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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 ☆

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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 [E_2], 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 E_2 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.

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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 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 autotransplantated of 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

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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–

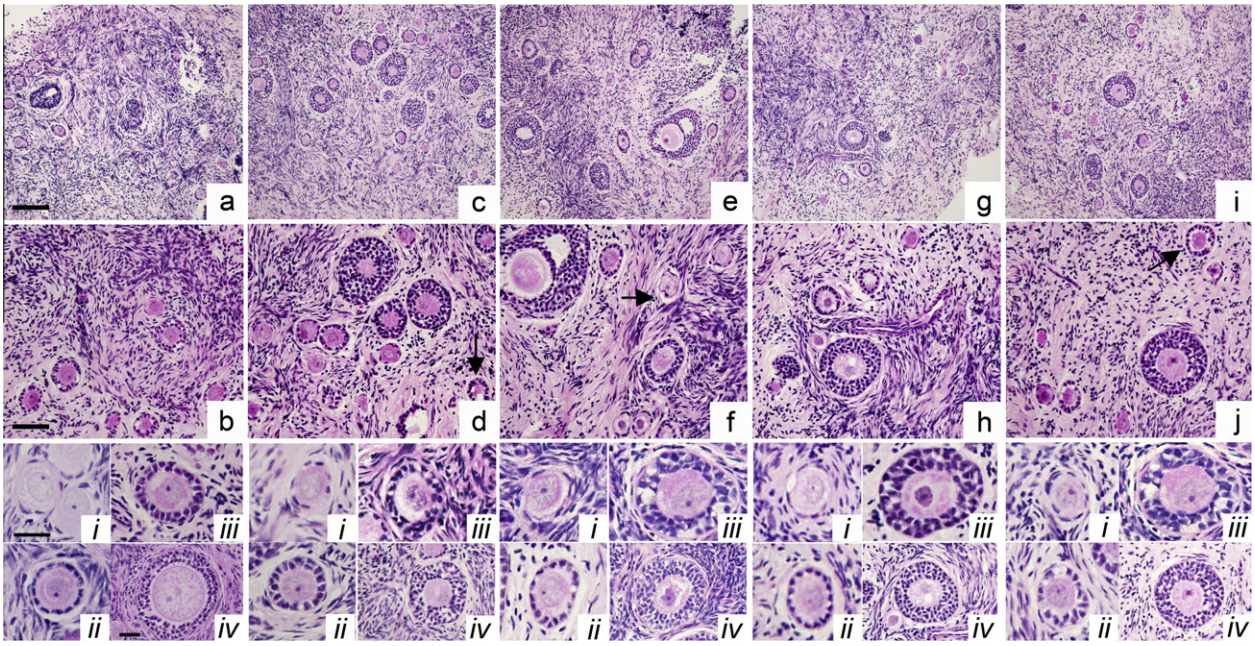

Fig. 1. Representative photomicrographs of fixed ovarian tissue from fresh (a, b), vitrified with short (3 min) CPA incubation without (V3; c, d) or with (VP3; e, f) polymers, and vitrified with long (8 min) CPA incubation without (V8; g, h) or with (VP8; i, j) polymers. Dense stroma was observed in 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 μm (a, c, e, g, i), 50 μm (b, d, f, h, j, *i*, *ii*, *iii*, *iv*).