cryopreservation treatments except that the proportion of primary follicles on D9 was decreased in frozen tissue when compared to fresh tissue ($P < 0.05$).

Effect of cryopreservation on immunohistochemistry for PCNA and TUNEL labeling

The effects of cryopreservation methods on preantral follicular populations labeling for PCNA and TUNEL were assessed via immunohistochemistry following culture (Fig. 4). A large majority of follicles evaluated was positive for PCNA, regardless

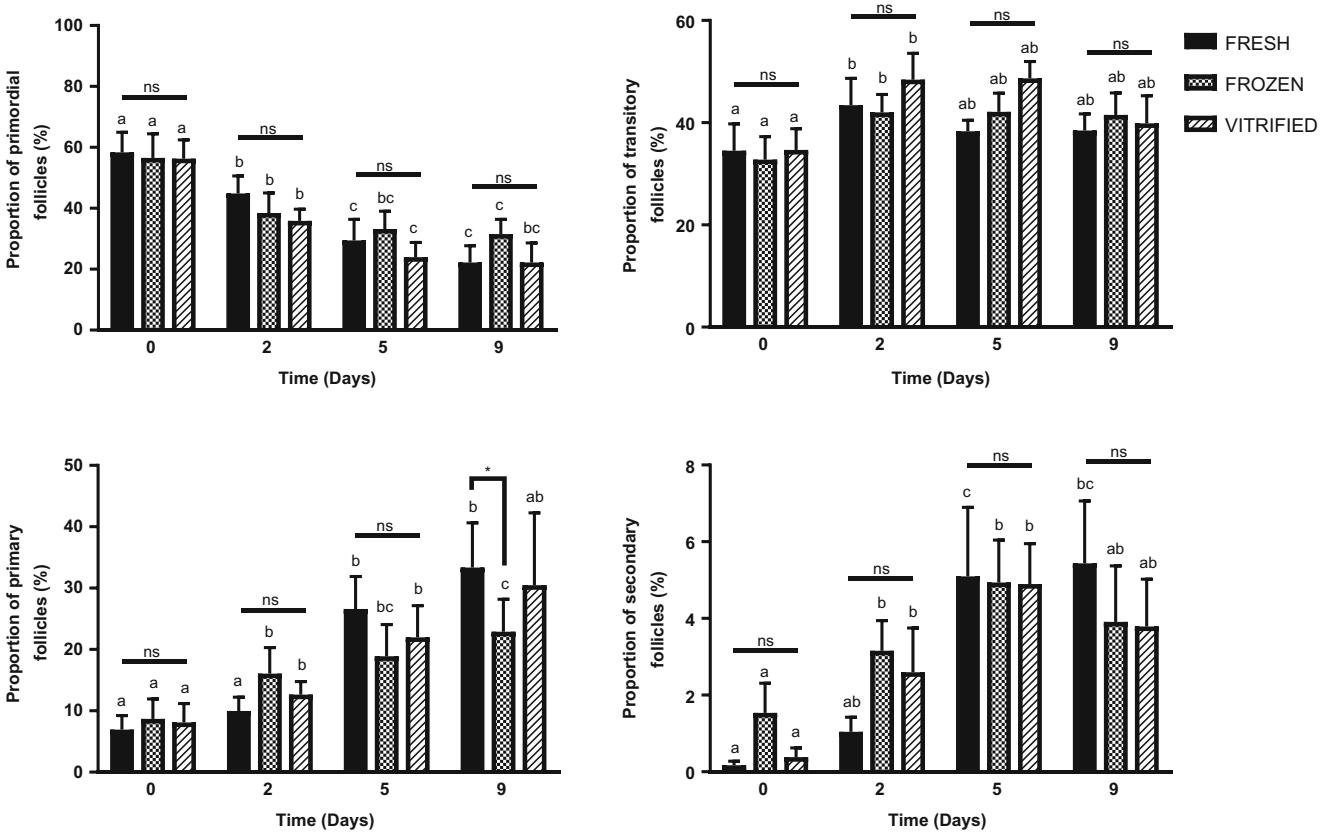

Fig. 3 Proportions of primordial, intermediate, primary, and secondary follicles after culture of fresh, frozen/thawed, or vitrified/warmed prepubertal sheep ovarian cortex fragments in wells. Data are presented as mean percentage ± SEM (given as indication). Within treatment, values with different superscript differ significantly, $P < 0.05$. For a given culture time, (*) indicates a significant difference between treatments

of follicle stage, with no significant effect of exposure to cryoprotectants (cytotoxicity test). For the primordial and intermediate follicles, no significant differences were observed by PCNA labeling according to culture duration. Furthermore, no significant differences were observed in the proportions of labeled follicles according to the treatment group (Fig. 5A). For all follicle stages, the culture of fragments during 7 days without medium renewal (negative control) confirmed a significant reduction of proportion of follicles positive for PCNA.

A large majority of follicles evaluated was negative for TUNEL, whatever the treatment or culture duration considered. When considering all follicle stages, the proportions of follicles positive for TUNEL varied in a large range (from a minimum of 8.5 ± 5.5% in frozen at day 0 to a maximum of 36.1 ± 15.6% in fresh at day 5, mean ± SEM) according to treatment or culture duration (Fig. 5B). However, when the variability of measure was considered for statistical analysis, differences observed between treatment groups and culture were not significant (interaction $P = 0.68$, treatment $P = 0.91$, and time $P = 0.26$). Similarly, when considering TUNEL labeling for primordial and intermediate populations of follicles, there was no effect of treatment ($P = 0.46$ and 0.81, for primordial and intermediate populations, respectively) or culture duration ($P = 0.44$ and 0.28, for primordial and intermediate populations, respectively).

Values of TUNEL labeling in stroma cells from fresh or cryopreserved ovine ovarian tissue according to culture time are shown in Fig. 5C. The proportion of stroma cells positive for TUNEL in fresh ovarian cortex fragments at the initiation of culture was extremely weak. The cumulated frequency of stroma cells positive for TUNEL was 0.3% at day 0 for fresh tissue (5/1658 total cells counted). Culture duration was associated with an increase in proportion of stroma cells positive for TUNEL when all data were considered ($P < 0.05$). The proportion of labeled stroma cells increased significantly from day 0 to day 9 for fresh tissue (0.4 ± 0.4 vs. 2.9 ± 1.3, mean ± SEM, $P < 0.05$). Overall, there was no effect of cryopreservation treatment on proportion of stroma cells positive for TUNEL.

Effect of cryopreservation on the synthesis of steroids

The concentrations of progesterone and estradiol in spent media were determined by EIA and are represented in Fig. 6. Estradiol levels increased significantly in culture media on

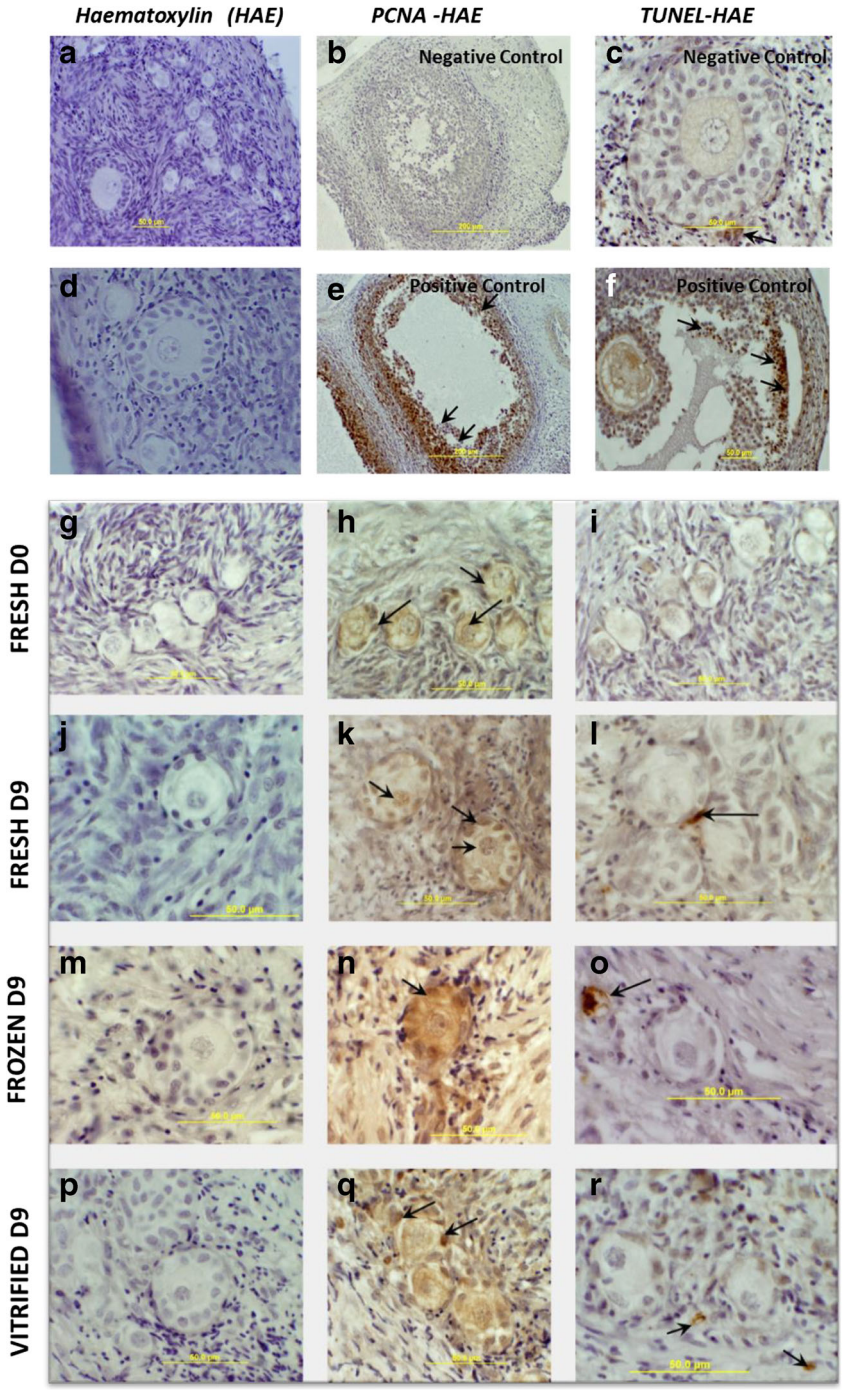

Fig. 4 (A) Population of follicles stained with hematoxylin. (B) Tertiary healthy follicle (D0 tissue) subjected to PCNA labeling but without primary antibody (PCNA negative control). (C) Healthy secondary follicle (D0 tissue) not labeled with TUNEL. The TUNEL marking signal is visible in the stroma. (D) Intermediate and secondary follicles stained with hematoxylin. (E) Same follicle as B, in an adjacent section, subjected to PCNA marking (PCNA + control). The staining is localized in the granulosa. (F) Tertiary follicle with numerous pyknotic cells with histology (atretic) and TUNEL labeled in the granulosa and in the oocyte (TUNEL + control). Fresh fragments at t0: (G) population of primordial and intermediate follicles stained with hematoxylin. (H) Primordial and intermediate follicles whose granulosa cells are labeled with PCNA. Cytoplasmic and nuclear staining of the oocyte can be detected. (I) Primordial and intermediate follicles not marked with TUNEL. Fresh fragments at 9 days of culture: (J) primary follicle. (K) Primary follicles with oocyte nuclei and granulosa cells labeled with PCNA. (L) A single cell of an intermediate follicle labeled with TUNEL. Fragments thawed at 9 days: (M) primary follicle. (N) Primary follicle whose granulosa cells are labeled with PCNA. (O) No TUNEL markings on the primary follicle but presence of a signal in the stroma. Fragments warmed at 9 days: (P) Primary and intermediate follicle. (Q) PCNA primary and intermediate follicles. (R) No TUNEL markings on the primary and intermediate follicle but presence of a signal in the stroma

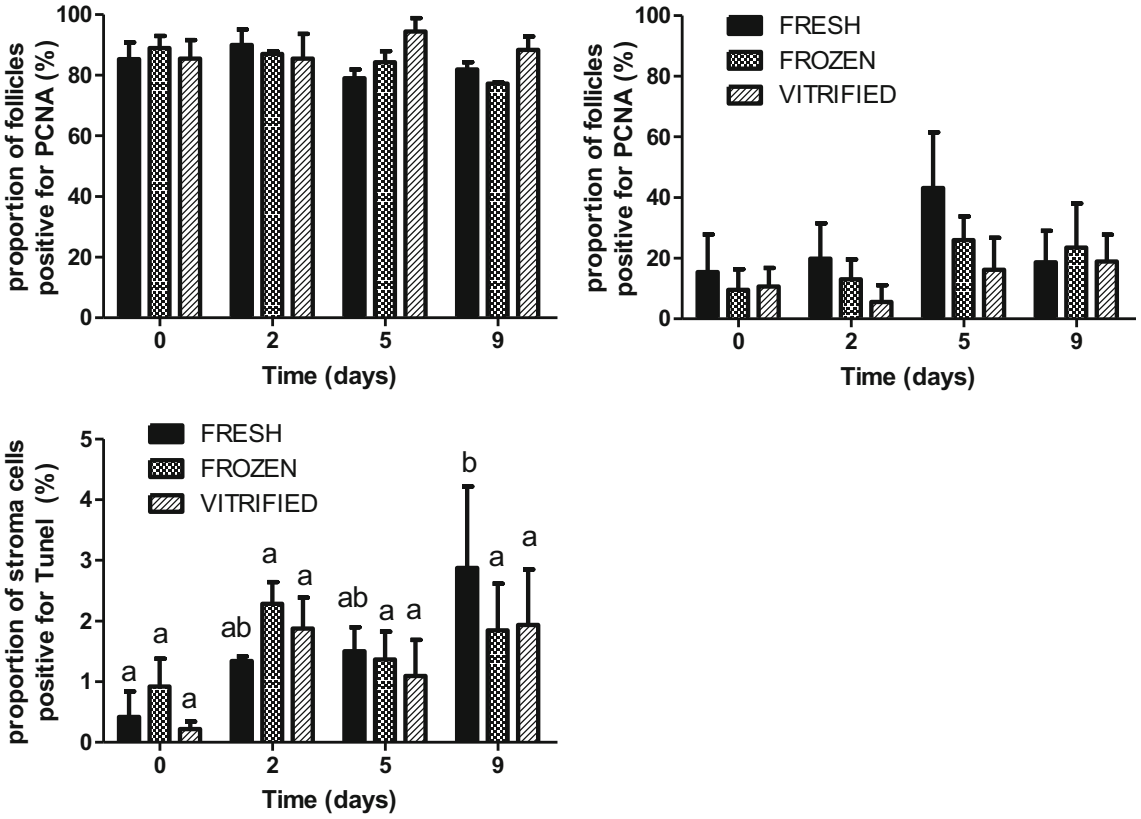

Fig. 5 Proportion of PCNA and TUNEL-positive follicles (A, B) and stroma cells (C) from fresh or cryopreserved ovine ovarian tissue according to culture duration. Data are presented as mean ± SEM. Within a treatment, values with different superscript differ significantly, $P < 0.05$

day 8 of culture ($P < 0.05$) and reached maximal concentrations at day 9 of culture for the three treatment groups. Overall, for a given culture time, estradiol concentrations did not differ significantly between treatment groups ($P = 0.103$). Maximal concentrations on day 9 did not differ significantly between the treatment group (165.9 ± 68.4 vs. 95.6 ± 26.8 vs. 169.5 ± 15 pg/mL for fresh vs. frozen vs. vitrified tissue, respectively, $P = 0.43$). Notably, estradiol secretion profiles presented a relatively important amount of variation between individuals.

As observed with estradiol, progesterone synthesis was also characterized by a high variability between individuals. Mean concentrations increased from day 2 to day 5 for fresh, frozen, and vitrified tissues. There was a significant effect of time but not treatment on progesterone secretion ($P < 0.0001$ and $P = 0.17$, respectively, two-way ANOVA). Progesterone concentrations were statistically increased by culture for the three culture conditions.

Discussion

There are very few reports of direct comparisons of cryopreservation between ovarian cortex using slow freezing and vitrification. The objective of the present study was to deepen understanding of the efficiency of ovarian cortex cryopreservation by non-equilibrium vitrification in comparison with the conventional slow-freezing method. Indeed, although vitrification protocols were recently successfully applied for human ovarian cortex cryopreservation, little is known on tissue viability beyond immediate morphological or cytotoxicity evaluation after thawing/warming. In the present study, using ovine as a model for the study of human folliculogenesis, we developed and characterized a culture system for ovarian cortex allowing both the survival and activation of primordial follicles. This culture system was employed to monitor the ability of tissue to sustain preantral follicular growth after cryopreservation using classical slow freezing or non-equilibrium vitrification. When considering follicular dynamics, granulosa cell proliferation, follicle/stroma apoptosis, and steroidogenesis under our culture conditions, our data clearly provide new evidence that the non-equilibrium vitrification method is at least as efficient as slow freezing.

When OTC protocols are developed for fertility preservation, xeno- or autografts represent the best options to assess efficiency of cryopreservation through long-term evaluation of tissue function [20]. However, in vitro culture systems can be comparable to complicated transplantation models [25], and so are particularly useful for monitoring the extent of primordial follicle activation and development, and reproductive potential of the tissue at thawing or warming [29].

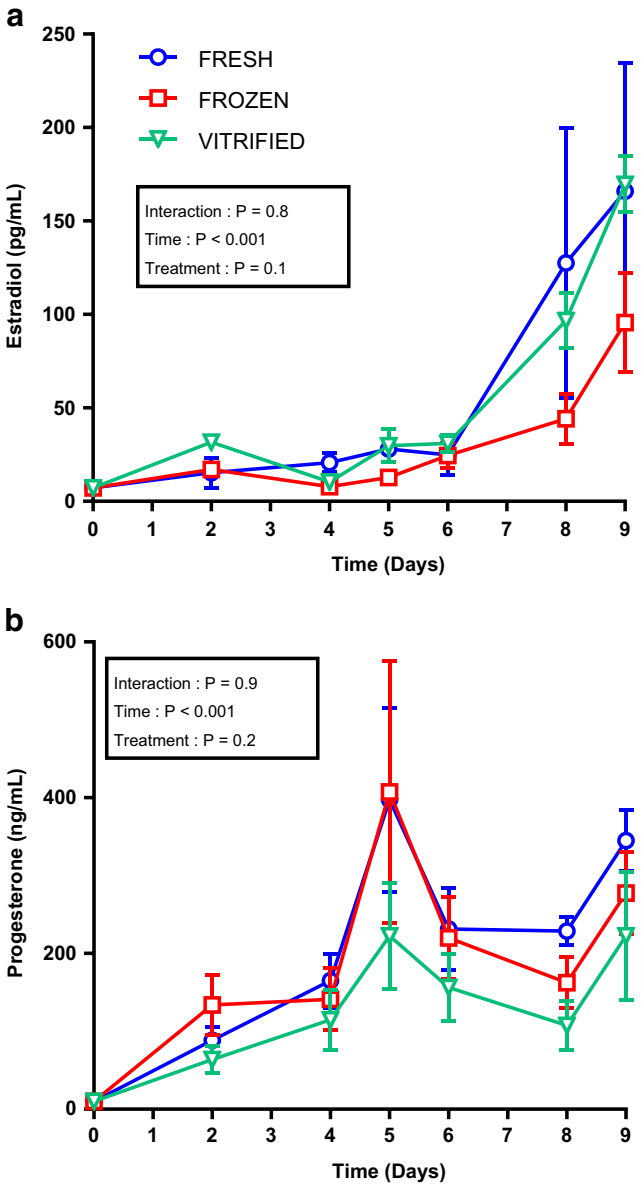

Fig. 6 Estradiol (**a**) and progesterone (**b**) secretions of by ovine ovarian cortex cultured for 9 days. Culture medium was renewed every 2 days. Ovarian tissue was cultured from fresh tissue (blue line) or following tissue cryopreservation through slow freezing (red line) or vitrification (green line). Values are mean ± SEM, $n = 4–6$

Interestingly, when culturing ovarian cortex fragments, the massive precocious activation of primordial follicles, reported to occur under challenging conditions in vitro [30], closely resembles early follicle loss generally observed after autotransplantation [10, 25, 31]. In the present study, when fresh ovarian cortex fragments were cultured in wells or in culture inserts, a clear activation of dormant follicle was observed as early as after 2 days of culture. In both culture systems, a significant decrease in the proportion of primordial follicles occurred whereas the proportion of intermediate and primary follicles increased significantly. The kinetics of follicular growth were similar between the two methodologies; however, the proportion of secondary follicles was significantly increased after 5 days for culture in wells whereas there was a tendency observed for the insert culture system. In our hands, culture in wells was easier to achieve and judged sufficiently effective to allow a good evaluation of follicular activation within ovarian cortical tissue after OTC.

The first OTC method assessed is a slow-freezing protocol adapted from Gosden et al. [5] and corresponds to the gold standard procedure employed worldwide for women ovarian tissue cryopreservation. The second, a non-equilibrium vitrification protocol, was established on the basis of the results obtained in previous studies in human [21, 32, 33]. In theory, vitrification represents a good alternative to slow freezing as it may better prevent potential cryodamage resulting from extracellular crystallization of the water that occurs during slow freezing. To allow sufficient cooling rates for vitrification and effective comparison between OTC treatments, both slow-freezing and vitrification protocols were performed using ovarian cortex strips in 0.5-mL straws. In all treatments (fresh, frozen-thawed, and vitrified-warmed fragments), similar patterns of follicular development during culture were observed. Following the commencement of culture, a global activation of primordial follicles occurred by day 2. This increase in primordial follicle activation appeared to continue until day 5, but slowed by day 9. The reduction in the proportion of primordial follicles was associated with increasing numbers of growing preantral follicles, suggesting that primordial follicle activation had occurred, and follicles were growing. However, on day 9, follicular development diverged in slow-frozen tissue when compared to fresh tissue with significantly less primary follicles in the cryopreserved tissue. Sustaining the primordial follicle pool is important for the long-term reproductive potential of the ovary, in addition to graft longevity [34]. These data suggest that the ovarian tissue cryopreserved whether by slow freezing or vitrification demonstrated comparable ability to fresh tissue in supporting follicular activation and growth. These findings are in accordance with previous studies in sheep or goat wherein vitrified tissue at warming showed similar proportions of normal follicles and comparable viability or developmental competence after culture when compared with fresh tissue [35, 36]. One study by Nikiforov et al. has compared the efficiencies of different vitrification methods opposed to conventional slow freezing in sheep model and reported overall, a superiority of slow-freezing methodology when histological evaluation and DNA fragmentation were considered at warming [26, 37]. However, follicular development or steroidogenesis was not evaluated after thawing or warming using culture or xenografts [26, 37], making it difficult to compare with the results from the present study. Recently, slow freezing was also reported to better preserve follicular integrity (morphology) than vitrification when chick embryo chorioallantoic membrane transplantation for 5 days was used to monitor tissue function

after cryopreservation [38]. These conflicting results may be explained by differences in vitrification protocols employed, cryoprotectants, exposure time to vitrification solutions, the device used for cryopreservation, and/or tissue evaluation methodology.

In the present study, follicle integrity was assessed using immunohistochemical evaluation of cell proliferation and apoptosis, targeting PCNA and TUNEL, respectively. PCNA is a protein involved in the optimization of DNA polymerase activity during DNA replication and has been largely employed as a marker of cell proliferation [39]. The vast majority of the primordial follicles were positive to the PCNA from the day 0. This result is in accordance with previous observations under similar conditions in sheep [29, 39] but is in contradiction with the results of Wandji et al. in the baboon [40] or Silva et al. in the goat [41] where a small proportion (< 10%) of primordial follicles were immunopositive for PCNA. It should be noted, however, that the intensity of the labeling observed in our study for primordial and intermediate follicles is lower than that observed for primary follicles and is similar to that observed by Wandji et al. [40]. For the three culture conditions tested, there was no significant change in the proportion of immunopositive follicles during culture, further demonstrating that cryopreservation had no significant effect on granulosa cell PCNA expression. This suggests that the follicles of cryopreserved tissue are capable of proliferation in a similar capacity as those from fresh tissue.

When follicular apoptosis was assessed in ovarian cortex using TUNEL, the proportions of immunopositive follicles observed at initiation or end of culture were in accordance with previous findings for fresh or cryopreserved tissues in sheep [42] or goat [43]. No significant differences were observed in TUNEL labeling in follicular populations after the two cryopreservation treatments when compared with fresh tissue, and the large majority of follicles evaluated in the present study was negative for TUNEL, also when culture duration was considered. These data may suggest that the follicular populations within the ovarian cortex did not suffer from apoptosis and may be considered healthy after cryopreservation whether by slow freezing or vitrification or after culture. However, different mechanisms of programed cell death have been suggested in follicle populations [44]. Indeed, during the prepubertal period in mice, when follicles die and are cleared from the ovary at a high rate, the primordial follicle cohort is not positively associated with nuclear condensation or cell shrinkage, activation of caspase 3, cleavage of poly(ADP ribose) polymerase 1 (PARP1), or fragmentation of DNA, suggesting that a non-apoptotic pathway can be involved in preantral follicle loss [44]. These results underline differences according to species but also underline possible differences in mechanisms involved in preantral as opposed to antral follicle death. In the present study, some primordial, intermediate, and primary follicles showed abnormal morphology (contracted oocyte and chromatin condensation) and appeared untagged by TUNEL, suggesting a possible absence of DNA fragmentation in some damaged follicles. Vitrification has been suggested as a superior option than cryopreservation by slow freezing to both preserve preantral follicles and stroma integrity [17–19]. Similarly, a recent study in sheep model reported a decrease in proliferation activity in stroma compartment after slow freezing when compared with vitrification [38]. In the present study, the proportion of stroma cells positive for TUNEL in fresh ovarian cortex fragments at the initiation of culture was extremely low. There was no effect of cryopreservation treatment on the proportion of stroma cells labeled by TUNEL at the initiation of culture. Culture duration increased the proportion of apoptotic cells within the stroma but this effect was independent from the two cryopreservation methods. Overall, results from histology and immunohistochemistry for PCNA and TUNEL suggest that both slow-freezing and vitrification protocols are efficient and preserve ovarian cortex follicular reserve, stroma integrity, and capacity to sustain basal folliculogenesis in vitro.

In parallel with the histological and immunological analyses, the presence of the steroids (estrogen and progesterone) in the culture media over time was measured. The steroidogenic activity of ovarian tissue may represent an interesting tool for evaluating the viability of the tissue after cryopreservation [45]. For all treatment groups, estradiol secretion followed the same trend, increasing after the sixth day of culture to reach a maximum on day 9 of culture, which is similar to that previously reported for cultured fresh tissue [39]. The increase in estradiol secretion during culture is probably due to an increase in the proportion of follicles reaching more advanced stages of development [46]. Progesterone levels secreted in the culture medium were not affected by cryopreservation treatment. Progesterone increased sharply until the fifth day of culture, then decreased significantly until day 8 before a second increase in synthesis on day 9 of culture. Similar increase in progesterone levels in spent media from human ovarian cortex culture has been reported [47, 48] and a premature luteinization of growing follicle has been proposed as a possible explanation to this phenomenon [49, 50]. However, the rapid rise in progesterone concentrations observed in the present study might also be explained by luteinization of damaged early antral follicles consecutive to dissection of ovaries. Indeed, the dissection of the ovarian cortex is likely to associate the destruction or rupture of some of the largest follicles present within cortex pieces, leading in turn to the luteinization of granulosa cells. The increase in progesterone synthesis noted at the end of culture is probably due to the development and luteinization of secondary and tertiary follicles within the cultured tissue. However, no significant differences were observed in estradiol or progesterone secretions between the fresh, thawed, and

warmed fragments, confirming similar steroidogenic activities of the tissue after cryopreservation.

On the basis of histological, immunohistochemical, and functional studies, carried out after the cryopreservation of sheep ovarian tissue by slow freezing or non-equilibrium vitrification and followed by in vitro culture, our data clearly provide new arguments illustrating that the vitrification protocol is at least as efficient as slow freezing. The results obtained under both cryopreservation treatments were generally found to be comparable to those observed for fresh tissue. Further studies, focusing on long-term tissue function using xenografts on immunodeficient mice or using autografts strategies will be performed at thawing or warming to better address the most effective approach for ovarian cryopreservation of this large mammal model.

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Author contributions YL designed and performed the experiments and analysis, wrote the manuscript, and secured the funding. LC performed the experiments and analysis and contributed to first draft of the manuscript. ND performed the experiments and LL performed the assay for estradiol. DM, PP, and PM contributed to experimental design and revised the manuscript. MJB performed the experiments and analysis, wrote, and revised the manuscript.

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