human reproduction

In vitro development of secondary follicles from cryopreserved rhesus macaque ovarian tissue after slow-rate freeze or vitrification

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BACKGROUND: Ovarian tissue cryopreservation is the only option for preserving fertility in prepubertal girls and cancer patients requiring immediate treatment. Following ovarian tissue cryopreservation, fertility can be restored after tissue transplant or *in vitro* follicle maturation.

METHODS: Macaque ($n = 4$) ovarian cortex was cryopreserved using slow-rate freezing (slow freezing) or vitrification. Tissues were fixed for histology or phosphohistone H3 (PPH3) analysis, cultured with bromodeoxyuridine (BrdU) or used for three-dimensional secondary follicle culture. Follicular diameter and steroid hormones were measured weekly.

RESULTS: Slow freezing induced frequent cryo-injuries while vitrification consistently maintained morphology of the stroma and secondary follicles. PPH3 was similar in fresh and vitrified, but sparse in slow-frozen tissues. BrdU uptake appeared diminished following both methods compared with that in fresh follicles. *In vitro* follicle survival and growth were greater in fresh than in cryopreserved follicles. Antrum formation appeared similar after vitrification compared with the fresh, but was reduced following slow freezing. Steroid production was delayed or diminished following both methods compared with fresh samples.

CONCLUSIONS: Secondary follicle morphology was improved after vitrification relative to slow freezing. Following vitrification, stroma was consistently more compact with intact cells typical to that of fresh tissue. BrdU uptake demonstrated follicle viability post-thaw/warming. For the first time, although not to the extent of fresh follicles, macaque follicles from cryopreserved tissue can survive, grow, form an antrum and produce steroid hormones, indicating some functional preservation. The combination of successful ovarian tissue cryopreservation with *in vitro* maturation of follicles will offer a major advancement to the field of fertility preservation.

Key words: cryopreservation / ovarian tissue / non-human primates / *in vitro* follicle culture / vitrification

Introduction

The 5-year survival rate for female cancers of all ages is 68%, and is increased to 82% when the patient is under 45 years old or of reproductive age (Altekruse, 2010). With advanced detection tools, patients are diagnosed and treated for cancer at a younger age and are more likely to survive. However, cancer treatments can deplete follicles in the ovary leading to premature ovarian failure, infertility and long-term health risks associated with menopause (Schmidt *et al.*, 2010). Currently, embryo cryopreservation is the preferred method of fertility preservation, although oocyte cryopreservation techniques are markedly improving (Noyes *et al.*, 2009); however, for female cancer patients who are pre-pubertal

or require immediate cancer therapy, ovarian tissue cryopreservation offers their only hope for future fertility. Other unique advantages offered by cryopreserving ovarian tissue include the preservation of thousands of oocytes and possible restoration of ovarian function temporarily if followed by autograph transplantation. In 2004, fertility preservation using ovarian tissue cryopreservation was made a reality in a cancer patient, who after chemotherapy, underwent autotransplantation of her cryopreserved tissues and subsequently, delivered a healthy baby (Donnez *et al.*, 2004); however, to date, there are only 13 live births reported (Donnez *et al.*, 2004; Meirow *et al.*, 2005; Demeestere *et al.*, 2007; Andersen *et al.*, 2008; Silber *et al.*, 2008; Demeestere *et al.*, 2010; Donnez *et al.*, 2011; Ernst *et al.*, 2010; Roux *et al.*, 2010; Sanchez-Serrano *et al.*,

2010), indicating an urgent need for further improvements of the current technique.

Two common methods for cryopreservation are slow-rate freezing (slow freezing) and vitrification. Conventional slow freezing utilizes a lower concentration of cryoprotectants, resulting in extracellular freezing without serious toxic and osmotic damage inside the cell (Gosden et al., 2010). On the other hand, vitrification requires a higher concentration of cryoprotective agents and a higher cooling rate. Once vitrified, a sample transforms into a glass-like state and has relatively insignificant intracellular and extracellular ice crystals, but the concentrated cryoprotectants can induce cellular toxicity and cause osmotic trauma (Fahy et al., 1990). The gold standard for cryopreservation has been slow-rate freezing; however, in recent years, vitrification has gained popularity due to promising results observed in vitrified embryos and oocytes (Kuwayama et al., 2005; Cobo et al., 2008; Abdelhafez et al., 2010; Cobo et al., 2010). For ovarian tissue cryopreservation, efforts have been made to compare the two methods, but results are controversial. Mixed results may be a reflection of inconsistent tissue size, heterogeneity of the follicle pool, different types and concentrations of cryoprotectants, and variable end-points used among studies, resulting in data favoring either slow-rate freeze (Gandolfi et al., 2006; Isachenko et al., 2009; Rahimi et al., 2009) or vitrification (Silber et al., 2010), or suggesting that both are equivalent (Yeoman et al., 2005; Wang et al., 2009).

Currently, live births are very rare after ovarian tissue transplantation likely due to ischemic damage as well as damage caused by slow freezing procedures. However, the greatest concern for ovarian tissue autotransplantation is the possibility of re-introducing malignant cells back to cancer patients (Sonmezer et al., 2005). This can be circumvented through in vitro maturation of follicles isolated from cryopreserved ovarian tissue. Recent development of a three-dimensional (3D) matrix through bio-engineering has the added advantage over a two-dimensional environment of maintaining in vitro structural integrity and allows isolated secondary follicles from the fresh ovary to develop an antrum and yield mature oocytes that are capable of fertilization and embryo development, and have resulted in live offspring in mice (Xu et al., 2006). Various culture systems have also been applied to pre-antral follicles from cryopreserved ovarian tissues in rodents and resulted in metaphase II oocytes (Newton and Illingworth, 2001), blastocysts (Smitz and Cortvrindt, 1998) and live pups (Wang et al., 2011). However, in vitro maturation of primate follicles is technically challenging due to the greater magnitude of growth (pre-antral follicle = 100–200 μm to pre-ovulatory follicle = 6–20 mm) and longer duration of folliculogenesis (months). When encapsulated in alginate and cultured, macaque secondary follicles can grow to the antral stage, produce steroids and growth factors, and yield healthy oocytes within 40 days (Xu et al., 2010). The ability to mature pre-antral follicles in vitro provides not only a research technique to evaluate follicular function following ovarian tissue cryopreservation, but ultimately, a potential clinical tool for fertility preservation.

In the current study, we used a non-human primate model which provides a plentiful supply of tissue for systematic comparison of slow-freezing and vitrification methods for ovarian tissue cryopreservation. In addition to examining the traditional morphological and histological end-points, we investigated cellular proliferation using phosphohistone H3 (PPH3) and bromodeoxyuridine (BrdU), and explored in vitro secondary follicle maturation using an encapsulated 3D culture system as a novel indicator of follicular function after cryopreservation.

Materials and Methods

Animals and ovary collection

The general care and housing of rhesus macaques (Macaca mulatta) at the Oregon National Primate Research Center (ONPRC) has been previously described (Wolf et al., 1990). The studies were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and all protocols were approved by the ONPRC Animal Care and Use Committee. Adult female rhesus monkeys exhibiting normal menstrual cycles ($n = 2$, age $= 6\frac{1}{2}$ and $9\frac{1}{2}$) or undergoing necropsy with unknown reproductive phase ($n = 2$, age $= 11$) were used in this study. Ovaries were collected from anesthetized monkeys (on Days 4–5 of the follicular phase, first day of menses is Day 1) by laparoscopy or during necropsy procedures. A blood sample was obtained prior to ovariectomy for steroid hormone measurements. The serum estradiol (E_2) level in animals during the follicular phase and prior to necropsy was 29 ± 2 and 41 ± 6 pg/ml, respectively, with a progesterone (P_4) level of <0.2 ng/ml in all animals. Ovaries were immediately transported to the laboratory on ice in 3-(N-morpholino) propanesulfonic acid (MOPS) buffered tissue holding media (CooperSurgical Inc., Trumbull, CT, USA).

Ovarian tissue processing

Ovaries were bivalved and the medulla removed with curved iris scissors in holding media supplemented with 15% (v/v) serum protein substitute (SPS, CooperSurgical Inc.) and 29 μg/ml of the antioxidant L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate (ascorbic acid phosphate, Sigma, St. Louis, MO, USA). Using a scalpel, the cortex was cut into $3 \times 3 \times (1–1.5)$-mm^3 cortical pieces. Each piece was examined under a dissecting scope for the presence of secondary follicles and only tissues ($n = 44 \pm 10$ per animal) that showed visible secondary follicles were used in the current experiment. Due to the considerable heterogeneity of follicle distribution in the primate ovary (Schmidt et al., 2003), the inconsistency of follicular density among tissues was minimized by selecting only cortical pieces with secondary follicles. All procedures for ovarian tissue processing were done on ice.

Conventional slow-rate freezing and thawing

The slow-freezing protocol was based on the method of Newton et al. (1996), and modified by Yeoman et al. (2005). Tissues were placed individually into cryovials loaded with 0.9 ml of freezing media containing 1.5 M ethylene glycol (EG) in MOPS-diluent with 0.1 M sucrose (customized by CooperSurgical, Inc.). After gentle vortexing, tissues were moved to a controlled-rate freezer (Freeze Control System, CryoLogic, Victoria, Australia) with temperature controlled by CryoGenesis V software (CryoLogic), and equilibrated at 5°C for 30 min. Samples were cooled at 2°C/min to −7°C, held for 20 min at −7°C, and when the actual temperature reached −7°C, manual seeding was performed using a cotton swab saturated with liquid nitrogen. Cooling was continued at 0.3°C/min until sample temperature reached −30°C, and cryovials were then plunged into liquid nitrogen for storage. To thaw, samples (maximum of three samples per handling) were placed at room temperature for 1 min and then plunged into a 37°C water bath for 2 min with gentle shaking. Tissues were transferred into 1.0 M EG-freezing media (diluted with holding media) for 10 min, 0.5 M EG-freezing media for 10 min and holding media alone for 5 min at room temperature.

Vitrification and warming

Tissues were equilibrated, three pieces per Petri dish, sequentially in vitrification solutions containing 1.2 M glycerol (10% glycerol, v/v, 8 min), 1.2 M glycerol + 3.6 M EG (10% glycerol + 20% EG, 8 min) and 3 M glycerol + 4.5 M EG (25% glycerol + 25% EG, 5 min) with shaking on ice (Ali and Shelton, 1993; Yeoman et al., 2005). Following the last incubation, individual tissues were blotted with sterile absorption spears (Fine Science Tools, Foster City, CA, USA) and transferred onto a piece of 8×4 mm^2 aluminum foil. The foil was immediately submerged into liquid nitrogen and transferred into a liquid nitrogen-filled cryovial for storage. For warming, each cryovial was removed from storage individually followed by immediate transfer of the tissue into 0.5 M sucrose (5 min, 37°C, in holding media), then 0.25 M sucrose (5 min), 0.125 M sucrose (5 min) and holding media (10 min, twice). Both vitrification and warming solutions were made with holding media and supplemented with 15% (v/v) SPS and 29 μg/ml of ascorbic acid phosphate.

Tissue processing, histology and PPH3

PPH3 was used as a mitotic marker for the evaluation of cellular proliferation (Brenner et al., 2003). Fresh, frozen and vitrified tissues ($n = 4–6$ per group) were fixed in 4% paraformaldehyde at 4°C overnight, processed for paraffin embedding and serial sectioned at 5 μm. A subset of tissue sections (every 20th section) was stained with hematoxylin and eosin (H&E) for histological analysis. Adjacent sections were prepared for immunostaining by antigen retrieval (pressure cooker, 10 mM citrate buffer, 10 min) and incubation with 0.3% H_2O_2 (30 min) and serum block (60 min). Non-immune serum or primary antibody against PPH3 (1:1000; rabbit polyclonal IgG, Upstate, Billerica, MA, USA) was applied and visualized with biotinylated secondary antibodies and DAB (Vector, Burlingame, CA, USA).

Tissue culture and BrdU uptake

BrdU is incorporated into newly synthesized DNA in dividing cells and was used for the evaluation of post-thaw and post-warm tissue viability in culture (Onions et al., 2008). Fresh, frozen and vitrified tissues ($n = 2$ per treatment group) were cultured in follicle culture media containing BrdU (0 or 50 μM, BD Pharmingen, Franklin Lakes, NJ, USA) for 48 h at 37°C in 5% CO_2 in atmospheric air. Tissues were cultured (one per well) in 24-well Lumox^{TM} culture dishes (Greiner Bio-One, Monroe, NC, USA) with a fluorocarbon film bottom that provides enhanced gaseous exchange. After culture, tissues were fixed, processed and sectioned as described above. Every 30th section was immunostained for BrdU (1:200; mouse monoclonal IgG, MP Biomedicals, Solon, OH, USA) using the above immunostaining protocol with an additional 2 M HCl blocking step (30 min) prior to the serum block procedure. Negative controls included tissue cultured in the absence of BrdU as well as primary antibody omission during the staining protocol.

Quantification of secondary follicles in tissue sections

Tissues were assessed in serial sections stained with H&E [$n = 4$ animals; 4 pieces/animal; every 20th section (100 μm intervals); 8–12 sections/piece]. Secondary follicles were counted and evaluated as normal or abnormal based on histology. Normal follicles were classified as those exhibiting direct contact between the oocyte and surrounding granulosa cells as well as between neighboring granulosa cells, and the absence of any contraction of the cytoplasm as well as pyknotic nuclei in the oocyte and granulosa cells. Abnormal oocytes were shrunken or filled with vacuoles, and abnormal granulosa cells showed pyknotic nuclei, enlarged space between neighboring cells or the presence of vacuoles.

Secondary follicles were also quantified in sections stained for PPH3 [$n = 4$ animals; 4 pieces/animal; every 20th section (100 μm intervals); 8–12 sections/piece] and BrdU [$n = 4$ animals; 2 pieces/animal; every 30th section (150 μm intervals); 5–7 sections/piece]. Follicles that showed at least one granulosa cell with positive staining for PPH3 or BrdU were counted as positive. Secondary follicles in adjacent sections stained with H&E and PPH3 were evaluated and assumed to be identical, whereas follicles examined in BrdU-labelled sections were of different cortical tissues. Only follicles with a visible oocyte were counted for accurate examination of their developmental stage.

Secondary follicle isolation, encapsulation and culture

A subset of fresh, frozen and vitrified tissues ($n = 3–4$/group/animal) were used for secondary follicle isolation. Secondary follicles ($n = 8–22$/group/animal, 120–250 μm in diameter, partly surrounded by stromal tissue) with a visible oocyte (round and centrally located within the follicle), an intact basement membrane and no antral cavity were mechanically isolated using 25-gauge needles. Isolated follicles were encapsulated in 0.25% alginate as previously described (Xu et al., 2010, 2011); these studies indicated that the 0.25% alginate increases the rate of follicle survival and number of follicles achieving 1 mm in diameter. Although follicles can survive and grow with 0.5% alginate as reported previously (Xu et al., 2009), it appeared that this alginate concentration is too rigid for follicles to expand to a larger size and allow the formation of a large antrum that resembles the one in vivo. Encapsulated follicles were transferred to individual wells of a 48-well plate containing 300 μl of alpha minimum essential medium (αMEM) culture media supplemented with 2.16 mg/ml glucose, 60 μl/ml SPS, 44 mIU/ml follicle-stimulating hormone, 0.5 mg/ml bovine fetuin, 29 μg/ml ascorbic acid phosphate, 5 μg/ml transferrin, 0.5 μg/ml insulin and 5 ng/ml sodium selenite (Sigma). A maximum of 12 encapsulated follicles were loaded per 48-well plate, while the remaining wells were loaded with 300 μl of sterile water to minimize media evaporation during long-term culture. Encapsulated follicles were cultured at 37°C in 5% CO_2 in atmospheric air for 5 weeks. Every 2 days, half of the culture media (150 μl) was exchanged with fresh culture media (prepared weekly) and stored at −20°C for subsequent hormonal measurements.

Follicle survival and growth

Follicle health and diameter were assessed using an Olympus CK40 inverted microscope attached to an Olympus DP11 digital camera (Center Valley, PA, USA). Follicles were considered to be degenerating if (i) the oocyte was no longer surrounded by a layer of granulosa cells, (ii) the oocyte became dark, (iii) the granulosa cells became dark and lost connection with one another, or (iv) the diameter of the follicle decreased. For each follicle, weekly photographs were taken and diameters measured using ImageJ (National Institutes of Health, Bethesda, MD, USA). The mean of two measurements per follicle (perpendicular to each other) was then calculated and reported as the follicle diameter. Follicle growth was defined as a significant increase in follicle diameter in comparison to the day of isolation (Week 0 or Day 1).

Hormone assays

Serum and weekly follicle culture media concentrations of E_2 and P_4 were determined by the Endocrine Technology and Support Core at the ONPRC using an Immulite 2000, a chemiluminescence-based automatic clinical ELISA-based platform (Siemens Healthcare Diagnostics, Deerfield, IL, USA) as previously described (Xu et al., 2010). The sensitivity of E_2 assays by the Immulite 2000 is 20 pg/ml and 0.2 ng/ml for P_4.

Concentrations of androstenedione (A_4) were measured by RIA using a DSL-3800 kit (Diagnostic Systems Laboratories, Inc., Webster, TX, USA) with 0.1 ng/ml sensitivity. The intrassay and inter-assay coefficients of variation with the Immulite 2000 are $<$15% for all assays. Values reported were corrected for culture medium blanks.

Data analysis and statistics

Data are presented as mean $\pm$ SEM and analyzed by combining observations to make one single count per animal giving an equal contribution from each animal. Follicle survival and antrum formation represent the percent (mean $\pm$ SEM) of four individual animals in each treatment group. One-way repeated measures analysis of variance (ANOVA) was performed for weekly follicle diameter and hormone production within treatments, chi square was used for proportional data and ANOVA was used for all other comparisons (SigmaPlot 11.0, Systat Software, Inc, San Jose, CA, USA). Differences were considered significant when $P \leq 0.05$. Data for numbers of secondary follicles in H&E, PPH3 and BrdU sections are presented as the average and sum of follicles counted in all four animals in each group and the percentage of follicles in each category relative to the total number of secondary follicles of the same group. Data for follicle counts were not analyzed statistically among different groups due to variation in follicle density within each cortical piece. In addition, follicles in both cryopreserved groups that had been completely lysed would not be detected in counting follicles for histology, BrdU and PPH3 end-points.

Results

Follicular morphology and stromal integrity

Follicular morphology was only examined in pre-antral follicles since antral follicles were rarely present in these cortical strips. Follicles in fresh tissue showed intact morphology with minimal cell-to-cell space between the oocyte and surrounding granulosa cells as well as between neighboring granulosa cells (Fig. 1a–d). A homogeneous and vacuole-free distribution of cytoplasm in oocytes was also observed in follicles from fresh tissue. There was no difference in morphology of primordial and primary follicles in frozen (Fig. 1h) or vitrified (Fig. 1l) tissue compared with those in fresh tissue (Fig. 1d). Morphology of secondary follicles, however, was consistently better preserved in vitrified tissue (Fig. 1j and k) compared with tissue after slow freezing (Fig. 1f and g). Observed mostly in frozen tissue and less in vitrified tissue, cryo-induced damage in secondary follicles included shrunken oocytes (Fig. 1e and f, arrow; o,q), vacuoles in oocytes (Fig. 1f and j, arrowhead; n,r) and granulosa cells (Fig. 1f, *; p), granulosa cells with pyknotic nuclei and enlarged space between the oocyte and surrounding granulosa cells as well as between neighboring granulosa cells, resulting in the apparent loss of cell density (Fig. 1e, X). Secondary follicles were categorized into ones showing damaged oocytes (Fig. 1n and o), granulosa cells (Fig. 1p) or both (Fig. 1q and r) and quantified. Total number of secondary follicles in H&E stained sections and the distribution (presented as %) of damaged follicles in fresh and cryopreserved tissues are presented in Table I. Damaged oocytes were observed in fresh tissue in all animals. While no statistical analysis was performed, there seem to be a greater proportion of damaged secondary follicles following both cryopreservation methods in comparison to fresh tissue. Specifically, damage observed in granulosa cells seemed to be more prominent after slow freezing when compared with vitrification.

Dense stromal cells and collagen matrix made up the architecture in fresh (Fig. 1a) and the majority of vitrified (Fig. 1i) tissues. In contrast, while the collagen matrix remained intact after slow freezing, abundant necrotic stromal cells with pyknotic nuclei were more frequently observed in comparison to fresh and vitrified tissue. In addition, abnormal space was observed throughout the stroma in slow-frozen tissues (Fig. 1e and g; #) and to a lesser extent in vitrified tissues.

PPH3 expression

In fresh tissue, abundant PPH3 expression was found in the nucleus of the majority of granulosa cells in healthy growing follicles (Fig. 2a and b), while atretic follicles exhibited very few positive granulosa cells (Fig. 2a, arrow). Following quantification, a high proportion of secondary follicles in all three groups showed some level (at least one granulosa cell showing positive staining) of PPH3 (Table I). However, based on our gross observations, fewer granulosa cells within a follicle appeared to express PPH3 in slow-frozen tissue (Fig. 2c and d) relative to the fresh tissue. In contrast, most growing follicles in vitrified tissue showed a similar PPH3 pattern (Fig. 2e and f) to the fresh tissue. Negative controls showed no positive staining (Fig. 2g and h).

BrdU uptake

Similar to PPH3 expression, BrdU uptake was observed in granulosa cells of primary, secondary and multilayer follicles with additional staining of some stromal cells in the fresh tissue (Fig. 3a and b). BrdU incorporation was also evident in growing follicles in frozen (Fig. 3c and d) and vitrified tissue (Fig. 3e and f), although to a lesser extent relative to fresh tissue (Table I). BrdU uptake in the stroma of cryopreserved tissue (Fig. 3c–f) was rarely observed. Negative controls, including tissue cultured in the absence of BrdU, as well as primary antibody omission during the staining protocol, both showed no positive staining (Fig. 3g and h).

Encapsulated 3D follicle culture: survival and growth

Total and average number of secondary follicles and their survival and antrum formation rates are summarized in Table II. Five-week survival rate for follicles from frozen and vitrified tissue was reduced ($P < 0.05$) relative to those from the fresh tissue. Antrum formation was observed by Weeks 3–4 in 67 ± 6% of fresh follicles that survived 5 weeks of culture. The ability to form antrum was diminished (13 ± 13%; Table II; $P < 0.05$) in the slow-freezing group, but remained similar in the vitrification group (43 ± 2%) in comparison to the fresh.

Follicle diameters were similar in all groups on the day of isolation (148 ± 5 µm). Follicles from fresh tissue showed increased diameters during Weeks 3 (360 ± 44 µm), 4 (461 ± 56 µm) and 5 (491 ± 51 µm; Figs 4 and 5, $P < 0.05$). Follicular growth was delayed in slow freezing and vitrification groups relative to the fresh (Figs 4 and 5). An increase in diameter was not observed until Week 4 (214 ± 16 µm) in the vitrification group and 5 (223 ± 16 µm) in the slow-freezing group, and these increases were less ($P < 0.05$) than that of the fresh group (Fig. 4). One follicle (VitF) from vitrified tissue showed a much different growth pattern (Fig. 5). VitF showed antrum formation during Week 5 and continued to grow in culture for a total of 10 weeks. The greatest diameter reached by this
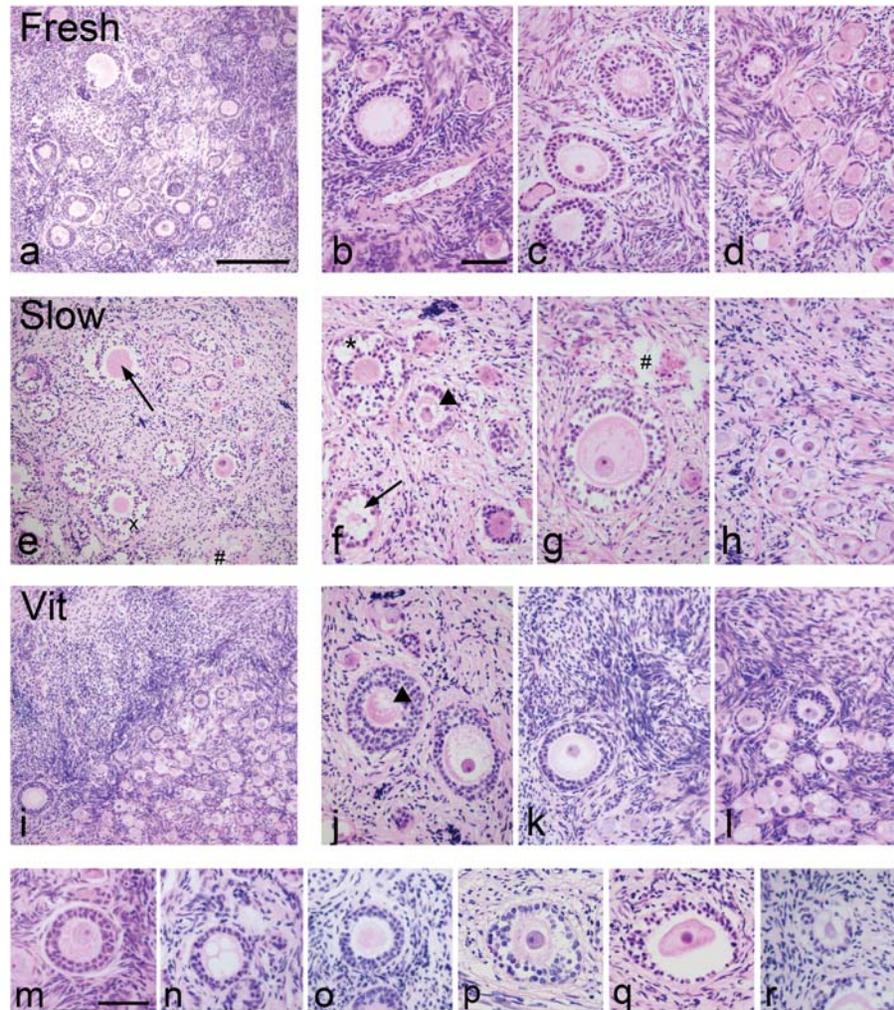
Figure 1 Fresh tissue showed healthy stroma (a) as well as primordial (d), primary (d) and secondary follicles (b, c, m). Post-thaw, slow freezing (Slow) induced cryo-injuries and cellular degeneration in stroma (e) and secondary follicles (f, g), but not in primary and primordial follicles (h). Tissues after vitrification (Vit) not only showed morphologically normal primordial and primary follicles (l), but also resulted in healthy stroma (i) and exhibited fewer damaged secondary follicles (j, k) in comparison to Slow. Cryo-damaged follicles exhibited shrunken oocytes (e, f, arrow; o, q), vacuoles in oocytes (f, j, arrowhead; n, r) and granulosa cells (f, *; p) and enlarged spaces between oocyte and granulosa cells as well as between neighboring granulosa cells (e, X; o–q). Damage to the stroma, more frequently observed following slow freezing in comparison to vitrification, included abundant lysed stromal cells and the presence of abnormal empty spaces throughout the extracellular matrix (e, g, #). Scale bar = 200 μm (a, e, i), 50 μm (b–d, f–h, j–r).

particular follicle was 512 μm, which exceeded that of any other follicles from the frozen or vitrified groups and was comparable to that of fresh follicles. Follicles in all three groups shown in Fig. 5 represent those that formed antrum; these follicles exhibited greater diameters than the average presented in Fig. 4.

Encapsulated 3D follicle culture: hormone production

E_2 production by follicles of fresh, frozen and vitrified tissue was similar at baseline (Week 0; 78 ± 3 pg/ml). Levels of E2 in fresh follicles increased above baseline during Week 3 (787 ± 302 pg/ml) and continued to rise through the end of culture (Week 5 = 12,558 ±

6,401 pg/ml; Fig. 6A, $P < 0.05$). Similar to our previous findings (Xu et al., 2010), E2 production by fresh follicles that formed an antrum correlated with follicle size and ranged from 502 to 51 720 pg/ml. In contrast, E_2 levels produced by follicles of slow-frozen and vitrified tissue did not increase above baseline until Week 4 (1,025 ± 1,000 pg/ml) for slow frozen and Week 5 (428 ± 73 pg/ml) for vitrified groups. The average E2 production from follicles of slow-frozen and vitrified tissue was less ($P < 0.05$) than the mean value produced by follicles from fresh tissue by Week 5 (Fig. 6A). However, these levels from the slow-frozen and vitrified tissues were similar to the lower end of the range of E2 levels produced by fresh tissue.

P_4 production (Fig. 6B) from follicles of fresh tissue raised above baseline (0.48 ± 0.13 ng/ml) after 1 week of culture (7.9 ±

Table I Average and total numbers as well as percentages of secondary follicles in fresh, frozen (Slow) and vitrified (Vit) tissues exhibiting damaged oocytes, granulosa cells or both and expressing phosphohistone H3 (PPH3) and BrdU uptake.

Morphological analysis	% Damaged (total number of follicles)			Average (total) number of follicles assessed
	Oocyte	Granulosa	Both	
Fresh	32 ± 2 (24)	3 ± 2 (3)	2 ± 1 (2)	19 ± 2 (74)
Slow freezing	91 ± 5 (64)	80 ± 10 (52)	77 ± 10 (50)	18 ± 6 (70)
Vitrification	65 ± 11 (57)	27 ± 12 (22)	26 ± 11 (21)	21 ± 4 (83)

Granulosa proliferation	% PPH3 (number of follicles)		Average (total) number of follicles assessed	% BrdU (number of follicles)		Average (total) number of follicles assessed
	Positive	Negative		Positive	Negative	
Fresh	95 ± 2 (72)	5 ± 2 (4)	19 ± 2 (76)	65 ± 9 (32)	35 ± 9 (17)	12 ± 5 (49)
Slow freezing	84 ± 9 (59)	16 ± 9 (9)	17 ± 6 (68)	25 ± 15 (18)	75 ± 15 (21)	10 ± 5 (39)
Vitrification	87 ± 6 (70)	13 ± 6 (7)	19 ± 4 (77)	54 ± 2 (28)	46 ± 2 (24)	13 ± 2 (52)

Data are presented as mean $\pm$ SEM of four individual animals in each treatment group.

1.5 ng/ml; $P < 0.05$) and continued to rise to Week 5 (72 ± 17 ng/ml). Similar to E_2, production of P_4 by follicles of slow-frozen and vitrified tissue was delayed to Week 4 (14.6 ± 6.3 ng/ml) for frozen and Week 3 (6.4 ± 2.2 ng/ml) for vitrified. By Week 5, P_4 was lower ($P < 0.05$) in both cryopreserved groups (Slow = 20.7 ± 8.5 ng/ml; Vit = 12.0 ± 4.0 ng/ml) relative to the fresh tissue (Fig. 6B).

Elevated levels of A_4 were observed during Week 4 (0.53 ± 0.15 ng/ml; $P < 0.05$) in follicles from fresh tissue, compared with the baseline level (0.06 ± 0.0004 ng/ml; Fig. 6C). In contrast, follicles from frozen and vitrified tissue did not show an appreciable level of A_4 throughout 5 weeks in culture (at Week 5: Slow = 0.14 ± 0.05; Vit = 0.08 ± 0.01 ng/ml; Fig. 6C). While there seemed to be an apparent rise in A4 production after 4 weeks in follicles from the slow-freezing group, this is due to the level observed in one follicle from the whole group, which accounts for the large standard error in the data. Nevertheless, the level produced by this follicle (0.37 ng/ml) from the slow-freezing group at Week 4 is less than that of the fresh follicles. Similarly, the rise in E2 and P4 after 4–5 weeks in follicles from the slow-freezing group, although not to levels of the fresh, is the result of hormone reproduction from the same individual follicle.

Again, as an exception, VitF showed a continuous rise in E_2, P_4 and A_4 levels (2635 pg/ml, 180 ng/ml and 0.96 ng/ml, respectively, in Week 10) that were comparable to those produced by follicles from fresh tissue at 4–5 weeks of culture.

Discussion

Both slow freezing and vitrification were able to preserve the morphology of primordial and primary follicles; however, secondary follicles and the stroma were consistently better preserved with vitrification based on gross histological observation. While stromal integrity has been suggested to be maintained following vitrification

(Keros et al., 2009; Silber et al., 2010; Xiao et al., 2010) and damaged following slow freezing (Gook et al., 1999, 2000; Keros et al., 2009) in previous studies, the current experiment is the first to show morphologically intact secondary follicles in macaque ovarian tissue after vitrification. Cryoprotectants (glycerol and EG) for the vitrification procedure were chosen for their lower cellular toxicities relative to others, and a similar protocol has been demonstrated with promising results in vitrification of embryos and tissues (Ali and Shelton, 1993; Yeoman et al., 2001, 2005). Different cryopreservation methods (Newton et al., 1996; Yeoman et al., 2005) tested in the current study appear to preserve different classes of pre-antral follicles when directly compared in macaque tissue.

Histologically, the majority of secondary follicles from frozen tissue showed damage in oocytes as well as granulosa cells (i.e. vacuoles, separation of oocyte and granulosa cells and loss of cell content). Damage in secondary follicles was observed to a lesser extent in vitrified tissues. In agreement with previous findings (Keros et al., 2009; Silber et al., 2010; Xiao et al., 2010), good preservation of stroma was observed in vitrified tissue and may imply better ability of the tissue to revascularize and to support follicular development if tissue transplantation and culture, respectively, are performed. In the current study, only cortical tissues (1–1.5 mm thick cortex) were included, although stroma density can be varied in different regions of the cortex. While stroma density appeared to be similar in some areas of vitrified and slow-frozen tissue (i.e. Fig. 1j and 1h), we observed with more consistency among all animals that stroma is more compact in vitrified tissues in comparison to slow-frozen tissue. In addition, even though dense stroma can also be found in areas of the slow-frozen tissue (i.e. Fig. 3c), stromal cells were mostly atretic with pyknotic nuclei. Stroma from tissues labeled with BrdU was not evaluated and cannot be compared with tissues used for histology and PPH3 staining because BrdU-labeled tissues were
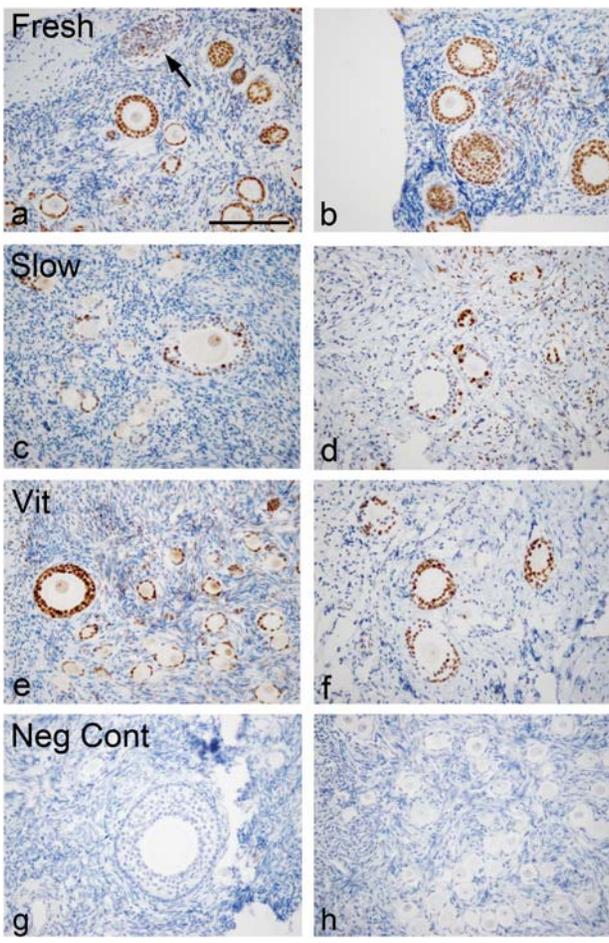
Figure 2 In fresh tissue, PPH3 (dark brown) was expressed in the majority of granulosa cells of growing follicles including follicles transitioning from primordial to primary as well as primary and secondary follicles (**a**, **b**). Light staining, as seen in most of the granulosa cells of atretic follicles, was considered non-specific and negative for the presence of PPH3 (a, arrow). After slow freezing (Slow), only a few granulosa cells showed PPH3 expression in primary and secondary follicles (**c**, **d**). After vitrification (Vit), some follicles showed similar PPH3 expression (**e**) in comparison to the fresh tissue, while others showed few granulosa cells with PPH3 expression (**f**). Primary antibody omission negative control (Neg Cont; **g**, **h**) showed no positive staining. Scale bar = 150 μm.

Figure 3 BrdU uptake was observed in stromal cells as well as in granulosa cells of growing follicles in fresh tissues (**a**, **b**). Tissues cryopreserved with both slow freezing (Slow; **c**, **d**) and vitrification (Vit; **e**, **f**) showed less BrdU uptake in the stroma as well as growing follicles relative to fresh tissue. Negative controls, including tissue cultured in the absence of BrdU, as well as primary antibody omission during the staining protocol, showed no positive staining (**g**, **h**). Scale bar = 200 μm.

in culture for 48 h and effects of culture to stromal histology is unclear. In addition, stromal damage followed by slow freezing has also been demonstrated by Gook et al. (1999, 2000) and Keros et al. (2009), who attributed the freezing technique as the cause of stromal necrosis and damaged extracellular matrix. Indeed, abnormal space throughout the stroma was more frequently observed in slow-frozen tissue in comparison with vitrified tissue. This may be due to the formation of extracellular ice during slow freezing, which is avoided during vitrification. While other studies did not show extensive damage to the stroma and follicles while evaluating the slow freezing protocol, some of these studies showed only a small section of the tissue where primordial and primary follicles are located, but the presence
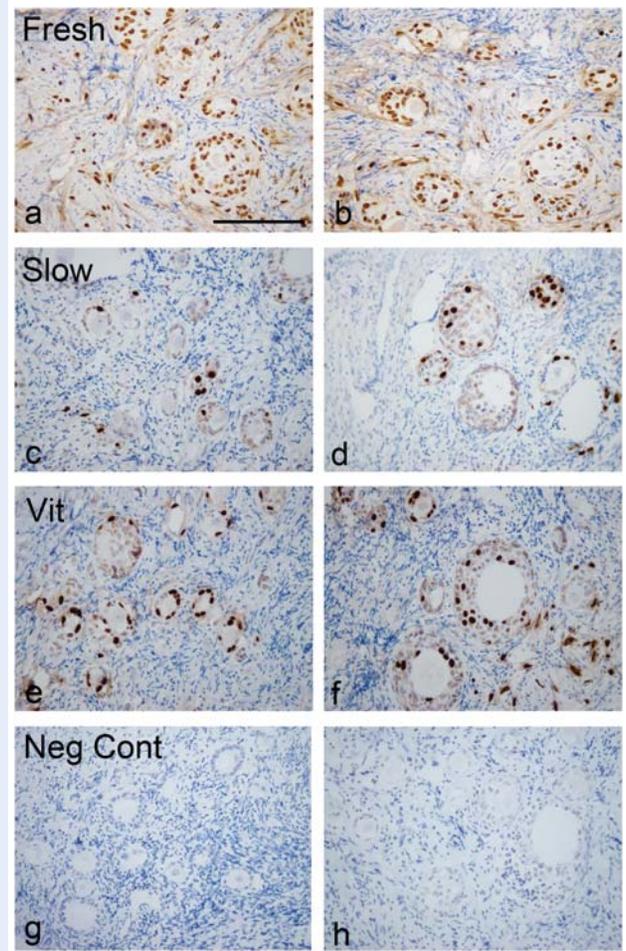
of secondary and multilayered follicles were rarely shown. If only a small tissue section of primordial and primary follicles were examined (Fig. 1h), morphology of follicles and stroma was comparable to the fresh; however, in a bigger section of the tissue, stromal and secondary as well as multilayered follicles were found to be damaged. We feel that our results represented an accurate demonstration of the tissue histology after freeze and thaw; however, morphometric analysis of stromal area will be necessary to quantify actual differences (Keros et al., 2009).

Challenges exist in evaluating survival and viability of cryopreserved ovarian tissue (Gook and Edgar, 2011). Descriptive analyses of tissue histology have been used in studies examining methods of ovarian tissue cryopreservation to indicate tissue health. In the current study, we have attempted to quantify the number of secondary follicles based on morphology. Our data suggest that a higher proportion

Table II Average number and total number (per animal, $n = 4$ animals) of secondary follicles and their survival rates (%) at 5 weeks and the ability to form antrum (%) among surviving follicles in follicles isolated from fresh, frozen (Slow) and vitrified (Vit) tissues.

	Fresh	Slow	Vit
Average (total) number of follicles	12 ± 2 (49)	19 ± 1 (75)	17 ± 2 (67)
Survival rate (number of follicles)	57 ± 7 (29)	9 ± 4 (7)[a]	20 ± 7 (12)[a]
Antrum formation rate (number of follicles)	67 ± 6 (19)	13 ± 13 (1)[a]	43 ± 2 (4)

Data are presented as mean ± SEM of four individual animals in each treatment group.

[a]Significant difference ($P \leq 0.05$) in comparison to the fresh group.
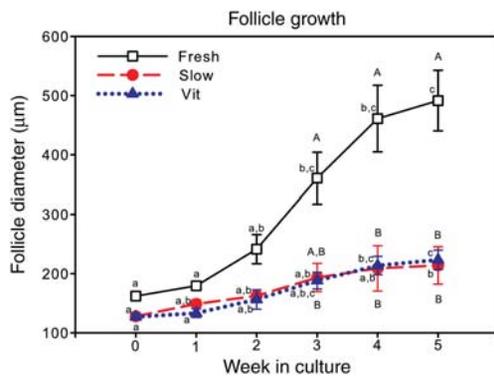

Figure 4 Weekly follicle diameter during 5 weeks in culture. Fresh follicles ($n = 29$) showed increased diameters in Week (wk) 3 and continued to grow, while follicles from the slow freezing (Slow; $n = 7$) and vitrification (Vit; $n = 12$) showed delayed and reduced growth in Week 5 when compared with the fresh. Lower case letters represent significant changes within the same group. Capital letters represent significant changes among different groups. Data are presented as mean ± SEM.

of secondary follicles show damage following slow freezing and vitrification in comparison to fresh tissue. Also, there seem to be more secondary follicles with granulosa cell damage after slow freezing compared with vitrification. However, we must take great caution in making generalized interpretation from these data. In primate ovaries, the distribution of follicles is heterogeneous and follicular density can vary more than two orders of magnitude in cortical tissues within the same ovary (Schmidt et al., 2003). Each cortical piece subjected to cryopreservation does not have the same number or the same classes of follicles, so it is invalid to compare numbers of follicles after cryopreservation to those in the fresh group or between different cryopreservation methods. In addition, follicles that may have lysed after cryopreservation would not be detected following counting, resulting in an overestimation of follicular survival. For these reasons, follicular quantification from histological

end-points is difficult to validate and will require systematic morphometric analysis of the entire tissue or counting total numbers of follicles in each individual tissue prior to cryopreservation, which would be difficult to perform, if not impossible, especially for primordial follicles.

Cellular proliferation in fresh and cryopreserved tissues was examined using PPH3 immunostaining and BrdU uptake, and interestingly, results from these two techniques were not identical. Quantitative data showed high percentages of secondary follicles expressing PPH3 in all three groups. However, since follicles that showed at least one granulosa cell with positive PPH3 staining were considered positive, quantitative results do not reflect partial PPH3 expression as observed in most secondary follicles of slow-frozen tissue in comparison to the fresh and vitrified tissue. Based on gross observation, PPH3 expression in vitrified tissues was abundant and similar to that of fresh tissue, whereas following slow freezing, PPH3 expression was sparse and present at a lesser extent in most follicles. The apparent difference in PPH3 expression between slow-frozen and vitrified tissue, however, may be manifested by the discrepancy in tissue-processing time needed for slow freezing (hours) and vitrification (minutes). Phosphorylation at Ser10 of histone H3 is crucial for the progression through mitosis during the cell cycle and tightly correlated with chromosome condensation during prometaphase and metaphase with its dephosphorylation occurring during anaphase (Preuss et al., 2003). Based on the time a cell needs for the dephosphorylation of histone H3, it is possible to have cells that are no longer viable but their histone H3 may still be phosphorylated if the vitrification procedure occurs very rapidly. The freezing procedure may also alter the antigen preservation and affect levels of PPH3. Therefore, another marker of cellular proliferation that is not altered by tissue processing and low temperature may be needed to confirm these findings.

Uptake of BrdU, a marker for chromosomal replication during the S phase of mitosis (Gratzner, 1982), was evident in frozen and vitrified tissue after culture suggesting viable granulosa and stromal cells in cryopreserved tissues. BrdU uptake was examined in tissues cultured for 48 h after thawing and may therefore be a more accurate reflection for viability post-thaw as well as for the ability of cells to recover from cryopreservation procedures. From both gross observation of the entire tissue and quantification of secondary follicles, BrdU uptake appeared to be less in both frozen and vitrified tissues in comparison to those of the fresh, suggesting cryo-induced damage from vitrified and frozen tissues and a need for further improvement in both cryopreservation techniques.

The current experiment is the first to demonstrate long-term secondary follicle viability and function in vitro after tissue cryopreservation using encapsulated 3D culture. In the current study, secondary follicles were isolated from fresh and cryopreserved tissue without collagenase treatment to avoid further damage to follicles (Itoh and Hoshi, 2000; Gook et al., 2004). During normal development, a secondary follicle grows in size by proliferation of granulosa cells and an increase in oocyte diameter. This growth is accompanied by the formation of an antrum and increased production of steroid hormones E_2 by granulosa cells as well as P_4 and A_4 by theca cells. Previously, morphology and growth of pre-antral follicles from cryopreserved human ovarian tissue has only been examined in frozen, not vitrified, tissues and tested up to 14 days in culture, and the function of these
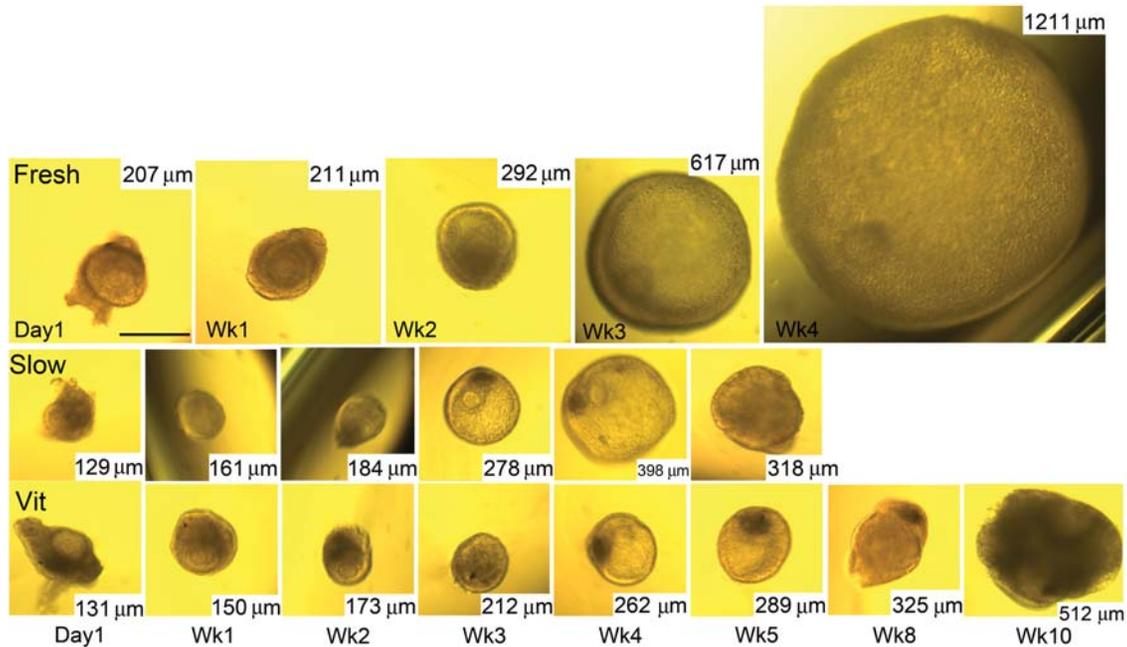
Figure 5 Follicles isolated from fresh tissues grew in size in 5 weeks of culture and formed an antrum by Week (wk) 3 or 4. Follicles isolated from frozen (Slow) and vitrified (Vit) tissue showed delayed growth but some were able to form an antrum. Follicles selected for this figure represented follicles that formed antrum, therefore exhibiting greater diameters than the average presented in Fig. 4. The series of images represent the growth pattern of a single follicle from each group during culture. Scale bar = 250 μm.

follicles was not investigated (Gook et al., 2004; Amorim et al., 2009). Others have placed cryopreserved tissues in short-term organ culture system and measured E_2 and P_4 production; interestingly, levels of E_2 (~300 pg/ml) and P_4 (~2 ng/ml) produced by individual tissues after 14 days in culture (Isachenko et al., 2002; Isachenko et al., 2007) were similar to those produced by one individual follicle in the current experiment. However, levels of both hormones are greatly influenced by follicular density and developmental stage of the follicles present, which can vary among cortical tissue samples; therefore, without knowing the number and types of follicles in the tissue, it is difficult to compare follicular functions based on hormone levels produced between individual follicles and tissues.

While results from encapsulated 3D follicle culture revealed cryo-induced functional damage in secondary follicles following both slow freezing and vitrification, follicles from vitrified tissue showed better preservation in the ability to form an atrum. During the 5-week 3D culture, follicle survival was reduced in slow-freezing and vitrification groups in comparison to the fresh follicles. Follicle growth, while diminished in comparison to the fresh, was delayed in follicles of vitrified and frozen tissues when compared with the fresh. The precise length of this delay cannot be determined since follicles in both cryopreserved groups never grew to the size of the fresh follicle. The ability to form an antrum among surviving follicles was reduced by slow freezing, but not vitrification, in comparison to the fresh follicles. However, it is possible that differences in antrum formation rate was not detected between fresh and vitrification or vitrification and slow-freezing groups due to low numbers of surviving follicles from both cryopreserved groups. This issue, as well as

histology of individual follicles that develop antrum during culture, will be further examined in future experiments with increased number of cultured follicles or improved cryopreservation methods.

Follicles from cryopreserved tissues were able to produce E_2 and P_4, although not to levels produced from the fresh, suggesting some intact, but diminished function of granulosa and theca cells in culture. Furthermore, A_4 levels did not increase in follicles from cryopreserved tissues. Previously, we found that A_4 production of follicles from fresh tissue increases at the beginning of antrum formation and continues to rise in parallel with E_2 as follicle grows (Xu et al., 2010), but the majority of follicles from cryopreserved tissues either failed to develop an antrum or did not grow after antrum formation. There was one exception when a follicle (VitF) from a vitrified tissue survived 10 weeks in culture and also grew to the size, as well as produced E_2, P_4 and A_4 levels, comparable to those of follicles isolated from fresh tissue, showing delayed but intact functions.

Currently, the biggest obstacle in ovarian tissue vitrification in humans is the lack of a standard protocol, and a closed system to eliminate cross-contamination from direct contact with liquid nitrogen (Bielanski and Vajta, 2009). Extracellular ice formation following the slow-freezing procedure can sometimes cause damage, especially when cell density is high and cell-to-cell connections are important (i.e. in tissue) (Pegg, 2010). Previous studies have demonstrated this phenomenon, termed 'packing effect'; in cell suspension, highly compact cells were found to be more susceptible than loosely associated cells to damage caused by freeze and thaw procedures (Pegg et al., 1984). In contrast, vitrification was designed to prevent all forms of ice crystals, therefore it should benefit tissue
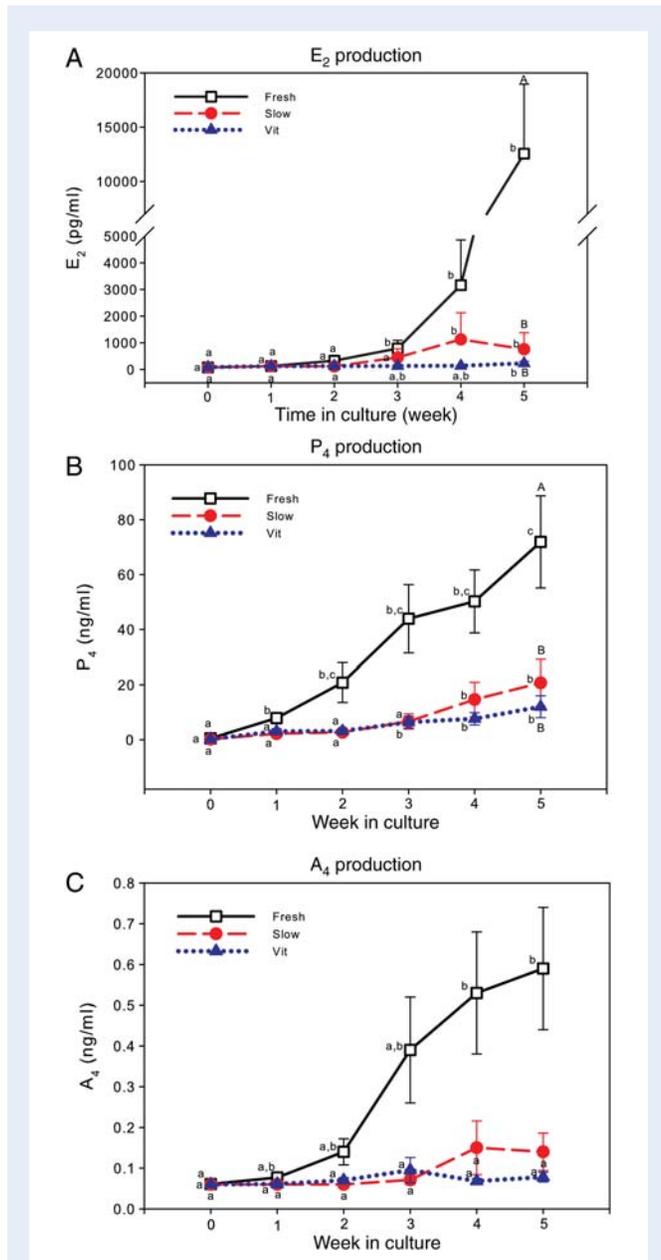
Figure 6 Weekly production of steroid hormones, estradiol (E2, Fig. 6A), progesterone (P4, Fig. 6B) and androstenedione (A4; Fig. 6C). Follicles from frozen (Slow) and vitrified (Vit) tissue showed reduced E2 and P4 productions during Week 5 when compared with fresh, while A4 levels remained minimal throughout culture. No differences in growth or steroid production were found between Slow and Vit. Lower case letters represent significant changes within the same group. Capital letters represent significant changes among different groups. Data are presented as mean $\pm$ SEM.

therefore difficult to improve existing protocols and compare results from previous studies in humans due to the different methods used among studies. The size of cortical strips has also varied among human studies (1×1 mm (Gandolfi *et al.*, 2006) to 10×10 mm (Kagawa *et al.*, 2009)) and sometimes within a study (Keros *et al.*, 2009). The type of cryoprotectant, exposure time and temperature used and even end-points evaluated, also has varied among various human studies (Isachenko *et al.*, 2009; Rahimi *et al.*, 2009; Xiao *et al.*, 2010). The non-human primate will continue to be an important model that can be utilized to systematically compare and optimize the vitrification protocol, as well as test novel cryoprotectants and various closed system devices for eventual clinical use.

Taken together, we have shown for the first time that although not to the extent of the fresh follicle, functional primate secondary follicles can be isolated from ovarian tissue after cryopreservation and these follicles can survive, grow, form an antrum and produce steroid hormones. Viable secondary follicles will allow the production of fertilizable oocytes if followed by *in vitro* follicle maturation, or facilitate the restoration of ovarian function if followed by tissue transplantation. However, our results also revealed that good histology does not equate to normal follicular function, reiterating the importance of coupling morphology results with functional end-points during the evaluation of ovarian tissue cryopreservation. Suboptimal results from tissue BrdU uptake as well as follicle growth and hormone production during encapsulated 3D culture indicate that further improvements to ovarian cryopreservation protocols are needed. Our histological results suggest that vitrification may be a better approach for ovarian tissue cryopreservation for the purpose of *in vitro* maturation of secondary follicles. Once an optimized cryopreservation protocol has been achieved in the non-human primate model, it can be used as a prototype to apply for human use. Combination of successful ovarian tissue cryopreservation with *in vitro* maturation of pre-antral follicles may offer significant advancement for fertility preservation, especially for prepubertal girls and premenopausal cancer patients who are at high risk for the presence of metastatic cancer cells in the ovary.

Authors' roles

All authors contributed to study design, execution, analysis, critical discussion and drafting or revising the manuscript. All authors have approved the final version and submission of this manuscript.

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cryopreservation (Fahy *et al.*, 2004; Pegg, 2010). The success of tissue cryopreservation, especially vitrification, is dependent upon the balance between CPA toxicity and concentrations of CPA needed for successful vitrification and optimal cooling and warming rate. Factors that can affect the outcome of vitrification include tissue size, CPA type and exposure time, as well as temperature. It is

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