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Animal

Mouse Ovarian Tissue Cryopreservation Has Only a Minor Effect on In Vitro Follicular Maturation and Gene Expression

Hung-Ching Liu,[1,2] Zhiming He,[1] and Zev Rosenwaks[1]

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***Purpose*:** To establish a protocol for ovarian tissue cryopreservation which can retain fertility potential after thawing and to evaluate the impact of cryopreservation on development and gene expression during folliculogenesis.
***Methods*:** A controlled randomized study in a clinical and academic research setting in a university medical center was conducted to study cryopreservation and in vitro maturation (IVM) of mouse ovarian follicles. Preantral follicles isolated from either fresh (Group A) or cryopreserved (Group B) murine ovarian tissues were used to test their fertility potential by in vitro culture–in vitro maturation (IVC-IVM). Expression of Graafian follicles derived from both groups were detected by DNA microarray techniques for comparison.
***Results*:** Although there were no significant differences in IVM outcomes and follicular gene expression between the two experimental groups, cryopreservation appears to induce the expression of heat shock proteins, DNA-damage-inducible protein 45 and death-related apoptosis genes (i.e., Fas and Fas-ligand).
***Conclusion*:** Cryopreservation may trigger biological events not amenable to normal cell function and follicular development. However, neither follicular development nor gene expression was dramatically changed after cryopreservation. These data suggest that although our current cryopreservation techniques yield competent follicles and mature oocytes, subtle changes observed in gene expression imply that the present cryopreservation techniques need to be further refined.

KEY WORDS: DNA chip; gene expression; in vitro maturation; ovarian tissue cryopreservation; preantral follicles.

INTRODUCTION

Ovarian tissue cryopreservation provides an opportunity for long-term preservation of oocytes. This technology can potentially be used to conserve fertility and may be particularly beneficial to young cancer patients who require treatment with chemo- and/or radiotherapy, and are at risk for premature ovarian failure.

Follicular viability after thawing has been evaluated morphologically and histologically (1,2), by ortho- or heterotopic transplantation as well as by in vitro culturing. Table I summarizes the experience with ovarian tissue cryopreservation.

In animal models, recipients of orthotopic grafts of frozen–thawed ovarian tissue have resumed ovarian cycles in mice (14), sheep (30) and humans (31). Some of these grafts have developed to the antral stage in mice (10), in sheep (13–15), and have even resulted in the birth of live offspring in mice (3,5–7), and in sheep (12). Heterotopic grafting of cryopreserved marmoset tissue (2), and elephant (11) tissue to severe combined immunodeficient (SCID) mice showed follicular development to the antral

[1] The Center for Reproductive Medicine and Infertility, Weill Medical College of Cornell University, 515 East, 71st Street, S 500, New York.
[2] To whom correspondence should be addressed; e-mail: hcliu@mail.med.cornell.edu.

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Table I. Progress and Outcomes of Ovarian Tissue Cryopreservation

Ovarian tissue	Cryoprotectant	Restoration method	Results	Ref.
Mouse	Glycerol	Orthotopic autografting	Successive litters	(3)
	DMSO	Orthotopic autografting	Live offspring	(46)
	DMSO	Orthotopic autografting	Live offspring	(5)
	DMSO	Orthotopic autografting	Live offspring	(6)
	DMSO	Orthotopic autografting	Live offspring	(7)
	DMSO	Orthotopic autografting	Live offspring	(8)
	DMSO	Orthotopic autografting	Restored cyclicity antral formation	(9)
	DMSO	Orthotopic autografting	Antral formation	(10)
Mammoset		Heterotopic xenografting	Antral formation	(2)
Elephant		Heterotopic xenografting	Antral formation	(11)
Sheep	PROH	Orthotopic autografting	Live offspring	(12)
		Orthotopic autografting	Antral formation	(13)
		Orthotopic autografting	Antral formation	(14)
		Orthotopic autografting	Antral formation	(15)
Human	Glycerol	Xenografting (Under the kidney capsules)	Follicular viability assessed by histological analysis	(1)
	+ PROH + EG		DMSO, PROH, EG is more effective than Glycerol	
	EG & Fresh	None	Assessed viability live/death stain viability were not affected by freezing	(16)
	Fresh tissue	Xenografting	Antral formation	(17)
	Fresh tissue	Xenografting (S.C.)	Antral formation	(18)
	DMSO	Autografting	Ovulate 3 months post stimulation with human menopausal gonadotropins	(19)
	PROH	Xenografting	Initiation of mitosis within primary follicles	(20)
	PROH	Autografting	Rising E2 and antral formation	(21)
	PROH & Sucrose	Xenografting	Antral formation	(22–24)
Mouse	Fresh tissue	Organ culture/in vitro maturation	1 live offspring	(25)
Mouse	Fresh tissue	Xenografting, then In vitro maturation	Maturation of oocytes in primordial follicles to MII oocytes	(26)
Human	DMSO & fresh	In vitro culture of isolated PMF	Viable in follicles but Atretic after culture	(27)
	DMSO or PROH + Sucrose	Organ culture within extracellular matrix	Secondary follicles with few antral formation	(28,29)

stages. In the human, histological and morphological analyses have shown that follicles retained their viability after cryopreservation (16), or following xenografting of frozen–thawed tissue (2). Antral follicles were first found in fresh ovarian tissue following xenografting to SCID mice (17,18). With advancing techniques, xenografts of human thawed tissue showed initiation of mitosis (21) or antral follicle formation (22–24). The first successful ovulation was reported following laparoscopic autotransplantation of frozen–thawed human ovarian tissue on the pelvic wall (19). In a subsequent case, frozen–thawed tissue was transplanted in the ovarian fossa. Patients not only resumed normal serum testosterone levels, but also ovulated in response to hMG stimulation (23). These results have given researchers hope that fertility may be restored following ovarian tissue banking.

Although in vitro ovarian culture techniques are still being refined, the greatest challenge appears to be in IVM of immature oocytes from both fresh and cryopreserved tissue. Optimal culture conditions for both animal and human tissue are currently under investigation. In mice, only a few pregnancies have resulted from in vitro culture (25,26). Eppig and O'Brien (25) were first to achieve a live-birth in the following organ culture (obtained from newborn mouse ovaries) and in vitro culture of isolated oocyte–granulosa cell

complexes (25). A two-step strategy of in-vivo transplantation with subsequent in vitro culture was also applied successfully (26). These investigators found that primordial follicles isolated from grafts after allotransplantation of newborn mouse ovaries could be matured to generate metaphase II oocytes (26). In the human, ovaries are more fibrous, and over 96% of follicles are in the primordial stage. These early-staged follicles are less differentiated, possess fewer organelles, and lack their zonae pellucidae and cortical granules. Moreover, they appear to be less sensitive to cryoinjury. For this reason, cryopreservation of primordial follicles appears to hold greater promise in the humans (16,27). Most primordial follicles remained viable after thawing as substantiated by live/death staining and electron microscopy. However, culturing primordial follicles in vitro remains difficult. Attempts have been made to isolate primordial follicles from fresh and cryopreserved human ovarian tissue. Unfortunately, most isolated primordial follicles become atretic after 1–3 weeks of culture in vitro (16,27). Organ cultures of thawed human cortical tissue, cultured in extracellular matrixes, retain better primordial follicle viability and are able to advance to preantral or even early antral stages of development (28,29).

Despite these rare successes, ovarian tissue cryopreservation remains experimental. Maturation of gametes after thawing has not yet been realized in humans and pregnancies have only been achieved in animal models (32). The loss of functional follicles may be due to damage resulting from the freeze–thaw process or possibly due to ischemia occurring after grafting. Further refinement in freezing and grafting techniques as well as optimization of in vitro follicle maturation techniques will make clinial application of ovarian tissue cryopreservation feasible.

Recently, a successful in vitro follicle maturation system (33,34) and sensitive oligonucleotide microarray-based hybridization (DNA chip) techniques (34) have been established in our Lab. This in vitro follicle maturation system, similar to that reported by others (35–37), allows the maturation of preantral follicles with recombinant gonadotropins in vitro (33,34). During the in vitro maturation period, cultured follicles undergo morphological and biochemical changes that mimic in-vivo changes. Oocytes recovered from this culture system can be fertilized, and can develop to the blastocyst stage (33,34). In this study, our intent was to use this in vitro follicle maturation technique to test the possibility of retaining fertility potential after thawing and to evaluate the efficiency of the established cryopreservation method. Biological responses of cells and/or follicles are dependent on cellular gene activation, expression, and production. Changes in gene expressions may occur prior to changes in cellular behavior. Therefore, we will also utilize our established sensitive DNA chip method, capable of performing high throughput analyses of gene expression profiles, to detect differences in the gene expression between Graafian follicles derived from fresh and from cryopreserved tissue. Studying these differential gene expression profiles may gain insight into gene regulation during folliculogenesis and may facilitate the development of strategies for optimizing cryopreservation techniques.

MATERIALS AND METHODS

Animals and Ovarian Tissue

The use of mice for this study was approved by our Institutional Animal Care and Use Committee under protocol #9803-494A.

Ovaries (~2 mm^3) from 14-day-old B6D2F1 mice (Jackson Laboratory, Bar Harbor, ME) were dissected free of fat and mesentery and cut into 4–6 pieces. The dissected tissue of each ovary will be allocated half into the fresh group and half into the cryo group. Tissue from the cryo group were preserved for a predetermined period of time according to the cryoprotocol described below. Preantral follicles isolated from fresh and frozen–thawed ovarian tissue were cultured and matured in vitro for comparison.

Freezing and Thawing of Ovarian Tissue

The standard slow-freezing–rapid-thawing protocol was used (12) for cryopreservation with minor modification. Ovarian tissues was dehydrated at 37°C in Dulbecco's phosphate buffer (D-PBS) with 15% fetal calf serum (FCS) (Gemini BioProducts, Casa Basas, CA) for 5 min; in D-PBS plus 0.25 M fructose, 10% egg yolk, 15% ethylene glycol (EG) for 5 min; in D-PBS plus 0.5 M fructose, 10% egg yolk, 15% EG for 5 min; and finally in D-PBS plus 1.0 M fructose, 10% egg yolk, 15% EG for 5 min. After dehydration, ovarian tissue was transferred into freezer vials (VWR Scientific, Edison, NJ) containing 0.5 mL of D-PBS plus 1.0 M fructose, 10% egg yolk, 15% EG, and incubated at room temperature for 10 min prior to freezing. After incubation, the freezer vials were cooled using a programmable freezer (KRYO 10, series III) (Planer

LTD, Sunburry on Thames, UK) with a slow rate protocol (-2°C/min from 25°C to -7.5°C, seeding manually, farther reduction of temperature to -40°C at -0.3°C/min; and finally to -180°C at -50°C/min). Frozen vials were subsequently stored in liquid nitrogen (-196°C) until ready for use.

The vials were thawed by rapid shaking in a water bath at 37°C. The ovarian tissue was immediately transferred from the vials to tissue culture dishes and washed three times as follows: 1) D-PBS plus 0.5 M fructos, 10% egg yolk, 15% EG (5 min); 2) D-PBS plus 5% DMSO, 10% egg yolk (5 min); and 3) Opti-MEM (Gibco BRL, Grand Island, NY) plus 15% FCS (5 min). Thawed ovarian tissue was then incubated in in vitro follicle maturation medium at 37°C, 5% CO_2 overnigt before mechanical isolation of preantral follicles for in vitro follicle maturation in microdroplets.

In Vitro Follicle Maturation, Ovulation, Fertilization, and Embryo Development

The methods used for isolation and in vitro follicle maturation has been published previously (33,34). Briefly, preantral follicles mechanically isolated from either fresh ovarian tissue (Group A) or thawed after cryopreservation (Group B) were matured in vitro in 20-μL microdroplets containing Opti-MEM with recombinant FSH (100 mIU/mL) (Sigma, St. Louis, MO) and LH (100 mIU/mL) (Sigma) plus 5% fetal calf serum (FCS) for 2 days, then transferred to Opti-MEM with FSH (100 mIU/mL) and LH (10 mIU/mL) plus 5% FCS for an additional 10 days until Graafian follicles were formed. Somatic cell proliferation and antral formation were assessed under the inverted phase microscope (Nikon Inc., Melville, NY). On day 12, ovulation was induced by administering human chorionic gonadotropins (hCG) (1.5 IU/mL) (Sigma) and epidermal growth factors (EGF) (100 μg/mL) (Sigma). In vitro ovulated oocytes were inseminated with spermatozoa and cultured in vitro for an additional 5 days after fertilization was observed.

During in vitro maturation, the time (in days) required for the studied follicles to acquire morphological changes were recorded and evaluated.

RNA Extraction and Labeling

For detection of gene expression profile, new groups of ovarian tissue from the fresh and cryo groups were treated identically as described above. After in vitro maturation, the fully grown preovulatory Graafian follicles ($\sim$350 mm in diameter) were collected and pooled (3 follicles per group). Total RNAs were extracted from each pool of Graafian with Tri-Reagent (Molecular Research Center Inc., Cincinnati, OH), following the addition of 2 μL of microcarrier gel TR (Molecular Research Center). The extracted total RNAs were then purified through phase separation with bromo-3-chloropropane (BCP), precipitated, and washed with 75% ethanol (Sigma). Purified total RNAs were aliquoted and stored at -70°C.

A special CIS-platinum compound consisting of four binding sites was used to label mRNAs directly. One of the four binding sites binds a digoxigenin molecule and another one carries a nitrate ligand which is cleavable in an aqueous solution. After incubation with nucleic acids, this CIS-platinum compound labeling with digoxigenin will bind covalently to the mRNA at the N7 position of their guanosine and adenosine bases, thus introducing digoxigenin into the mRNAs directly. A commercially available Dig-Chem-Link® kit (Roche Boehringer Mannheim, Indianapolis, IN) was used to label mRNAs extracted from both experimental groups according to the manufacturer's suggestions. One microgram of extracted mRNA was incubated with 1-μL Dig-Chem-Link and 50 μL of water for 30 min at 85°C. The reaction was stopped by adding 5 μL of stop buffer provided in the set. Labeled mRNAs were further purified through CENTRE SEP Spin columns (Princeton Separations, Adelphi, NJ) before use for hybridization.

Hybridization and Detection

In general, conditions for hybridization and the method for detection were the same as previously published (34). Hybridization was performed overnight at 68°C utilizing a commercially available mouse cDNA expression microarray (Clontech Lab, Palo Alto, CA, Cat.# 7741-1). After stringent washes, the arrays were detected with Dig Luminescent Detection Kit (Roche Molecular Biochemical, Indianapolis, IN) according to the manufacturer's instructions. Chemiluminescence signals generated from CDP-star substrate conversion were scanned by Fluor-S-MAZ multiimager system (Bio-Rad, Hercules, CA) for 3–10 min. The digital images from both groups were evaluated by Borland Delphi 3.0 software. All data were normalized with the controls of mouse genomic DNAs and the housekeeping genes which were arrayed along the edges of the arrays. After normalization, the intensities of the signals were measured and used to monitor gene expression.

Repeats of This Experiment and Comparison of Expression Between Fresh and Cryo Groups

Detection of gene expression were repeated three times with three different pools of samples for confirmation. The mean and standard deviation of each expressed gene was calculated and studied. For each gene, the ratio of mean expression of the frozen group to that in the fresh group (i.e., expression cryo/fresh or expression B/A) were calculated. According to the manufacturer, genes were considered to be significantly. suppressed if expression B/A was less than 0.5, significantly elevated if expression B/A was between 2.0–23.0, or induced if the expression B/A was greater than 300.

Statistics

The survival rate (%) (number survived per number of isolated preantral follicles), MII formation rate (%) (number fertilized per number of survived follicles), fertilization rate (%) (number of fertilized number of MII per number of inseminated oocytes), and blastocyst formation rate (%) (number of blastocyst per number of fertilized oocyte) were analyzed by chi-square analysis. The difference was considered as statistically significant if the P value was less than 0.05.

RESULTS

Morphology of Ovarian Follicles After Thawing

After thawing, ovarian tissue was observed under the inverted phase microscope (Nikon, Melville, NY). The ovarian tissue from 14-day-old B6D2F1 mice was predominantly composed of preantral follicles consisting of a centrally located oocyte surrounded by layers of granulosa cells within the basal membrane. Generally, 15–20 preantral follicles were found in each fresh or frozenthawed ovarian slice (Fig. 1(a)). After thawing, viable preantral follicles were characterized as bright, intact, round follicles having a close contact between granulosa cells and oocytes (Fig 1(b)). Cryoinjury was evidenced by the presence of dark, shrinking follicles which became arrested or atretic after 3 days culture in vitro (Fig 1(c)). Most (>80%) of the preantral follicles remained viable after the frozen–thawed and retained their ability to be matured in vitro with the capability of generating mature metaphase II (MII) oocytes.

In Vitro Maturation Outcome

Preantral follicles isolated from both fresh and thawed ovarian tissue underwent similar morphological changes involving thecal cell adhesion (Fig. 2(a)), granulosa cell outgrowth (Fig. 2(b)), antral formation (Fig. 2(c)), and in vitro ovulation (Fig. 2(d)). Graafian follicles derived from both groups were capable of generating MII oocytes (Fig. 2(e)) after administering hCG/EGF and the in vitro ovulated oocytes were able to be fertilized and develop into 2-cell (Fig. 2(f)), 4-cell (Fig. 2(g)), and blastocyst (Fig. 2(h)) staged embryos after culture in vitro 1, 2, and 5 days respectively. After the experiment was repeated twice, the summarized outcome of in vitro follicle maturation is depicted in Table II. There was no significant differences in the survival rate ($p = 0.057$) per isolated

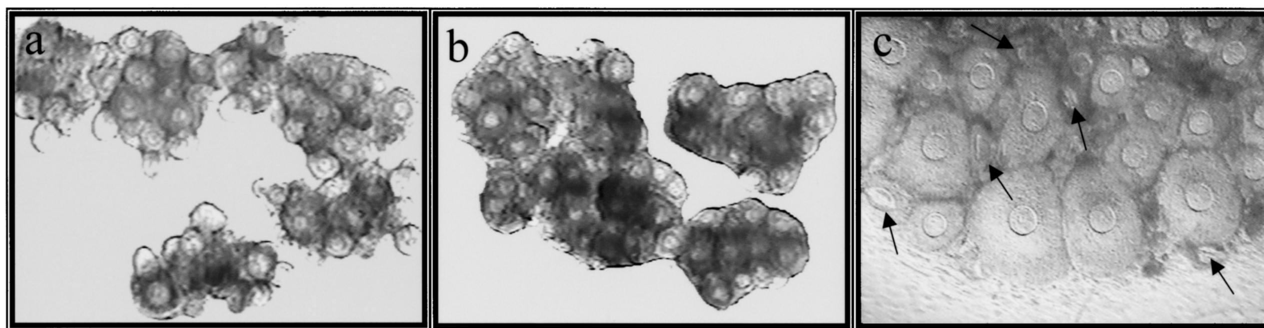

Fig. 1. Morphology of ovarian follicles pre- and postcryopreservation. Fresh ovarian tissue composed predominantly of preantral follicles consisting of a centrally located oocyte surrounded by layers of granulosa cells within the basal membrane (a). After thawing, ovarian tissues maintained tissue integrity and most of the preantral follicles remained viable as characterized by bright, intact, round follicles having close contact between granulosa cells and oocytes (b). Cryoinjury was evidenced by the presence of dark, shrinking follicles which became arrested or atretic after 3 days culture in vitro (c, cryoinjured follicles are indicated by arrows).

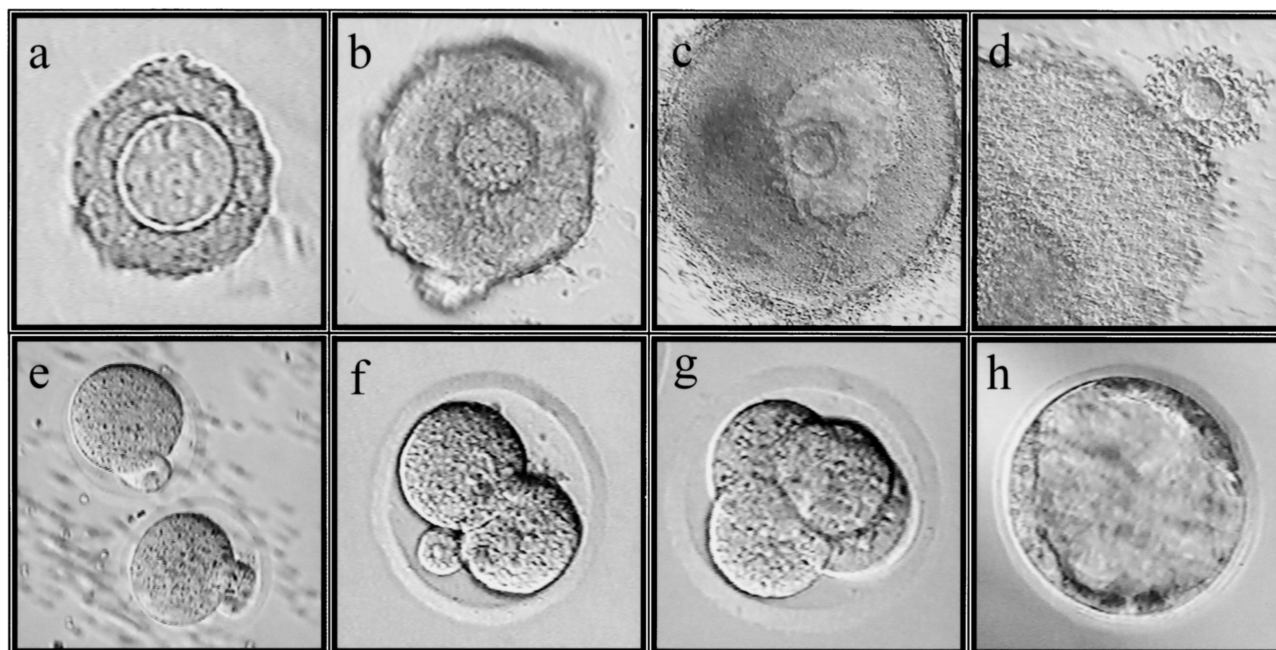

Fig. 2. Maturation of preantral follicles isolated from frozen–thawed tissue. Isolated follicles (2a) underwent normal morphological changes involving granulosa cell outgrowth (2b), antral formation (2c), and in vitro ovulation (2d), The ovulated mature oocytes (2e) can be fertilized and develop to 2-cell (2f), 4-cell (2g), and blastocyst-stage (2h) embryos after culture in-vitro for 1, 2, and 5 days respectively.

follicle, fertilization rate ($p = 0.936$) per MII oocyte formed, and blastocyst formation rate ($p = 0.297$) per fertilized oocytes between the cryopreserved and the fresh groups. However, there was a significant difference in MII formation rate ($p = 0.0031$) per survived follicle. In addition, the time required to trigger morphological changes in the cryo group were in general, delayed by 1 day (Table III). This result may indicate that the follicles had been slightly affected as was indicated by the reduction of MII oocyte formation.

Gene Expression Profiles

Gene expression profiles were similar in both groups (Fig. 3). It appears that the number of genes detected and the mean level of expression in both groups were very similar. Sixty-one and 66 genes were detected in the fresh and cryo groups, respectively. The mean and standard deviation of 61 genes expressed in both groups were 21.83 ± 18.25 and 21.12 ± 17.86 for groups A and B, respectively. When analyzing each gene by the ratio of mean expression in the cryo group/mean expression in the fresh group, we found that 44 were not significantly different in terms of expression (i.e., ratio of expression B/A was between 0.5 and 2.0), 11 were suppressed (ratio of expression B/A was <0.5), and six were elevated (ratio of expression B/A > 2.0) and five were induced because they were only expressed in the cryo group (Fig. 4). It is of interest to note that among the six elevated and five

Table II. Outcome of In Vitro Follicle Maturation

Ovarian tissue	Group A (fresh) no. (%)			Group B (cryopreserved) no. (%)			p-value
	Experiment 1	Experiment 2	Total	Experiment 1	Experiment 2	Total	
A. # Isolated preantral follicles	50	106	156	77	92	169	
B. # Survived (%A)	45 (90)	94 (88.7)	139 (89.1)	65 (84.4)	73 (79.3)	138 (81.7)	0.057
C. # Metaphase II oocytes (% B)	24 (53.3)	41 (39)	65 (46.8)	23 (35.4)	26 (28)	49 (29)	0.0031*
D. # Fertilized (% C)	16 (66.6)	15 (37)	31 (47.7)	12 (57.1)	11 (42)	23 (46.9)	0.936
E. # Blastocysts (% D)	12 (75)	11 (73)	23 (74.2)	5 (4.1)	9 (82)	14 (60.9)	0.297

*Significant difference between Group A and Group B.

Table III. Time Required for Morphological Changes During In Vitro Maturation

Type of ovarian tissue	Time required (days)			
	Thecal cell attachment	Glandular cell growth	Antral formation	In vitro ovulation
Fresh	1	3	6	10
Cryo	1.5	4	7	11

induced gene, three were heat shock protein (HSP84, HSP86, 78 ka glucose-regulated protein which is 60% homologous to HSP 78), one was DNA-damage-inducible protein 45, and two were apoptosis-related genes (Fas, Fas-ligand). Of 11 suppressed genes, one was a protein kinase (LIM domain kinase I), four were receptors (interleukin-receptor gamma subunit, ubiquitously expressed nuclear receptor 2, activin A receptor type IB, orphan receptor), and one was a cytokine (inhibin activin beta-A subunit).

DISCUSSION

Cryopreservation of ovarian tissue is an emerging technology. Advances have been made in establishing optimal conditions for cryopreservation and restoration of thawed tissue by either transplantation or in vitro culture. In this report, we describe a novel system for ovarian tissue cryopreservation and in vitro restoration of fertility after thawing. Dehydration and equilibration at high temperatures (37 and 25°C, respectively), mixture of ethylene glycerol, fructose, egg yolk as cryoprotectant, slow freezing and rehydration with media containing DMSO may have contributed to the success of this cryopreservation method.

A soluble protectant with high permeation is critical for cryopreservation. Incubating tissues/cells with protectants allows permeation of cryoprotectants for dehydration. Dehydration can minimize ice formation and reduce osmotic stress, thus avoiding cryoinjury. Dehydration and equilibration at high temperatures can facilitate cryoprotectants reaching to the center of the tissue efficiently. Newton *et al.* used proton nuclear magnetic resonance imaging analysis to demonstrate that ethylene glycol, a high-soluble protectant, had high permeation into human ovarian tissue at high temperature (1).

The addition of sucrose (e.g. PROH) to protectants was found to enhance protection against cell damage (22), possibly by reducing osmotic stress. In our protocol, fructose was added to achieve the same protective effect. Our results clearly indicate that fructose may be superior to sucrose because fructose is a smaller molecule and possess a higher permeation rate.

Egg yolk was incorporated for the purpose of reducing deleterious changes in cell membrane and cytoskeleton during the freeze–thaw process. We speculate that egg yolk may bind to the cell membrane or cytoplasmic organelles to increase their flexibility to protect against cryoinjury. Cryodegradation of cytoplasmic proteins may also be reduced with the dilution of proteins in the egg yolk preparation.

Among rapid freezing (i.e., plunging directly into liquid nitrogen), intermediate freezing (i.e., suspended over liquid nitrogen for 12 h), or slow freezing protocols, the highest oocyte/follicle survival rate in

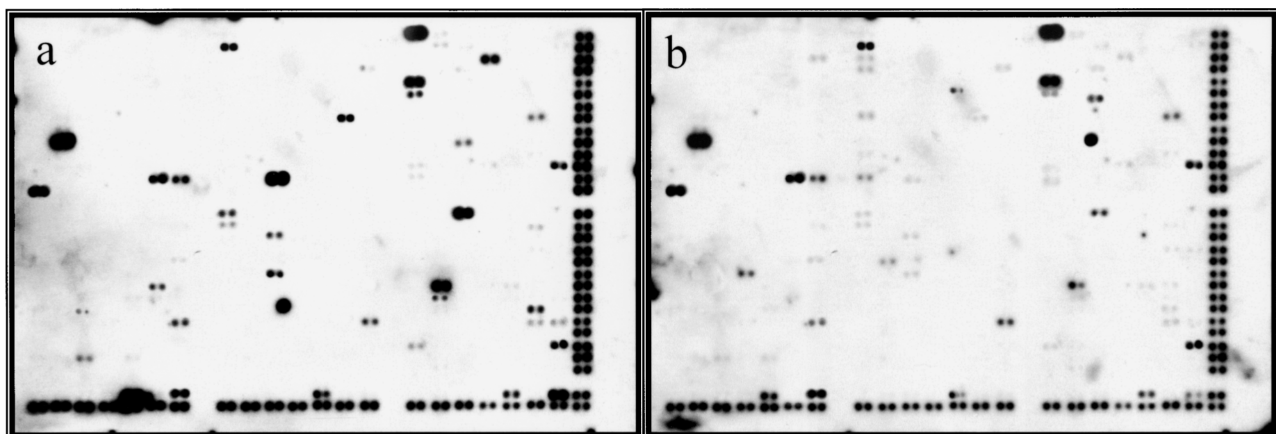

Fig. 3. Gene expression profiles on DNA chips. Antral follicles derived from fresh (A), and cryopreserved (B) ovarian tissues were detected as described in the text. The digital images of gene expression profiles obtained from fresh and cryopreserved tissues were illustrated.

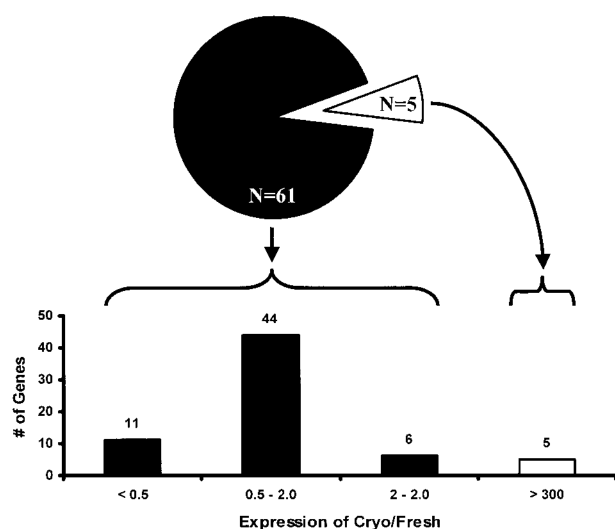

Fig. 4. Gene transcripts in antral follicles derived from fresh and cryopreserved tissues. Sixty-one and 66 genes were detected in the fresh and cryopreserved groups, respectively. Genes were classified according to their ratio of expression in the frozen to that in the fresh group (i.e., expression cryo/fresh or expression B/A). Genes were considered to be significantly suppressed if the ratio was less than 0.5, elevated if the ratio was between 2.0–23.0, or induced if the ratio is greater than 300.

thawed ovarian tissue was achieved with slow freezing and the poorest was observed with rapid freezing (23). For this reason, the standard slow freezing–rapid-thawing protocol was incorporated in our cryopreservation regimen.

Rehydration is an area which is least addressed. In fact, rehydration is as important as dehydration. During the period of rehydration, the shrunken tissues/cells will be gradually expanded through a stepwise washing. Optimal rehydration is also critical. Rapid expansion will result in the bursting of cells/tissues and fragmentation and incomplete expansion results in flat, shrunken follicles with gaps between the oocytes and somatic cells. These gaps would greatly reduce the restoration of follicular growth which require the interactions between granulosa cells, theca cells, and oocytes (33).

Only through complex inter- and intracellular communication, somatic cells as well as oocytes underwent dramatic morphological changes accompanied by a series of biochemical and genetic changes (33). Therefore, viable follicles and tissue integrity with close contact between oocytes and somatic cells are equally important for reviving follicle development during in vitro maturation.

DMSO seems to play an important role during rehydration. It remains unclear what role DMSO plays. It may effectively pump out protectants from cells to complete the rehydration process. With the presence of DMSO during the washing process, the follicles could be fully expanded to gain tissue integrity. In this condition, almost all follicles were viable and close contact between oocytes and somatic cells were observed. Whereas, in the absence of DMSO in washing, a few number of follicles were dark and shrinking and became arrested or atretic after 3 days culture in vitro (Fig. 1(c)).

After cryopreservation with the above-described protocol, follicle viability and developmental potential were examined by using an in vitro follicle maturation system. Preantral follicles isolated from both fresh and thawed tissue underwent similar follicular development, including thecal cell adhesion, granulosa cell outgrowth, antral formation, and in vitro maturation (Fig 2). More importantly, both groups generated a high yield of competent oocytes, which were able to be fertilized, developed to blastocyst-staged embryos (Fig. 2). Our data suggest that this in vitro follicle maturation system appears to be a reliable method for assessing tissue viability and for monitoring the efficiency of the tested cryopreservation protocol. The in vitro maturation outcome may not be significantly different between the fresh and cryopreserved groups (Table II). However, there was a significant difference in the blastocyst formation rates per survived follicles between these two experimental groups ($p = 0.0068$). In addition, a dlay in all levels of morphological changes (i.e., granulosa cell outgrowth, antral formation, and ovulation in vitro) after cryopreservation was observed (Table III). This may hint that some degree of cryoinjury did occur during cryopreservation.

Our data clearly shows that in vitro follicle maturation can serve as a way to restore fertility after cryopreservation. It may offer the best prospect for using frozen–banked ovarian tissue because it can completely eliminate inherent problems associated with either autografting (e.g., possible recurrence of cancer) or xenografting (transmission of animal diseases) (38,39). In addition, after in-vitro ovulation, the wasted biomaterials (such as granulosa cells, thecal cells, and cumulus cells) can be utilized to detect possible contamination of microscopic metastatic cells if genetic markers are available. Thus, tumor or diseasefree embryos can be prescreened before embryo transfer to avoid the risk of tumor recurrence, or disease occurrence. For example, a number of characteristic chromosomal abnormalities in leukemia and lymphomas have been defined at the molecular level (40).

DNA chip technology is an expanding and dynamic arena. It allows for the rapid detection of gene expression profiles of hundreds of thousands of genes simultaneously (41–45). This powerful method, capable of performing high throughput analysis of differential gene expression, is often used to identify genes involved in critical cellular function (45–49). Here, we intend to apply this method to detect differential gene expression profiles of Graafian follicles derived from fresh and cryopreserved tissue to know the impact of cryopreservation at the genetic level. In the past, we have refined the method to increase the sensitivity of detection (34). Using this modified method with a commercially available mouse cDNA expression microarray, gene expression in mouse Graafian follicles matured in vitro were studied. Of 588 known genes studied, only 61 were detected and their level of expression has been defined (34).

In this study, the DNA chip technique was further refined by labeling with digoxigenin directly on the extracted mRNA instead of on the reverse-transcribed cDNAs. To our surprise, the new method did not improve the sensitivity of detection and when applied to detect Graafian follicles derived from fresh tissue, the same 61 genes detected by the previous method (34) were also detected in this study. The consistency of gene expression profile in Graafian follicles may facilitate the identification of differential gene expression profiles in different preservation and developmental environments. With the new detection method, though the number of expressed genes and their level of expression were similar in both groups, minor changes between the two groups were observed. More genes were detected (66 vs. 61) in the cryopreserved group (Fig. 3). Most (44/61) of the detected genes expressed similarly in both the studied groups. After cryopreservation, 11 were significantly suppressed, 6 were significantly elevated, and 5 extra genes were induced (Fig. 3). Interestingly, many of the induced as well as the elevated genes were related to DNA reparation, apoptosis, and stress protection proteins, whereas many of the suppressed genes were associated with receptors or protein kinases. These genetic changes suggest that cryopreservation triggers biological events not amenable to nornal cell function and follicular development. These subtle changes in gene expression imply that the present cryopreservation techniques may need to be further refined. Upregulation of the suppressed genes and downregulation of the elevated and newly induced genes may improve cryopreservation techniques and outcome. In addition, the significance of these changes in gene expression after cryopreservation deserves further investigation.

In conclusion, we have established a system that can retain ovarian tissue integrity and follicle viability after cryopreservation and more importantly, which can also restore fertility function of thawed ovarian tissue by in vitro follicle maturation. Gene expression profiles of antral follicles derived from fresh and cryopreserved tissues revealed only minor differences. Cryopreservation appears to induce the expression of stress proteins (i.e., heat shock proteins), apoptosis-related genes (Fas, Fas-ligand), and DNA damage genes (DNA-damage-inducible protein 45), and to suppress the expression of some receptors and protein kinases. However, neither follicular development nor gene expression was dramatically changed after cryopreservation. These data suggest that although our current cryopreservation techniques yield competent follicles and mature oocytes, subtle changes observed in gene expression imply that present cryopreservation techniques need to be further refined. This investigation underscores the importance of developing sensitive markers of tissue viability and survival after cryopreservation.

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