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Synthetic polymers improve vitrification outcomes of macaque ovarian tissue as assessed by histological integrity and the *in vitro* development of secondary follicles☆

Alison Y. Ting, Richard R. Yeoman, Maralee S. Lawson, and Mary B. Zelinski*

Division of Reproductive Sciences, Oregon National Primate Research Center, Beaverton, OR 97006, United States

Abstract

Ovarian tissue cryopreservation is the only proven option for fertility preservation in female cancer patients who are prepubertal or require immediate treatment. However it remains unclear which cryopreservation protocol is best in cases where the tissue may contain cancerous cells, as these should be matured *in vitro* rather than autografted. This study evaluated different cryoprotectant exposure times and whether the addition of synthetic polymers (Supercool X-1000, Z-1000 and polyvinylpyrrolidone [PVP K-12]) to the vitrification solution is beneficial to tissue morphology, cellular proliferation and subsequent *in vitro* function of secondary follicles. Pieces of macaque ($n = 4$) ovarian cortex were exposed to vitrification solution containing glycerol (25%, v/v) and ethylene glycol (25%, v/v) for 3 or 8 min, without (V3, V8) or with (VP3, VP8) polymers (0.2% [v/v] X-1000, 0.4% Z-1000 and 0.2% PVP). Fresh and vitrified tissues were fixed for histology and phosphohistone H3 (PPH3) analysis, or used for secondary follicle isolation followed by encapsulated 3D culture. Five-week follicle survival and growth, as well as steroid hormones (estradiol [E₂], progesterone, androstenedione) were measured weekly. Morphology of the stroma and preantral follicles as well as PPH3 expression, was preserved in all vitrified tissues. Vitrification with polymers and shorter incubation time (VP3) increased *in vitro* follicle survival and E₂ production compared to other vitrified groups. Thus, a short exposure of macaque ovarian tissue to a vitrification solution containing synthetic polymers preserves morphology and improves *in vitro* function of secondary follicles.

Keywords

Synthetic polymers; Ovarian tissue; Vitrification; Nonhuman primates; *In vitro* follicle culture

Introduction

It is generally accepted that women are born with a finite supply of oocytes that decreases with age and is depleted by menopause [19,53]. This natural decline in fertility is dramatically accelerated with chemotherapy or radiation. Life-saving, cancer treatments destroy ovarian follicles, leaving young female cancer patients with devastating sequelae such as premature ovarian failure, infertility and long term health risks associated with menopause [38,46]. While oocyte retrieval, *in vitro* fertilization, embryo and oocyte

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*Corresponding author. Fax: +1 503 690 5563.

cryopreservation, and embryo transfer are routine and well established protocols, these techniques are not always suitable for female cancer patients in particular those who are pre-pubertal or require immediate cancer therapy. For such patients, ovarian tissue cryopreservation offers some hope for future fertility. The first live birth from autotransplanted frozen ovarian tissue was reported in 2004 [15] and only 15 more live births have been reported since then [3,11,12,14,16–18,36,43,44,49].

Tissue transplantation is currently the only proven option to restore fertility using cryopreserved ovarian tissue. However, for patients with possible malignant cells residing in the ovary, tissue reimplantation bears the risk of re-seeding cancer cells back to the patient and can lead to disease recurrence [42]. Theoretically, this risk can be circumvented by maturing ovarian follicles under *in vitro* conditions to obtain competent oocytes that can be fertilized and undergo normal embryo development by assisted reproductive technology. In rodents, *in vitro* matured oocytes and subsequent live births have been achieved from non-cryopreserved primordial, primary as well as secondary stage follicles [for review, see 40], and from cryopreserved preantral follicles [10,25]. In primates, maturation of follicles *in vitro* becomes much more challenging due to the magnitude of follicular growth and its lengthy process. Maturation of human primordial and primary follicles has been attempted using a two step culture system; however, oocyte competency has yet to be achieved from this technique [50]. Mature oocytes that are capable of fertilization have been produced from secondary follicles of nonhuman primates using an encapsulated three dimensional (3D) culture system [58,59].

Although current success in producing fertilizable oocytes from *in vitro* matured primate preantral follicles are limited to the use of secondary and multilayer follicles, these follicles have not been the focus for evaluating or improving ovarian tissue cryopreservation procedures. Current emphasis in optimizing techniques for ovarian tissue cryopreservation tends to focus on preserving primordial and primary follicles for tissue transplants. We have recently shown that morphologically, secondary follicles are better preserved following ovarian tissue vitrification in comparison to slow freezing [51]. However, isolated secondary follicles from vitrified tissue, while morphologically normal, showed delayed growth and reduced hormone production in culture in comparison to fresh follicles, indicating a need for further optimization of the vitrification protocol.

Natural polymers such as antifreeze proteins and glycoproteins (AFGPs) are found in polar fish and cold weather insects to promote tolerance from freezing and survival in the icy environment [5,34]. AFGPs are thought to inhibit ice growth by binding to small ice crystals [7] or to inhibit ice formation by binding to heterogeneous nucleators [57]. To mimic ice blocking properties of AFGPs, natural and synthetic polymers have been used as non-permeating components in vitrification solutions and have shown promising results in several systems [9,35,47]. Fahy et al. [20] have developed several synthetic polymers including a copolymer of PVA (polyvinyl alcohol, super cool X-1000), polyvinylpyrrolidone (PVP) K12, and polyglycerol (super cool Z-1000). These polymers decrease the concentration of permeating cryoprotectant required for vitrification, improve amorphous state stability of low-toxicity vitrification solutions, and inhibit occurrence of devitrification [56,57]. The addition of one or more of these polymers improves the outcome for vitrified mouse embryos [4], rat kidney slices [9], and tissue-engineered bone [32]. Supercool X-1000 was also used during vitrification of human ovarian tissue [27] and porcine oocytes [33]; however, the effect of this polymer was not studied due to a lack of polymer-free controls. Furthermore, when a higher molecular weight PVP was used in vitrification solution for cynomolgus and human ovarian tissue [26,30], results demonstrated improved morphology as well as intact mitochondria observed with electron microscopy.

Using a nonhuman primate model, the current study evaluated different cryoprotectant exposure times and whether the addition of synthetic polymers (Supercool X-1000, Supercool Z-1000 and PVP) to the vitrification solution is beneficial to tissue morphology, cellular proliferation and subsequent function of secondary follicles *in vitro*. Cellular proliferation is present at a high level in granulosa cells of growing follicles and is determined by staining for phosphohistone H3 (PPH3). Phosphorylation of histone H3 at Ser10 is crucial for the progression through mitosis during the cell cycle, and tightly correlated with chromosome condensation during prometaphase and metaphase with dephosphorylation occurring during anaphase.

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 [55]. Briefly, animals were pair caged in a temperature-controlled (22 °C) light-regulated 12L:12D room and fed food and water ad libitum. 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. Ovaries were collected by laparoscopy from anesthetized adult (14–16 years old; 7.1–8.2 kg), female rhesus monkeys ($n = 4$) exhibiting normal reproductive cycles. A blood sample was obtained prior to ovariectomy for steroid hormone (estradiol [E₂] and progesterone [P₄]) measurements. Serum E₂ and P₄ levels prior to the laparoscopy were 69 ± 9 pg/ml and 0.3 ± 0.1 ng/ml, respectively. Ovaries were placed into 3-(N-morpholino) propanesulfonic acid (MOPS)-buffered tissue holding media (HM; CooperSurgical Inc., Trumbull, CT, USA) and immediately transported to the laboratory at 37 °C. All chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise stated.

Ovarian tissue processing

Ovaries were halved 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). Using a scalpel, the cortex was cut into $3 \times 3 \times (1-1.5)\text{-mm}^3$ pieces. Due to the considerable heterogeneity of follicle distribution in the primate ovary, each piece was examined under a dissecting scope for the presence of secondary follicles and only tissues that showed visible secondary follicles ($n = 32 \pm 6$ pieces per animal) were used in the current experiment. For each animal, tissues were randomly divided into fresh and four vitrification groups (6–8 pieces per group). Each animal contributed equally to each treatment group and all evaluation endpoints. All procedures for ovarian tissue processing were carried out at 37 °C.

Vitrification and warming

Tissues were equilibrated sequentially, first in a solution containing 1.2 M glycerol (10% glycerol, v/v) followed by 1.2 M glycerol + 3.6 M EG (10% glycerol + 20% EG), neither equilibration solution contained added polymers. The equilibrated tissue pieces were then transferred into vitrification solution (VS) containing 3 M glycerol + 4.5 M EG (25% glycerol + 25% EG) [1,31,60] with or without polymers (0.2% [v/v] Supercool X-1000, 0.4% Supercool Z-1000 and 0.2% PVP K-12; 21st Century Medicine, Fontana, CA, USA). Tissues were equilibrated at 37 °C for either 3 min each in equilibration solution followed by 1 min in the VS in the absence (V3) or presence (VP3) of polymers (short protocol), or 8 min each in equilibration solution followed by 3 min in the VS in the absence (V8) or in the presence (VP8) of polymers (long protocol). Individual tissues were blotted with sterile

absorption spears (Fine Science Tools, Foster City, CA) and transferred onto a piece of 8×4 mm² aluminum foil. The foil was immediately submerged into liquid nitrogen (LN₂) and transferred into a LN₂-filled cryotube (1.8 ml, internal thread, Nunc, Roskilde, Denmark) for storage. For warming, each cryovial was individually removed from LN₂ followed by immediate transfer of the foil containing the tissue into holding media supplemented with 0.5 M sucrose (5 min), 0.25 M sucrose (5 min), 0.125 M sucrose (5 min), and holding media (10 min, twice). The warmed tissues were put in holding media at 37 °C for 30 min before fixation. All incubations in CPA-containing or sucrose solutions were carried out at 37 °C with shaking. Previously, we have carried out ovarian tissue processing and equilibration of vitrification solutions on ice. The working temperature was changed from ice to 37 °C due to the sensitivity of oocytes to chilling injuries [21]. No difference in follicle growth and tissue histology was found when tissues were processed at 37 vs. 4 °C (data not shown). All solutions were made in HM supplemented with 15% (v/v) SPS and 29 µg/ml ascorbic acid phosphate.

Tissue processing, histology and PPH3

Fresh and vitrified tissues ($n = 4-6$ per treatment group) were fixed in 4% paraformaldehyde at 4 °C overnight, processed for paraffin embedding and serial sectioned at 5 µm. Every 20th section was stained with hematoxylin and eosin (H&E) for histological analysis. Adjacent sections were prepared for immunostaining by antigen retrieval (boiling, 10 mM citrate buffer, 10 min) and incubation with 0.3% H₂O₂ (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). PPH3 was used as a mitotic marker for the evaluation of cellular proliferation, which is present at a high level in granulosa cells of growing follicles [6]. In addition, PPH3 stains cells that have entered the S phase of mitosis, thus truly representing dividing cells as opposed to Ki-67 which indicates cells that have just entered the M phase of mitosis that may not necessarily divide.

Quantification of secondary and multilayer follicles in tissue sections

Secondary follicles were counted and evaluated in each H&E stained section ($n = 4$ animals; 4 pieces/animal; 8–12 sections/piece [100 µm intervals]). Secondary follicles were defined as follicles with two complete layers of granulosa cells surrounding the oocyte whereas multilayer follicles were defined as follicles with at least three complete layers of granulosa cells surrounding the oocyte with an absence of antrum [24]. Secondary follicles were counted and evaluated as normal or abnormal based on morphology. Normal follicles were classified as those exhibiting cell-to-cell 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 or granulosa cells. 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). Follicles that showed at least one granulosa cell with positive staining for PPH3 were counted as positive for mitotic activity. Only follicles with a visible oocyte were counted for accurate examination of their developmental stage. Secondary follicles in adjacent sections stained with H&E and PPH3 were evaluated and assumed to represent the same follicle.

Secondary follicle isolation and 3D culture

A subset of fresh and vitrified tissues ($n = 3-4$ /group/animal) were used for secondary follicle isolation. Following the last warming solution, vitrified tissues were transferred into culture media containing αMEM, 3% SPS and 29 µg/ml ascorbic acid at 37 °C with 20% O₂. Each tissue was taken out of the culture environment immediately prior to follicle isolation to avoid possible stress induced by tissue exposure to less stable environment

(laboratory bench). Typically, follicle isolation was completed within 2 h per animal. Follicles were isolated from fresh and cryopreserved tissue without collagenase treatment to avoid further damage to follicles [23,29]. Secondary follicles ($n = 8\text{--}12/\text{group/animal}$, $n = 194$ total, $120\text{--}250\text{ }\mu\text{m}$ in diameter, partially 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 [59]. Briefly, follicles were transferred individually into $5\text{ }\mu\text{l}$ 0.25% (w/v) sterile sodium alginate (in PBS) and the droplets were cross-linked in 50 mM CaCl_2 and 140 mM NaCl solution for 1 min. Encapsulated follicles were transferred to individual wells of a 48-well plate (NUNC) containing 300 μl of alpha minimum essential medium (αMEM) culture media supplemented with 2.16 mg/ml glucose, 60 $\mu\text{l}/\text{ml}$ SPS, 44 mIU/ml follicle stimulating hormone (NV Organon, Oss, Netherlands), 0.5 mg/ml bovine fetuin (fetal plasma protein), 29 $\mu\text{g}/\text{ml}$ ascorbic acid phosphate, 5 $\mu\text{g}/\text{ml}$ transferrin, 0.5 $\mu\text{g}/\text{ml}$ insulin, and 5 ng/ml sodium selenite. Encapsulated follicles were cultured at $37\text{ }^\circ\text{C}$ in 5% CO_2 in atmospheric air for 5 weeks. Every 2 days, half of the culture media was exchanged with fresh culture media (prepared weekly) and stored at $-20\text{ }^\circ\text{C}$ for subsequent hormonal measurements.

Follicle growth and survival

During culture, 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 granulosa cells (the oocyte is separated from and no longer inside the follicular wall), (ii) the oocyte became dark, (iii) the granulosa cells became dark, or (iiii) the diameter of the follicle decreased. For each follicle, weekly photographs were taken, and diameters measured using ImageJ (National Institutes of Health, Bethesda, MD). The mean of two measurements per follicle (perpendicular to each other) was then calculated and documented as the follicle diameter.

Hormone assays

Serum collected from each animal prior to ovariectomy 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). The sensitivity of assays by the Immulite 2000 is 20 pg/ml for E_2 , 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 interassay 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 analyzed by combining observations to make one single count per animal giving an equal contribution from each animal presented and as mean $\pm$ SEM. For numbers of secondary and multilayer follicles in H&E and PPH3-stained sections, data 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 follicles of the same group. Percentages of follicles that exhibit normal oocyte, granulosa cells or both among different treatment groups were analyzed by Chisquare test.

For 3D culture, the percentage of follicular survival and antrum formation is presented as percent rates (mean $\pm$ SEM). The 5-week survival rate was analyzed using ANOVA (SigmaPlot 11.0, Systat Software, Inc., San Jose, CA, USA) while no statistical analysis was

performed for antrum formation rate due to the low number of follicles that formed an antrum in any group. One way repeated measures analysis of variance was performed for weekly follicle diameter and hormone production within treatments, whereas for data among treatments at a single time point, one way analysis of variance was performed. Differences were considered significant when $p < 0.05$.

Results

Descriptive morphology of the stroma, and primordial and primary follicles

Microscopic visualization of H&E-stained sections of fresh tissue revealed intact morphology with tight contact between the oocyte and surrounding granulosa cells as well as between neighboring granulosa cells in primordial and primary follicles (Fig. 1a and b). A uniform distribution of cytoplasm in oocytes was observed in follicles of fresh tissue (Fig. 1b). Immediately after warming, the majority of primordial and primary follicles from all four vitrification groups showed morphology similar to fresh tissue (Fig. 1d, f, h, j, *i* [primordial], and *ii* [primary]). Occasionally, damage was observed in primordial and primary follicles in vitrified tissues. Damage included shrunken (Fig. 1j, arrow) and vacuolated (Fig. 1d, arrow) oocytes, as well as abnormal space between the follicle and stroma (Fig. 1f, arrow). The cortex of fresh tissue (Fig. 1a) and tissues vitrified using different protocols (Fig. 1c, e, g and i) all had dense and compact stroma.

Morphology and quantification of secondary and multilayer follicles

The total number of secondary and multilayer follicles and percentage of morphologically normal follicles in fresh and cryopreserved tissues was evaluated from H&E stained sections (Fig. 2). Interestingly, not all secondary and multilayer follicles in fresh tissue exhibited morphology that was classified as “normal”. Most of these “abnormalities” in fresh tissue were found in the oocyte. Only $74 \pm 3\%$ and $57 \pm 4\%$ of oocytes in secondary and multilayer follicles, respectively, exhibited “normal” morphology (Fig. 2). In oocytes of secondary follicles, the abnormality was predominantly the presence of vacuoles, whereas in oocytes of multilayer follicles, they included the presence of vacuoles, shrunken cytoplasm and loss of cytoplasm (empty zona pellucida, Fig. 3). By contrast the granulosa cells of most secondary and multilayer follicles in fresh tissue pieces were morphologically normal ($98 \pm 2\%$ and $88 \pm 4\%$, respectively; Fig. 2).

After warming, more than half of all secondary follicles in V8 and VP3 exhibited morphologically “normal” oocytes (Fig. 2). However, in comparison to the fresh control, the proportion of normal oocytes in secondary follicles was reduced ($p < 0.05$) in all vitrified groups. The percentage of normal oocytes in secondary follicles was lowest when VP8 was used as compared to fresh and other vitrified groups. The difference in percentages of normal oocytes between groups was not observed ($p > 0.05$) in multilayer follicles. After warming, the percentage of secondary and multilayer follicles with morphologically preserved granulosa cells was not different ($p > 0.05$) to those in fresh tissue (Fig. 2). Follicles with abnormal granulosa cells displayed vacuoles (Fig. 3c and g) and dying cells with pyknotic nuclei resulting in abnormal space between neighboring granulosa cells (Fig. 3d).

PPH3 expression

In fresh tissue, abundant PPH3 expression was found in the nucleus of the majority of granulosa cells in healthy growing follicles (primary, secondary, multilayer) as well as some stromal cells (Fig. 4a). In vitrified tissues, most growing follicles showed a similar expression pattern of PPH3 (Fig. 4c–f) to the fresh tissue. Negative control showed no PPH3

staining (Fig. 4b). Following quantification of PPH3 immunoreactivity in secondary and multilayer follicles, levels of PPH3 appeared similar among all groups (Fig. 5).

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 1. The five-week survival rate of follicles from the V3 and V8 groups was reduced ($p < 0.05$) relative to those from the fresh tissue. The addition of polymers to the VS had different effects in the short and long equilibration groups. Follicle survival in the VP3 group was similar to that of fresh follicles and improved ($p < 0.05$) in comparison to that of the V3 group. None of the follicles in the VP8 group survived 5 weeks of culture. Antrum formation was observed by weeks 3–4 in $52 \pm 21\%$ of fresh follicles that survived 5 weeks of culture (Fig. 6). Among surviving follicles from V3 and VP3 groups, $8 \pm 8\%$ and $33 \pm 24\%$, respectively, developed an antrum during culture (Table 1). None of the follicles in the V8 group formed an antrum within 5 weeks of culture (Table 1 and Fig 6).

Follicle diameters were similar in all groups on the day of isolation ($P > 0.05$, $148 \pm 5 \mu\text{m}$). Follicles from fresh tissue showed increased diameters during week 2 and continued to grow to week 5 (Figs. 6 and 7, $p < 0.05$). Follicle diameters also increased ($p < 0.05$) during week 2 in follicles from V3 and VP3 groups. However, the follicle diameters at week 5 of V3 follicles showed an apparent decrease ($P = 0.094$) when compared to that of the fresh controls. In addition, the diameters of V3 and VP3 follicles at week 5 were similar to the diameter of the fresh follicles at week 3, suggesting a delayed growth. Diameters of V8 follicles remained unchanged throughout the 5 weeks of culture (Figs. 6 and 7).

Encapsulated 3D follicle culture: hormone production

E_2 production by fresh follicles increased ($p < 0.05$) above baseline (week 0) during week 2 and continued to rise ($p < 0.05$) through 5 weeks of culture (Fig. 7). In contrast, E_2 levels produced by follicles of the V3 and V8 group were detectable, but minimal, throughout 5 weeks of culture. Although there was no growth in the VP8 group, the use of polymers in the shorter vitrification protocol resulted in E_2 production by VP3 follicles similar to that of fresh follicles throughout culture. P_4 production (Fig. 7) by follicles of fresh tissue elevated ($p < 0.05$) above baseline after 2 weeks of culture, and plateaued between weeks 3–5. Production of P_4 by VP3 follicles showed a similar pattern when compared to fresh follicles throughout the culture period. V3 follicles also showed increased ($p < 0.05$) P_4 production during weeks 2–5 in comparison to the baseline level; however, P_4 levels produced by these follicles tended to be less than that of fresh in weeks 4 and 5. Levels of P_4 in the V8 group remained at baseline throughout culture. A_4 levels were elevated ($p < 0.05$) above baseline during week 5 in fresh follicles (Fig. 7). Similarly, production of A_4 by VP3 follicles increased ($p < 0.05$) above baseline to levels of fresh follicles during weeks 4 and 5. Production of A_4 in V3 and V8 follicles was detectable, but remained at baseline throughout 5 weeks of culture.

Discussion

The addition of nonpermeating polymers (X-1000, Z-1000 and PVP K12) to the vitrification solution in combination with a short (3 min) CPA exposure time allowed a small proportion of secondary follicles, isolated from vitrified macaque ovarian tissue, to grow and produce steroids during 3D culture. Successful vitrification is dependent upon tissue geometry, CPA type, concentration, and equilibration time, which is a function of temperature. Glycerol and EG, the permeating CPAs used in the current experiment, are both considered to be weak glass-forming agents; therefore, a higher concentration is needed for solutions to vitrify.

However, glycerol and EG induce relatively low toxicity in cells and tissues in comparison to other CPAs [1]. The low toxicity may be due to the relatively high equilibration temperature (37 °C) coupled with a relatively short equilibration time (3 min), resulting in minimal cell damage observed in macaque ovarian tissue in the current experiment.

Unlike the conventional slow freezing procedure which results in the formation of extracellular ice, vitrification is designed to prevent all forms of ice crystals while samples become glass-like, therefore provides benefits during tissue cryopreservation where cell-to-cell connections are important [20,39]. Diversity of cell types in the ovary and the importance of extracellular connections within follicles make vitrification an attractive alternative to slow freezing. As shown in embryo and oocyte cryopreservation, the vitrification procedure is effective, less time consuming and economical and can be performed in virtually any laboratory. Tissue pieces are less easy to vitrify because of their size; therefore, polymers may be valuable by enhancing the capacity of the solution to vitrify, without requiring excessively high CPA concentrations. Polymers tested in the current study could potentially be quickly translated into clinical use because of their proven safety. The US Food and Drug Administration has approved PVP for many uses as a component in toothpaste and envelope adhesive [37]. PVA (X-1000) as a lubricant has also been used in eye drops and hard contact lens solution [13]. In addition, polyglycerol (Z-1000) is frequently used in the food processing industry [54]. Viable secondary follicles could facilitate the production of fertilizable oocytes if followed by *in vitro* follicle maturation, or improve the restoration of ovarian function if followed by tissue transplantation.

Morphologically, the addition of polymers in the VP3 group improved the preservation of oocytes in secondary follicles in comparison to the V3 group. However, with the longer equilibration time (more than doubled) in the V8 and VP8 groups, tissue vitrified with polymers (VP8) have fewer follicles with morphologically normal oocytes compared to tissue vitrified without polymers (V8). Furthermore, the follicles isolated from the VP8 tissues failed to survive in the current 3D culture system. Even though polymers are considered to be present only in the extracellular space and induce minimal toxicity to the cell, previous studies have demonstrated time-dependent [27] and dose-dependent [7] toxicity by polymers, possibly due to excess cell shrinkage leading to disruption of cellular organelles or damage of the membrane. While damage in granulosa cells of secondary and multilayer follicles was observed following vitrification, cryo-induced damage predominantly appeared in the oocyte. Our histological data revealed morphologically normal stroma, as well as primordial and primary follicles, in all vitrification groups. In contrast, normal morphology of secondary and multilayered follicles was observed by the addition of polymers and short CPA exposure time (VP3).

Levels of PPH3 in granulosa cells in all vitrification groups also appeared similar to that of the fresh tissue, suggesting intact mitotic functions. [41]. However, based on the time a cell needs for 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 more rapidly than dephosphorylation. Therefore, PPH3 expression may not be an ideal endpoint to conveniently demonstrate the viability of ovarian follicles after cryopreservation. Furthermore, in the current experiment, tissues were fixed soon after warming and may not reflect their true mitotic capability. For future studies, it may be beneficial to delay tissue fixation for cells to re-establish their mitotic state after warming. Alternatively, cellular proliferation can be examined by 5-bromo-2'-deoxyuridine uptake [51]. A limitation of all proliferation assays is that they only reveal somatic cell function but not the health of the oocyte. There is currently no secreted marker for assessing oocyte viability or function during 3D culture.

The evaluation of growth and hormone production of intact secondary follicles using a 3D encapsulated culture system showed that vitrification with short CPA exposure time in the presence of polymers (VP3) maintained follicular function *in vitro*. This is important because patients whose ovarian tissue may contain malignant cells and may have to rely on *in vitro* culture rather than grafting and *in vivo* growth. During normal *in vivo* and *in vitro* development, a secondary follicle grows in size due to granulosa cell proliferation and an increase in oocyte diameter. This growth is accompanied by the formation of an antrum and increased production of steroid hormones E₂ by granulosa cells, as well as P₄ and A₄ by theca cells. The vitrified follicles grew in diameter in culture, indicating viable granulosa/theca cells; however, only the VP3 group produced steroid hormones at concentrations approximating those of the control, non-vitrified follicles, suggesting diminished function of granulosa/theca cells of the V3, V8 and VP8 groups. Secondary follicles from the VP3 group had lower survival and growth rate in comparison to that of fresh follicles. Survival of V8 follicles was similar to that of V3 follicles, but none of the V8 follicles formed an antrum and their follicle diameter did not increase throughout culture. The finding that none of the follicles in the VP8 group survived 5 weeks of culture, may indicate toxicity from prolonged exposure to the polymers. Interestingly, while not statistically significant, the average follicle diameter from groups V3 and V8 on the day of isolation seemed to be smaller in comparison to that of the fresh and VP3 groups. It is possible that the larger secondary follicles (>150 µm) did not survive the vitrification procedure in V3 and V8 groups and that smaller follicles that survived the procedure did not grow and secrete hormones as well as the larger follicles in the fresh and VP3 groups. Therefore, by increasing CPA exposure time, follicular function was reduced, possibly due to CPA toxicity and excess dehydration.

Although studies in the mouse have shown that cryopreserved isolated follicles can form viable oocytes capable of developing to term [8,10,25], it has been suggested that the stroma is crucial for follicular development by providing physical/mechanical and molecular supports [52]. In the current study, cultured secondary follicles are partially isolated and surrounded by some stromal tissue because collagenase was not used during isolation. Therefore, levels of steroid hormone production as well as follicular growth and antrum formation rate may be affected by the health of surrounding stromal cells. However, the culture system used in the current study was designed to support follicular growth, and evidence of stromal growth/development has not been observed. Nevertheless, the viability and health of stromal cells after cryopreservation will be evaluated in future studies. Previously, morphology and growth of preantral follicles from cryopreserved human ovarian tissue has only been examined in frozen (not vitrified) tissues and only tested up to 14 days in culture, while the function of these follicles was not investigated [2,23]. Others have placed cryopreserved tissues in short term organ culture systems and measured E₂ and P₄ production [27,28]. 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 stromal density, source of steroid production, 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 a mature oocyte was not produced from vitrified tissue following 3D culture of secondary follicles in the current experiment, we have shown that long term culture of isolated secondary follicles from cryopreserved tissue is a feasible and reliable method for the evaluation of follicular growth and steroidogenesis. Thus, this 3D follicle culture system can serve as a useful bioassay to rapidly assess/screen ovarian tissue cryopreservation protocols prior to *in vivo* studies.

There are currently two major obstacles in ovarian tissue vitrification in humans. One challenge involves evaluating survival and viability of cryopreserved ovarian tissue solely

based on histology [22]. Assessment of follicular function has been mainly demonstrated in the few cases of live births following slow-freezing, but there are no human live births reported from vitrified tissue. In monkey and human 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 [45]. Since each cortical piece subjected to cryopreservation does not have the same number or the same classes of follicles, it is inaccurate to compare numbers of follicles after cryopreservation to those in the fresh group or between different cryopreservation methods. Furthermore, proportional data for follicular survival that are often used in other studies should also be interpreted with caution since follicles that lyse after cryopreservation would not be detected during counting, thereby resulting in an overestimation of follicular survival, especially in vitrified groups. In the current study, culturing of secondary follicles in a 3D matrix was an additional tool utilized to assess follicular function. Further studies examining function after transplantation of vitrified tissues are needed. The other obstacle of ovarian tissue vitrification is the lack of a standard protocol that demonstrates optimal ovarian function and consistent outcome, and the lack of a closed system that eliminates possible cross contamination from direct contact of tissue with liquid nitrogen or leakage of liquid nitrogen into the cryotube. Sheikhi et al. [48] described a closed system where ovarian fragments are vitrified in a closed cryotube and stored in liquid nitrogen vapor. However, whether or not a closed cryotube used by Shekhi et al. or for slow freezing is a true closed (sealed) system for storage in liquid nitrogen or liquid nitrogen vapor is still debatable due to possible leakage of liquid nitrogen or airborne contaminants into the vial during storage. The nonhuman primate will continue to be an important model that can be utilized to systematically compare and optimize the vitrification protocol, as well as to test novel cryoprotectants and various closed system devices for eventual clinical use.

Taken together, we have shown that preantral follicles can be morphologically and functionally preserved following vitrification of macaque ovarian tissue using glycerol, EG and synthetic polymers with optimal CPA exposure time. However, morphology after cryopreservation does not correlate with secondary follicle viability and function. Over exposure of ovarian tissues to permeating as well as nonpermeating CPAs results in reduced function of secondary follicles. Secondary follicles from tissue vitrified with optimal condition can be isolated, survive, grow, form an antrum and produce steroid hormones in a 3D culture system. Once an optimized cryopreservation protocol has been achieved in the nonhuman primate model, it can be used as a prototype for human application. Additionally, the combination of successful ovarian tissue cryopreservation with *in vitro* maturation of preantral follicles may offer significant advancements 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.

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Fig. 1.

Representative photomicrographs of fixed ovarian tissue from fresh (a, b), vitrified with short (3 min) CPA incubation without (V3; c, d) or with (VP3; e, f) polymers, and vitrified with long (8 min) CPA incubation without (V8; g, h) or with (VP8; i, j) polymers. Dense stroma was observed in fresh (a) and all vitrified groups (c, e, g, i). Fresh ovarian tissue showed intact primordial (*i*), primary (*ii*), secondary (*iii*), and multilayered (*iv*) follicles with healthy oocyte and densely compact granulosa cell layers (b). Following vitrification, morphology of granulosa cells of different classes (*i*, *ii*, *iii*, *iv*) of follicles were mostly preserved in all groups (d, f, h, j); however, infrequently, damage including shrunken (j, arrow) or vacuolated oocytes (d, arrow), as well as abnormal space between the follicle and stroma (f, arrow) was observed. Scale bar = 100 μ m (a, c, e, g, i), 50 μ m (b, d, f, h, j, *i*, *ii*, *iii*, *iv*).

Fig. 2.

Percentages (mean $\pm$ SEM, $n = 4$) of normal oocyte, granulosa cells or both found in secondary and multilayer follicles of fresh tissue and tissue vitrified with short (3 min) CPA incubation without (V3) or with (VP3) polymers, and vitrified with long (8 min) CPA incubation without (V8) or with (VP8) polymers. Numbers inside the bar represent total number of follicles counted in each group. Different letters on top of the bar represent significant differences among treatment groups. No statistical difference was found in percentage of normal granulosa cells or normal oocyte and granulosa cells among treatment groups.

Fig. 3. Representative photomicrographs of normal secondary (a) and multilayer (e) follicles. Abnormal morphology observed in secondary and multilayer follicles included vacuoles in the oocyte (b, f) and granulosa cells (c), shrunken oocytes (f–h), as well as degenerating granulosa cells (d) and oocytes (h). Scale bar = 50 μ m.

Fig. 4. Representative photomicrographs of fixed ovarian tissue stained for phosphohistone H3 (PPH3). In fresh tissue, PPH3 (dark brown nuclei) was expressed in the majority of granulosa cells of growing follicles and some stromal cells (a). A similar pattern for PPH3 staining was observed in all groups of vitrified tissue with short (3 min) CPA incubation without (V3; c) or with (VP3; d) polymers, and with long (8 min) CPA incubation without (V8; e) or with (VP8; f) polymers. Follicles showing negative PPH3 staining were observed in some vitrified tissues (c and f, arrow). No positive staining (b) was observed when primary antibody was omitted (negative control). Scale bar = 50 μ m.

Fig. 5. Percentages (mean $\pm$ SEM, $n = 4$) of secondary and multilayer follicles expressing phosphohistone H3 (PPH3) in fresh tissue and tissue vitrified with short (3 min) CPA incubation without (V3) or with (VP3) polymers, and vitrified with long (8 min) CPA incubation without (V8) or with (VP8) polymers. Numbers inside the bar represent total number of follicles counted in each group. No statistical difference was found among treatment groups.

Fig. 6.

Weekly photomicrographs of isolated secondary follicles during 3D culture. Follicles from fresh tissue showed increased diameter over time and formed an antrum by week (wk) 4. Follicles isolated from tissues vitrified with short (3 min) CPA incubation without (V3) or with (VP3) polymers, and with long (8 min) CPA incubation without polymers (V8) showed delayed growth, but some were able to form an antrum (V3, wk5; VP3, wk3). None of the follicles isolated from VP8 vitrified tissue survived 5 weeks in culture. Note that follicles isolated from vitrified tissues were frequently surrounded by stromal tissue. The series of images represent the growth pattern of a single follicle from each group during culture. Scale bar = 250 μ m.

Fig. 7. Weekly follicle diameter and steroid hormone production (E_2 , P_4 , A_4) during 5 weeks (wks) in culture. Data are presented as mean $\pm$ SEM of isolated follicles ($n = 4$ animals, 8–12/group/animal) that survived (see Table 1). Lower case letters represent significant changes within the same group over 5 weeks in culture. Capital letters represent significant changes among different groups within a given week of culture.

Survival (at 5 weeks) and antrum formation during 3D culture in follicles isolated from fresh, V3, V8, VP3, and VP8 tissues. Data represent the average number (mean $\pm$ SEM, per animal, $N = 4$ animals) of secondary follicles and their 5-week survival rates (%) and the ability to form antrum (%) among those that survived. Data in parentheses represents the total number of follicles.

Table 1

	Fresh	V3	VP3	V8	VP8
Average (total) number of follicles	9 $\pm$ 1 (37)	11 $\pm$ 1 (42)	11 $\pm$ 0 (44)	10 $\pm$ 1 (39)	8 $\pm$ 3 (32)
Survival rate (total number of follicles)	33 $\pm$ 14 (10)	8 $\pm$ 8 (3) ^a	14 $\pm$ 6 (6) ^a	10 $\pm$ 6 (3)	0 (0)
Antrum formation rate (total number of follicles)	52 $\pm$ 21 (5)	8 $\pm$ 8 (1) ^b	33 $\pm$ 29 (3) ^c	(0)	N/A (0) ^d

^aIndicates significant difference ($p = 0.05$) in comparison to the fresh group.

^b $N = 4$ animals. Follicles from 3 out of 4 animals did not form an antrum.

^c $N = 4$ animals. Follicles from 2 out of 4 animals did not form an antrum.

^dNone of the follicles survived in this group; therefore, antrum formation rate was not calculated.