Journal of Reproduction and Fertility (1994) **100**, 123–129

Quick freezing of unfertilized mouse oocytes using ethylene glycol with sucrose or trehalose

A. A. Rayos, Y. Takahashi, M. Hishinuma and H. Kanagawa

Department of Theriogenology, Faculty of Veterinary Medicine, Hokkaido University, Sapporo 060, Japan

Unfertilized mouse oocytes were frozen by directly plunging them into liquid nitrogen vapour after equilibration in a freezing medium containing 3 mol ethylene glycol l^{-1} with 0.25 mol sucrose or trehalose l^{-1} for 5–40 min. After thawing and dilution of the cryoprotectant, oocytes of normal morphology were inseminated *in vitro* and the effect of equilibration period on the rates of fertilization and development *in vitro* was examined. Regardless of the equilibration in the freezing medium, no significant difference was observed on the fertilization rate of frozen–thawed oocytes. However, higher fertilization and higher normal fertilization rates were obtained with equilibration in 3 mol ethylene glycol l^{-1} with either 0.25 mol sucrose l^{-1} or trehalose for 20 and 40 min than with 5 and 10 min equilibration. Development rates to two-cell embryos and expanded blastocysts of *in vitro* fertilized frozen–thawed oocytes that were equilibrated in the freezing medium for 20 and 40 min were significantly higher ($P < 0.05$ or $P < 0.01$) than with 5 min equilibration. Development *in vivo* was assessed by transferring blastocysts derived from unfertilized oocytes frozen by the optimum treatment (20 min equilibration in the freezing medium before freezing) into the uterine horns of day 3 pseudopregnant female recipients. The development rate of frozen–thawed oocytes to the blastocyst stage after insemination *in vitro* was significantly lower than that of the non-frozen control ($P < 0.001$). However, transfer of the blastocysts derived from frozen–thawed oocytes to the uterine horns of the recipients resulted in fetal development and implantation rates similar to those of the control. The overall development rates to fetuses of blastocysts derived from *in vitro* fertilization of mouse oocytes frozen after 20 min equilibration in 3 mol ethylene glycol l^{-1} with 0.25 mol sucrose l^{-1} or trehalose were 20.3 and 22.5%, respectively.

Introduction

Success in the cryopreservation of mouse embryos with development to normal young after transfer of embryos to recipients was reported by Whittingham *et al.* (1972). Since then, several studies have been performed using mouse embryos as experimental models, and the embryos of at least 15 mammalian species have been successfully cryopreserved (Leibo, 1989).

In combination with other reproductive technologies, cryopreservation of oocytes can be used for the management of human infertility, livestock breeding and conservation of rare genetic stocks. Cryopreservation of unfertilized mouse oocytes by slow cooling has been reported by Tsunoda *et al.* (1976), Parkening *et al.* (1976), Whittingham (1977), Fuller and Bernard (1984), Glenister *et al.* (1987) and Ko and Threlfall (1988). However, compared with the success rate obtained with embryos, the development rates of frozen–thawed oocytes are still low.

Received 8 March 1993.

Earlier studies on oocyte cryopreservation used the slow or conventional cooling procedures. Attempts at improving the techniques have been directed to the simplification of the freezing procedures. Nakagata (1989), Kono *et al.* (1991) and Shaw *et al.* (1991) reported high development rates of vitrified-warmed mouse oocytes after fertilization *in vitro*. Surrey and Quinn (1990) successfully cryopreserved unfertilized mouse oocytes by a combined process of dehydration of the oocyte with sucrose and permeation with dimethyl sulfoxide before plunging the oocytes into liquid nitrogen (ultrarapid freezing). In the studies of Rayos *et al.* (1992a, b), early stage mouse embryos were successfully cryopreserved after determining the optimum equilibration period in 3 mol ethylene glycol l^{-1} with 0.25 mol sucrose l^{-1} or lactose before plunging them into liquid nitrogen vapour (quick freezing). Unfertilized mouse oocytes have low permeability to cryoprotectants (Mazur *et al.*, 1976; Jackowski *et al.*, 1980); equilibration in the freezing medium is therefore of critical importance for survival before quick freezing (Szell and Shelton, 1986; Takahashi and Kanagawa, 1990).

This study was conducted to examine the effect of equilibration period in 3 mol ethylene glycol l^{-1} with 0.25 mol sucrose l^{-1} or trehalose on the post-thaw, post-dilution survival, fertilization *in vitro* as well as *in vivo* development rates of unfertilized mouse oocytes cryopreserved by a quick freezing method.

Trehalose, a non-reducing disaccharide of glucose, which has been found to maintain membrane integrity during dehydration (Crowe and Crowe, 1984), was used as a non-permeable cryoprotectant in combination with glycerol in the rapid (Krag *et al.*, 1985) and conventional (Honadel *et al.*, 1988) freezing of early preimplantation mouse embryos. Compared with sucrose, trehalose was also reported by Kim *et al.* (1986) to improve the viability of mouse morulae frozen ultrarapidly. The use of trehalose as a cryoprotectant for the cryopreservation of unfertilized oocytes was mentioned briefly by Bernard *et al.* (1990). This study also compares trehalose with sucrose as a non-permeable cryoprotectant in the quick freezing of unfertilized mouse oocytes.

Materials and Methods

Experimental animals

Female F_1 hybrid (C57BL/6J × CBA) mice, 6–8 weeks of age and 20–25 g, were used as oocyte donors. Female ICR strain mice, 10–12 weeks of age and 30 to 40 g, were used as recipients. They were maintained in a room with controlled lighting 14 h light (05:00 to 19:00 h):10 h dark cycle and given commercial feed and water *ad libitum*. Female F_1 mice were induced to superovulate by i.p. injections of 5 iu pregnant mares' serum gonadotrophin (PMSG) (Serotrophin: Teikoku Zoki, Tokyo) and 5 iu hCG (Gonatrophin: Teikoku Zoki) given 48 h apart. Fourteen to fifteen hours after hCG injection, they were killed by cervical dislocation and unfertilized oocytes were released from excised oviducts into modified phosphate-buffered saline (PB1; Whittingham, 1971) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Inc., Grand Island, NY) and containing hyaluronidase (150 U ml^{-1}; bovine testis type I-S: Sigma Chemical Co., St Louis, MO) for 5–10 min. When the cumulus cells were detached, the oocytes were washed three times in fresh PB1 medium with 10% FBS and pooled in sterile plastic dishes (Nunc, Nunclon, Kamstrup) until used. Only oocytes with normal morphology were used in this study.

Freezing procedure

A modified freezing method described by Takahashi and Kanagawa (1985, 1990) was used in this study. Briefly, the oocytes were pipetted into a plastic dish containing the freezing medium (3 mol ethylene glycol l^{-1} with either 0.25 mol sucrose l^{-1} or trehalose) in PB1 with 10% FBS. After the oocytes had settled at the bottom of the dish, 10–15 oocytes together with the freezing medium were drawn into a 0.25 ml French straw (IMV, L'Aigle) which was heat sealed and equilibrated for 5, 10, 20 and 40 min. The equilibration period included the time the oocytes were in the dish containing the freezing medium. The volume of the freezing medium containing the oocytes was 135 µl and was separated by air bubbles on each side and the remainder of the straw was filled with the same medium. After the assigned equilibration period was attained, the oocytes were frozen in liquid nitrogen vapour (about − 170°C), by placing the sealed straw horizontally on a styrofoam plate (140 mm × 60 mm × 5 mm) with a stainless steel mesh on its upper surface and floating in a liquid nitrogen bath. Two minutes later, the straw was plunged into liquid nitrogen and stored for 1–120 days.

Thawing and dilution

Thawing was performed in a water bath at 37°C for 20 s. The contents of the straw were then expelled into a Petri dish and the oocytes were immediately pipetted into 0.5 mol l^{-1} of the same sugar used in the freezing medium for one-step dilution of the cryoprotectant. After 5 min, the oocytes were washed three times with PB1 with 10% FBS and oocytes with normal morphology were transferred into 0.4 ml of TYH medium (Toyoda *et al.*, 1971) supplemented with 4 mg BSA ml^{-1} (> 98% (GE) Sigma Chemical Co.) under paraffin oil for 30 min in an atmosphere of 5% CO_2 in air at 37°C before fertilization *in vitro*.

Fertilization in vitro

In vitro fertilization was carried out according to the method of Toyoda *et al.* (1971). All oocytes with normal morphology after freezing and thawing (surviving oocytes) as well as the non-frozen control, underwent insemination *in vitro*. Semen was collected from the cauda epididymides, one from each of two mature F_1 (C57BL/6J × CBA) males, allowed to disperse in 0.4 ml TYH medium under paraffin oil and incubated in an atmosphere of 5% CO_2 in air at 37°C for 30 min. Thereafter, the sperm concentration was determined by means of a haemocytometer and the spermatozoa were incubated for a further 1–1.5 h. A suitable volume (4–10 µl) of spermatozoa was added to the medium containing the oocytes and incubated for 6 h, providing a final sperm concentration of 150 cells $µl^{-1}$.

The fertilization rate was assessed by removing frozen–thawed as well as the non-frozen control oocytes 6 h after insemination, mounting them on a slide, fixing with 10% neutral formalin overnight, washing with 99.5% ethanol, staining with 0.25% aceto-lacmoid, and then examining the slides under a phase-contrast microscope for evidence of fertilization. Oocytes were considered as fertilized when they had an enlarged sperm head(s) or male pronucleus(ei) with corresponding sperm tail(s). The frequency of normal fertilization was determined as a ratio of oocytes with a pair of pronuclei and corresponding sperm tail to the total number of oocytes evaluated.

Culture in vitro

In a separate experiment, frozen–thawed and control oocytes of normal morphology also underwent insemination *in vitro* as described above. The oocytes were then washed three times with Whitten's medium (Whitten, 1971) supplemented

Table 1. The effect of equilibration period in 3 mol ethylene glycol l^{-1} with 0.25 mol sucrose l^{-1} on the fertilization rate of mouse oocytes cryopreserved by a quick freezing method

Equilibration period (min)	Number of oocytes frozen	Number (%) surviving after thawing and dilution and inseminated *in vitro*	Number (%) fertilized†	Number (%) normally fertilized‡	Overall normal fertilization rate (%)§
5	142	74 (52.1)[a]	57 (77.0)**	42 (56.8)***	29.6[a]***
10	125	84 (67.2)[b]	67 (79.8)**	51 (60.7)***	40.8[ab]***
20	133	103 (77.4)[b]	87 (84.5)	71 (68.9)**	53.4[b]***
40	127	98 (77.2)[b]	83 (84.7)	66 (67.3)**	52.0[b]***
Non-frozen control		94	88 (93.6)	82 (87.2)	87.2

†Number fertilized: number of oocytes inseminated *in vitro*.
‡Number normally fertilized: number of oocytes inseminated *in vitro*.
§Number normally fertilized: number of oocytes frozen.
Values with different superscripts in the same column are significantly different ([ab]$P < 0.01$).
Values with asterisks are significantly different from the control (**$P < 0.01$; ***$P < 0.001$).

with 3 mg BSA ml^{-1} 6 h after insemination *in vitro* before they were cultured in 100 µl of Whitten's medium with 100 µmol EDTA l^{-1} (Kanto Chemical Co., Tokyo) under paraffin oil at 37°C in 5% CO_2 in air. After 19 h of incubation (24 h after insemination), the culture was examined under inverted microscope and the number of two-cell embryos was recorded. Development to the expanded blastocyst stage was assessed 110–120 h after insemination.

All manipulations were performed at room temperature (20–25°C). Survival of the frozen–thawed oocytes was assessed at the following level: proportion of oocytes of normal morphology after thawing and dilution, development to two-cell embryos and to expanded blastocysts.

Parthenogenetic activation

Parthenogenetic activation of oocytes after exposure to a cryoprotectant, cooling, thawing and dilution has been reported by Tarkowski (1975), Shaw and Trounson (1989) and Kono *et al.* (1991). Oocytes frozen after equilibration in 3 mol ethylene glycol l^{-1} with 0.25 mol sucrose l^{-1} or trehalose for 5–40 min were incubated in TYH medium for 6 h without sperm and cultured in Whitten's medium for 4 days, to determine whether oocyte activation occurs after quick freezing and thawing.

Development in vivo

Blastocysts derived from oocytes frozen by the optimum treatment (20 min equilibration in 3 mol ethylene glycol l^{-1} with either 0.25 mol sucrose l^{-1} or trehalose) were transferred into the uterine horns of day 3 pseudopregnant female ICR recipients (six to nine embryos per horn) to assess viability *in vivo*. The recipients were killed on day 18 of pregnancy, and the number of live and resorbing fetuses, as well as the implantation sites were determined. Blastocysts developing from non-frozen oocytes after insemination and culture *in vitro* were also transferred into the uterine horns of the recipients to serve as controls.

Statistical analysis

Experiments were repeated four times and data on survival after thawing and dilution, fertilization *in vitro*, and development *in vitro* and *in vivo* of the frozen–thawed oocytes were analysed by χ^2 test.

Results

Morphology of oocytes after thawing and dilution

More than 90% of the frozen oocytes were recovered after thawing and no significant difference was observed in the post-thaw recovery, regardless of the equilibration period. After thawing and dilution, the number of oocytes of normal morphology frozen after 10, 20 or 40 min equilibration in 3 mol ethylene glycol l^{-1} with either 0.25 mol sucrose l^{-1} or trehalose was significantly higher ($P < 0.01$) than with 5 min equilibration (Tables 1–4). No significant difference on the survival (normal morphology) of frozen mouse oocytes after thawing and dilution was observed irrespective of the sugar (sucrose or trehalose) used in combination with ethylene glycol.

Fertilization rate

No significant difference on the fertilization rate among mouse oocytes equilibrated in 3 mol ethylene glycol l^{-1} with either 0.25 mol sucrose l^{-1} or 0.25 mol trehalose l^{-1} before plunging into liquid nitrogen vapour was observed (Tables 1 and 2). However, higher fertilization and higher normal fertilization rates were obtained with 20 and 40 min equilibration in the freezing medium than with 5 or 10 min equilibration. Moreover, the fertilization rates of the oocytes cryopreserved after 20 and 40 min equilibration were not significantly different from the control value, while the fertilization rates of oocytes cryopreserved after 5 or 10 min in the freezing medium were significantly lower ($P < 0.01$) than those of the controls. Most of the frozen–thawed oocytes that had

Table 2. The effect of equilibration period in 3 mol ethylene glycol l^{-1} with 0.25 mol trehalose l^{-1} on the fertilization rate of mouse oocytes cryopreserved by a quick freezing method

Equilibration period (min)	Number of oocytes frozen	Number (%) surviving after thawing and dilution and inseminated *in vitro*	Number (%) fertilized†	Number (%) normally fertilized‡	Overall normal fertilization rate (%)§
5	123	68 (55.3)^{a}	53 (77.9)**	35 (55.9)***	30.9^{a}***
10	114	76 (66.7)^{ab}	59 (77.6)**	45 (59.2)***	39.5^{ab}***
20	101	72 (71.3)^{b}	62 (86.1)	50 (69.4)**	49.5^{b}***
40	124	38 (71.0)^{b}	75 (85.1)	60 (68.4)**	48.4^{b}***
Non-frozen control		94	88 (93.6)	82 (87.2)	87.2

†Number fertilized: number of oocytes inseminated *in vitro*.
‡Number normally fertilized: number of oocytes inseminated *in vitro*.
§Number normally fertilized: number of oocytes frozen.
Values with different superscripts in the same column are significantly different ($^{ab}P < 0.01$).
Values with asterisks are significantly different from the control (**$P < 0.01$; ***$P < 0.001$).

Table 3. The effect of equilibration period in 3 mol ethylene glycol l^{-1} with 0.25 mol sucrose l^{-1} on the *in vitro* development of mouse oocytes cryopreserved by a quick freezing method

Equilibration period (min)	Number of oocytes frozen	Number (%) surviving after thawing and dilution and inseminated *in vitro*	Number (%) developed to two-cell†	Number (%) developed to expanded blastocyst‡	Overall rate of development to expanded blastocyst (%)§
5	216	110 (50.9)^{a}	65 (59.1)^{c}	52 (47.3)^{a}	24.1^{a}
10	204	148 (72.5)^{b}	95 (64.2)^{cd}	77 (52.0)^{ab}	37.7^{b}
20	220	169 (76.8)^{b}	118 (69.8)^{d}	99 (58.6)^{ab}	45.0^{b}
40	225	172 (76.4)^{b}	122 (70.9)^{d}	100 (58.1)^{b}	44.4^{b}
Non-frozen control		161	146 (90.7)***	141 (87.6)***	87.6***

†Number developed to two-cell embryos: number of oocytes inseminated *in vitro*.
‡Number developed to expanded blastocysts: number of oocytes inseminated *in vitro*.
§Number developed to expanded blastocysts: number of oocytes frozen.
Values with different superscripts in the same column are significantly different ($^{ab}P < 0.01$; $^{cd}P < 0.05$).
Values in the same column without asterisks are significantly different from the control (***$P < 0.001$).

undergone fertilization were normally fertilized, possessing a pair of pronuclei, but their normal fertilization rate was significantly lower than that of the controls ($P < 0.01$ or $P < 0.001$). Moreover, the rate of polyploidy of the frozen–thawed oocytes was significantly higher than in the controls ($P < 0.01$). The highest overall fertilization rate was obtained with 20 min equilibration in 3 mol ethylene glycol l^{-1} with either 0.25 mol sucrose l^{-1} (53.4%) or 0.25 mol trehalose l^{-1} (49.5%).

Development in vitro

Development rates to two-cell stage embryos of *in vitro* inseminated frozen–thawed oocytes that were equilibrated in the freezing medium for 20 or 40 min were significantly higher ($P < 0.05$) than with 5 min equilibration (Tables 3 and 4). However, development of oocytes equilibrated in the freezing medium for 10 min before quick freezing to two-cell embryos was not significantly different from development after 5, 20 or 40 min equilibration. Similarly, development to the expanded blastocyst stage of oocytes equilibrated for 20 or 40 min in 3 mol ethylene glycol l^{-1} with either 0.25 mol sucrose l^{-1} ($P < 0.01$) or 0.25 mol trehalose l^{-1} ($P < 0.05$) before quick freezing was significantly higher than with equilibration for 5 min. However, a high percentage (82–85%) of two-cell embryos obtained after insemination *in vitro* of frozen–thawed oocytes subsequently developed to the expanded blastocyst stage, regardless of the equilibration period. Development of the control oocytes after insemination *in vitro* to two-cell embryos and expanded blastocysts was significantly higher ($P < 0.001$) than for the frozen–thawed oocytes. The highest overall development rates to expanded blastocysts were attained when oocytes were frozen after equilibration for

Table 4. The effect of equilibration period in 3 mol ethylene glycol l^{-1} with 0.25 mol trehalose l^{-1} on the *in vitro* development of mouse oocytes cryopreserved by a quick freezing method

Equilibration period (min)	Number of oocytes frozen	Number (%) surviving after thawing and dilution and inseminated *in vitro*	Number (%) developed to two-cell†	Number (%) developed to expanded blastocyst‡	Overall rate of development to expanded blastocyst (%)§
5	225	119 (52.9)[a]	70 (58.8)[c]	61 (51.3)[c]	27.1[a]
10	224	158 (70.5)[b]	106 (67.1)[cd]	83 (52.5)[cd]	37.1[ab]
20	227	153 (67.4)[b]	112 (73.2)[d]	98 (64.1)[e]	43.2[b]
40	230	152 (66.1)[b]	117 (77.0)[d]	96 (63.2)[de]	41.7[b]
Non-frozen control		161	146 (90.7)***	141 (87.6)***	87.6***

†Number developed to two-cell embryos: number of oocytes inseminated *in vitro*.
‡Number developed to expanded blastocysts: number of oocytes inseminated *in vitro*.
§Number developed to expanded blastocysts: number of oocytes frozen.
Values with different superscripts in the same column are significantly different ([ab]$P < 0.01$; [cde]$P < 0.05$).
Values in the same column without asterisks are significantly different from the control (***$P < 0.001$).

Table 5. *In vivo* development of mouse oocytes quickly frozen using 3 mol ethylene glycol l^{-1} with 0.25 mol sucrose l^{-1} or trehalose

Group	Number of oocytes frozen	Number (%) surviving after thawing and dilution and inseminated *in vitro*	Number (%) developed to blastocysts and transferred to recipients†	Number (%) of fetuses‡	Overall fetal development rate (%)§
Frozen–thawed					
Sucrose	202	134 (66.3)	78 (58.2)[a]	41 (52.6)	20.3[a]
Trehalose	215	148 (68.8)	84 (56.8)[a]	44 (52.4)	20.5[a]
Non-frozen control		130	112 (86.2)[b]	63 (56.3)	48.5[b]

†Number of blastocysts: number of surviving oocytes inseminated *in vitro*.
‡Number of live fetuses: number of blastocysts transferred.
§Number of live fetuses: number of oocytes frozen.
[ab]$P < 0.001$.

20 min in 3 mol ethylene glycol l^{-1} with either 0.25 mol sucrose l^{-1} (45.0%) or 0.25 mol trehalose l^{-1} (43.2%).

Parthenogenetic activation

Parthenogenetic activation or cleavage of frozen–thawed oocytes, irrespective of the freezing medium used (3 mol ethylene glycol l^{-1} with either 0.25 mol sucrose l^{-1} or 0.25 mol trehalose l^{-1}) and the equilibration period, was not observed following 4 days of culture in Whitten's medium.

Development in vivo

The development *in vivo* of unfertilized mouse oocytes quickly frozen after 20 min equilibration in 3 mol ethylene glycol l^{-1} with either 0.25 mol sucrose l^{-1} or 0.25 mol trehalose l^{-1} is shown (Table 5). Development of frozen–thawed oocytes after insemination and culture *in vitro* to the blastocyst stage was significantly lower than in the controls ($P < 0.001$). Blastocysts derived from frozen–thawed and non-frozen control oocytes were transferred into the uterine horns of pseudopregnant recipients. The proportions of the blastocysts derived from frozen–thawed oocytes that developed to 18-day live fetuses (52.6 and 52.4%) were similar to the control values (56.8%). Moreover, the implantation rates between the blastocysts derived from frozen–thawed oocytes were not significantly different from the control (79.5 and 82.1% versus 85.7%). The overall rates of development to fetus of blastocysts derived from *in vitro* fertilization of mouse oocytes frozen after 20 min equilibration in 3 mol ethylene glycol l^{-1} with 0.25 mol sucrose l^{-1} or 0.25 mol trehalose l^{-1} were 20.3 and 20.5%, respectively.

Discussion

For the first time, unfertilized mouse oocytes were successfully frozen by a combined process of dehydration of the oocytes with sucrose or trehalose and permeation with ethylene glycol before plunging them into liquid nitrogen vapour.

The study reported here also confirmed reports of Szell and Shelton (1986), Takahashi and Kanagawa (1990) and Mazur (1990) that equilibration in a freezing medium containing high concentrations of cryoprotectant is of critical importance for the survival of embryos or oocytes (Surrey and Quinn, 1990) frozen at an extremely rapid rate than it is for those frozen by slow freezing. Abas *et al.* (1990) and Rayos *et al.* (1992a, b) indicated that optimum survival can be attained with 5 min equilibration in 3 mol ethylene glycol l^{-1} with 0.25 mol sucrose l^{-1} or 0.25 mol lactose l^{-1} for mouse morula, and 10 min for one-, two-, four- and eight-cell embryos, thus allowing sufficient time for the cryoprotectant to permeate into the embryonic cells. The study reported here showed that equilibration of unfertilized mouse oocytes for 20 or 40 min in the freezing medium before freezing results in a high proportion of oocytes of normal morphology, fertilization and development to two-cell embryos and blastocysts after thawing and dilution. The need for longer equilibration in the freezing medium as a requisite for high survival of mouse oocytes could be explained by the different permeabilities of fertilized and unfertilized oocytes (Jackowski *et al.*, 1980). The permeability of unfertilized oocytes to the cryoprotectant is low, but increases as development proceeds to the blastocyst stage after fertilization (Mazur *et al.*, 1976; Schneider and Mazur, 1984). The time required for embryos (5–10 min) may be insufficient for the permeation of the cryoprotectant into the oocytes to the extent that the cryoprotective agent can exert its protective effect. Although most of the frozen oocytes were of normal morphology after thawing, more oocytes frozen after short (5 min) equilibration may have suffered membrane damage that was not evident under the dissecting microscope. Thus, the number of degenerated frozen–thawed oocytes increased after dilution of the cryoprotectant.

Smorag *et al.* (1990) reported that trehalose has a better stabilizing effect on the cell membranes of one- and two-cell rabbit embryos than does sucrose and results in improved viability. Moreover, mouse morula equilibrated in glycerol–trehalose solution before plunging into liquid nitrogen had a higher development rate to expanded blastocysts than did those equilibrated in glycerol–sucrose solution (Kim *et al.*, 1986). In our study, however, no significant difference was observed on the survival, fertilizability and developmental capacity of frozen–thawed oocytes, regardless of the sugar used. This finding indicates that sucrose or trehalose in combination with ethylene glycol are effective in the quick freezing of unfertilized oocytes. Observations by Heyman *et al.* (1986) did not demonstrate the beneficial effect of trehalose in the rapid freezing of bovine non-mature oocytes and one-cell embryos and viability remained at the level obtained with sucrose. Thus, the possible stabilizing effect of trehalose on the cell membranes may be species- and cell-stage dependent.

Unfertilized mouse oocytes cryopreserved after 20 min equilibration in the freezing medium had a higher rate of normal fertilization than did 5, 10 or 40 min of equilibration. This finding was similar to the proportion of surviving frozen–thawed oocytes that developed to two-cell embryos 24 h after insemination and culture *in vitro*. However, subsequent development *in vitro* to expanded blastocysts of two-cell embryos derived from frozen–thawed oocytes was similar, regardless of the equilibration period. This finding shows that a high proportion of frozen–thawed oocytes that undergo normal fertilization can develop into two-cell embryos and subsequently into expanded blastocysts.

The fertilization and development rates of frozen–thawed oocytes were significantly lower than those of the controls, indicating that some membrane damage may have occurred during freezing because of intracellular ice formation and thawing, which could have interferred in the subsequent fertilization and development of the oocyte (Whittingham, 1977). Membrane damage may result from osmotic forces, including overexpansion of the cell membranes after loss of surface area during dehydration (Steponkus and Weist, 1979) and disruption of membrane integrity by mass movement of water (Muldrew and McGann, 1990). Failure of fertilization may also be due to a still undefined modification to the zona pellucida or the vitelline membrane during freezing and thawing which inhibits the entry of spermatozoa (Carroll *et al.*, 1989, 1990; Wood *et al.*, 1992).

An increased frequency of polyploidy has been reported by Carroll *et al.* (1989) in frozen–thawed oocytes compared with the non-frozen controls. Their findings showed that this was caused mainly by the retention of the second polar body and not by polyspermy. In contrast, Kono *et al.* (1991) reported that the increased incidence of polyploidy was due mainly to polyspermic fertilization in vitrified–warmed oocytes compared with the controls. This was in agreement with our present study in which the incidence of polyspermy, as indicated by the presence of two or more male pronuclei or enlarged sperm head(s), was significantly higher than in the controls.

During normal fertilization, fusion of a spermatozoon with the vitelline membrane of the oocyte leads to a cortical granule reaction. Sperm-specific receptors on the zona pellucida are inactivated by the constituents of the cortical granules and the molecular structure of the zona pellucida is altered. The vitelline membrane also becomes structurally changed by the reaction and these modifications block further sperm penetration. Metabolic changes brought about by freezing or thawing might cause the failure of the normal cortical granule reaction and allow polyspermic fertilization (Glenister *et al.*, 1987; Wood *et al.*, 1992). Moreover, physical damage to the vitelline membrane or to the zona pellucida during freezing and thawing may enable more than one spermatozoa to enter the egg (Whittingham and Adams, 1976).

There was no indication that any procedure involved in the freezing and thawing in the study reported here stimulated parthenogenetic activation. This finding was in agreement with reports by Whittingham (1977) and Shaw *et al.* (1991), who used conventional and vitrification methods, respectively. However, this result was in contrast to studies of Kono *et al.* (1991), who reported 2–22% parthenogenetic activation in vitrified–warmed oocytes.

Live 18-day normal fetuses were obtained after transfer of blastocysts derived from frozen–thawed oocytes that were inseminated and cultured *in vitro* at a rate similar to the control. Moreover, the implantation rates between the blastocysts obtained from frozen–thawed oocytes and the controls were not significantly different. Acceptable rates of oocyte survival, fertilizability *in vitro* and embryonic development *in vitro* and *in vivo* were obtained in this study and are comparable or superior to those of oocytes frozen by conventional (Tsunoda *et al.*,

1976; Parkening *et al.*, 1976; Whittingham, 1977; Fuller and Bernard, 1984; Glenister *et al.*, 1987; Ko and Threlfall, 1988) or ultrarapid (Surrey and Quinn, 1990) freezing methods. However, our rates were lower than those from studies in which oocytes were cryopreserved by vitrification (Nakagata, 1989; Kono *et al.*, 1991; Shaw *et al.*, 1991).

The study reported here demonstrated that ethylene glycol in combination with either sucrose or trehalose can be used effectively in the quick freezing of unfertilized mouse oocytes. The quick freezing technique represents an efficient, inexpensive and viable option for the cryopreservation of mouse oocytes.

This study was supported in part by a Grant-in-Aid for Scientific Research (No. 02556039) from the Ministry of Education, Science and Culture, Japan.

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