The Effect of Cryoprotectants on the Survival and *In Vitro*-Growth of Cryopreserved Mouse Preantral Follicles

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ABSTRACT

The aim of this study was to examine more effective cryoprotectant for the cryopreservation of mouse preantral follicles. Enzymetically isolated preantral follicles from 12-day-old mice were cryopreserved by a slow freezing protocol with 1.5 M propanediol (PROH), dimethyl sulphoxide (DMSO) or glycerol (GLY) and then grown and matured *in vitro* for 11 days after thawing. The survival of preantral follicles immediately after freezing and thawing was not different among the PROH (68.2%), DMSO (72.4%) and GLY (72.1%). After grown and matured *in vitro*, the rates of survival and metaphase II oocytes were 54.9% and 36.6% for PROH which was significantly higher rates ($p<0.05$) compared with the rates obtained from DMSO (16.9% and 9.0%) and GLY (16.3% and 7.5%). The diameter of metaphase II oocytes from preantral follicles frozen in PROH (67.4±1.8 μm) was significantly ($p<0.05$) smaller than that of the fresh preantral follicles (69.1±2.3 μm). The results from the present study revealed that PROH is more suitable cryoprotectant for the cryopreservation of mouse preantral follicles.

(Key words : Cryoprotectant, Mouse, Preantral follicles, Slow freezing)

INTRODUCTION

At birth the mammalian ovary contains many thousands of follicles but most follicles become atretic during their growth and maturation. Very few, highly selected viable oocytes are produced during the reproductive lifespan of the female (Carroll *et al.*, 1990). The ovarian follicle management system is therefore highly inefficient and has evolved for intense competition between gametes (Hartshorne, 1997). Cryopreservation of preantral follicles enables the storage of large numbers of preantral follicles and preserves the structural integrity of somatic and reproductive cells (Lin *et al.*, 2008). Previous attempts to preserve large numbers of preantral follicles involved the cryopreservation of ovarian tissues, which resulted in the birth of offspring after orthotopic transplantation 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 isolated from the frozen-thawed ovarian grafts (Liu *et al.*, 2001). In spite of these successes, a large proportion of preantral follicles are damaged during freezing and thawing.

Research about the cryopreservation of isolated preantral follicles has been conducted less frequently than have been studies of the freezing of ovarian tissue. Grafting of frozen-thawed preantral follicles embedded in collagen gels resulted in the birth of pups after *in vitro* fertilization of oocytes grown and matured *in vivo* (Carroll *et al.*, 1990). Cryopreserved mouse preantral follicles grew to maturity *in vitro* and developed to the blastocyst stage after *in vitro* fertilization (Cortvrindt *et al.*, 1996).

Most previous studies on cryopreservation of mouse preantral follicles using slow freezing used dimethyl sulphoxide (DMSO) as a cryoprotectant (Carroll *et al.*, 1990; Carroll and Gosden, 1993; Cortvrindt *et al.*, 1996). However, the efficiency of cryopreservation, transplantation and *in vitro* culture of preantral follicles frozen in DMSO were generally lower than those obtained in fresh preantral follicles.

The aim of the present study was to examine the effect of three common cryoprotectants (1,2-propanediol (PROH), DMSO and glycerol (GLY)) on the survival and *in vitro* growth of mouse preantral follicles after cryopreservation.

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MATERIALS AND METHODS

Isolation of Preantral Follicles

The ovaries were aseptically removed from the 12-day-old ICR female mice. The ovaries were immersed into Leibovitz L-15 medium (Gibco-BRL, Carlsbad, CA, USA) containing 1 mg/ml collagenase (Type 1A; Sigma, St. Louis, MO, USA) and 0.2 mg/ml DNase I (Sigma, St. Louis, MO, USA) for 20 min at 37℃ and repeatedly drawn in and out of the pipette until the ovaries were dissociated into individual follicles. The preantral follicles (100~120 μm in diameter) were selected based on the following criteria: (i) intact round follicular structure with 2~3 layers of granulosa cells and (ii) the oocyte had to be visible, round, and centrally located within the follicle. All the selected preantral follicles were randomly allocated to the control or frozen groups.

Solutions for Freezing and Thawing

Freezing and thawing solutions were prepared in Leibovitz L-15 medium supplemented with 10% fetal bovine serum (FBS). The cryoprotectants were PROH (Sigma, St. Louis, MO, USA), DMSO (Sigma, St. Louis, MO, USA) and GLY (Sigma, St. Louis, MO, USA). An equilibration solution was used in equilibration step for the freezing procedure with each cryoprotectant. Equilibration solutions were 1.5 M PROH, 1.5 M DMSO, and 1.5 M GLY. Two equilibration solutions were used in two different steps for the thawing procedure with each cryoprotectant. The equilibration solutions were: 1.0 M and 0.5 M PROH; 1.0 M and 0.5 M DMSO; 1.0 M and 0.5 M GLY.

Freezing and Thawing of the Preantral Follicles

The same freezing-thawing procedure was used for the three cryoprotectants. The preantral follicles were exposed to 1.5 M cryoprotectant, loaded in 0.25 ml straw, and then charged in a controlled programmed freezing machine (Kryo 10; Planer, Middlesex, UK). Not more than 10 preantral follicles were loaded into each straw. The preantral follicles were equilibrated in the freezing mixture for 15 min at 4℃ and then cooled to −7℃ at a rate of −2℃/min. After manual seeding at −7℃, the temperature was lowered to −40℃ at −0.3 ℃/min. Before transfer for storage in liquid nitrogen, the preantral follicles were very rapidly cooled to −110 ℃ at a rate of −50℃/min.

For thawing, the straws were held in air for 10 sec and then plunged into a water bath at 37℃ for a few seconds until the ice melted. The cryoprotectant was removed from the preantral follicles in two steps: 5 min in 1 M cryoprotectant, and 5 min in 0.5 M cryoprotectant. Finally, the preantral follicles were washed for 15 min in Leibovitz L-15 medium supplemented with 10% FBS at 37℃.

Morphologically normal preantral follicles with an intact oocyte surrounded by a continuous layer of granulose cells were collected and cultured for *in vitro* growth and maturation. Freshly collected preantral follicles were treated similarly and used as controls.

In Vitro Growth and Maturation of the Preantral Follicles

The culture medium was αMEM medium (Gibco-BRL, Carlsbad, CA, USA) supplemented with 5% FBS, 100 mIU/ml human menopausal gonadotrophins (HMG; Humegon, Organon, Oss, Netherland). The preantral follicles were cultured on Transwell-COL membrane inserts (3.0 μm pore size, 24.5 mm diameter; Costar, Cambridge, MA, U.S.A) in six well cluster dishes for 10 days at 37℃ and 5% CO_2 in air. Half of the medium was changed every 2 days. After 10 days of growth *in vitro*, preantral follicles were allowed to mature for 16~18 hours in αMEM medium supplemented with 1.5 IU/ml hCG (Profasi, Sereno, Aubonne, Switzerland).

Measurement of Oocyte Diameter

After maturation, oocyte-cumulus cell complexes from matured preantral follicles were collected and adherent cumulus cells were removed by pipetting in Leibovitz L-15 medium containing 0.1% hyaluronidase. The maturation status and the diameter of the oocytes, excluding the zona pellucida, were examined with an inverted microscope and the ocular micrometer. As a control, *in vivo*-grown oocytes were collected from the ovaries of 22-day-old ICR mice and matured in the same medium in which *in vitro*-grown oocytes were matured.

Statistical Analysis

The statistical significance of the data was analyzed using a Student's *t*-test and chi-square (x^2) test. A statistical significance was established at the $p<0.05$ level.

RESULTS

Morphological Survival of Preantral Follicles after Cryopreservation

Fig. 1 shows the morphological survival rates of preantral follicles frozen in the three different cryoprotectants. After thawing, the survival rates were 68.2% (n=71/104), 72.4% (n=89/123) and 72.1% (n=80/111) for PROH, DMSO and GLY, respectively.

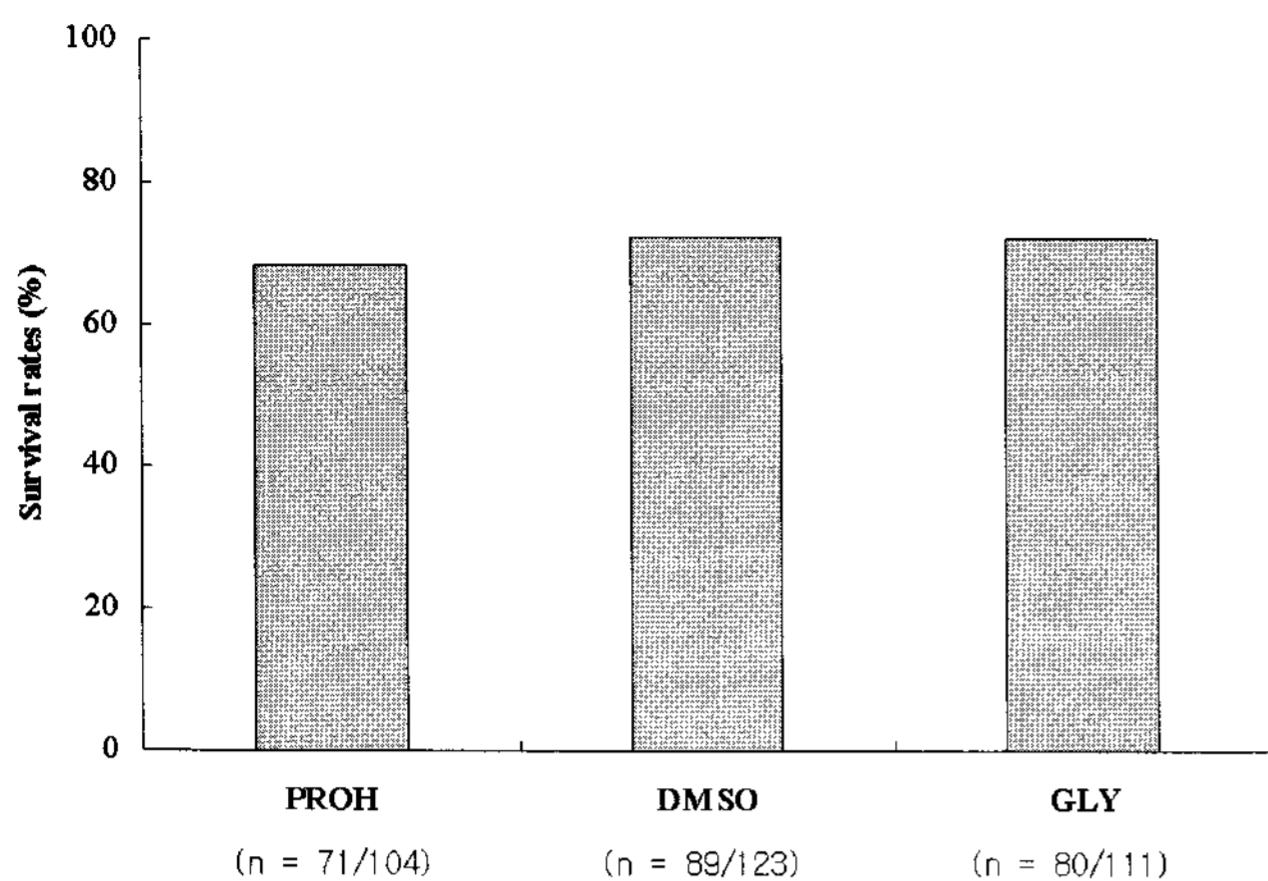The major morphological abnormalities observed just

Fig. 1. Morphological survival of mouse preantral follicles after cryopreservation using different cryoprotectants.

Fig. 2. Survival of mouse preantral follicles cryopreserved using different cryoprotectants after *in vitro* growth. [a,b,c] $p<0.05$.

after thawing were the formation of clear space between the granulose cell layer and oocyte or within the granulose cell mass, the collapse of the oocyte within the follicle and the extrusion of denuded oocyte from the follicle.

In Vitro Growth of Preantral Follicles after Cryopreservation

Morphologically normal preantral follicles obtained from the frozen-thawed and fresh preantral follicles were cultured on Transwell-COL membrane inserts. The percentage of fresh preantral follicles which survived 10 days of *in vitro* growth was 77.7% (Fig. 2). Following culture from preantral follicles frozen-thawed in PROH, DMSO or GLY, respectively, 54.9, 16.9 and 16.3 % of preantral follicles survived. The survival rates of PROH treatment group was significantly higher ($p<0.05$) than that of DMSO and GLY treatment groups.

In Vitro Maturation of Preantral Follicles after Cryopreservation
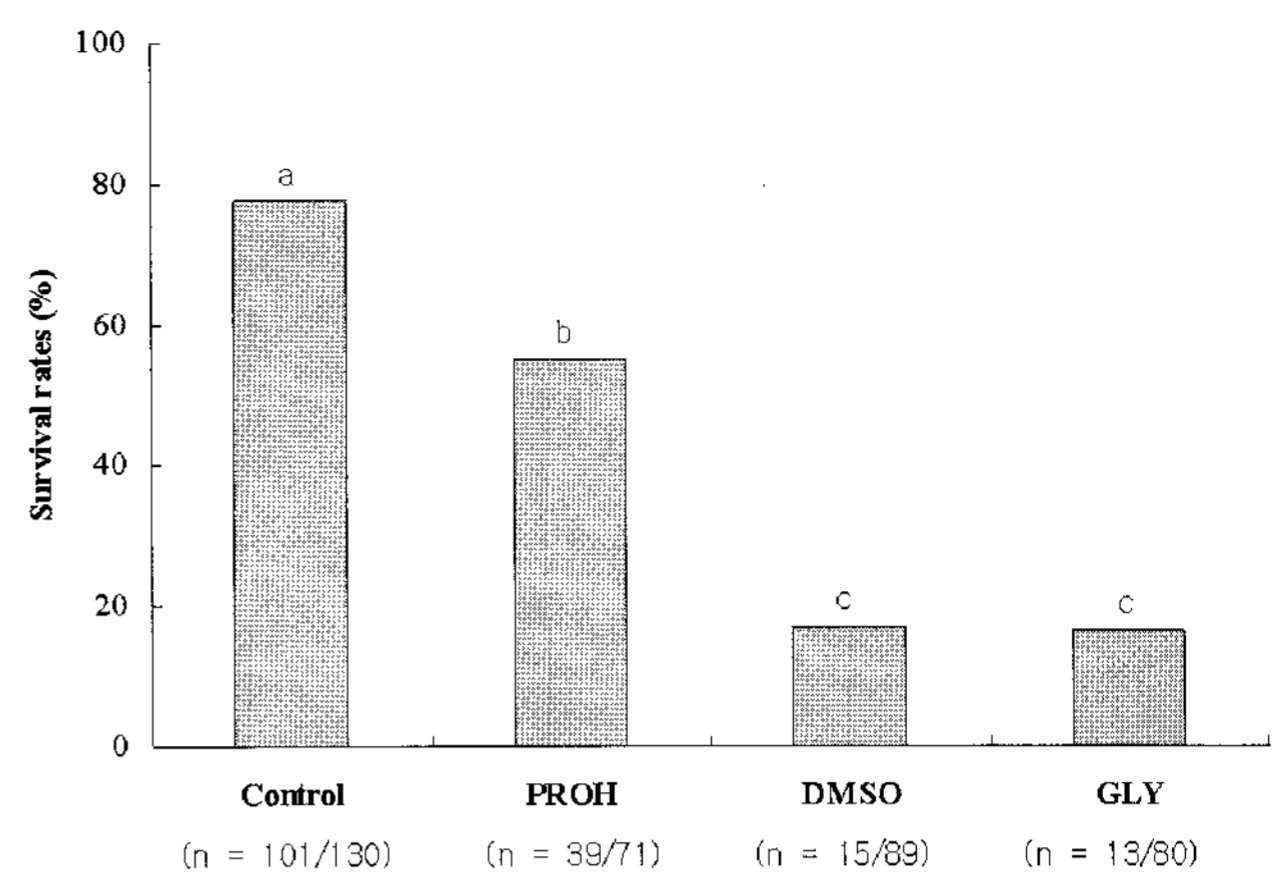
After *in vitro* maturation, the proportion of mature

oocytes that reached metaphase II stage was 36.6% for PROH treatment group which was significantly higher rates ($p<0.05$) compared with the rate obtained from DMSO (9.0%) and GLY (7.5%) treatment groups (Table 1). The proportion of mature oocytes in PROH treatment group did not differ that of fresh control (40.8%).

Diameter of Mature Oocytes

The metaphase II oocytes derived from *in vivo* and fresh and PROH frozen-thawed preantral follicles were used for measuring oocyte diameter. The diameter of metaphase II oocytes from frozen group (67.4±1.8 μm) was significantly smaller than that of the fresh (69.1±2.3 μm) and *in vivo* (71.7±2.1 μm) groups (Fig. 3).

DISCUSSION

This study was carried out to determine optimal cryoprotectant for effective cryopreservation of mouse preantral follicles using slow freezing. Successful freez-

Table 1. *In vitro* maturation of mouse preantral follicles after cryopreservation using different cryoprotectants

Cryoprotectant	No. of follicles cultured	GV (%)[1]	GVBD (%)[2]	Meta II (%)[3]
Control	130	36 (27.7)	12 (9.2)	53 (40.8)[a]
PROH	71	6 (8.5)	7 (9.9)	26 (36.6)[a]
DMSO	89	4 (4.5)	3 (3.4)	8 (9.0)[b]
GLY	80	5 (6.3)	2 (2.5)	6 (7.5)[b]

[1] Germinal Vesicle.
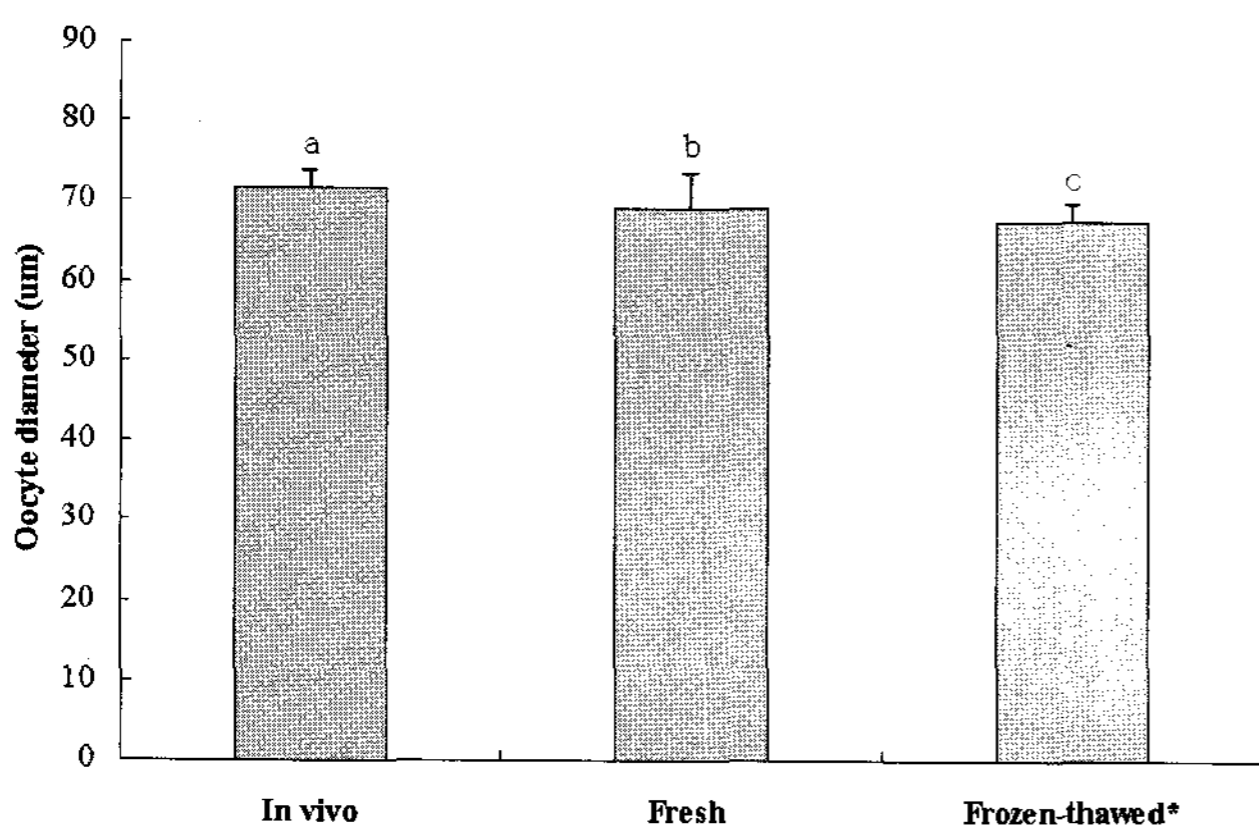[2] Germinal Vesicle Break-Down.
[3] Metaphase II.
[a,b] $p<0.05$.

Fig. 3. Diameter of oocytes in fresh and cryopreserved mouse preantral follicles after *in vitro* growth and maturation. * Mouse preantral follicles were frozen in 1.5 M PROH. [a,b,c] $p<0.05$.

ing procedures rely on the removal of intracellular water to avoid the lethal effects of the formation of large amounts of intracellular ice during cooling and warming (Candy *et al.*, 1997). Cryoprotectants are used to remove and/or substitute for the intracellular water before begins. The ability of cryoprotectants to permeate cell membranes varies and thus affects the concentration of cryoprotectant that can penetrate a cell and the rate of permeation in and out of the cell (Candy *et al.*, 1997).

In the present study, preantral follicles from the ICR mice ovary were cryopreserved in PROH, DMSO or GLY. About 70% of preantral follicles appeared morphologically normal immediately after freezing and thawing and showed survival rate of cryopreserved preantral follicles was not difference between the PROH, DMSO and GLY. This finding is consistent with previous observations. More than 70% of follicles survived in mouse preantral follicles frozen in 1.5 M DMSO (Carroll *et al.*, 1990; Cortvrindt *et al.*, 1996). In domestic cats preantral follicles frozen in DMSO and PROH, 33% and 29% of follicles appeared normal immediately after freezing and thawing (Jewgenow *et al.*, 1998).

In the present study, the cryopreserved preantral follicles were cultured on collagen-coated Transwell-COL membrane inserts (Eppig and Schroeder, 1989; Kim *et al.*, 2004). This culture system allowed the attachment and maintenance of the complexes with only minimal migration of the granulosa cells from the oocytes. The comparison of survival rate of cryopreserved preantral follicles after 10 days of *in vitro* culture showed that PROH freezing had a significantly higher survival rates than DMSO or GLY freezing. The metaphase II rate of oocytes within cryopreserved preantral follicles after *in vitro* growth and maturation was also significantly higher in PROH freezing than in DMSO or GLY freezing. One explanation for the poor survival and mature (metaphase II) oocyte rates recorded after

freezing preantral follicle in DMSO and GLY may be that the solute has a slow permeation rate, thus oocyte and their surrounding granulosa cells are not adequately protected from freezing damage. Isolated mouse oocytes are known to be less permeable to GLY than DMSO and PROH (Payner *et al.*, 1995, 1996). The low permeability of cells to GLY may increase the risk of osmotic stress during thawing and dilution as water enters the cell more quickly than GLY is lost (Candy *et al.*, 1997). Previous studies showed that about 40% of oocytes in mouse preantral follicles frozen in DMSO developed to metaphase II oocytes (Carroll *et al.*, 1990; Cortvrindt *et al.*, 1996). This result is inconsistent with our observation. This is probably due to the difference of method for preantral follicle isolation, exposure time in cryoprotectant, equilibration temperature, and method for *in vitro* culture.

The diameter of oocytes is an important index for the cytoplasmic maturation and developmental competency of oocytes (Kim *et al.*, 2005). In the present study, the diameter of metaphase II oocytes from preantral follicles cryopreserved in 1.5 M PROH was significantly smaller than that of the fresh. This result is probably due to the damage of granulosa cells surrounding oocytes during freezing and thawing. Throughout the oocyte growth in ovary, granulosa cells are coupled with oocytes via heterologous gap junctions (Anderson and Albertini, 1976) through which small molecules such as energy substrate, nucleotides and amino acids are transferred into the oocytes (Heller *et al.*, 1981; Brower and Schultz, 1982). It is therefore important that active metabolic cooperativity is maintained between the oocytes and granulosa cells through gap junctions under the *in vitro* culture conditions (Hirao *et al.*, 1994).

In conclusion, the results of the present study demonstrated that PROH is the most effective cryoprotectant for the cryopreservation of mouse preantral follicles. Further studies are needed to verify developmental capacity of *in vitro* grown and matured oocytes derived from cryopreserved preantral follicles after *in vitro* fertilization.

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