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創新的卵巢組織玻璃化冷凍，對於細胞凋亡、顯微結構及  
原位移植懷孕能力之研究  
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# Novel direct cover vitrification for cryopreservation of ovarian tissues increases follicle viability and pregnancy capability in mice

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**BACKGROUND:** Cryopreservation of ovarian tissue is valuable for fertility preservation. We develop an innovative vitrification method using less concentrated cryoprotectants and direct application of liquid nitrogen to the ovarian tissue (direct cover vitrification, DCV) to improve its efficiency. **METHODS:** Ovaries of 5- to 6-week-old C57BL/6J mice were randomly allocated to four groups: DCV, conventional vitrification, slow-freezing and non-frozen controls. **Experiment 1:** observing the follicle morphology. **Experiment 2:** assessing viability. **Experiment 3:** investigating the ultrastructure. **Experiment 4:** examining the follicle number after grafting. **Experiment 5:** ascertaining pregnancy potential by allogeneic orthotopic transplantation. **RESULTS:** The percentages of morphologically normal or viable follicles from DCV were significantly greater than those achieved from conventional vitrification and slow freezing ( $P < 0.01$ ). The ultrastructure of primordial follicles from DCV appeared better than that achieved from conventional vitrification and slow freezing. After grafting, the follicle number from DCV was greater than conventional vitrification ( $P = 0.001$ ) and slow freezing ( $P = 0.021$ ). The pregnancy rate of DCV was higher than conventional vitrification ( $P < 0.01$ ). The litter size from DCV was comparable with that from non-frozen graft and was significantly greater than that achieved from conventional vitrification and slow freezing ( $P < 0.01$ ). **CONCLUSIONS:** DCV is highly efficient for cryopreservation of ovarian tissue. Using less concentrated cryoprotectants appears to reduce toxicity. Direct cover by liquid nitrogen maximizes cooling that could facilitate vitrification and prevent ice crystal injury.

*Key words:* cryopreservation/ovarian tissue/transplantation/ultrastructure

## Introduction

The progress of chemotherapy and radiotherapy has much improved the survival rate of young cancer patients. Yet these treatments are toxic to the gonads. In recent years, cryopreservation technology for mature oocytes has advanced significantly, resulting in better survival, fertilization and pregnancy potential (Porcu *et al.*, 2000; Fabbri *et al.*, 2001; Chen *et al.*, 2005). While cryopreservation is used to preserve the fertility of female cancer patients (Porcu *et al.*, 2004; Chen *et al.*, 2005), mature oocytes are collected following a period of gonadotrophin stimulation, requiring a delay in the treatment of cancer. Furthermore, mature oocytes are available in limited numbers.

Ovarian tissue cryopreservation has several potential advantages over mature oocyte cryopreservation (Amorim *et al.*, 2003). It preserves both steroidogenic and gametogenic functions. Primordial follicles are present in large numbers in ovarian tissue. The collection of ovarian tissue can be performed independent

of the stage of the menstrual cycle, without postponement of cancer treatment. It is also suitable for pre-pubertal girls.

The slow-freezing method using a programmed cryomachine is traditionally employed for the cryopreservation of embryos, oocytes or ovarian tissues (Trounson and Mohr, 1983; Gosden *et al.*, 1994; Porcu *et al.*, 2000; Donnez *et al.*, 2004; Oktay *et al.*, 2004). These procedures usually take several hours. Vitrification is an alternative method. With high concentrations of cryoprotectants and a faster cooling rate, it transforms cells into an amorphous glassy state, instead of ice crystal formation (Rall and Fahy, 1985; Mazur *et al.*, 1992; Chen *et al.*, 2000a,b). Vitrification is time saving and does not require special equipment. Recently, vitrification has also been applied to ovarian tissues (Sugimoto *et al.*, 2000; Salehnia *et al.*, 2002; Migishima *et al.*, 2003; Yeoman *et al.*, 2005).

Cryovials or tubes are commonly used to hold ovarian tissues for slow freezing or vitrification. They have the advantage of keeping a larger amount of ovarian tissues in physically

isolated containers. However, for achieving vitrification of a larger volume of vitrification solution and tissues by plunging the container into liquid nitrogen, the concentration of cryoprotectants must be higher. Thus, toxicity of the vitrification solution also increases. Sugimoto *et al.* (2000) used 10-mm (outer diameter) glass tubes as containers for vitrification of rat ovarian tissues. They found that the grafts from vitrified ovarian tissues had significantly fewer follicles than did non-frozen grafts. Migishima *et al.* (2003) used 1-ml cryotube for vitrification of mouse ovarian tissue. They achieved lower litter size for vitrified grafts than that achieved for non-frozen grafts.

To improve the efficiency and efficacy of cryopreservation, we developed a new vitrification method using less concentrated cryoprotectants and direct application of liquid nitrogen to the ovarian tissue (direct cover vitrification, DCV). We hypothesized that less concentrated cryoprotectants would reduce toxicity and that applying liquid nitrogen directly to the ovarian tissue would maximize cooling to facilitate vitrification and prevent ice crystal injury. We systematically examined the effects of DCV on mouse ovarian tissue by histological examination, viability tests, transmission electron microscopy (TEM), heterotopic autograft and orthotopic allograft. **We compared these parameters with conventional vitrification and slow freezing.**

## Materials and methods

### Animals

Five- to six-week-old female C57BL/6J mice (black fur) were obtained from the Animal Center of National Taiwan University Hospital. All mice were housed under a 14-h light/10-h dark regime at 22–24°C, with food and water supplied without restriction. After anaesthesia by inhalation of ethyl ether (Nihon Shiyaku Industries, Osaka, Japan), the ovaries were removed through small dorsolateral skin incisions and placed in Dulbecco's phosphate-buffered solution (DPBS) (Gibco, Grand Island, NY, USA) with 20% fetal bovine serum (FBS) (Sigma, St Louis, MO, USA). The size of an ovary was approximately 1.2 × 1.5 × 1.5 mm. The skin was closed with 9-mm Michel clips (Becton-Dickinson, Lincoln Park, NJ, USA). The Animal Care and Use Committee of our institute approved this study.

### DCV

DPBS medium was traditionally used as base media for the preparation of cryoprotectants in our laboratory (Chen *et al.*, 2000a,b, 2005). HEPES–human tubal fluid (HTF) medium or tissue culture medium-199 can also be used as base media (Isachenko *et al.*, 2004; Huang *et al.*, 2005; Takahashi *et al.*, 2005). The ovary was pretreated with an equilibration solution (0.8 ml) consisting of 7.5% (v/v) ethylene glycol (EG) and 7.5% (v/v) dimethylsulphoxide (DMSO) in DPBS with 20% FBS for 10 min at room temperature. For example, 10 ml of equilibration medium comprised 0.75 ml of EG, 0.75 ml of DMSO and 8.5 ml of DPBS plus 20% FBS medium. It was then transferred to a vitrification solution (0.8 ml) consisting of 15% EG, 15% DMSO and 0.5 M sucrose for 2 min. This vitrification solution was used very successfully in recent publications on blastocyst and oocyte vitrification (Chian *et al.*, 2004; Hiraoka *et al.*, 2004a,b).

The ovary was then placed on a piece of gauze to remove the surrounding vitrification medium. The ovary was put in a 1.8-ml plastic standard cryovial (Nunc, Roskilde, Denmark). Liquid nitrogen was directly applied onto the ovary for vitrification (Figure 1). The cap of



**Figure 1.** Graphic representation of the direct cover vitrification (DCV) method. Liquid nitrogen was directly applied onto the ovary for vitrification.

the cryovial was closed. The lid did not have a hole. The vial was then placed into a liquid nitrogen tank.

For thawing, the vial was taken out of liquid nitrogen, and the cap was opened. It took ~20 s after opening the vial for all the liquid nitrogen to disappear. Then, the ovary quickly was moved into 1 ml of 1 M sucrose and kept for 5 min. It was serially transferred into 0.5 M, 0.25 M sucrose and DPBS medium for 5 min each. The procedures were carried out in a sterile hood. The ovary was subjected to subsequent examination or transplantation.

### Conventional vitrification

The vitrification solution was prepared on the basis of formula used by Salehnia *et al.* (2002). It was made of DPBS plus 20% FBS containing 30% (w/v) Ficoll 70 (Sigma), 0.5 M sucrose, 10.7% (w/v) acetamide and 40% (v/v) EG. The ovary was transferred to the vitrification solution at room temperature and held for 5 min (Salehnia *et al.*, 2002). It was loaded into a 0.5-ml standard straw (IMV, L'Aigle, France). The straw was placed directly into liquid nitrogen.

For thawing, the straw was warmed in air for 5 s and then in a water bath for 20 s at room temperature. After warming, the contents of the straw were expelled into a Petri dish, and the ovary was serially transferred into 1 ml of 1 M, 0.5 M, 0.25 M sucrose and DPBS medium for 5 min each.

### Slow-freezing method

The ovary was put into 1 ml of 1.5 M 1,2-propanediol (PROH) plus 0.1 M sucrose in DPBS with 20% FBS for 25 min. It was then transferred into a 1.8 ml cryovial containing 1 ml of freezing medium of the same solution. The cryovial was transferred into an automated vertical freezer (Kryo 10 series III) (Planer Products, Middlesex, UK). The starting temperature was 22°C. It was slowly reduced to –7°C at a rate of 2°C/min. Ice nucleation was induced manually at –7°C. After holding for 10 min, the cryovial was cooled slowly to –30°C at a rate of 0.3°C/min and then rapidly to –150°C at a rate of 50°C/min. After 10 min of temperature stabilization, the cryovial was transferred into a liquid nitrogen tank.

After thawing, the ovary was placed in 1.0 M PROH and 0.2 M sucrose for 5 min. Then, it was serially transferred to 0.5 M PROH plus 0.2 M sucrose, 0.2 M sucrose and DPBS medium for 5 min each.

### Experiment 1: analysis of follicle morphology

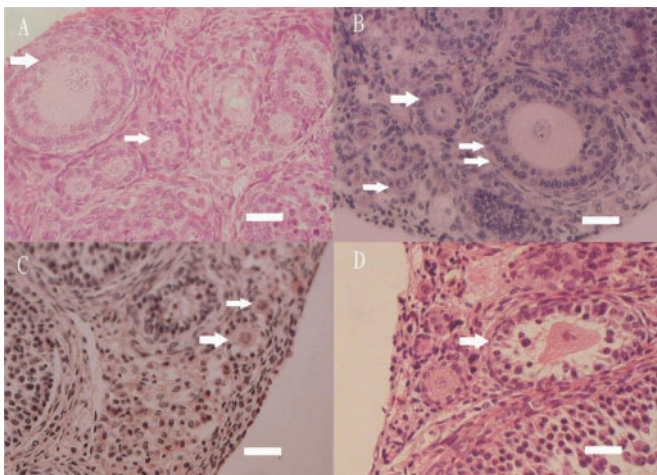
To assess the integrity of follicles after freezing and thawing, we examined the follicle morphology by histology. The ovaries removed

from female C57BL/6J mice were randomly allocated to four groups: non-frozen controls, DCV, conventional vitrification and slow freezing. Six samples from six different ovaries in each group were examined. The non-frozen controls and specimens after thawing were fixed in Bouin's solution. The specimens were embedded in paraffin blocks and serially sectioned into 7- $\mu\text{m}$  slices. They were stained with haematoxylin and eosin (H&E) and observed with light microscopy ( $\times 400$ ). Follicles were classified as follows: (i) primordial follicles with one layer of flattened granulosa cells surrounding the oocyte; (ii) primary follicles with one layer of cuboid granulosa cells and (iii) secondary follicles with two or three layers of granulosa cells (Gougeon, 1986). Antral follicles were not included in this study.

To avoid counting follicles more than once, in every third section, only follicles with a visible nucleus were counted (Cleary *et al.*, 2001). The definition of normality of follicles was based on the criteria proposed by Lucci *et al.* (2004). The follicles classified as morphologically normal were those containing an intact oocyte surrounded by well-organized granulosa cells (Figure 2A and B). In contrast, follicles were classified as degenerate if they had one or more of the following aspects: a pyknotic oocyte nucleus, shrunken ooplasm or disorganized granulosa cells (Figure 2C and D).

#### Experiment 2: examination of follicle viability

To evaluate the viability of follicles, we used Trypan Blue stain for follicles isolated from frozen-thawed ovarian tissues and non-frozen controls. Five samples from five different ovaries in each group were evaluated. The ovaries in each group were cut into small pieces. They were transferred to a 5-ml centrifuge tube containing 1 ml of HTF medium with 10% FBS plus 200 IU/ml type I collagenase (Sigma). The ovarian tissues were incubated in 5%  $\text{CO}_2$  at 37°C for 1 h and mechanically disrupted using a Pasteur pipette every 15 min. The digestion was terminated by adding 4 ml of HTF medium with 50%



**Figure 2.** Histological pictures of mouse ovarian tissues before and after cryopreservation. (A) In non-frozen ovarian tissue, the morphologically normal primary (small arrow) and secondary follicles (large arrow) consisted of intact oocytes and compact granulosa cells. (B) In ovarian tissue subjected to direct cover vitrification (DCV), the primordial follicle (small arrow), primary follicle (large arrow) and secondary follicle (double arrows) were morphologically normal. (C) In ovarian tissue subjected to conventional vitrification, the primordial (small arrow) and primary follicles (large arrow) were morphologically abnormal. The follicles had shrunken oocytes with pyknotic nuclei. (D) In ovarian tissue subjected to slow freezing, the abnormal secondary follicle (arrow) had detached granulosa cells. The oocyte was shrunken, with a pyknotic nucleus. Scale bar = 50  $\mu\text{m}$ .

FBS. After centrifuging at 400 g for 5 min, the pellet was resuspended in 100  $\mu\text{l}$  of HTF medium and mixed with 40  $\mu\text{l}$  of 4% Trypan Blue (Sigma). The suspension was spread thinly in a Petri dish and covered with oil for examination with an inverted microscope ( $\times 400$ ). Follicles were scored as being alive if the oocyte and the surrounding granulosa cells were clear (Figure 3A and B). Follicles were scored as being non-viable if the oocyte or the surrounding granulosa cells had blue coloration, demonstrating the inability to exclude the dye (Figure 3C and D).

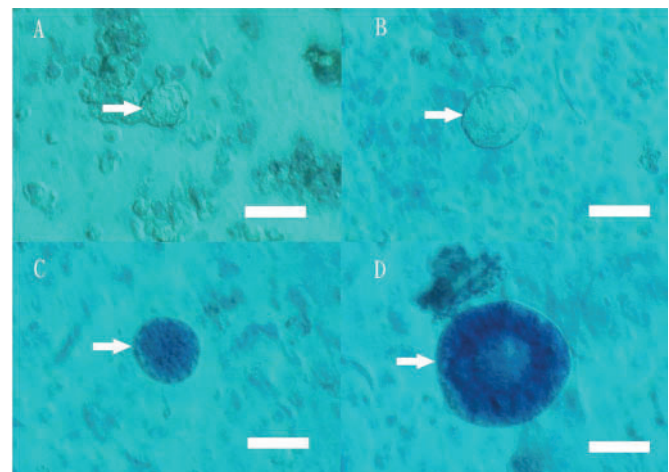
#### Experiment 3: investigation of ultrastructure of follicles

To ascertain the ultrastructural changes of follicles, we examined the frozen-thawed and non-frozen specimens by TEM using four samples from four different ovaries in each group. The ovarian tissue was fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 3 h at room temperature. The specimen was washed with the same buffer for 10 min and post-fixed in 1% osmium tetroxide for 1 h. After dehydration in an increasing ethanol concentration, the ovarian tissue was embedded in Epon 812 resin (TABB Laboratories, Aldermaston, UK). Semi-thin sections (1  $\mu\text{m}$ ) were stained with 1% toluidine blue for locating the follicles. Ultrathin sections (70 nm) were obtained and mounted on a nickel grid and were stained with alcoholic uranyl acetate together with aqueous lead citrate.

They were examined by TEM at magnifications ranging from  $\times 3000$  to  $\times 15\,000$ . The ultrastructural examination was focused on the primordial follicle with intact cytoplasmic membrane and nuclear envelope. The normal primordial follicle had a round oocyte tightly enclosed by the surrounding elongated granulosa cells (Figure 4A). Mitochondria with normal cristae and other membranous organelles such as Golgi apparatus and endoplasmic reticulum could be found in the cytoplasm of oocyte (Figure 4B and C). The follicular oocyte with a percentage of swollen mitochondria greater than 30% was defined as abnormal (Figure 4D).

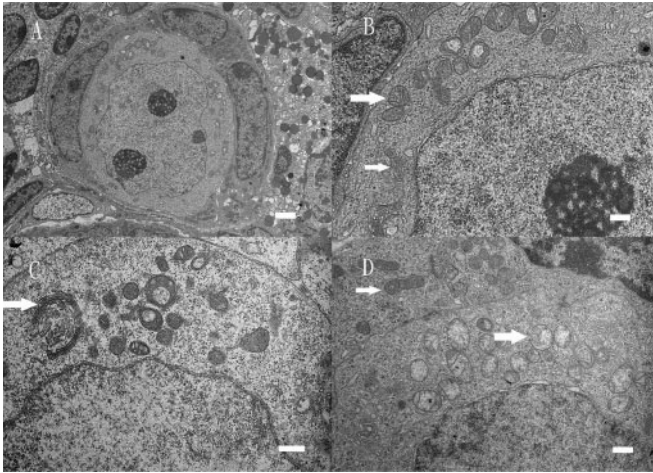
#### Experiment 4: autologous transplantation of ovaries beneath the renal capsule

To assess the follicles surviving from freezing-thawing and grafting, we transplanted frozen-thawed and non-frozen ovaries autologously



**Figure 3.** Trypan Blue staining for viability of follicles of non-frozen and frozen-thawed mouse ovarian tissues. (A) The primordial follicle (arrow) isolated from non-frozen ovarian tissue was alive, as shown by exclusion of Trypan Blue. (B) The primary follicle (arrow) from ovarian tissue subjected to direct cover vitrification (DCV) was also alive. (C) The primary follicle (arrow) from ovarian tissue subjected to conventional vitrification was dead, as shown by blue staining (the cells could not exclude the dye). (D) The secondary follicle (arrow) from ovarian tissue subjected to slow freezing was also dead. Scale bar = 50  $\mu\text{m}$ .





**Figure 4.** Transmission electron microscopy of primordial follicles in non-frozen and frozen–thawed mouse ovarian tissues. **(A)** The primordial follicle of non-frozen ovarian tissue had a round oocyte tightly connected to the surrounding granulosa cells. The cytoplasmic membrane and nuclear envelope were intact. Scale bar = 2  $\mu$ m. **(B)** In the higher power view of this oocyte, mitochondria (large arrow) and smooth endoplasmic reticulum (small arrow) distributed in the cytoplasm were morphologically normal. The cristae of mitochondria were clearly visible. Scale bar = 500 nm. **(C)** The primordial follicle of ovarian tissue subjected to direct cover vitrification (DCV) showed the oocyte with a normal mitochondrial pattern. An intact Golgi apparatus (arrow) was seen. Scale bar = 500 nm. **(D)** The primordial follicle of ovarian tissue subjected to conventional vitrification showed the oocyte with increased swollen mitochondria and disappearance of cristae (large arrow). The mitochondria (small arrow) in the surrounding granulosa cells were normal. Scale bar = 500 nm.

beneath renal capsules. A dorsolateral incision was made into the skin and peritoneum to expose the kidney. The kidney was exteriorized, and a small hole was torn in the capsule. The ovary was then inserted under the capsule. The kidney was returned to its normal anatomical position. The contralateral side was performed in a similar manner. The skin was closed with Michel clips. Two weeks after grafting of ovaries, mice were killed by cervical dislocation. The ovaries were removed and placed in Bouin's fixative and sectioned for H&E staining and follicle counts. Eight samples from eight different ovaries in each group were used for calculating graft follicle number.

#### **Experiment 5: allogeneic orthotopic transplantation of ovaries**

We further investigated the pregnancy potential of frozen–thawed and non-frozen ovaries after transplantation. Female C57BL/6J mice were randomly allocated into the four groups. Non-frozen and frozen–thawed ovaries were orthotopically transplanted into allogeneic female recipient mice of C57BL/6J-Tyr<sup>c-2J</sup>-J albino strain (Behringer, 1994). Fifteen mice with 30 different ovaries in each group were transplanted. The recipient female mice were oophorectomized by removing ovaries from ovarian bursae before transplantation. Three weeks after grafting, recipients were paired with fertile albino male mice of C57BL/6J-Tyr<sup>c-2J</sup>-J strain. Mating was confirmed by the presence of a vaginal plug, and then the female was housed individually. If no vaginal plug was seen, females were left in continuous pairings with males for 28 days. The pregnant female mice were allowed to litter, and the size of each litter was recorded. The pups have black eyes and fur (Figure 5).

#### **Statistics**

The percentages of follicles with normal morphology or viability were calculated in each experiment. Follicular counts and litter sizes are



**Figure 5.** Female C57BL/6J-Tyr<sup>c-2J</sup>-J albino recipients delivered and bred pups that were derived from allogeneic transplants of frozen–thawed ovarian tissue [by direct cover vitrification (DCV)] of female C57BL/6J mice. The heterozygous newborn mice had black eyes and fur.

reported as means  $\pm$  SD. The Mann–Whitney *U*-test was used to compare the study and the control groups. The Bonferroni method was used for multiple comparison adjustments. Comparability between the fecundity of the study groups and control recipients was tested with the Fisher's exact test.

## **Results**

### **Follicle morphology**

Percentages for normal morphology of various developmental follicle stages after treatment with different freezing methods and non-frozen controls are presented in Table I. The percentages of normal morphology for primordial, primary and secondary follicles in the non-frozen controls were significantly higher than those of the groups of DCV, conventional vitrification and slow freezing. The percentages of normal morphology of primordial, primary and secondary follicles in the DCV group were significantly greater than conventional vitrification and slow freezing.

### **Follicle viability**

Percentages of viable follicles for frozen–thawed ovarian tissues and non-frozen controls are presented in Table II. The percentage of viability of primordial follicles of the DCV group was comparable with that of non-frozen controls but was significantly greater than that achieved from conventional vitrification and slow freezing. For primary and secondary follicles, the percentages of viability in non-frozen controls were significantly higher than those of the groups of DCV, conventional vitrification and slow freezing. There was significantly greater viability in the DCV group than that achieved from conventional vitrification and slow freezing.

### **Ultrastructure of follicles**

The results from TEM showed that the 16 primordial follicles observed in non-frozen controls were all ultrastructurally normal.

**Table I.** Percentages of morphologically normal follicles of various stages in the groups of direct cover vitrification (DCV), conventional vitrification, slow-freezing and non-frozen controls

Groups	DCV	Conventional vitrification	Slow freezing	Non-frozen
Primordial (1099 ± 155)	95.1 ± 1.7 <sup>a</sup> (1161 ± 186)	83.0 ± 2.8 (1056 ± 129)	88.2 ± 3.2 (1049 ± 182)	99.0 ± 0.4 <sup>b</sup> (1131 ± 124)
Primary (284 ± 52)	88.8 ± 2.6 <sup>a</sup> (250 ± 24)	76.8 ± 1.2 (304 ± 58)	78.6 ± 3.3 (271 ± 45)	95.0 ± 1.5 <sup>b</sup> (309 ± 60)
Secondary (143 ± 34)	74.3 ± 9.3 <sup>a</sup> (127 ± 24)	51.7 ± 8.2 (156 ± 42)	49.3 ± 4.6 (141 ± 31)	92.1 ± 1.9 <sup>b</sup> (148 ± 39)

Percentage data are shown as mean ± SD. Values in parenthesis are mean ± SD of follicles observed per ovary, with the mean of the four groups given in column 1.

<sup>a</sup> $P < 0.01$ , compared with conventional vitrification and slow freezing.

<sup>b</sup> $P < 0.01$ , compared with DCV, conventional vitrification and slow freezing.

**Table II.** Percentages of viable follicles of various stages in the groups of direct cover vitrification (DCV), conventional vitrification, slow-freezing and non-frozen controls

Groups	DCV	Conventional vitrification	Slow freezing	Non-frozen
Primordial (544 ± 107)	92.3 ± 2.7 <sup>a</sup> (509 ± 121)	59.6 ± 7.1 (572 ± 67)	77.8 ± 4.0 <sup>b</sup> (536 ± 87)	95.4 ± 1.8 <sup>a</sup> (557 ± 158)
Primary (93 ± 26)	85.2 ± 4.3 <sup>a</sup> (94 ± 15)	42.1 ± 4.9 (108 ± 46)	70.5 ± 5.4 <sup>b</sup> (86 ± 16)	92.0 ± 2.7 <sup>a,c</sup> (82 ± 15)
Secondary (40 ± 14)	69.4 ± 7.7 <sup>a</sup> (33 ± 10)	27.6 ± 6.0 (40 ± 17)	38.8 ± 7.9 (40 ± 16)	91.9 ± 2.2 <sup>a,c</sup> (47 ± 14)

Percentage data are shown as mean ± SD. Values in parenthesis are mean ± SD of follicles observed per ovary, with the mean of the four groups given in column 1.

<sup>a</sup> $P < 0.01$ , compared with conventional vitrification and slow freezing.

<sup>b</sup> $P < 0.01$ , compared with conventional vitrification.

<sup>c</sup> $P < 0.02$ , compared with DCV.

The number of primordial follicles with normal ultrastructure in the DCV group (13/15, 87%) was comparable with non-frozen controls (16/16, 100%) (Figure 4). However, compared with the conventional vitrification (4/15 or 27%) ( $P = 0.003$ ) and slow-freezing (6/14, 43%) ( $P = 0.021$ ) groups, the incidence of primordial follicles with normal ultrastructure was higher in the DCV group.

### Follicle number after transplantation

The sum of primordial, primary and secondary follicles in each graft represents the follicle number. The mean follicle number of the DCV group (828 ± 141, range 630–1107) was similar to that of non-frozen ovarian grafts (857 ± 156, range 648–1175). The follicle number of the DCV group was significantly greater than that of the conventional vitrification (380 ± 128, range 187–593) ( $P = 0.001$ ) and slow-freezing (601 ± 162, range 410–879) ( $P = 0.021$ ) groups.

### Pregnancy capability after grafting

The numbers of pregnant recipients and pups derived from grafted non-frozen or frozen–thawed ovaries are summarized in Table III. Overall, 176 pups were delivered, 170 of which had black eyes. Six pups (3.4%) with red eyes were excluded from results. The pregnancy rate of females receiving frozen–thawed

ovaries from DCV was similar to that of those receiving non-frozen ovaries. However, the pregnancy rate of females receiving ovaries from the DCV or non-frozen groups was significantly higher than that from conventional vitrification. Pups delivered by recipients in the group of DCV (4.3 ± 2.4) were comparable with the non-frozen group (4.4 ± 2.2). They were significantly greater than those delivered in the groups of conventional vitrification (0.7 ± 1.1) and slow freezing (2.0 ± 1.9).

### Discussion

The DCV cryopreservation method was highly efficient at increasing morphologically normal and viable follicles from cryopreserved ovarian tissue, compared with slow freezing and conventional vitrification. Direct cover of the ovarian tissues with liquid nitrogen maximized the cooling rate, facilitating vitrification and reducing ice crystal formation. When thawing, the vitrified tissues were directly put into a dilution solution that maximized the warming rate to minimize devitrification. After transplantation, the follicular counts and litter size of the DCV group were significantly greater than those of the slow-freezing and conventional vitrification groups. In all experimental groups, a decrease in the percentage of morphologically normal or viable follicles with increased stage of follicular development was observed. The cause for this decrease may be

**Table III.** Fecundity of the female C57BL/6J-Tyr<sup>c-2J</sup>-J recipients transplanted with non-frozen or frozen–thawed ovaries of female C57BL/6J mice, examined by natural mating

Groups	Direct cover vitrification	Conventional vitrification	Slow freezing	Non-frozen controls
Recipients	15	15	15	15
Pregnant recipients [ <i>n</i> (%)]	13 (87) <sup>a</sup>	5 (33)	9 (60)	14 (93) <sup>a</sup>
Number of pups	64	10	30	66
Pups/recipient (range)	4.3 ± 2.4 <sup>b</sup> (0–8)	0.7 ± 1.1 (0–3)	2.0 ± 1.9 (0–5)	4.4 ± 2.2 <sup>b</sup> (0–8)

<sup>a</sup> $P < 0.01$ , compared with conventional vitrification.

<sup>b</sup> $P < 0.01$ , compared with conventional vitrification and slow freezing.

due to the larger size of follicles with increased stage of development that was more difficult for sufficient dehydration.

Using cryoloops instead of straws for vitrification resulted in significant improvement in the cryopreservation of oocytes and blastocysts (Cai *et al.*, 2005; Takahashi *et al.*, 2005). With a small amount of less concentrated cryoprotectants and direct contact with liquid nitrogen, the cryoloop technique achieved high thermal change and reduced the toxicity from the cryoprotectants. However, the cryoloop is not appropriate for handling larger amounts of tissue, such as an ovary. Unlike oocytes or embryos that have to be transferred under a microscope, the ovarian tissues can be manipulated without a microscope. Therefore, we used cryovials for the development of the DCV method that can hold larger amounts of tissue for vitrification, with direct contact by liquid nitrogen. We employed less-concentrated vitrification medium consisting of 15% EG, 15% DMSO and 0.5 M sucrose. This formula of cryoprotectants was used by Chian *et al.* (2004) for vitrifying bovine oocytes, with a survival rate of 92%. In the conventional vitrification solution, a much higher EG concentration (40%) combined with a polymer like Ficoll was used, which was considered to be more toxic. A clear drop in the outcome using conventional vitrification may be related to the higher concentration of cryoprotectants used in this protocol. We further demonstrated that using the less-concentrated vitrification solution achieved better outcome for ovarian tissues by the DCV method.

Salehnia *et al.* (2002) applied conventional vitrification for mouse ovarian tissue by plunging a cryovial into liquid nitrogen. The tissue was exposed to a more concentrated vitrification solution. They found that the examination of the ultrastructure of the oocytes of primary follicles in vitrified ovarian tissues revealed increased numbers of swollen mitochondria with disappearance of cristae. In our study, the mitochondrial morphology of oocytes of primordial follicles in the DCV group was comparable with that of the non-frozen controls. The incidence of ultrastructurally abnormal oocytes with swollen mitochondria, indicating cells under stress, was higher in the conventional vitrification and slow-freezing groups than in the DCV group.

Yeoman *et al.* (2005) performed vitrification of cortical pieces of monkey ovarian tissues suspended in cryoprotectants, by dropping these pieces from a serological pipette into a shallow container of liquid nitrogen. The solid drops were then picked up with forceps and sealed in liquid nitrogen-filled cryovials. They achieved survival rates for ovarian follicles similar to slow freezing. However, the volume of the vitrification solution surrounding the tissue pieces could slow the cooling by liquid nitrogen. It could also reduce the warming rate during thawing. In the mouse model, we verified that the follicle viability and the litter size from the DCV method were significantly greater than those from slow freezing.

## Conclusion

We developed DCV and explored the effects of DCV on mouse ovarian tissue in the five different experiments using histology, viability tests, TEM, heterotopic autograft and orthotopic allograft. The DCV method had greater percentages of

morphologically normal follicles in the histological examination, compared with conventional vitrification and slow freezing. *In vitro* viability tests showed larger survival of follicles in the DCV group. The DCV method also preserved better ultrastructure of oocytes of primordial follicles, indicating less cellular stress. *In vivo* viability of follicles after autotransplantation beneath the renal capsule revealed higher follicular counts in the DCV group. After allogeneic orthotopic transplantation, the *in vivo* function of frozen-thawed ovaries treated by DCV was comparable with that of non-frozen ovaries regarding the pregnancy capability and the litter size and was better than that achieved with conventional vitrification and slow freezing. Therefore, the DCV method was highly efficient at increasing follicular survival and pregnancy of cryopreserved mouse ovarian tissues. It also resulted in less ultrastructural injury. The principle of the DCV method allows the cryopreservation of many tissue pieces, and the size of containers may also be increased. The effects of DCV on the ovarian tissues of other species, including humans, deserve further investigation. The possibility of vitrifying pieces of other tissues, such as liver, may merit further studies.

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