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Markers of growth and development in primate primordial follicles are preserved after slow cryopreservation

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Abstract

Objective—To investigate the effect of slow cryopreservation on the morphology and function of primate primordial follicles within ovarian tissue slices.

Design—Fresh monkey ovarian tissue was frozen by slow cryopreservation and thawed for analysis of morphological and functional parameters.

Setting—University-affiliated laboratory.

Animals—Rhesus monkey ovarian tissue.

Intervention(s)—None

Main Outcome Measure(s)—Histological analysis, follicle counting, assessment of protein abundance and localization.

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Authors have nothing to disclose

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Capsule:

Molecular markers (actin subunits and pSmad2) of follicles health were preserved in monkey primordial follicles after ovarian tissue cryopreservation.

Result(s)—After freezing and thawing, 89% of the primordial follicles maintained their laminar-based architecture, with sizes close to those of fresh fixed follicles ($p>0.05$). Molecular markers of early follicle health (activin subunits and the phosphorylated form of the signaling protein Smad2 (pSmad2)) were present in fresh and frozen-thawed primordial follicles. Stroma cells but not follicles had a higher level of TUNEL staining. Granulosa cells within the follicles of frozen-thawed ovarian tissue cultured for 48 hours had the capacity to proliferate and sustained expression of the activin subunits and nuclear pSmad2.

Conclusion(s)—This study provides evidence that markers of early follicle growth and development are preserved after slow cryopreservation and thaw, with little effect on follicle morphology and function.

Keywords

slow cryopreservation; primordial follicles; rhesus monkey; stromal cell; activin subunits; pSmad2

Introduction

Cryopreservation of oocytes or ovarian tissue provides a potential option for fertility preservation of women facing the loss of reproductive function, either due to diseases such as cancer or treatments, including radiation and chemotherapy (1-5). Cryopreserved and thawed ovarian tissue has been successfully reimplanted and has produced live births, with lower success rates for frozen tissue vs. fresh transplant (4-9). Ovarian tissue is cryopreserved using conventional freezing (slow) or vitrification (rapid). Although studies on each of these techniques report varying levels of success, there is evidence suggesting that slow cryopreservation is more efficient and produces a greater number of surviving and functional follicles after thawing (10-11). The viability of primordial follicles has been confirmed after slow cryopreservation and thawing, whether they had been frozen within ovarian tissue or as individually isolated follicles (12-13).

Activin is involved in mouse and human follicle formation, and promotes germ cell and granulosa cell proliferation (14-17). The null gene mutation of activin results in gonadal dysgenesis or inappropriate follicle function (18). Smad2 is a downstream target of activin and is phosphorylated as a consequence of activation. Also activin subunits and pSmad2 are detected in the cat primordial follicles (19). Thus, activin-psmad2 signaling pathway may play an important role in primordial follicle development. Here, we test the effects of ovarian tissue cryopreservation technique on the molecular markers and developmental capacity of nonhuman primate primordial follicles.

Materials and Methods

Collection of ovarian tissue

Four female monkeys (0~5 years old) were used here. The general care and housing of rhesus monkeys was provided by the Division of Animal Resources at the Oregon National Primate Research Center (ONPRC). Animals were treated in accord with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and protocols were approved by the ONPRC Institutional Animal Care and Use Committee.

Cryopreservation and thawing of ovarian tissue

Freezing of monkey ovarian tissue strips was performed according to the method described by Gosden et al (20) with some modifications. Pieces of ovarian cortical tissue (5×1×1 mm) were transferred into 1.5 ml cryovials containing 1 ml of 1.5 M ethylene glycol, 0.1 M sucrose and 10% (v/v) serum substitute in Leibovitz L-15 media. Ovarian tissue was equilibrated for 30

min at 4 °C. The cryovials were frozen using a programmable freezer with a cooling rate of 2 °C per min from 4 °C to -7 °C. At -7 °C, seeding was manually performed, then cooling was resumed at a rate of 0.3 °C per min from -7 °C to -40 °C, and then 10 °C per min from -40 °C to -140 °C. Finally, the cryovials were plunged into liquid nitrogen for storage.

For thawing, the cryovials were exposed to room temperature for 30 sec and immersed in a water bath at 37 °C until the ice completely melted. Then media and pieces of tissue were poured into a culture dish. Ovarian cortical tissues were rehydrated in four steps, 10 min per step, at room temperature into the following thawing solutions: thawing solution I: 10% serum protein substitute SPS, 0.1 M sucrose, 5.57% ethylene glycol (EG); thawing solution II: 10% SPS, 0.1 M sucrose, 2.79% EG; thawing solution III: 10% SPS, 0.1 M sucrose, 0% EG; thawing solution IV: 10% SPS, 0 M sucrose, 0% EG.

Histological and immunological analysis and follicle classifications

Monkey ovarian tissue from the fresh group, frozen-thawed group or cultured frozen-thawed group were fixed and five- μ m sections were cut for hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC). A healthy primordial follicle is defined as previously described (21-22), which consists of an oocyte completely encapsulated by one layer of flattened pre-granulosa cells. H&E images were acquired on a Nikon E600 microscope using a Spot Insight Mosaic 11.2 color digital camera (Diagnostic Instruments, Sterling Heights, MI) and ADVANCED SPOT IMAGING software (Version 4.6, Universal Imaging, Downingtown, PA). The basement membrane surrounding the granulosa cell layer was considered to be the outer boundary of the follicle. IHC was performed and visualized as previously described (15).

Antibodies

Primary antibodies used were rabbit polyclonal anti-human β A- and β B-subunit antibodies (a gift from W. Vale and J. Vaughn, The Salk Institute, La Jolla, CA) and a rabbit polyclonal anti-phosphorylated-Smad2 antibody was purchased from Zymed Laboratories (South San Francisco, CA). The antibody for detection of proliferating cell nuclear antigen (PCNA) is rabbit polyclonal from Santa Cruz Biotechnology. Laminin antibody is from Sigma. Dilutions of the primary antibodies against the β A- and β B-subunit, phosphorylated Smad2 (pSmad2), PCNA and Laminin were 1:100, 1:100, 1:50, 1:100 and 1:50 respectively. The secondary antibodies used were biotinylated goat anti-rabbit (Vector Laboratories, Inc., Burlingame, CA). Negative controls were obtained by omitting the primary antibody.

Identification of apoptotic cells

TUNEL staining was performed using the TACS.XL *in situ* apoptosis detection kit (catalog #TA200, R&D, Minneapolis, MN) following the manufacturer's protocol. Negative controls consisted of omitting the terminal deoxynucleotidyl transferase (TDT) enzyme.

Counting of healthy and immunopositive follicles

Only follicles that contained an oocyte nucleus were counted. Follicles were counted in four different areas (same power field) per section, six random sections per sample, and 2-3 ovarian tissue samples per animal were used here. Follicle counts were repeated in four different animals. The percentage of healthy follicle is calculated as number of healthy follicles out of total follicles number. And the percentage of immunopositive follicles is out of total healthy follicles number. Follicles with either an oocyte or single granulosa cell stained positive by IHC were considered to be immunopositive follicles (23-24).

Culture of frozen-thawed ovarian cortical tissues

Frozen ovarian cortical tissues were thawed and cultured as described previously (21,25). Tissues were cut into small, thin pieces of approximately 0.5 mm³ and transferred in a culture plate insert (Millicell-CM, 0.4-μm pore size; Millipore Corp) in a 24-well cell culture plate. Ovarian tissue slices on the membrane were covered with a thin film of medium, and 400 μl of culture medium was added in the compartment below the membrane insert. Up to four ovarian slices were placed in each well. The culture medium was minimal essential medium (αMEM) supplemented with 10 mIU/ml recombinant human follicle-stimulating hormone (rhFSH) [A. F. Parlow, National Hormone and Peptide Program, National Institute of Diabetes and Digestive and Kidney Diseases], 3 mg/ml BSA, 1 mg/ml bovine fetuin [Sigma-Aldrich, St. Louis, MO], 5 ng/ml insulin, 5 ng/ml transferrin and 5 ng/ml selenium. The ovarian tissue was incubated at 37 °C, in 5% CO₂ for 48 h.

Statistical analysis

At least three replicates were performed for each experiment on each animal. Comparisons for diameter of follicle, oocyte, nuclear were made between fresh and frozen-thawed groups using a Student t-test, with $p < 0.05$ considered statistically significant. The percentage of healthy follicles, PCNA-positive follicles or cells and TUNEL-positive follicles or cells were analyzed using Fisher's exact test, with $p < 0.05$ considered statistically significant. Values are given as mean $\pm$ S.E.M.

Results

Morphologic assessment of primordial follicles before and after slow cryopreservation

Fresh or frozen-thawed monkey ovarian tissues were sectioned for H&E staining to determine the effect of slow cryopreservation and thawing on the morphology of primordial follicles. In the fresh group, 98% (n=540) of primordial follicles had a complete architecture, and the oocyte was surrounded by a single layer of pre-granulosa cells with a round nucleus (Fig 1-A, E). After cryopreservation, 89% (n=470) of thawed primordial follicles had a complete architecture and compact structure (Figs 1-B, C, E). Moreover, the laminin-stained basement membrane (Fig 1-D) was maintained in primordial follicles after cryopreservation. For the healthy follicles from fresh group (n=530) or frozen-thawed group (n=419), the average diameters of nuclei (13.4 μm vs 13.2 μm), oocytes (29.3 μm vs 29.4 μm) and primordial follicles (35.9 μm vs 35.1 μm) between them were not significantly different ($P < 0.05$) (Fig 1-F).

Effect of cryopreservation on apoptosis and expression of activin and pSmad2 of primordial follicle cells

To determine the amount of apoptosis in monkey primordial follicles after slow cryopreservation and thawing, TUNEL staining was performed. Sections of fresh and frozen-thawed monkey ovarian tissue showed very little TUNEL-positive staining in the primordial follicles (Fig 2-A, B), with no difference between the two groups ($5 \pm 1.1\%$ vs $8 \pm 1.9\%$, $P > 0.05$) (Fig 2-C). However, many more stromal cells are positive by TUNEL-staining post cryopreservation ($25 \pm 2.4\%$ vs $5 \pm 0.9\%$; $P < 0.05$) (Fig 2-A, B, C).

To investigate whether cryopreservation and thawing impacted the localization of the activin-signaling pathway in early follicles, the activin β_A -subunit, and β_B -subunits and pSmad2 were measured in fresh and frozen-thawed monkey ovarian tissues. All three proteins were present in primordial follicles from both groups (Fig 2-D). The activin β_A -subunit was detected in oocytes and pre-granulosa cells in fresh and frozen-thawed tissues, however the staining was weaker in the cytoplasm of oocyte in frozen-thawed tissue (Figure 2D, panels a,d). Activin

β_B -subunit expression had a similar pattern in primordial follicles in fresh and frozen-thawed ovarian tissue (Figure 2D, panels b,e). Phosphorylated Smad2 immunostaining was detected in the nuclei of oocytes and the pre-granulosa cells of primordial follicles in both groups (Figure 2D, panels c,f).

Effect of cryopreservation and thawing on proliferation and abundance of activin/smad2 in rhesus primordial follicles post culture

We investigated differences in follicle or stromal cell proliferation pre- and post-culture of frozen-thawed tissue. PCNA was detected in some oocytes and many granulosa cells in transiting primordial follicles in frozen-thawed monkey ovarian tissue after 48 hours of culture (Fig 3-B) and more follicles have PCNA-positive staining post culture compared with non-culture group ($78 \pm 9.2\%$ vs $7 \pm 0.7\%$, $P < 0.05$) (Fig 3-A,B,C). Moreover, more PCNA-staining positive stromal cells were found in cultured tissue ($25 \pm 4.1\%$ vs $4 \pm 0.6\%$, $P < 0.05$) (Fig 3A,B, C). Furthermore, the activin β_A -subunit (Fig 3-D), activin β_B -subunit (Fig 3-E) and pSmad2 (Fig 3-F) were expressed in early stage follicles after 48 hours of culture.

Discussion

This study demonstrates that nonhuman primate primordial follicles can undergo slow cryopreservation and thawing with little effect on their morphology or markers of early follicular development. Most primordial follicles in frozen-thawed tissue maintained complete and healthy follicle structure with round oocytes surrounded by a layer of flattened pre-granulosa cells and an intact laminar membrane, and had nuclear, oocyte and follicle diameters similar to those of primordial follicles in fresh ovarian tissue. The abundance and localization of proteins important in early follicle development (activin subunits and pSmad2) were also similar in fresh and frozen-thawed tissues. Finally, after short-term *in vitro* tissue culture, primordial follicles in frozen-thawed ovarian tissue exhibited a high level of follicle survival and proliferation.

A controlled-rate freezing process has been successfully applied in the rodent, human and other species, and has proven to be an effective method for preservation of ovarian tissue (26-29). The present study examined the application of this slow cryopreservation technique to nonhuman primate (rhesus monkey) ovarian tissue. After thawing, 89% of the primordial follicles had complete follicle architecture, consistent with the findings of others applying this technique in other species. Chen et al (30) reported that 88% of the primordial follicles were morphologically normal after slow freezing in mice, and Jeremias et al (31) reported that 91% of primordial follicles survived after cryopreservation. Also no difference was reported in oocyte or follicle size before and after freezing human ovarian tissue (32). Our observation of low apoptotic activity in frozen-thawed ovarian tissue is similar to previous findings in mice and humans (32-34). Moreover, after short-term culture, most follicles not only survive and proliferate, but also sustain activin-psmad2 expression, which phenocopy the normal fresh follicles characters. These results suggest that slow cryopreservation does not induce primordial follicle apoptosis or disrupt the three-dimensional architecture and somatic-germ cell interactions that are crucial for oocyte growth and development.

To date, the majority of studies of ovarian tissue cryopreservation have described the effects on follicle morphology and survival (12,32,34). We further investigated whether cryopreservation affects primordial follicle function, specifically the activin-pSmad2 pathway, which has been shown to play an important role in regulating the formation and development of rodent ovarian follicles in the female (14-19,36). Previous studies in our laboratory showed that the activin β_A - and β_B -subunits are expressed in both oocytes and somatic cells in primordial follicles, and that activin A promotes primordial follicle formation when administered to neonatal mice (15). The current study demonstrated that the activin signaling

pathway is present in primordial follicles and remains intact after slow freezing and thawing of macaque ovarian tissue. There was a modest reduction in activin A staining in oocyte cytoplasm post freezing which was restored during the short culture period. The patterns of activin β B-subunits and phosphorylated Smad2 were similar in fresh, thawed and cultured tissues. One potential conclusion from this study is that the activin signaling pathway is relevant marker for follicle health, which can be used as cryopreservation methods are improved in coming years.

In contrast, TUNEL staining of stromal cells was higher in frozen-thawed ovarian tissue compared with fresh tissue and the tissue responds with an increase in proliferation. Keros et al (35) reported that the stroma was not preserved as expected by slow-programmed freezing of human ovarian tissue. Ovarian tissue is composed of different cell types, and it might be expected that each cell type has different requirements for optimal survival. Stromal cells not only support the three-dimensional environment necessary for maintaining follicle architecture, they also produce various factors that promote follicle growth and development (36). While changes in stromal cell health after freezing may not affect follicle function after short-term culture, it may negatively affect the growth and development of follicles over the long term, which may partially explain the observed follicle loss and low efficiency of implantation using follicles from frozen-thawed tissue (26,37-40). These data suggest that better methods for protecting the stroma cells from programmed cell death and inappropriate proliferation are necessary to develop.

In conclusion, our results suggest that, after slow cryopreservation and thawing, monkey primordial follicles retain their morphology, plus markers of functional development during short-term culture. The goal of these studies is to develop an ovarian tissue cryopreservation technique that will allow thawed primordial follicles to be matured *in vivo*, through retransplantation of thawed tissue, or *in vitro*, and produce oocytes competent for fertilization and culminating in live births (41). Identifying good molecular and functional markers for primordial follicles in nonhuman primate ovarian tissue is the first step towards this goal.

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Figure 1. Morphologic assessment of primordial follicles in monkey ovarian tissue pre or post cryopreservation

(A) In fresh tissue, the normal primordial follicle indicated by the arrowhead contains a germinal vesicle (GV)-stage oocyte surrounded by a layer of flattened pre-granulosa cells. (B) In frozen-thawed monkey ovarian tissue, the primordial follicle indicated by the arrowhead is similar in appearance to that of follicles in the fresh group at the same magnification and at high magnification (C). (D) Laminin expression was detected in frozen-thawed monkey ovarian tissues, and a primordial follicle with a complete basement membrane is indicated by arrowhead, the inset in figure D shows laminin-staining follicle in high magnification. Moreover, healthy rate (E) and morphological parameters (F) of primordial follicles between fresh and frozen-thawed groups were compared. Values are from four animals. Different superscripts with each column indicate statistically significant differences ($p < 0.05$). Each image here is a representative section from each group. Bar = 50 μm .

Figure 2. Apoptotic activity and localization of activin subunits and phosphorylated Smad2 in fresh and frozen-thawed primordial follicles

TUNEL staining was detected in fresh (A) and frozen-thawed (B) monkey ovarian tissues. No difference between the groups with regard to staining in primordial follicles (arrowhead), more TUNEL-positive staining was observed in stromal cells of the frozen-thawed group (arrow) as showed in (C). Moreover, Activin β_A -subunit, Activin β_B -subunit and pSmad2 were detected in fresh and frozen-thawed ovarian tissues (D). All three proteins localize mainly in both oocytes and pre-granulosa cells. a-c are fresh groups, d-f are frozen-thawed groups. Each image here is a representative section from each group. Bar = 50 μ m in figure A and B. Bar = 10 μ m in figure D.

Figure 3. Proliferation detection and expression of the activin β _A- and β _B-subunits and psmad2 in cultured monkey ovarian cortical tissue

Frozen ovarian cortical pieces were thawed and cultured for 48 h *in vitro*, then fixed and sectioned at 5 μ m for PCNA staining and IHC. Most follicles showed no PCNA staining in granulosa cells and oocytes in frozen-thawed uncultured tissue (A), but strong PCNA staining was measured in cultured tissue (B). More PCNA-immunostaining follicles (B, arrowhead) and stromal cells (B, arrow) were detected after culture (C). And immunostaining of activin β _A-subunit (D), activin β _B-subunit (E) and psmad2 (F) (arrows) were seen in growing follicles in cultured tissue. Activin β _A-subunit and β _B-subunit localize in the oocyte and granulosa cells, and psmad2 in the nuclei of both oocytes and granulosa cells. Each image here is a representative section from each group. Bar = 50 μ m in figure A and B. Bar = 25 μ m in figure D, E and F.