***In vitro* follicular development of cryopreserved mouse ovarian tissue**

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Abstract

In a previous report, we showed that follicles isolated from frozen/thawed mouse ovarian tissues reached the mature follicle stage on the 12th day of culture. However, the developmental ability was lower than that of fresh ovarian tissue. The purpose of this study was to define a culture system with some technical modification for preantral follicles isolated from frozen/thawed ovarian tissue and to confirm cell injury. Ovaries obtained from three-week-old female mice were cryopreserved by the rapid freezing method. Preantral follicles isolated from frozen/thawed ovarian tissues were cultured for 12–16 days. The follicles were then stimulated with human chorionic gonadotropin. *In vitro* fertilization was performed on the released cumulus–oocyte complexes (COCs). Preantral follicle viability was assessed by supravital staining using Hoechst 33258. Using this stain cell death was found in part of the granulosa cells of a follicle obtained from frozen/thawed ovarian tissue. On the 14th and 16th days of culture, the diameters of follicles isolated from frozen/thawed ovaries were larger than on the 12th day of culture. The released COCs were fertilized and developed to the blastocyst stage in 15.8% (12/76) of the oocytes taken from the fresh group, and in 0% (0/73), 2.9% (2/69) and 19.1% (22/115) of the oocytes taken from the frozen/thawed group that had been cultured for 12, 14 and 16 days respectively. The preantral follicles isolated from frozen/thawed mouse ovarian tissues developed slowly compared with the freshly prepared preantral follicles. During prolonged culture from 12 to 16 days, these follicles obtained the potential to fertilize and develop to the blastocyst stage.

Reproduction (2005) **130** 187–192

Introduction

Cryopreservation of female gametes is an important technology for medical and scientific applications. There are two methods of obtaining mature oocytes from frozen/thawed ovarian tissues. One is transplantation and the other is isolation and culture of the follicles (or oocytes) obtained from ovarian tissues *in vitro*.

In 1953, Parkes and Smith reported success with the cryopreservation of ovarian tissues. Since then, normal offspring have been obtained from mice after orthotopic transplantation of cryopreserved ovarian tissues (Parrott 1960, Cox *et al.* 1996, Gunasena *et al.* 1997, Sztejn *et al.* 1998). There is a risk that if cancer cells are present in the ovarian tissue at the time of cryopreservation, they may survive and will be transplanted. There are also ethical and moral problems with transplants of human ovarian tissue into immunodeficient animals (xeno-graft) for clinical applications. Thus *in vitro* growth of follicles (or oocytes) isolated from frozen/thawed ovarian tissues is desirable.

In the murine model, there are some reports that preantral follicles have been grown to the antral phase *in vitro* (Qvist *et al.* 1990, Nayudu & Osborn 1992), were matured to metaphase II after human chorionic gonadotropin (hCG) stimulation (Cortvrindt & Smitz 1998), were fertilized and developed into blastocysts (Cortvrindt *et al.* 1996a, Demeestere *et al.* 2002) and resulted in live births after embryo transfer (Spears *et al.* 1994). In 2001, Newton and Illingworth reported the survival and *in vitro* growth of murine follicles after isolation from ovarian tissues cryopreserved by a slow freezing method. They indicated that follicles isolated from frozen/thawed tissue produced mature oocytes, but at the end of the culture period the diameter of the frozen/thawed follicles was smaller than that of fresh ones.

In most previous studies, slow freezing methods using a programmed freezer have been employed for the cryopreservation of ovarian tissues (Parrott 1960, Cox *et al.* 1996, Gunasena *et al.* 1997, Sztejn *et al.* 1998, Newton &

Illingworth 2001). Rall and Fahy (1985) reported an extremely rapid method called 'vitrification', in which embryos suspended in a high concentration of cryoprotectant solution were rapidly placed into liquid nitrogen. They used dimethylsulfoxide, acetamide and propylene glycol as the cryoprotectant. Ethylene glycol has been reported to be less toxic to embryos (Kasai *et al.* 1990, 1996, Mukaida *et al.* 1998) or oocytes (Rayos *et al.* 1994, Kuleshova *et al.* 1999) than other compounds. We have demonstrated that mature oocytes can be obtained after culture of follicles isolated from ovarian tissues frozen/thawed by a rapid freezing method (Segino *et al.* 2002, 2003). The size of the follicles obtained from the frozen/thawed ovarian tissues was smaller than those from freshly prepared controls after 12 days of culture. In addition, their developmental rates were lower compared with those of fresh follicles. In this paper we describe our studies of cell injury following cryopreservation and the time course of culture of preantral follicles obtained from frozen/thawed ovarian tissues.

Materials and Methods

Animals and collection of ovarian tissue

Three-week-old female C57BL/6N × DBA/2N F1 mice (B6D2F1; CLEA Japan, Inc., Tokyo, Japan) were used in this study. They were housed under controlled conditions (14 h light:10 h darkness) with food and water available *ad libitum*. The mice were killed by cervical dislocation and the ovaries were collected in Dulbecco's phosphate buffered saline (Sigma, St Louis, MO, USA) supplemented with 10% v/v fetal bovine serum (FBS; Sigma), 0.133 g/l CaCl₂·2H₂O, 0.1 g/l MgCl₂·6H₂O, 1.0 g/l D-glucose, 0.036 g/l sodium pyruvate, 100 U/ml penicillin G, 0.1 g/l streptomycin sulfate and 0.1 g/l kanamycin sulfate (m-DPBS).

Rapid freezing

Five female mice were killed to obtain ovaries. These experiments were performed on four replications. The enveloping tissues were dissected from the collected ovaries and they were cut in half with a scalpel. They were cryopreserved by the modified rapid freezing method (Segino *et al.* 2002, 2003), which was based on a protocol for the rapid freezing of mouse embryos (Kasai *et al.* 1990). Briefly, they were treated in the following manner: EFS10 (10% v/v ethylene glycol, 27% w/v Ficoll and 0.45 mol/l sucrose) for 10 min at 25 °C, EFS20 (20% v/v ethylene glycol, 24% w/v Ficoll and 0.4 mol/l sucrose) for 10 min at 4 °C and EFS40 (40% v/v ethylene glycol, 18% w/v Ficoll and 0.3 mol/l sucrose) for 5 min at 4 °C. Finally they were put into cryotubes with a small amount of cryoprotectant solution. Throughout all steps, ovarian pieces were continuously mixed with 1 ml cryoprotectant solution on a shaker. The cryotubes were then exposed to the vapor phase above liquid nitrogen. After 3 min, the cryotubes were

plunged into liquid nitrogen and stored for 1–20 days. All cryoprotective solutions were prepared using m-DPBS.

Thawing

Following pulling up from liquid nitrogen, the ovarian tissues were placed in the air for 30 s at room temperature and then rinsed in EFS20 for 5 min at 4 °C, EFS10 for 5 min at 25 °C and 0.5 mol/l sucrose for 10 min at 25 °C. Finally, they were put into m-DPBS and washed 3 times. During all these steps they were continuously mixed using a shaker.

Hoechst 33258 staining

The viability of preantral follicles was assessed by supravital staining using Hoechst 33258 (bis-benzimide; Wako Pure Chemical Industries Co., Ltd, Osaka, Japan) as described in the literature (Jewgenow *et al.* 1998, Itoh & Hoshi 2000). This staining has proved useful for the characterization of cell-membrane integrity by dye exclusion from viable cells. A preantral follicle obtained from frozen/thawed or fresh ovarian tissue was stained with 10 µg/ml Hoechst 33258 in culture medium for 15 min at 37 °C. Then, the follicle was evaluated under phase contrast microscopy with fluorescence excitation (Olympus, Tokyo, Japan).

In vitro culture of preantral follicles

Preantral follicles were obtained from the thawed ovaries by mechanical dissection with a 29-gauge insulin needle (Terumo, Leuven, Belgium) and transferred to fresh m-DPBS. They were 100–130 µm in diameter, spherical in structure, and consisted of two or three layers of granulosa cells and a visible oocyte. Selected follicles were washed three times before culture. A three-week-old female C57BL/6N × DBA/2N F1 mouse was killed to obtain fresh ovaries as a control.

Mechanically isolated preantral follicles were individually cultured in alpha minimal essential medium (αMEM; Life Technologies, Inc., Rockville, MD, USA), supplemented with 100 mU/ml human follicle stimulating hormone (hFSH, Fertinorm P; Serono, Geneva, Switzerland), 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenium (ITS; Sigma) and 5% v/v FBS (Cortvridt *et al.* 1996a). Twenty follicles were individually cultured in a culture dish (60 mm tissue culture dish; Falcon, Becton Dickinson, Oxford, Oxon, UK), containing a 10 µl droplet covered with 5 ml mineral oil (Sigma), at 37 °C in a humidified atmosphere of 5% CO₂ in air. On the second day of culture, 10 µl medium were added to each droplet and thereafter half of the medium was exchanged every second day. On the 1st, 4th, 8th, 12th, 14th and 16th days of culture, oocyte and follicle diameter excluding the theca stroma was estimated by measuring two perpendicular diameters (length and width) with an ocular micrometer under the inverted microscope (Olympus).

Releasing of cumulus–oocyte complexes

On the 12th, 14th or 16th day of culture, cumulus–oocyte complexes (COCs) were released from the antral follicles by the addition of 2.5 U/ml hCG (Gonotropin 3000; Teikoku Zoki, Co. Ltd, Tokyo, Japan) and 5 ng/ml epidermal growth factor (EGF; Sigma). Seventeen hours later, the released oocytes were classified as follows: GV when the germinal vesicle was present, germinal vesicle broken down (GVBD), or metaphase II (MII) when the first polar body was extruded.

In vitro fertilization

Preantral follicles obtained from 6 fresh ovarian tissues ($n = 140$) and 24 frozen/thawed ovarian tissues ($n = 308$) were cultured in 3 replications. Expanded COCs released from the antral follicles by the addition of hCG and EGF were placed in human tubal fluid medium (HTF; Irvine Scientific, Santa Ana, CA, USA) containing 0.4% w/v bovine serum albumin (BSA; Sigma). Caudal epididymal spermatozoa, collected from an adult B6D2F1 male, were used for insemination at concentrations of 1×10^6 sperm/ml. Oocytes were removed from the fertilization drops after 4–5 h and cultured in fresh medium for 120 h.

Hormonal assays

On the 12th, 14th or 16th day of culture, conditioned media from individual follicles were collected for single follicles and stored at -30°C . Estradiol-17 β in these media were measured by enzyme immunoassay (17 β -estradiol ELISA kit; IBL, Hamburg, Germany). This assay has been described in the literature (Cortvrindt *et al.* 1996a, Liu *et al.* 2002).

Statistical analysis

Category variables were assessed by calculating chi-square or Fisher's exact test in the case of small cell frequencies. Follicle diameter and hormonal levels were compared between the two groups using Student's *t*-test. All values are presented as means $\pm$ S.E.

Results

Viability of preantral follicles

A viable preantral follicle obtained from a fresh ovary with granulosa cells and a germinal vesicle in the oocyte showed no staining with Hoechst 33258. In contrast, a little staining of the granulosa cells was found in a follicle obtained from frozen/thawed ovarian tissue but there was no staining of the oocyte (Fig. 1a,b).

Growth of preantral follicles in vitro

The number of preantral follicles isolated from individual fresh and frozen/thawed ovaries was 22.4 ± 1.68 and 13.4 ± 0.73 respectively (Fig. 2a). After 4 days of culture, the fresh ovarian follicles had increased in diameter while

the size of the frozen/thawed ovarian follicles had not changed. After 12 days of culture, the diameter of follicles isolated from fresh and frozen/thawed ovaries were $620.6 \pm 12.67 \mu\text{m}$ and $477.0 \pm 20.0 \mu\text{m}$ respectively. Frozen/thawed ovarian follicles were cultured for a maximum of 16 days. On the 14th and 16th day of culture, the diameters were $560.3 \pm 20.5 \mu\text{m}$ and $632.2 \pm 17.0 \mu\text{m}$ respectively (Fig. 2b, Fig. 3).

The oocyte diameter of a selected follicle was measured in tissue taken from both the fresh ($57.5 \pm 0.46 \mu\text{m}$, $n = 88$) and the frozen/thawed ($56.1 \pm 0.56 \mu\text{m}$, $n = 81$) group. After 12 days of culture, the oocytes finally reached a diameter of $70.9 \pm 0.59 \mu\text{m}$ and $70.2 \pm 0.46 \mu\text{m}$ respectively. On the 14th and 16th day of culture, the diameters of the oocytes isolated from the frozen/thawed ovary were $72.7 \pm 0.51 \mu\text{m}$ and $73.4 \pm 0.67 \mu\text{m}$ respectively.

On the 12th day of culture, 90.7% of the follicles in the fresh group survived. On the 12th, 14th and 16th day of culture, the survival rate of follicles isolated from frozen/thawed ovaries was 65.0%, 69.5% and 69.5% respectively (Table 1). During follicular growth, morphological degeneration was determined by extrusion of denuded oocytes and darkening of the ooplasm.

At the end of the culture period, the follicles were stimulated with hCG and EGF for 17 h to induce ovulation. COCs were expanded and were released from the follicle (Fig. 2c). The rate of released COCs from freshly isolated ovaries was 97.6%. On the 12th, 14th and 16th day of culture, the rates of released COCs from

Figure 1 A preantral follicle isolated from (a) fresh and (b) frozen/thawed ovarian tissue stained with Hoechst 33258. Denatured cells were stained. Scale bar = 100 μm .

Figure 2 (a) A preantral follicle isolated from frozen/thawed ovarian tissue. (b) An antral follicle after 16 days of culture. (c) An antral follicle stimulated with hCG/EGF showing cumulus–oocyte complex extraction. (d) Blastocyst stage embryos in the frozen/thawed group. Scale bars: a = 50 μ m, b and c = 200 μ m, d = 100 μ m.

frozen/thawed ovaries were 84.3%, 91.2% and 93.8% respectively (Table 1).

In vitro fertilization rate

Expanded COCs were used for *in vitro* fertilization and preimplantation development. The fertilization rates were

Figure 3 The diameter of preantral follicles and estradiol-17 β concentrations in conditioned medium. The diameters of preantral follicles isolated from fresh ($n = 88$) and frozen/thawed ($n = 81$) ovarian tissues were measured. In the fresh group, estradiol-17 β levels were measured by enzyme immunoassay on the 12th ($n = 11$) day of culture. In the frozen/thawed group, levels were measured on the 12th ($n = 7$), 14th ($n = 7$) and 16th ($n = 7$) day of culture. Values are means $\pm$ S.E. (* $P < 0.001$). Significant differences are marked with fresh and frozen/thawed groups.

73.7% in the fresh group, and 57.5%, 62.3% and 73.0% in the frozen/thawed group cultured for 12, 14 and 16 days respectively. Blastocysts were observed in 15.8% of the oocytes taken from the fresh group, and in 2.9% and 19.1% in the frozen/thawed group cultured for 14 and 16 days respectively (Table 2, Fig. 2d).

Hormonal assays

The concentration of estradiol-17 β in conditioned medium from fresh and frozen/thawed ovarian tissue is shown in Fig. 3. Estradiol-17 β production by follicles isolated from fresh tissue was significantly greater than that produced by those isolated from frozen/thawed tissue (3091.5 ± 162 pg/ml vs 1339.6 ± 203 pg/ml). The production of estradiol-17 β increased progressively up to 12 or 16 days. On the 14th and 16th day of culture, the estradiol-17 β concentration of follicles isolated from frozen/thawed ovaries was 2346.1 ± 435 pg/ml and 3138.5 ± 472 pg/ml respectively. The estradiol-17 β levels were not statistically different between 12 days of culture in the fresh group and 16 days of culture in the frozen/thawed group. Estradiol-17 β is a lipid-soluble sterol that can easily diffuse into the surrounding oil. Liu *et al.* (2002) indicated that $<1\%$ of the follicular secretory product was diffused to adjacent oil. Therefore, estradiol-17 β concentrations in the collected media were used to monitor follicular estradiol-17 β production *in vitro*.

Discussion

The survival rates of cryopreserved embryos depend upon several mechanisms related to cell injury, such as the chemical toxicity of the cryoprotectant, intracellular ice formation, fracture damage, and osmotic swelling during the removal of the cryoprotectant (Mukaida *et al.* 1998).

Table 1 Acquisition of competent oocytes after *in vitro* maturation of preantral follicles. Preantral follicles isolated from fresh ovaries were cultured for 12 days. Preantral follicles isolated from frozen/thawed ovaries were cultured for 12, 14 or 16 days.

Follicles	No. of follicles			No. of oocytes		
	Cultured	Survived	Released COCs	GV	GVBD	MII
Fresh ovary	140	127 ^a (90.7%)	124 ^a (97.6%)	9 (7.3%)	9 ^a (7.3%)	106 ^a (85.5%)
Frozen/thawed ovary						
Cultured for 12 days	177	115 ^c (65.0%)	97 ^c (84.3%)	9 (9.3%)	27 ^c (27.8%)	61 ^c (62.9%)
Cultured for 14 days	131	91 ^c (69.5%)	83 ^b (91.2%)	7 (8.4%)	18 ^b (21.7%)	58 ^b (69.9%)
Cultured for 16 days	210	146 ^c (69.5%)	137 ^{NS} (93.8%)	10 (7.3%)	22 ^b (16.1%)	105 ^{NS} (76.6%)

Significant differences are compared with fresh group (a–b: $P < 0.05$, a–c: $P < 0.001$). NS, no significant differences. COCs, cumulus-oocyte-complexes; GV, germinal vesicle; GVBD, germinal vesicle breakdown; MII, metaphase II.

The ovary is a complex structure composed of several different types of cells. By comparison with an oocyte or embryo, which is a single unit, cryopreservation of ovarian tissue is more difficult because different cell types have different requirements for optimal survival. We have shown that cell death was found in a part of the granulosa cells of a follicle obtained from frozen/thawed ovarian tissue. The lower survival rate of follicles isolated from frozen/thawed ovaries was ascribed to initial cell death of the granulosa cells.

Our culture system is based upon an established method by Cortvrindt *et al.* (1996a). They indicated that optimal maturity was obtained after 12–14 days, and that on the 16th day of culture there was a significantly lower maturity rate and a lower final yield of GVBD. In our experiment, the preantral follicles isolated from frozen/thawed ovarian tissue developed slowly compared with the freshly prepared preantral follicles. On the 12th day of culture, the diameters of follicles isolated from fresh ovaries were larger than those from frozen/thawed ovaries, and quantification of the estradiol-17 β in the conditioned medium revealed lower production from the frozen/thawed follicles compared with the control follicles.

On the 16th day of culture, the diameter and estradiol-17 β production of follicles isolated from frozen/thawed ovaries reached the same level as those of fresh follicles on the 12th day. This might be a reflection of the number of intact granulosa cells present as a result of the initial cell death that occurred during the freeze/thaw process (Cortvrindt *et al.* 1996b, Newton & Illingworth 2001).

In all experimental groups, MII stage oocytes were observed in more than 60% of follicles after hCG/EGF stimulation. However, blastocysts were observed in the oocytes taken from the fresh group and the frozen/thawed group cultured for 14 and 16 days but not when cultured for 12 days. Oocyte maturity is often assessed only in terms of nuclear maturity, a visible parameter that can be easily observed under an inverted microscope. The processes of oocyte development involved in the acquisition of competence to undergo fertilization and preimplantation development are often referred to as cytoplasmic maturation. Eppig and Schroeder (1989) reported that oocytes matured and fertilized *in vitro* after isolation from small antral follicles are less likely to complete preimplantation development than oocytes from large antral follicles. Oocytes have to reach their full size and competence to undergo both nuclear and cytoplasmic maturation and thus support future completion of development.

In conclusion, we have demonstrated that cryopreservation of mouse ovarian tissues by rapid freezing is successful in allowing the oocytes to maintain their ability to undergo meiosis and preimplantation development. The freeze/thaw process may have some effects on growth suppression of the oocytes themselves and of granulosa cells. However, this impairment is within the range from which recovery is possible. Patients undergoing potentially sterilizing therapy for cancer need to protect their fertility before treatment. Recent studies demonstrate good rates of follicular survival and normal morphology after cryopreservation, but there are major uncertainties about the

Table 2 Fertilization rate and development of oocytes matured *in vitro* into the 2-cell, morula and blastocyst stages.

Follicles	No. of oocytes		No. of embryos		
	Inseminated	Fertilized	2-cell	Morula	Blastocyst
Fresh ovary	76	56 ^a (73.7%)	52 ^a (68.4%)	24 ^a (31.6%)	12 ^a (15.8%)
Frozen/thawed ovary					
Cultured for 12 days	73	42 ^b (57.5%)	31 ^b (42.5%)	7 ^c (9.6%)	0 ^c
Cultured for 14 days	69	43 ^{NS} (62.3%)	43 ^{NS} (62.3%)	14 ^{NS} (20.3%)	2 ^b (2.9%)
Cultured for 16 days	115	84 ^{NS} (73.0%)	79 ^{NS} (68.7%)	34 ^{NS} (29.6%)	22 ^{NS} (19.1%)

Significant differences were compared with fresh group (a–b: $P < 0.05$, a–c: $P < 0.001$). NS, no significant differences.

best use of the tissue afterwards. Our study using mouse ovaries may provide the basis of clinical applications to human folliculogenesis for female gamete conservation.

Acknowledgements

We thank our colleagues and the present and past members of our laboratories who contributed to the research presented in this study. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 7 October 2004

First decision 23 December 2004

Revised manuscript received 20 April 2005

Accepted 18 May 2005