Birth of pups after transfer of mouse embryos derived from vitrified preantral follicles

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Preantral follicles mechanically isolated from the ovaries of 12-day-old mice were exposed to 2 mol ethylene glycol l⁻¹ for 2 or 5 min and then to a vitrification solution containing 6 mol ethylene glycol l⁻¹ and 0.3 mol raffinose l⁻¹ for 0.5, 1.0 or 2.0 min before vitrification. The vitrified and fresh preantral follicles were treated with collagenase, and the oocyte–granulosa cell complexes (OGCs) obtained were cultured *in vitro* for 10 days in membrane inserts. Preantral follicles exposed to 2 mol ethylene glycol l⁻¹ for 5 min and then to the vitrification solution for 0.5 or 1.0 min showed the highest survival rates after warming. The follicular loss after warming was approximately 20%. After *in vitro* culture, the proportion of viable OGCs from the vitrified follicles was 10% lower than that of the fresh

preantral follicles. There were no differences in the rates of maturation, fertilization and subsequent development to blastocysts between the oocytes derived from vitrified follicles and those derived from fresh preantral follicles; however, the developmental competence of the oocytes derived from both vitrified and fresh preantral follicles grown *in vitro* was lower than that of oocytes grown *in vivo*. One of the five recipient mice that received 20 blastocysts derived from vitrified preantral follicles gave birth to six live pups. The results of the present study demonstrate for the first time that mouse preantral follicles can be vitrified and that some of the embryos derived from vitrified preantral follicles can develop to live pups.

Introduction

Several follicle culture systems that support oocyte development *in vitro* have been developed and the appropriate combinations of these approaches could permit the growth of follicles from all stages of folliculogenesis *in vitro* (Hartshorne, 1997). So far, birth of offspring from preantral follicles cultured completely *in vitro* has been achieved only in mice (Eppig and Schroeder, 1989; Spears *et al.*, 1994; Eppig and O'Brein, 1996; Cortvrindt *et al.*, 1998). The culture of follicles for growth *in vitro* and subsequent maturation and fertilization *in vitro* in combination with cryopreservation of follicles will allow exploitation of the large number of follicles in mammalian ovaries and the cryobanking of female genetic material.

Previous attempts to preserve large numbers of mammalian oocytes involved the cryopreservation of ovarian tissues, which resulted in the birth of offspring after orthotopic grafting of frozen–thawed ovarian tissues in mice (Parrott, 1960; Cox *et al.*, 1996; Gunasena *et al.*, 1997) and sheep (Gosden *et al.*, 1994). Mouse pups were also produced after sequential grafting of cryopreserved whole ovaries under the kidney capsule and *in vitro* culture of

preantral follicles derived from the frozen–thawed ovarian grafts (Liu *et al.*, 2001). Grafting of frozen–thawed preantral follicles embedded in collagen gels (Carroll *et al.*, 1990) and plasma clots (Carroll and Gosden, 1993) resulted in the birth of pups after *in vitro* fertilization of oocytes matured *in vivo* (Carroll *et al.*, 1990) or after natural mating (Carroll and Gosden, 1993). Cryopreserved mouse preantral follicular oocytes grew to maturity *in vitro* and developed to the blastocyst stage after *in vitro* fertilization (Cortvrindt *et al.*, 1996a). Although offspring have not been obtained from cryopreserved preantral follicles cultured entirely *in vitro*, the *in vitro* production of oocytes derived from cryopreserved preantral follicles has the potential to produce large numbers of embryos and offspring from genetically valuable animals. The development of cryopreservation techniques for preantral follicles could promote the utilization of *in vitro* embryo production systems.

Mouse oocytes at metaphase II (MII) have been cryopreserved successfully by rapid freezing (Nowshari *et al.*, 1994; Rayos *et al.*, 1994) and vitrification (Shaw *et al.*, 1991; Wood *et al.*, 1993; O'Neil *et al.*, 1997). Preantral follicles have been cryopreserved only by slow freezing (Carroll *et al.*, 1990; Carroll and Gosden, 1993; Cortvrindt *et al.*, 1996a). The development of a reliable, simple and efficient vitrification procedure for ovarian follicles at all developmental stages is required to enhance our effort to preserve ovarian follicles.

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Vitrification offers a simple approach to the cryopreservation of cells but requires high concentrations of cryoprotectant, which is known to damage cells because of both osmotic and toxic effects. Incorporation of non-permeable macromolecules, such as sugar, polyvinylpyrrolidone, polyethylene glycol and Ficoll, promotes vitrification of a solution and reduces the chemical toxicity of permeable cryoprotectants (Fahy *et al.*, 1984; Rall and Fahy, 1985; Kasai, 1997). Exposure of mouse embryos (Zhu *et al.*, 1993, 1994) and matured oocytes (Hotamisligil *et al.*, 1996; Kasai, 1997) to a low concentration of permeable cryoprotectant followed by brief exposure to the vitrification solution reduces possible osmotic stress and chemical toxicity of the permeable cryoprotectant.

Ethylene glycol has a low toxicity and is commonly used as a penetrating cryoprotectant in combination with non-permeating compounds in the vitrification of mouse (Miyake *et al.*, 1993; Hotamisligil *et al.*, 1996) and cattle oocytes at the MII stage (Otoi *et al.*, 1998; Dinnyés *et al.*, 2000). A vitrification solution containing 6 mol ethylene glycol l⁻¹ and 0.3 mol raffinose l⁻¹ was used by dela Peña *et al.* (2001) in the vitrification of mouse MII oocytes. Exposure of mouse MII oocytes to a low concentration of permeable cryoprotectant (2 mol ethylene glycol l⁻¹) before exposure to the vitrification solution increased the viability of vitrified-warmed oocytes (dela Peña *et al.*, 2001). The optimum period of exposure to the vitrification solution was influenced by the period of exposure to a low concentration of permeable cryoprotectant (Wood *et al.*, 1993; Kasai *et al.*, 1997; dela Peña *et al.*, 2001). The optimum condition achieved previously for mouse oocyte vitrification may not be suited to the vitrification of morphologically complex preantral follicles.

In the present study, mouse preantral follicles were exposed to a low concentration of ethylene glycol before exposure to the vitrification solution containing ethylene glycol and raffinose, and the effects of exposure to ethylene glycol and to the vitrification solution on the viability of mouse preantral follicles were investigated. In addition, *in vitro* growth, maturation and fertilization capacity of oocytes derived from vitrified preantral follicles were determined. Finally, the *in vivo* development of blastocysts derived from preantral follicles vitrified under the optimum condition was determined.

Materials and Methods

Experimental animals

Female C57/BL6J and male CBA mice purchased from Japan SLC Inc (Shizuoka) were housed and bred in the animal housing facility in the Graduate School of Veterinary Medicine, Hokkaido University following the guidelines set by the University. The mice were kept in light and temperature controlled conditions (12 h light:12 h dark, 22 ± 2°C) and given chow pellets and water *ad libitum*. Female F₁ offspring, 12 days of age, were killed by cervical dislocation.

Ovaries were collected into Leibovitz's L-15 medium (L-15, Gibco BRL, Grand Island, NY) supplemented with 4 g BSA l⁻¹ (Fraction V, Sigma Chemical Co., St Louis, MO), 75 mg benzyl penicillin l⁻¹ and 50 mg streptomycin sulphate l⁻¹ (L-15 + BSA) at 37°C.

Isolation of preantral follicles

Preantral follicles were mechanically isolated from the mouse ovaries using a 25 G needle attached to 1 ml syringe in L-15 + BSA at 37°C. Morphologically normal preantral follicles with two to three layers of granulosa cells and centrally located spherical oocytes were used in the experiment.

Vitrification and warming

For vitrification, groups of approximately 30 preantral follicles were exposed to 2 mol ethylene glycol l⁻¹ in L-15 + 10% (v/v) fetal calf serum (FCS, Gibco; L-15 + FCS) for 2 or 5 min and then to a vitrification solution that was composed of 6.0 mol ethylene glycol l⁻¹ and 0.3 mol raffinose l⁻¹ in L-15 + FCS. The follicles were rinsed in a 100 µl drop of vitrification solution before transfer to a 40 µl droplet of the vitrification solution at room temperature (22–25°C). The drop containing the follicles was drawn into a 0.25 ml French straw (IMV, L'Aigle). The straws were cooled in liquid nitrogen vapour by placing them horizontally on a styrofoam plate with stainless steel mesh on its upper surface and floating in liquid nitrogen (Takahashi and Kanagawa, 1990). After 2 min, the straws were plunged into liquid nitrogen and stored for 1–120 days. The exposure periods to the vitrification solution elapsed from the time the follicles were initially rinsed in a 100 µl drop of vitrification solution until they were placed on the styrofoam plate in liquid nitrogen vapour. For loading the straw, approximately 100 µl L-15 + BSA with 1 mol sucrose l⁻¹ was aspirated into the straw followed by a short column of air and approximately 10 µl of the vitrification solution. The vitrification solution (40 µl) containing the follicles was aspirated and separated by an air space on each side. The remainder of the straw was filled with L-15 + BSA with 1 mol sucrose l⁻¹.

For warming of vitrified samples, the straws containing preantral follicles were held in air at room temperature for 10 s and for an additional 20 s in water at 20°C. The contents of the straw were expelled and mixed gently into 1 ml of 1 mol sucrose l⁻¹ in L-15 + BSA and held there for 10 min at room temperature. The samples were incubated in 3 ml L-15 + BSA at 37°C for 5 min and then kept in fresh L-15 + BSA for 5–10 min at the same temperature before collagenase treatment.

Preantral follicle culture

Vitrified or fresh preantral follicles were incubated in L-15 supplemented with 2 mg collagenase ml⁻¹ (from *Clostridium histolyticum*, 200 U mg⁻¹, Wako Pure Chem

Industries Ltd, Osaka) for 10 min at 37°C to obtain oocyte–granulosa cell complexes (OGCs) for *in vitro* culture. OGCs were washed in L-15 + BSA three times. The complexes were held in a follicle culture medium for 10–15 min at 37°C and their morphology was evaluated. OGCs were defined as morphologically normal if the oocyte was spherical and surrounded with two to three layers of granulosa cells, and if there were no visible clear spaces between the oocyte and granulosa cell layers or within the granulosa cell mass.

The culture system for enzymatically isolated preantral follicles (Eppig and Schroeder, 1989) was used with a slight modification. Approximately 110 morphologically normal OGCs derived from vitrified and fresh preantral follicles were cultured *in vitro* in Transwell-COL membrane inserts (3.0 µm pore size, 12 mm diameter, Corning Costar Corp, Cambridge, MA) fitted in Costar 12-well cluster dish with 2 ml follicle culture medium: Waymouth medium (Gibco) supplemented with 5% (v/v) FCS, 2 mmol hypoxanthine l⁻¹ (Sigma), 0.23 mmol sodium pyruvate l⁻¹ and 50 mg gentamycin sulphate ml⁻¹. The OGCs were cultured for 10 days at 37°C in an atmosphere of 5% O₂, 5% CO₂ and 90% N₂. At 48 h intervals, approximately 1 ml fresh medium was exchanged for the same volume of the spent medium.

Maturation, fertilization and subsequent development *in vitro*

Thirty to forty-five morphologically normal cumulus–oocyte complexes (COCs) derived from OGCs cultured *in vitro* and from follicles grown *in vivo* (control) were induced to mature in a 100 µl drop of follicle culture medium without hypoxanthine but with 1 iu porcine FSH ml⁻¹ (Antorin, Denka Pharmaceutical Co Ltd, Kanagawa) and 10 ng human recombinant epidermal growth factor (EGF) ml⁻¹ (Sigma). COCs were incubated for 14–16 h at 37°C in a humidified atmosphere of 5% O₂, 5% CO₂ and 90% N₂. COCs collected from antral follicles of adult F₁ mice (C57BL/6JxCBA, 24 days old) 48 h after the treatment with 5 iu eCG (Teikoku Hormone Mfg Co. Ltd, Tokyo) were used as *in vivo* grown controls.

After maturation culture, oocytes surrounded with cumulus cells were inseminated *in vitro* adopting the procedure described by Toyoda *et al.* (1971). Spermatozoa collected from the epididymis of 3–6 month old F₁ (C57BL/6JxCBA) mice were incubated for 1 h in a 0.4 ml drop of TYH medium under paraffin oil in a humidified atmosphere of 5% CO₂ in air at 37°C. The composition of TYH medium is as follows: 119.37 mmol NaCl l⁻¹, 4.78 mmol KCl l⁻¹, 1.71 mmol CaCl₂ l⁻¹, 1.19 mmol KH₂PO₄ l⁻¹, 1.19 mmol MgSO₄ l⁻¹, 25.07 mmol NaHCO₃ l⁻¹, 1.0 mmol sodium pyruvate l⁻¹, 5.56 mmol glucose l⁻¹ (Toyoda *et al.*, 1971) supplemented with 4.0 g BSA l⁻¹, 75 mg benzyl penicillin l⁻¹ and 50 mg streptomycin sulfate l⁻¹. An aliquot of sperm suspension was added to each insemination drop of 0.4 ml TYH medium containing 35–50 COCs to give a final sperm concentration of 1.5 × 10⁵ cells ml⁻¹. The drops were

incubated in a humidified atmosphere with 5% O₂, 5% CO₂ and 90% N₂ at 37°C. After 4–5 h of incubation, groups of approximately 20 presumptive zygotes freed from cumulus cells were transferred to 25 µl drops of potassium simplex optimized medium (KSOM; Erbach *et al.*, 1994) under paraffin oil and cultured *in vitro* for 120 h under the same culture condition.

Evaluation of maturation, fertilization and subsequent development *in vitro*

After maturation *in vitro*, oocytes were freed from cumulus cells by pipetting. They were then fixed with a mixture of ethanol and acetic acid (3:1), stained with 1% (w/v) aceto-orcein, washed with aceto-glycerol and examined under a phase-contrast microscope to determine their nuclear status.

Oocytes were fixed with neutral formalin (10%, v/v), immersed in 95% alcohol, stained with 0.25% (w/v) aceto-lacmoid and examined using phase-contrast microscopy to assess fertilization. Oocytes were defined as penetrated when they had an enlarged sperm head or male pronucleus with a corresponding sperm tail. Oocytes with a pair of male and female pronuclei with a corresponding sperm tail were considered normally fertilized.

Cleavage and development to the blastocyst stage of oocytes inseminated *in vitro* were determined at 24 and 120 h after insemination, respectively. The total number of cells of the blastocysts obtained after 120 h of culture was determined using an air-dry procedure described by Takahashi and First (1992) with slight modifications. Briefly, blastocysts were treated with 1% (w/v) sodium citrate and fixed with a mixture of methanol:acetic acid:distilled water (5:1:4) and a mixture of methanol and acetic acid (3:1). After staining with 3% (v/v) Giemsa solution, the total number of cells of the blastocysts was counted under a light microscope.

Embryo transfer

Transfer of embryos was carried out following the procedure described by Hogan *et al.* (1994). Recipient ICR mice (7–9 weeks old, Japan SLC) were anaesthetized with 90 µg sodium pentobarbital g⁻¹ body weight (Nembutal Injection, Dainippon Pharmaceutical Co, Ltd, Osaka) on day 4 of pseudopregnancy. Eight to ten blastocysts obtained after 96–120 h of culture were transferred to each uterine horn of recipient mice. Recipient mice were allowed to carry their litters to term.

Experimental studies

In the first experiment, the effects of periods of exposure to 2 mol ethylene glycol l⁻¹ and to the vitrification solution on follicular survival after vitrification were determined. Preantral follicles were exposed to 2 mol ethylene glycol l⁻¹ for 2 or 5 min and the vitrification solution for 0.5, 1.0 or 2.0 min, and were then vitrified. Survival of the

Table 1. Viability of mouse preantral follicles after exposure to 2 mol ethylene glycol (EG) l⁻¹ and to a vitrification solution (VS) containing 6 mol EG l⁻¹ and 0.3 mol raffinose l⁻¹ before cooling

Exposure to 2 mol EG l ⁻¹ (min)	Exposure to VS before cooling (min)	Number of follicles vitrified (replicates)	Percentage of follicles recovered after warming	Percentage of follicles that survived after warming
2	0.5	95 (3)	97.0 ± 3.0	35.8 ± 0.8 ^a
	1	92 (3)	97.9 ± 1.8	37.9 ± 1.8 ^{ab}
	2	100 (3)	95.0 ± 3.5	42.0 ± 4.9 ^b
5	0.5	97 (3)	98.0 ± 1.8	80.4 ± 1.0 ^c
	1	91 (3)	96.8 ± 3.1	77.0 ± 2.2 ^c
	2	86 (3)	98.9 ± 1.8	39.6 ± 1.0 ^{ab}

^{abc}Within a column, values (means ± SD) with different superscripts are significantly different ($P < 0.01$).

vitrified–warmed preantral follicles was assessed on the basis of their morphology just after warming.

Subsequent experiments were carried out using the preantral follicles vitrified after exposure to 2 mol ethylene glycol l⁻¹ for 5 min and to the vitrification solution for 1 min.

In the second experiment, OGCs derived from vitrified–warmed preantral follicles were cultured for 10 days to determine their viability. OGCs from fresh preantral follicles were cultured as controls. Before and after *in vitro* culture, the granulosa cells were stripped from some OGCs by repeated pipetting to measure the diameter of the oocyte under an inverted microscope with an ocular micrometer.

In the third experiment, *in vitro* maturation, fertilization and subsequent development of oocytes derived from COCs of vitrified follicles were determined separately. COCs collected from antral follicles of adult mice were used for *in vitro* maturation, fertilization and development as *in vivo* grown controls.

Finally, the development *in vivo* of blastocysts derived from vitrified preantral follicles was determined by uterine transfer to five recipient mice in two trials.

Statistical analysis

The data on the effects of periods of exposure to 2 mol ethylene glycol l⁻¹ and subsequent exposure to vitrification solution were analysed using two-way ANOVA followed by Fisher's protected least significant difference (PLSD) as *post hoc* test. Comparisons of viability of OGCs from vitrified and fresh preantral follicles were made using Student's *t* test. Other data were analysed by one-way ANOVA followed by Fisher's PLSD. Data analyses were performed using StatView software (Abacus Concepts Inc, Berkeley, CA).

Results

Survival of vitrified preantral follicles after warming

The recovery of preantral follicles after warming was high (ranging from 95 to 99%) regardless of the treatments (Table 1). There was a significant interaction between the

effects of periods of exposure to 2 mol ethylene glycol l⁻¹ and to the vitrification solution on follicular survival after warming ($P < 0.01$). When preantral follicles were exposed to ethylene glycol for 2 min, survival after warming increased with prolonged (2 min) exposure to the vitrification solution; however, after 5 min of exposure to ethylene glycol, survival was higher after exposure to the vitrification solution for shorter periods (0.5 and 1.0 min, $P < 0.01$).

The major morphological abnormalities observed just after warming were the formation of clear space between the granulosa cell layer and oocyte or within the granulosa cell mass, the collapse of the oocyte within the follicle and the extrusion of denuded oocytes from the follicles.

In vitro growth of OGCs from vitrified preantral follicles

Morphologically normal preantral follicles vitrified by the optimum treatment (5 min in 2 mol ethylene glycol l⁻¹ and 1 min in vitrification) were treated with collagenase. After collagenase treatment, the proportions of morphologically normal OGCs obtained from the vitrified and fresh preantral follicles were not significantly different (Table 2). However, after culture *in vitro*, the viability of OGCs from vitrified follicles was lower than that of the fresh follicle group ($P < 0.01$).

On day 2 of *in vitro* culture, the viable OGCs from both vitrified and fresh follicles were found attached to the collagen insert, whereas degenerating or collapsed OGCs floated in the medium during the first medium change. Growth of OGCs as a result of granulosa cell proliferation was first observed on day 4 of culture. During the growth of OGCs *in vitro*, morphological impairments were characterized by disruption of the complexes, oocyte extrusion, darkening of ooplasm and granulosa cells indicative of cell degeneration. The diameter of oocytes in freshly isolated preantral follicles was $54.1 \pm 0.7 \mu\text{m}$ (mean ± SD, $n = 60$). After 10 days of culture, the diameter of oocytes from the vitrified follicles ($69.8 \pm 0.5 \mu\text{m}$, $n = 45$) was similar to that of the fresh follicles ($70.6 \pm 0.6 \mu\text{m}$, $n = 50$), and all of the oocytes possessed a visible germinal vesicle.

Table 2. Viability of vitrified mouse preantral follicles after enzymatic treatment and *in vitro* culture

Type of follicle	Number of follicles treated (replicates)	Percentage of follicles that survived after	
		collagenase treatment	culture for 10 days
Vitrified*	432 (3)	93.8 ± 2.3	70.6 ± 0.5 ^a
Fresh	492 (3)	95.9 ± 1.1	80.6 ± 3.2 ^b

*Follicles were exposed to 2 mol ethylene glycol l⁻¹ for 5 min and to vitrification solution for 1 min before cooling. After vitrification and warming, only morphologically normal preantral follicles were treated with collagenase and cultured *in vitro* for 10 days.

^{ab}Values (means ± SD) with different superscripts are significantly different ($P < 0.01$).

Table 3. *In vitro* maturational competence of mouse oocytes derived from vitrified or fresh preantral follicles and from antral follicles grown *in vivo*

Source of oocytes*	Number of oocytes examined (replicates)	Percentage of oocytes at different nuclear stages				
		GV	GVBD	MI	AI–TI	MII
Vitrified preantral follicles	180 (4)	23.9 ± 5.4 ^a	12.0 ± 3.7	8.6 ± 1.3 ^c	6.1 ± 3.8	49.5 ± 6.8 ^a
Fresh preantral follicles	140 (4)	23.9 ± 4.3 ^a	11.3 ± 1.0	5.0 ± 2.6 ^{cd}	3.6 ± 4.3	56.3 ± 3.6 ^a
Antral follicles grown <i>in vivo</i>	137 (4)	2.5 ± 5.0 ^b	8.1 ± 4.4	3.7 ± 2.9 ^d	0.8 ± 1.7	83.9 ± 12.1 ^b

*Vitrified preantral follicles were exposed to 2 mol ethylene glycol l⁻¹ for 5 min and to vitrification solution for 1 min before cooling. Vitrified and fresh preantral follicles were cultured *in vitro* for 10 days before being subjected to oocyte maturation *in vitro*. Cumulus–oocyte complexes collected from antral follicles grown *in vivo* were directly subjected to oocyte maturation *in vitro*.

^{ab} and ^{cd} within a column, values (means ± SD) with different superscripts are significantly different ($P < 0.01$ and $P < 0.05$, respectively).

GV: germinal vesicle; GVBD: germinal vesicle breakdown; MI: metaphase I; AI–TI: anaphase I–telophase I; MII: metaphase II.

In vitro maturation, fertilization and development

The proportions of oocytes that reached the MII stage (Table 3), normally fertilized with a pair of male and female pronuclei (Table 4) and developed to the blastocyst stage (Table 5) did not differ between the vitrified and fresh preantral follicles grown *in vitro*. However, the above values were lower than those of the follicles grown *in vivo* ($P < 0.01$). Similarly, the number of cells of blastocysts derived from the oocytes of vitrified and fresh preantral follicles was lower than those of the antral follicles grown *in vivo* ($P < 0.01$). At day 17 after embryo transfer, one of the five recipient mice that received 20 blastocysts derived from vitrified preantral follicles gave birth to six live pups: two females and four males. No abnormalities were observed in any of the six pups at necropsy 22 days after birth (3 days after weaning).

Discussion

Mouse MII oocytes that were vitrified using the same protocol as in the present study showed a high rate of survival when they were exposed to 2 mol ethylene glycol l⁻¹ for 2 min and then to vitrification solution for 1 min, or when they were exposed to 2 mol ethylene glycol l⁻¹ for 5 min and then to vitrification solution for 0.5 min (dela Peña *et al.*, 2001). Prolonged (2 or 5 min) exposure of MII oocytes to the vitrification solution after 2 or 5 min

exposure to ethylene glycol reduced their developmental capacity (dela Peña *et al.*, 2001). In the present study, exposure of mouse preantral follicles to 2 mol ethylene glycol l⁻¹ for 2 min resulted in low survival, regardless of the period of exposure to the vitrification solution; however, exposure to 2 mol ethylene glycol l⁻¹ for 5 min followed by exposure to the vitrification solution for 0.5 or 1.0 min yielded a high follicular survival.

The difference in the cryobiological characteristics between preantral follicles and MII oocytes could be attributed to their morphological and functional differences. In comparison with the oocyte, which is a single unit, the follicle is a complex structure comprising the granulosa and theca cells, basal lamina and the oocyte, which may affect the ability of the cryoprotectant to permeate to the centre of the follicle, the movement of water during cellular dehydration and rehydration, and the removal of the cryoprotectant during dilution. Moreover, cell permeabilities may change as the follicles develop (Candy *et al.*, 1997), so that the co-efficient of permeability of the growing oocyte in the preantral follicle may be different from that of fully grown or mature oocytes in the antral follicles. In the present study, 5 min exposure to 2 mol ethylene glycol l⁻¹ may have allowed enough dehydration and initial permeation of ethylene glycol into the follicles so that a brief (0.5 or 1.0 min) exposure to the vitrification solution might have induced a small amount of ethylene glycol influx and further dehydration, which concentrates the intracellular solutes

Table 4. *In vitro* fertilization capacity of mouse oocytes derived from vitrified or fresh preantral follicles and from antral follicles grown *in vivo*

Source of oocytes*	Number of oocytes inseminated (replicates)	Percentage of oocytes			Total penetration rate (%)
		with 2 PN	with ESH	polyspermy	
Vitrified preantral follicles	191 (4)	51.0 ± 5.5 ^a	6.6 ± 5.2	3.9 ± 1.6	61.4 ± 9.3 ^a
Fresh preantral follicles	140 (4)	54.1 ± 2.6 ^a	3.8 ± 4.5	3.4 ± 2.6	61.3 ± 3.7 ^a
Antral follicles grown <i>in vivo</i>	186 (4)	71.0 ± 2.6 ^b	4.0 ± 3.1	1.9 ± 2.2	76.9 ± 2.9 ^b

*Vitrified preantral follicles were exposed to 2 mol ethylene glycol l⁻¹ for 5 min and to vitrification solution for 1 min before cooling. Vitrified and fresh preantral follicles were cultured *in vitro* for 10 days. Morphologically normal cumulus–oocyte complexes (COCs) collected after 10 day culture were subjected to oocyte maturation and fertilization *in vitro*. COCs collected from antral follicles grown *in vivo* were subjected to oocyte maturation before *in vitro* fertilization.

^{ab}Within a column, values (means ± SD) with different superscripts are significantly different ($P < 0.01$).

2 PN: a pair of male and female pronuclei; ESH: an enlarged sperm head.

Table 5. Developmental capacity of mouse oocytes derived from vitrified or fresh preantral follicles and from antral follicles grown *in vivo* after insemination *in vitro*

Source of oocytes*	Number of oocytes inseminated (replicates)	Percentage of oocytes developed to [†]		Number of cells in blastocyst (n)
		two-cell	blastocyst	
Vitrified preantral follicles	197 (4)	48.7 ± 2.3 ^a	21.8 ± 1.7 ^a	31.5 ± 4.4 ^a (32)
Fresh preantral follicles	160 (4)	52.0 ± 2.5 ^a	22.5 ± 1.8 ^a	29.5 ± 4.4 ^a (30)
Antral follicles grown <i>in vivo</i>	153 (4)	61.4 ± 1.7 ^b	48.8 ± 6.0 ^b	47.2 ± 6.3 ^b (34)

*Vitrified preantral follicles were exposed to 2 mol ethylene glycol l⁻¹ for 5 min and to vitrification solution for 1 min before cooling. Vitrified and fresh preantral follicles were cultured *in vitro* for 10 days. Morphologically normal cumulus–oocyte complexes (COCs) collected after 10 day culture were subjected to oocyte maturation, fertilization and subsequent *in vitro* culture for 5 days. COCs collected from *in vivo* grown antral follicles were subjected to oocyte maturation before fertilization and subsequent *in vitro* culture.

[†]Percentage of two-cell embryos and blastocysts were based on the number of oocytes inseminated.

^{ab}Within a column, values (mean ± SD) with different superscripts are significantly different ($P < 0.01$).

producing a cytoplasm capable of vitrification (Rall, 1987). The degree of cryoprotectant permeation and dehydration achieved with 2 min exposure to 2 mol ethylene glycol l⁻¹ and subsequent exposure to the vitrification solution may be inadequate to induce vitrification of mouse preantral follicles.

After warming, 20% of vitrified preantral follicles failed to survive, which is comparable with that in preantral follicles cryopreserved by slow freezing (Carroll *et al.*, 1990; Carroll and Gosden, 1993; Cortvrindt *et al.*, 1996a). The additional 10% loss of OGCs during *in vitro* culture can be accounted for by vitrification as the follicular survival was judged by gross morphology immediately after warming.

Cryopreservation of preantral follicles requires not only the survival of the granulosa cells and oocytes but also the maintenance of gap junctions between the granulosa cells and oocytes, and among the granulosa cells (Eppig, 1977). The morphological criteria used to determine follicular survival could not assess the state of these gap junctions; however, the similarity in ability of the oocytes derived from both vitrified and fresh follicles to mature, be fertilized and develop to the blastocyst stage indicates that sufficient numbers of gap junctions were kept intact allowing adequate

transport of essential factors during *in vitro* culture. Results of the present study also indicate that the integrity of the zona pellucida and ooplasm was not altered by vitrification. The maturation and fertilization rates of oocytes from the vitrified preantral follicles were comparable with those of oocytes derived from fresh preantral follicles and those cryopreserved by conventional slow freezing (Cortvrindt *et al.*, 1996a). Birth of live pups demonstrated that some of the oocytes derived from vitrified preantral follicles had the competence to achieve full-term development.

In the present study, the vitrified preantral follicles were treated enzymatically, as preliminary trials showed a poor survival of fresh whole follicles in membrane insert without enzymatic treatment. The culture system for the *in vitro* growth of oocyte–granulosa cell complexes in an insert (Eppig and Schroeder, 1989), which has the potential to generate large numbers of vitrified oocytes that can be fertilized, was adopted. After collagenase treatment and a subsequent 10 day culture period, the oocytes from both vitrified and fresh preantral follicles grew to a size comparable with that of oocytes grown *in vitro* in the previous study (Eppig and O'Brein, 1998). However, the developmental competence of the oocytes derived from

both vitrified and fresh preantral follicles was lower than that of oocytes grown *in vivo* and that of oocytes grown *in vitro* originating from preantral follicles cultured in medium containing FCS but without FSH (Eppig and O'Brien, 1998). Although the exact reasons for the low developmental capacity of the oocytes grown *in vitro* is not clear, it appears that the *in vitro* follicle culture condition in the present study is sub-optimal. Further studies are required, and should be directed to the improvement of the preantral follicle culture conditions and to the use of whole follicle culture systems (Spears *et al.*, 1994; Cortvrindt *et al.*, 1996b) for the growth and development of vitrified preantral follicles *in vitro*.

In conclusion, the results of the present study demonstrate for the first time that mouse preantral follicles can be vitrified and that some of the oocytes originating from vitrified preantral follicles can grow and develop to live pups after maturation and fertilization *in vitro*.

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