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Novel needle immersed vitrification: a practical and convenient method with potential advantages in mouse and human ovarian tissue cryopreservation

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BACKGROUND: Ovarian tissue cryopreservation may be a potential method of preserving fertility in women who have experienced gonadotoxic treatments. To improve the efficiency of existing cryopreservation, we developed a practical and convenient vitrification method named needle immersed vitrification (NIV), which required a less concentrated and minimum volume of vitrification solution. METHODS: Mouse ovaries and human ovarian cortex fragments were vitrified using the NIV method, the slow-freezing method or the dropping vitrification method. Their morphology, ultrastructure and viability were analyzed and compared with fresh group. RESULTS: Primordial follicles in human and mouse ovarian tissues vitrified by NIV were well preserved. In mice, the percentages of normal morphological primary and secondary follicles were greater in the NIV group than that in the slow-freezing group or dropping vitrification group ($P < 0.001$). Ultrastructure of the stromal cells was preserved better in the NIV group than the slow-freezing or the dropping vitrification group in both human ($P = 0.039$, $P = 0.023$, respectively) and mouse (both $P < 0.001$) models. The viability assessment on frozen–thawed human ovarian tissue strips revealed that the follicles and the stroma had a satisfactory viability in the NIV group. In mouse model, the ovarian functional restoration in the NIV group was the best among three freezing groups, which was demonstrated by follicle counting in grafts after transplantation ($P = 0.009$ and $P = 0.010$ versus slow freezing and dropping vitrification, respectively). The cleavage rate of oocytes from grafts of the NIV group was most similar to that observed in the fresh group. CONCLUSIONS: The NIV method could facilitate vitrification process, maximize the cooling rate and reduce the toxicity of the vitrification solution with a minimal volume of less concentrated cryoprotectants. NIV was practical and convenient for cryopreservation of ovarian tissues.

Keywords: ovarian tissue; vitrification; mouse; human

Introduction

The recent advance in cancer diagnosis and treatment has improved the survival of patients with malignant diseases. The growing population of long-term female cancer survivors may experience infertility and premature menopause because of the gonadotoxic chemotherapy and/or radiotherapy (Meirow, 2000). Several options can be available to the preservation of female fertility, such as oocyte cryopreservation and embryo cryopreservation. Cryopreservation of ovarian tissues as an alternative or a supplement method for preserving both steroidogenic and gametogenic functions has been studied by researchers (Oktay and Karlikaya, 2000; Callejo *et al*., 2001; Kim *et al*., 2003, 2004a; Donnez *et al*., 2004, 2006; Meirow *et al*., 2005, 2007; Schmidt *et al*., 2005; Demeestere *et al*., 2006). It is suitable for the patients who cannot delay anticancer treatment and the pre-pubertal girls who are unfit for ovarian stimulation. Large primordial follicles in ovarian cortex are less susceptible to cryoinjury. The structural integrity and the developmental potential of the primordial follicles could be well preserved in the stored ovarian cortex (Hovatta *et al*., 1996; Oktay *et al*., 1998, 2000; Gook *et al*., 2000, 2001; Van den Broecke *et al*., 2001a,b). Functional survival of the human cryopreserved ovarian tissues has been demonstrated by ovarian tissue transplantation (Oktay and Karlikaya, 2000; Callejo *et al*., 2001; Kim *et al*., 2003, 2004a; Gook *et al*., 2003, 2005; Donnez *et al*., 2004, 2006; Meirow *et al*., 2005, 2007; Schmidt *et al*., 2005; Demeestere *et al*., 2006). To date, two healthy children have been born after the orthotopic transplantation from the cryopreserved tissue (Donnez *et al*., 2004; Meirow *et al*., 2005).

Cryopreservation of human ovarian tissues has generally been performed using the slow-freezing method. However, a

rapid, simple and inexpensive method, vitrification, has recently been applied to preservation of ovarian tissues. Vitrification is an ultrarapid cooling process that can produce a glasslike solidification of cells by extreme elevation in viscosity. Programmed freezer is not needed and cellular injury caused by ice crystals can be avoided. Vitrification has already been successfully applied to preserve human blastocyst and oocyte (Takahashi *et al*., 2005; Antinori *et al*., 2007). Ovarian tissue preservation by vitrification has been reported in rodents, domestic animals, non-human primates and human (Sugimoto *et al*., 2000; Migishima *et al*., 2003; Hasegawa *et al*., 2004, 2006; Bordes *et al*., 2005; Yeoman *et al*., 2005; Chen *et al*., 2006; Gandolfi *et al*., 2006; Li *et al*., 2007; Santos *et al*., 2007). The birth of pups using vitrified ovaries and grafting or *in vitro* culture has been reported in mice (Chen *et al*., 2006; Hasegawa *et al*., 2006) and ewes (Bordes *et al*., 2005). Vitrification may be an effective alternative to slow-freezing method in human ovarian tissue cryopreservation. However, data on the vitrification of human ovarian tissues remain limited, therefore further investigation is required.

In this study, we developed a new vitrification method, needle immersed vitrification (NIV), which used a special carrier to hold the ovarian tissues and place them into liquid nitrogen directly. This method would maximize the cooling rate to facilitate vitrification and avoid ice crystal injury. We compared its protective effects on mouse and human ovarian tissues with other cryopreservation methods. The effects of the methods were identified by histological analysis, ultrastructural examination by transmission electron microscope (TEM) and viability staining. Mouse heterotopic allograft procedure was used as an animal model to evaluate the functional restoration of the cryopreserved ovarian tissues.

Materials and Methods

Animals and tissue collection

Kuming white mice were purchased from the Animal Center of Sichuan Chinese Materia Medica Institute. Sixteen-day-old female mice were used as donors, and 10–12-week-old male mice were used as recipients. The sperm from 12- to 14-week-old male mice were used for IVF. The mice were housed under a 12:12 h light–dark cycle regimen at 22–24°C. Food and water were freely available. The project had been approved by Institutional Animal Care and Use Committee of Sichuan University.

Ovaries from 16-day-old mice killed by cervical dislocation were aseptically removed and collected into the Leibovitz medium (L-15, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco), 50 μg/ml of streptomycin and 50 IU/ml of penicillin (Gibco). The ovaries were cleaned of surrounding tissues and placed in a sterile Petri dish for the next procedure.

Human specimen preparation

Human ovarian tissues were collected from five women (median age, 31 years; range, 21–37). Of patients, three had undergone oophorectomy for their specific conditions, including endometrial cancer (two patients) and breast cancer (one patient). The other two ovarian tissue samples were obtained from ovarian biopsy from patients who underwent ovary transposition because of cervix cancer. All the patients signed an informed consent form that was approved by Institutional Ethics Committee of West China Medical Center, Sichuan University, which also approved the current study. The collected tissue was placed into the L-15 medium supplemented with 10% FBS. When transferred to laboratory, the ovarian medulla was removed to obtain a 1 mm thick cortex slice. The tissue was cut into strips $\sim$1–2.5 mm^2 in size in fresh L-15 medium supplemented with 10% FBS. The samples were confirmed as showing no ovarian metastasis by an independent pathologist before data analysis.

Cryopreservation procedures

Human ovarian cortex fragments and intact ovaries from mice were assigned to three different cryopreservation procedures as described below. Before cryopreservation, one or two pieces of human ovarian cortex were fixed for morphological and ultrastructure assessment. The untreated mouse ovaries were also used as fresh control for morphological and functional analysis.

Slow-freezing protocol

A slow-freezing protocol put forward by Gosden *et al*. (1994), with some modifications, was used to perform ovarian tissue cyropreservation. According to the protocol, 2–3 intact mouse ovaries or 2–3 pieces of human ovarian cortex fragments were placed in a 1.8 ml cryovial (Nunc, Roskilde, Denmark) containing 1 ml of L-15 medium supplemented with 0.1 M sucrose (Sigma-Aldrich, St Louis, MO, USA), 10% FBS and 1.5 M dimethyl sulfoxide (DMSO, Sigma-Aldrich). After a 30 min exposure to the cryoprotectant solution at 4°C, the cryovials were placed in the programmable freezer (Biomed Freezer Kryo 10, Series II, Planer, UK). Slow-cooling protocol was performed with the starting temperature of 4°C. The vials were cooled at a rate of 2°C/min to −7°C and held for 5 min, seeded manually and maintained at −7°C for another 10 min. Then the temperature was cooled to −40°C at a rate of −0.3°C/min and was further cooled to −140°C at −10°C/min. Finally, the vials were transferred to liquid nitrogen for storage. Samples stored in liquid nitrogen for a minimum of a week.

For thawing, the cryovials were removed from liquid nitrogen, held in air for 20 s and transferred to a water bath (37°C) for 20–30 s. The contents of cryovials were emptied into the L-15 medium supplemented with 0.1 M sucrose, 10% FBS and 1.0 M DMSO for 5 min and washed in a stepwise manner (1.0 M DMSO + 0.1 M sucrose, 0.5 M DMSO + 0.1 M sucrose, 0.1 M sucrose) for 5 min each.

Vitrification

Direct dropping vitrification by using Pasteur pipette. The vitrification procedure used a containerless liquid nitrogen emersion system following the procedure described by Yeoman *et al*. (2005) and Li *et al*. (2007) with some modifications. Tissues were initially incubated for 5 min in 10% (v/v) ethylene glycol (EG, Sigma-Aldrich) and 10% (v/v) DMSO in Dulbecco phosphate-buffered saline medium (DPBS, HyClone, Thermo Fisher Scientific Inc., USA) with 20% FBS, then in 20% EG and 20% DMSO for another 5 min. After dehydration, the tissues were immediately drawn into a Pasteur pipette. Individual drops were released directly into a shallow container of liquid nitrogen by shaking gently. These solid drops then were collected with precooled forceps, sealed in liquid nitrogen-filled cryovials and stored in liquid nitrogen for at least 1 week.

Needle immersed vitrification. The ovarian tissues were dehydrated by using a two-step regimen: (i) an equilibration solution consisting of 7.5% (v/v) EG and 7.5% (v/v) DMSO in DPBS supplement with 20% FBS for 10 min at room temperature and (ii) a vitrification solution consisting of 15% EG, 15% DMSO and 0.5 M sucrose for

2 min. This vitrification solution was used successfully in recent publications on blastocyst, oocyte and mouse ovarian vitrification (Chian *et al*., 2004; Hiraoka *et al*., 2004; Chen *et al*., 2006).

A special carrier named 'acupuncture needle' (Cloud & Dragon Medical Device Co. Ltd, China) was used in this NIV method. Acupuncture is a traditional Chinese medical system, including the use of very fine needles inserted into the certain points of human body to release any uncomfortable feeling. The acupuncture needle consists of two parts: a stainless steel filiform (thread-like) needle and a handle. The long needle can hold several ovarian tissue samples in a row, and thus all the samples can be exposed to cryoprotectants and then immersed into liquid nitrogen under the same conditions. This can maximize the cooling rate and simplify the vitrification process. Needles which were 0.18 mm diameter and 13 mm long for the mouse ovaries, or 20 mm long for the human ovarian cortex fragments were used in present study.

To vitrify human ovarian cortex fragments, 4–5 pieces of ovarian tissue strips were held in a row by a needle in the L-15 medium supplemented with 10% FBS (Fig. 1A). We used forceps to clamp the handle of the needle and transport ovarian tissues held by the needle to the equilibration solution and vitrification solution in turn. After two steps of dehydration procedure, the ovarian tissues carried by the needles, held by the forceps, underwent the following procedures: (i) they were placed on an aseptic absorbent gauze to remove the remaining vitrification solution; (ii) then, they were plunged in liquid nitrogen directly (Fig. 1B); (iii) finally, they were put into liquid nitrogen-filled cryovials and stored in liquid nitrogen for at least 1 week. To vitrify 16-day-old mouse ovary, the intact ovaries were placed in the needle in a sterile Petri dish containing L-15 medium supplemented with 10% FBS and 3–4 ovaries in each needle were dehydrated (Fig. 1C) and then plunged into liquid nitrogen directly.

For thawing, the solid drops or the needles holding ovarian tissues were taken out of the vial using forceps and quickly immersed into 1 M sucrose solution, which had been pre-warmed at 37°C for 5 min. They were serially transferred into 0.5 and 0.25 M sucrose solution for 5 min each and were incubated in DPBS supplemented with 20% FBS (Fig. 1D) for ~20 min at 37°C with 5% CO_2 before the subsequent procedure was begun.

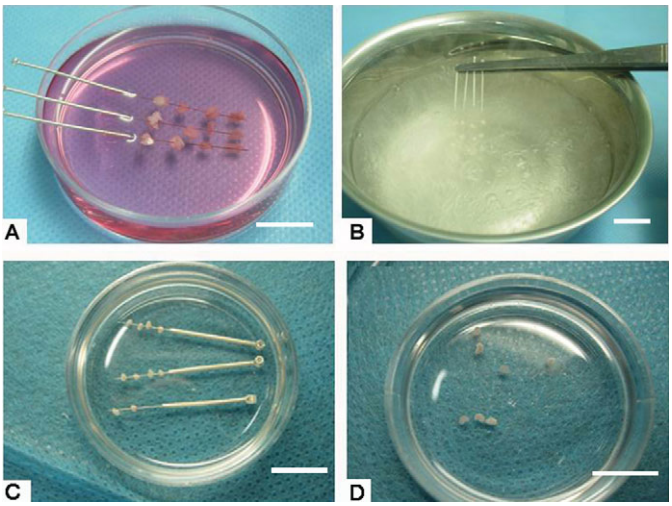

Figure 1: Graphical description for the novel method of NIV.
(**A**) The long needle held several pieces of human ovarian cortex in a row in L-15 medium. (**B**) Forceps were used to clamp the handle of the needle and the ovarian tissues were directly immersed into liquid nitrogen. (**C**) The 16-day-old mouse ovaries were held in a row by a needle, placed in the dehydration solution. (**D**) The 16-day-old mouse ovaries finished the thawing process (Bar = 10 mm).

Histological analysis by hematoxylin and eosin

Mouse ovaries from different frozen–thawed groups and fresh control group were fixed in 10% neutral-buffered formalin solution for 24 h at 4°C, dehydrated, paraffin-embedded, and serially sectioned at 5 μm thickness. The sections were mounted on glass slides and stained with hematoxylin and eosin (H&E). Every fifth section of each ovary was analyzed for follicle counting. Six samples from six different ovaries in each group were examined. To avoid counting follicles more than once, only follicles with a visible nucleus were counted.

The primordial follicles were defined as those containing one layer of flattened, or a mixture of flattened and cuboidal, pre-granulosa cells surrounding the oocytes; the primary follicles as those with one-layer cuboidal follicular cells; the secondary follicles as those where the oocytes were surrounded by two or more layers of cuboidal granulosa cells without an antrum (Gougeon, 1986). The antral follicles were only counted in the sections of the mouse ovarian grafts.

The fresh and the frozen–thawed human ovarian cortex pieces underwent the histological analysis in the same way. Every 10th section of each sample was assessed for follicle morphology. Only primordial follicles were analyzed. Structural normality was defined as a follicle that was spherical in shape with an even distribution of granulosa cells, intact theca and spherical oocyte while abnormality was a follicle that had a pyknotic oocyte nucleus, shrunken ooplasm or disorganized granulosa cells (Fig. 2).

Ultrastructural evaluation

After thawing, the intact mouse ovaries and the human ovarian cortex strips were prepared for the ultrastructural evaluation. The fresh specimens were used as controls. The tissues were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 1–7 days at 4°C. After being rinsed in phosphate-buffered saline (PBS), the samples

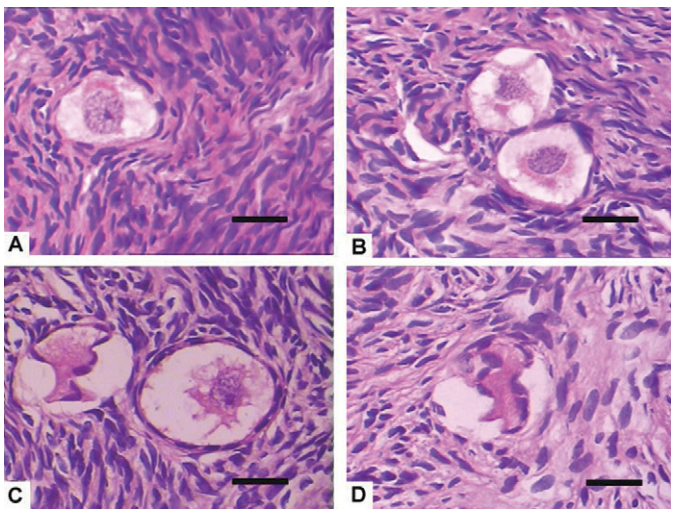

Figure 2: Morphological presentation of the human primordial follicles stained by H&E before and after cryopreservation.
(**A**) Normal appearance of the primordial follicles taken from fresh ovarian tissue before cryopreservation. (**B–D**) Appearance of the ovarian tissues taken from cryopreserved groups: (B) a typical example of two non-damaged primordial follicles after NIV; (C) the non-damaged (right) and the damaged (left) follicles after slow freezing; (D) a typical example of the damaged follicle after dropping vitrification: follicular cells were firmly adhered to the shrunk and damaged oocyte (original magnification ×400, Bar = 10 μm).

were post-fixed with 1% osmium tetroxide in PBS. Then, the samples were dehydrated in ethanol of an increasing concentration, immersed in propylene oxide overnight for solvent substitution, embedded in Epon 812 and sectioned. Semi-thin sections were stained with 1% toluidine blue. Ultra-thin sections (60–80 nm) were stained with aqueous lead citrate and alcoholic uranyl acetate. They were examined and photographed using TEM (HITACHI, H-600IV, Japan).

The oocytes, the granulosa cells of the primordial follicles and the stromal cells surrounding follicles were evaluated separately. The following elements were evaluated for qualitative assessment of ultrastructural preservation for oocytes and granulosa cells: nuclear content, membrane integrity, cytoplasm density, intramitochondrial matrix, cytoplasmic organelles (quality, type and microtopography) and intercellular contacts (between oocytes and follicular cells). The stromal cells were evaluated by their nuclear content and the integrity of the extracellular matrix (ECM).

Viability assay

Human ovarian tissue was placed in a Petri dish containing fresh collection medium and was cut with a scalpel knife into strips 200–300 μm thick under a stereomicroscope at 37°C. The live-dead fluorescent staining was done with carboxyfluorescein diacetate, succinimidyl ester (CFSE, 100 μg/ml, Sigma-Aldrich) and propidium iodide (100 μg/ml; Sigma-Aldrich) for 45 min at 37°C in the dark. The tissue strips were washed twice in DPBS, mounted between coverslips and evaluated under a fluorescence microscope (LEICA DM1000, Leica Microsystem, GmbH Wetzlar, Germany). The cytoplasm of all live cells appears bright green. Follicles were shown up as bright green large dots in the weakly stained stromal cortex.

To assess viability of vitrified mouse ovaries, the allografting procedure was performed as below.

The mouse ovary heterotopic allografting procedure

The fresh and the frozen–thawed ovaries from the 16-day-old mice were transplanted as grafts. The heterotopic allografting procedure used intact gonads and non-immunosuppressed male mice recipients according to Waterhouse *et al*. (2004). The male mice recipients were anesthetized by intraperitoneal injection of 10% chloral hydrate (0.004 ml/g). Surgery was carried out under strictly aseptic conditions. The left kidney was exteriorized through a small dorsohorizontal incision. A small hole was torn in the kidney capsule using a pair of fine watchmakers' forceps, and two ovaries were inserted beneath the capsule. The kidney was returned to the body cavity. Finally, the body wall incision and skin were closed.

The operation was carried out by two surgeons. One performed the operation, whereas the other gave assistance by assigning each mouse to the different experimental groups or the control group, and passing the ovarian grafts to the operating surgeon. The assistant was instructed not to reveal which group the ovarian grafts belonged to.

Seventeen days after transplantation, the kidney with ovarian grafts was removed and fixed for counting the number of follicles.

Functional analysis of the frozen–thawed mouse ovary after the heterotopic allografting procedure

For functional analysis, 2 weeks after transplantation, the mice recipients were treated with 7.5 IU pregnant mare serum gonadotrophin (PMSG, ShuSheng, Ningbo Second Hormone Factory, China) and 7.5 IU hCG (Livzon, China) 48 h later. Ten hours after hCG injection, the mice were killed by cervical dislocation and the grafts were collected into equilibrated (5% CO_2 at 37°C) α-minimum essential medium (MEM) supplemented with 10% FBS under mineral oil (Sigma-Aldrich). The oocytes were released by puncturing large antral follicles with a 26-G needle and collected into fresh equilibrated α-MEM maturation medium supplemented with 10% FBS, 1 IU/ml PMSG and 1 IU/ml hCG. Oocytes with an expanded cumulus were fertilized *in vitro* ~2 h after oocyte collection. Oocytes with compact cumulus cells and germinal vesicles were cultured at 37°C with 5% CO_2 for 16–18 h before being fertilized *in vitro*.

The 12–14-week-old male mice were killed by cervical dislocation and the testes were removed. The cauda of the epididymis was dissected free and cut into strips. Strips were placed into 1 ml human tubal fluid medium (HTF, SAGE BioPharma, USA) with 3% bovine serum albumin (Sigma-Aldrich) in 6 ml Falcon tubes (Becton Dickinson, USA). The sperm were incubated at 37°C, 100% humidity, 5% CO_2 in air for 30–60 min. Insemination drops were placed into 35 mm Falcon dishes (Becton Dickinson) and were covered with pre-equilibrated mineral oil.

IVF was performed in the equilibrated HTF medium using sperm capacitated in HTF for 1 h. Oocytes were left with sperm in the fertilization medium for 2 h and were washed before being moved to the equilibrated embryo culture medium (Quinn's Advantage Cleavage Medium, SAGE BioPharma) at 37°C with 5% CO_2 for 24 h. The numbers of 2-cell embryos were recorded.

Statistical analysis

The one-way analysis of variance was used to analyze the difference in percentages of morphologically normal follicles between the fresh and the frozen–thawed ovarian tissues and to evaluate the difference in follicle number after the heterotopic allografting procedure. Percentage data and follicle number were presented as mean ± SD. The chi-square test was used to analyze the difference in the proportion of ultrastructurally normal follicles and stromal cells and to evaluate the difference in the cleavage rates among the four experimental groups. *P*-value of <0.05 was considered statistically significant.

Results

The histological analysis on the follicles

The sections from human ovarian cortex fragments treated by different freezing methods and fresh controls were analyzed for morphological assessment of the follicles. A total of 334 primordial follicles were counted and analyzed. The percentages of morphologically normal primordial follicles from the frozen–thawed human ovarian cortex fragments and fresh controls were compared (Table I). The primordial follicles were preserved in a satisfactory morphology. There were no significant differences in the number of morphologically normal primordial follicles among the freezing protocols ($P = 0.591$). The percentage of the morphologically normal primordial follicles in the fresh group was significantly higher than that of the frozen–thawed groups ($P < 0.001$).

The percentages of the morphologically normal follicles in the different developmental stages of the frozen–thawed mouse ovaries and fresh controls were also compared in Table I (antral follicles were not included). The primordial follicles in the cryopreserved ovarian sections had a comparable morphological appearance with the fresh controls. The majority of the pre-antral follicles were preserved in a good morphology after cryopreservation (Fig. 3). The percentages of morphologically normal follicles in the different stages of the fresh controls were significantly higher than those in frozen–thawed groups ($P < 0.001$). The percentages of the

Table I. Percentage of morphologically normal human primordial follicles and mouse follicles (various stages) in groups of frozen–thawed and fresh tissues.

Groups	Human	Mouse		
	Primordial follicles	Primordial follicles	Primary follicles	Secondary follicles
Slow-freezing	82.93 ± 2.31^{a}	83.63 ± 1.41^{b}	74.00 ± 1.85^{c1}	43.5 ± 3.29^{d1}
Dropping vitrification	81.34 ± 3.72^{a}	82.63 ± 2.39^{b}	73.25 ± 2.31^{c2}	42.75 ± 2.12^{d2}
NIV	83.16 ± 2.70^{a}	84.38 ± 3.34^{b}	$81.75 \pm 1.83^{c1,c2}$	$68.25 \pm 1.93^{d1,d2}$
Fresh	90.70 ± 2.50	92.80 ± 1.30	90.40 ± 1.52	86.4 ± 1.40

Percentage data expressed as mean ± SD; NIV, needle immersed vitrification.
[a,b]No difference among the three groups: [a]$P = 0.591$ and [b]$P = 0.390$.
[c1,c2]NIV versus slow-freezing and dropping vitrification group: both $P < 0.001$.
[d1,d2]NIV versus slow-freezing and dropping vitrification group: both $P < 0.001$.

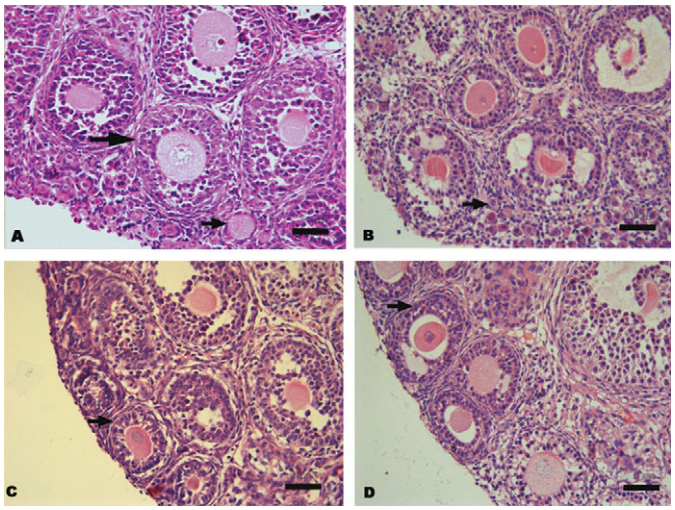

Figure 3: Morphological presentation of the 16-day-old mouse ovarian tissue stained by H&E before and after cryopreservation. (**A**) In fresh mouse ovarian tissue, the morphologically normal primary follicles (small arrow) and the secondary follicles (large arrow) consisted of intact oocytes and compact granulosa cells. (**B**) In the ovarian tissues cryopreserved by NIV, the primordial follicles (small arrow), the primary follicles and the secondary follicles were all morphologically normal. (**C**) In the ovarian tissues cryopreserved by slow-freezing, the pre-antral follicles (arrow) were similar in appearance to the NIV group and the fresh group. (**D**) In the ovarian tissues cryopreserved by dropping vitrification, abnormal secondary follicles with the retracted cytoplasm (arrow) in oocytes were observed (original magnification ×400, Bar = 25 μm).

morphologically normal primary and secondary follicles in the NIV group were significantly greater than those in the dropping vitrification and the slow-freezing groups ($P < 0.001$).

Ultrastructural evaluation

The primordial follicles, the stromal cells and the collagen bundles surrounding the follicles in human and mice ovarian tissues were analyzed by TEM. The ultrastructural evaluation was performed by two experienced electron microscopists. Oocytes and the granulosa cells with intact cytoplasmic membrane and nuclear envelope, an adequate density of the cytoplasm and intramitochondrial matrix, and with <30% of damaged cytoplasmic organelles were recorded as normal. Stromal cells showing a well-distributed nuclear content and >50% intact ECM were recorded as normal.

After slow freezing and vitrification, most cell organelles in the oocytes were well preserved. Mitochondria, smooth endoplasmic reticulum and rough endoplasmic reticulum had a normal morphological appearance (Fig. 4A). In the frozen–thawed tissues, some mitochondria in the granulosa cells and the stroma cells were swollen and their cristae had disappeared (Fig. 4B). The stromal cells with an enlarged intercellular space and disappearance of collagen bundles were more easily observed in the dropping vitrification and the slow-freezing groups compared with the NIV group (Figs 4C and D and 5). The number of the oocytes, the granulosa cells of the primordial follicles and the stromal cells with a normal ultrastructure were listed (Table II). All the three freezing methods could preserve oocytes with normal ultrastructure in mouse and human ovarian tissues. Differences in preservation of granulosa cells were not significant among the three freezing groups (human: $P = 0.472$, mouse: $P = 0.185$). For cryopreservation of the stromal cells, the NIV method achieved a significantly better result compared with the slow-freezing (human: $P = 0.039$, mouse: $P < 0.001$) and the dropping vitrification (human: $P = 0.023$, mouse: $P < 0.001$) methods.

Ovarian viability assessment

Human ovarian strips taken from different freezing groups and fresh group (strips from two patients) were observed under a fluorescence microscope with the conventional fluorescein long pass filter. The cytoplasm of all live cells appeared bright green, whereas the nucleus of the dead cells appeared red. This could be observed simultaneously. The viable follicles were observed to have bright green large spherical structures in the weakly stained stromal cortex (Fig. 6, arrow) and they could be seen in all frozen–thawed groups. Following this procedure, the viability of follicles and stromal compartments could be observed at the same time. However, statistical analysis was not performed for viable follicle counts owing to the variation in density in different samples and the small sample size. We also observed that in the frozen–thawed tissues, the green fluorescence did not appear to be as uniform as that in fresh tissues. The red dye staining the nucleus of dead stromal cells appeared more frequently, especially in the dropping vitrification and the slow-freezing groups when compared with the NIV group.

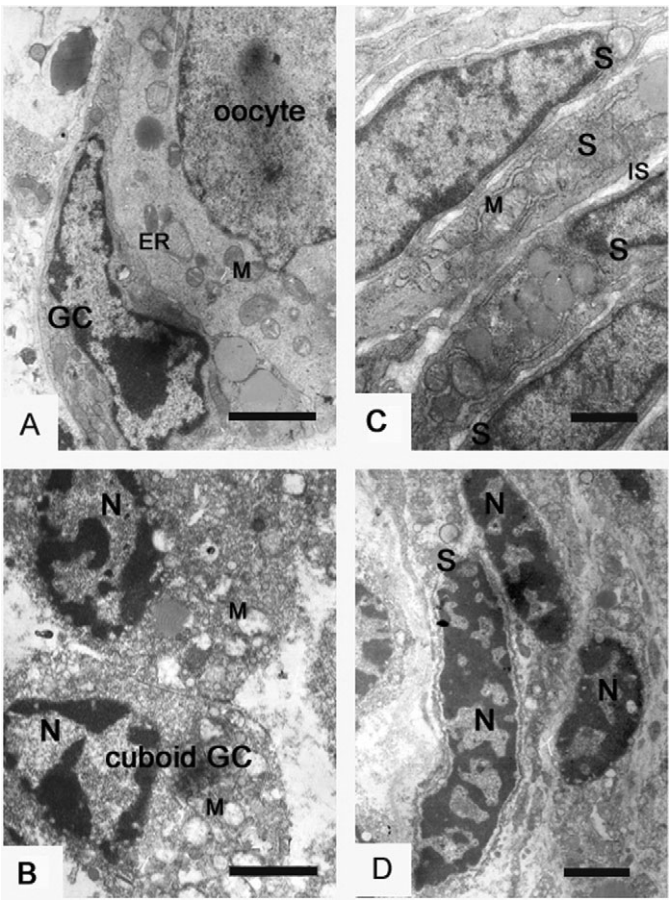

Figure 4: Transmission electron microscopic examination of the fresh and the frozen–thawed mouse ovarian tissues.
(**A**) Well preserved primordial follicle with round oocyte was tightly enclosed by surrounding flat granulosa cells in the NIV group. Mitochondria (M) and smooth endoplasmic reticulum (ER) distributed in the cytoplasm of oocyte were morphologically normal (A ×5000, Bar = 2 μm). (**B**) The cuboid granulosa cells in primary follicle had the indented nuclei (N) that contained peripheral patches of heterochromatin and numerous swollen M in cytoplasm in the dropping vitrification group (C ×8000, Bar = 2 μm). (**C**) The stromal cells (S) in fresh ovarian tissues had a normal intercellular space (IS). Cristae of M were clearly observed (B ×12 000, Bar = 1 μm). (**D**) The S had a change of N pycnosis and the collagen bundles disappeared in the slow-freezing group (D ×6000, Bar = 2 μm).

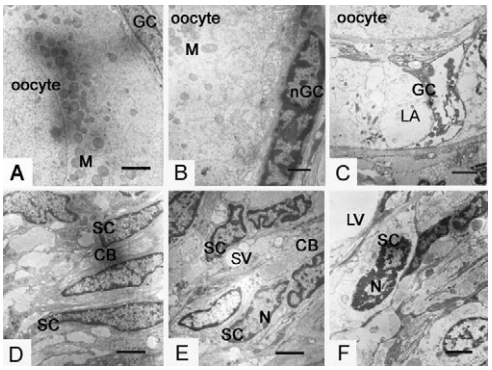

Figure 5: Transmission electron microscopic examination of the fresh and the frozen–thawed human ovarian tissues.
(**A**) The primordial follicle from fresh tissues had an abundance of M with normal cristae in the cytoplasm of the oocyte. Flat pre-granulosa cells (GC) showed distinct cell membranes and nuclei. The nuclear content was normally distributed (A ×5000, Bar = 2 μm). (**B**) The primordial follicle from the NIV group was observed. Most of the M in oocyte cytoplasm were normal in morphology. The indented nuclei containing peripheral patches of heterochromatin were observed in pre-granulosa cells (nGC) (B ×8000, Bar = 1 μm). (**C**) The primordial follicle from the dropping vitrification group was observed. The preservation of follicle GC was poor. The cytoplasm in GC consisted of a large area that was devoid of organelles (LA), with no M or ribosomes (C ×5000, Bar = 2 μm). (**D**) Stromal cells (SC) and a dense matrix of collagen bundles (CB) were found in fresh ovarian cortex, with a normal intercellular space (D ×6000, Bar = 2 μm). (**E**) The SC and the collagen bundles in the NIV group had moderately dispersed nuclear chromatin (N), with few small vacuoles (SV) in the cytoplasm. The intercellular space was slightly expanded compared with that in the fresh group. The CB were left behind (E ×6000, Bar = 2 μm). (**F**) The SC in the slow-freezing group had easily seen large vacuoles (LV). The intercellular space was obviously expanded. The SC had markedly condensed N, and the CB disappeared (F ×5000, Bar = 2 μm).

Ovarian functional restoration by means of heterotopic allografting procedure in mouse model

The ovarian grafts were retrieved 17 days after transplantation. The grafts that developed with an enlarged volume were easy to identify under the kidney capsule (Fig. 7A and B). Histological examination of retrieved grafts revealed that follicles had proceeded to pre-antral and early antral follicle stages. Normal development of follicles and formation of corpora lutea was observed in fresh ovarian grafts (Fig. 7C). Growing follicles in the cryopreserved grafts showed a similar histological appearance to those in the NIV group (Fig. 7D). The number of follicles in ovarian grafts after treatment with different freezing methods and fresh controls were presented in Table III. The average total follicle number in the NIV group was almost the same to that in the fresh ovarian grafts ($P = 0.225$). The numbers of growing follicles and total follicles in the NIV group were significantly greater than those in the slow-freezing and the dropping vitrification groups ($P = 0.009$ and $P = 0.010$, respectively).

The mice recipients in different freezing groups and the fresh group received exogenous gonadotrophin stimulation (two mice in each group). All of the grafts were retrieved. A total of 64 oocytes recovered from four groups were used for the *in vitro* maturation and/or IVF procedure. Two-cell embryos in the different groups were recorded. The 2-cell cleavage rates of the oocytes collected from frozen–thawed grafts were 28%, 30% and 36% in the slow-freezing, the dropping vitrification group and the NIV group, respectively. The 2-cell cleavage rate of oocytes collected from fresh grafts was 42%. These data indicated that the oocytes in frozen–thawed grafts in the NIV group have comparable developmental competence to those in the fresh grafts ($P = 0.71$). So, the viability assessment for frozen–thawed ovary by means of heterotopic allografting procedure showed that restoration of ovarian function in NIV groups was most similar to fresh grafts.

Discussion

Vitrifying ovarian tissues by direct immersion into liquid nitrogen using a minimal volume of cryoprotectant could maximize

Table II. Ultrastructural evaluation of the primordial follicles.

Groups	Primordial follicles					
	Oocytes		Granulosa cells		Stromal cells	
	Human	Mouse	Human	Mouse	Human	Mouse
Slow-freezing	10/11 (91%)[a]	12/14 (85%)[b]	21/38 (55%)[c]	22/40 (53%)[d]	40/100[e1]	28/100[f1]
Dropping vitrification	7/8 (87.5%)[a]	12/16 (75%)[b]	16/30 (53%)[c]	23/44 (52%)[d]	39/100[e2]	26/100[f2]
NIV	8/8 (100%)[a]	10/12 (83%)[b]	19/28 (68%)[c]	27/38 (71%)[d]	55/100[e1,e2]	48/100[f1,f2]
Fresh	5/5 (100%)[a]	9/10 (90%)[b]	21/23 (91%)	32/36 (89%)	97/100	93/100

[a,b]No difference among the four groups: [a]$P = 0.540$ and [b]$P = 0.638$.
[c,d]No difference among the three groups: [c]$P = 0.472$ and [d]$P = 0.185$.
[e1,e2]NIV versus slow-freezing/NIV versus dropping vitrification: [e1]$P = 0.034$ and [e2]$P = 0.023$.
[f1,f2]NIV versus slow-freezing/NIV versus dropping vitrification: [f1]$P = 0.004$ and [f2]$P < 0.001$.

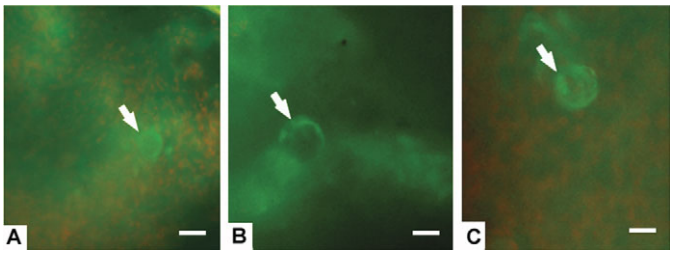

Figure 6: Viability assessment on human ovarian strips in the fresh group and the frozen–thawed groups.
(**A**) The viable follicles showed a bright green large spherical dot (arrow) in the weakly stained stroma for all freezing groups. The red fluorescent dye stained the nuclei of dead SC, which were not homogeneously distributed (A ×200, Bar = 50 μm). (**B**) Viable primordial follicles were observed in the fresh strips. (**C**) Typical viable follicles were observed in the extensively damaged stroma independent of the freezing group (B and C ×400, Bar = 25 μm).

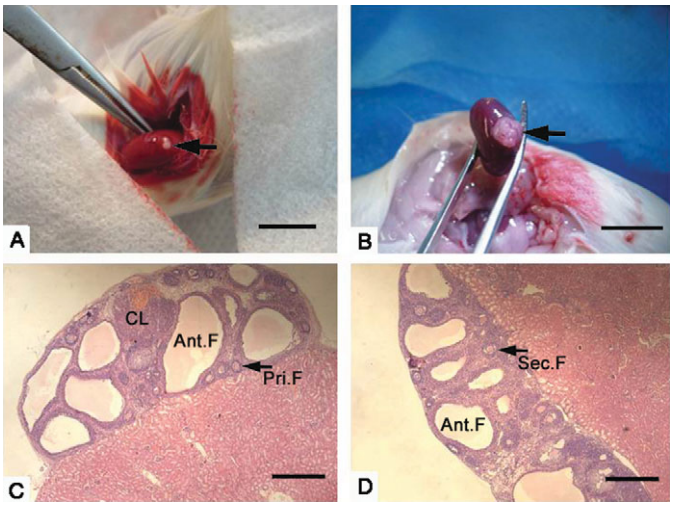

Figure 7: The frozen–thawed ovaries in the NIV group undergoing ovary heterotopic allografting, and histological appearance of grafts in the fresh group and the NIV group.
(**A**) The frozen–thawed ovary was laid beneath left renal capsule of the recipient mouse (arrow); (**B**) 17 days after the transplantation, the grafts were easily identified under renal capsule (arrow); (**C**) histological appearance of the fresh graft under renal capsule and (**D**) histological appearance of the NIV graft under renal capsule (A and B, Bar = 10 mm; C and D ×50, Bar = 100 μm) CL, corepus luteum; Ant.F., antral follicle; Sec.F., secondary follicle; Pri.F., primary follicle.

the cooling rate and reduce toxicity of the vitrification solution with less-concentrated cryoprotectants. Held by an acupuncture needle, the ovarian tissue pieces can be exposed to cryoprotectants synchronously. So, NIV can facilitate vitrification process especially when a large number of ovarian cortex fragments require to be cryopreserved.

Ovary has different heterogeneous cellular components. Diffusion rates of the cryoprotectants throughout the cells are different and the ice crystal formation potential is specific in each cell and tissue type (Fuller and Paynter, 2004). Thus, ovarian cryopreservation involves a compromise between effects on the oocytes, follicular cells and stroma. As we know, mammalian ovarian follicles are embedded in and surrounded by the stromal tissue, composed of stromal cells, blood vessels, nerves and ECM. The development of the follicles in the ovary is a complicated process. The stromal cells can transform to the theca interna and externa outside the basal lamina of follicles, which is believed to play an influencing role in granulosa cell proliferation and differentiation. ECM can regulate the distribution of extracellular signaling molecules and modulate signaling events at the cell surface that influence cell proliferation, adhesion and motility. The primordial follicles cultured within organs could grow better than isolated follicles in human and mouse (Hovatta *et al*., 1999; Liu *et al*., 2000). Supplementation with ECM could improve both stored and fresh human primordial and primary follicles in organ culture (Hovatta *et al*., 1997). These findings suggested that ovarian stromal cells and ECM might play a supportive role in follicle development. In ovarian tissue cryopreservation, the stromal cells appeared more vulnerable to cryoinjury than primordial follicles (Kim *et al*., 2004b). So, it seemed that evaluation of the effect of freezing/thawing protocols on both follicle and its surrounding stromal cells was essential.

Conventional programmed cryopreservation of human ovarian tissues, with low concentration of the cryoprotectant and a slow-cooling rate, could achieve reasonable follicle survival but seemed inadequate to preserve ovarian cortex (Gook *et al*., 2000). Ovary has different heterogeneous cellular components that have different properties. This makes it difficult to select an appropriate cooling speed for the whole tissue. In a comparison between conventional cryopreservation and

Table III. Follicle number after transplantation using the heterotopic allografting procedure in mice.

Groups	Number of Recipent mouse	Number of retrieved ovarian grafts/*n* grafted (%)	Number of primordial and primary follicles per graft	Number of second, antral and ovulatory follicles per graft	Number of total follicles
Slow-freezing	4	7/8 (87.5%)	470 ± 105^{a1} (range 357–612)	116 ± 11^{b1} (range 100–122)	585 ± 114^{c1} (range 457–748)
Dropping vitrification	4	8/8 (100%)	480 ± 124^{a1} (range 334–643)	118 ± 17^{b1} (range 98–137)	597 ± 137^{c1} (range 435–761)
NIV	4	8/8 (100%)	$659 \pm 117^{a1,a2}$ (range 514–783)	$132 \pm 18^{b1,b2}$ (range 121–150)	$791 \pm 121^{c1,c2}$ (range 615–932)
Fresh	3	6/6 (100%)	740 ± 104^{a2} (range 596–861)	144 ± 25^{b2} (range 103–170)	884 ± 125^{c2} (range 599–1031)

Follicle number expressed as mean ± SD.
[a1]Difference among the three groups: $^{a1}P = 0.006$.
[a2]No difference between fresh and NIV group: $^{a2}P = 0.203$.
[b1]Difference among the three groups: $^{b1}P = 0.009$.
[b2]No difference between fresh and NIV group: $^{b2}P = 0.223$.
[c1]Difference among the three groups: $^{c1}P = 0.010$.
[c2]No difference between fresh and NIV group: $^{c2}P = 0.225$.

vitrification techniques in tissue-engineered blood vessels, vitrification method showed a much better result in preserving multicellular tissues (Dahl *et al*., 2006). This phenomenon suggested that the vitrification method might have potential advantage in preserving ECM and multiple cell components.

Vitrification is an ultrarapid cooling technique with no ice crystal formation (that can damage the cells or the tissues), but a higher concentration of the cryoprotectant may increase the toxicity to the living cells, especially in conventional vitrification with straws. To decrease the concentration of the cryoprotectants, increasing the cooling rate is a feasible strategy. In the oocyte vitrification, a number of innovations have been introduced to increase the cooling rate. For example, a minimal volume of cryoprotectant solution containing the oocyte can be exposed directly to liquid nitrogen in either a thin open straw (Vajta *et al*., 1998), which has since been modified to the cryotop (Kuwayama *et al*., 2005), or on an electron microscopy grid (Martino *et al*., 1996), which has subsequently been modified to the cryoloop (Lane *et al*., 1999). Using cryoloops and cryotops, instead of straws for vitrification resulted in significant improvement in the preservation of oocytes and blastocysts (Kuwayama *et al*., 2005; Takahashi *et al*., 2005; Antinori *et al*., 2007). To vitrify ovarian tissues, different methods that put samples in direct contact with liquid nitrogen were used, such as the copper grid (Isachenko *et al*., 2003) or the polyester sheets (Hasegawa *et al*., 2006). Recently, Chen *et al*. (2006) employed vitrification method by direct covering liquid nitrogen (DCV) to mouse ovary in cryovial. A less-concentrated vitrification medium consisting of 15% EG (v/v), 15% DMSO (v/v) and 0.5 M sucrose has been used and increased follicle viability and pregnancy capability in mice were reported (Chen *et al*., 2006). However, such low concentration of cryoprotectants had not been applied in human ovarian tissue vitrification. In current study, we used this solution and achieved satisfactory results in cryopreservation of mouse and human ovarian tissue.

Making ovarian tissues directly contact with liquid nitrogen by polyester sheets (cryotop) or copper grid is able to increase the cooling rate and reduce the toxicity of cryoprotectants. However, these methods are not appropriate for handling a greater volume or/and a greater amount of tissues, such as the human ovarian cortex fragments. The DCV method was also a good choice for increasing the cooling rate. Yeoman *et al*. (2005) performed vitrification of cortical pieces of monkey ovarian tissues by dropping these pieces into a shallow container of liquid nitrogen. However, the vitrification solution attached to the tissue pieces and the formation of nitrogen vapor might slow down the cooling and warming speed during freezing/thawing procedure. Dropping the individual strips in liquid nitrogen could be time-consuming and might increase the exposure time of tissues to dehydrating solution. In current study, we compared these two different vitrification methods to evaluate their efficacy in mouse or human ovarian cryopreservation.

The result of the present study has indicated that using the very fine needles to manipulate the tissue strips during vitrification process can maximize the cooling rate. With less-concentrated vitrification solution, ovarian morphological analysis showed that the oocytes and the surrounding granulosa cells were well preserved in their histology and ultrastructure after cryopreservation. The ultrastructure examination on mouse and human ovarian tissues showed that a better effect was achieved in preservation of the stromal cells and the collagen bundles. *In vitro* viability assessment on human ovarian tissue strips showed the follicles and the stroma maintaining a satisfactory viability. However, statistical analysis was not performed for living follicles counting due to variation in densities of living follicles in different samples and the small sample size. *In vivo* viability evaluation by mouse heterotopic allograft procedure showed the function of frozen–thawed ovaries manipulated by needles was comparable with that of fresh ovarian grafts, in terms of follicle development.

The 16-day-old mouse ovary $\sim 18 \times 15 \times 10$ mm in size was similar to the size of vitrified human ovarian cortex fragments. In the animal models for ovarian cryopreservation studies, the mouse ovary maybe the smallest one, which is

very fragile with a loose collagen distribution in the stroma. In the present study, better preservation of stroma and survival of grafts could be observed in NIV group when compared with the slow freezing group or the dropping vitrification group. On the basis of the mouse model, we performed this preliminary study in human ovarian tissue vitrification. It suggested that the NIV method might be more suitable for the human ovarian tissue cryopreservation.

Moreover, the NIV method is relatively simple, convenient to manipulate and time-saving. All the tissue slices held by needles could be exposed to cryoprotectants synchronously. Because of the minimum volume of cryoprotectants attached to tissue slices, the cooling rate can be maximized and the warming rate can be equally increased, so that the potential toxicity and osmotic effect of cryoprotectants can be greatly reduced. Besides, the NIV method can be applicable for vitrifying a large number of tissue slices at the same time. NIV is an open vitrification method that puts ovarian tissues in direct contact with liquid nitrogen, like dropping vitrification, DCV or methods in which cryotops, cryloops and copper grids were used. Exposure of living tissues directly to liquid nitrogen may introduce risks of bacterial or viral infection. Separating cooling and storage phases of vitrification may be a possible solution to decrease the danger of liquid nitrogen-mediated contamination (Kuwayama, 2007). For a future clinical application of the NIV method, the cooling procedure should be performed in a small volume of sterilized liquid nitrogen. After vitrification, the device would be sealed into a sterile pre-cooled vial for hermetic isolation at storage. Anyway, we think that the NIV method is a potentially superior strategy for the vitrification of ovarian tissues. On the basis of our current preliminary study, further investigations would be made, such as including assessment of ovarian endocrine and reproductive function restoration.

In conclusion, our results have indicated that the oocytes and follicular cells preserved by the NIV method could retain a comparable morphological appearance with the slow-freezing method and the dropping vitrification method. It was noteworthy that the ultrastructure of the stromal cells was preserved better in NIV group than in the slow-freezing group or the dropping vitrification group in human or mouse. Viability assessment on frozen–thawed human ovarian tissue strips cryopreserved by NIV showed that the follicles and stroma maintained a satisfactory viability. In mouse model, the ovarian functional restoration in the NIV group was the best among the three freezing groups, which was confirmed by follicle counting in grafts after transplantation. Cleavage rate of the oocytes from grafts in the NIV group was most similar to that in the fresh group. The NIV method could maximize the cooling rate, facilitate the vitrification process and reduce the toxicity of the vitrification solution with a minimal volume of less concentrated cryoprotectants. Moreover, the NIV method was much more convenient and time-saving in preserving a large number of samples than the existing freezing methods. It is therefore suggested that the NIV method has potential advantages for research and clinical use, such as establishing a human oocyte bank. Further investigation would be needed.

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