STUDIES ON THE QUICK FREEZING OF MOUSE EARLY-STAGE EMBRYOS AND UNFERTILIZED OOCYTES USING ETHYLENE GLYCOL

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CONTENTS

GENERAL INTRODUCTION

Over the past twenty years, the cryopreservation of embryos and oocytes has become a useful tool for interrupting and controlling reproductive cyclicity. Most research and application has concentrated on three species: mouse, cattle and humans. Mouse embryos and oocytes are used for basic and applied research on cryobiological mechanisms. Applications include banks of embryos or oocytes to protect inbred, congenic, and transgenic strains against loss by disease, genetic contamination, or natural disaster (LEIBO, 1989). Cryopreservation is an integral component of the cattle embryo transfer industry. The transport of germ plasm as embryos rather than live animals is more economical, convenient, poses less risk of disease transmission, and has added advantage of allowing imported stocks to develop in recipients well-adapted to local conditions. Human embryos or oocytes are frequently cryopreserved as an adjunct to in vitro fertilization and other assisted techniques for treatment of infertility.

The procedure of cryopreservation include initial exposure to and equilibration in the cryoprotectant, cooling to subzero temperature, storage in liquid nitrogen (LN_2), thawing, and finally dilution and removal of the cryoprotectant with return to a physiological environment that will allow further development. The cells must maintain structural integrity throughout the cryopreservation procedure. Major factors known to affect survival include the species,

developmental stage, the cryoprotectant, and the method of cryopreservation (FRIEDLER et al., 1988).

Embryos can survive freezing only when suspended in molar concentrations of solutions of compounds referred to as cryoprotectants. Effective cryoprotectants have low molecular weight, high solubility, and low toxicity at high concentrations (SCHNEIDER & MAZUR, 1984; LEIBO, 1989). Cryoprotectants are classified into permeating (glycerol, dimethyl sulfoxide, propylene glycol, ethylene glycol, other alcohols) and nonpermeating (polyvinylpyrrolidone, sucrose, lactose, trehalose, other sugars). Permeating cryoprotectants are thought to give protection against solution effects because of their colligative properties, and provide protection against injury from ice crystals. On the other hand, nonpermeating cryoprotectants are known to cause dehydration during equilibration or limit cellular swelling during dilution.

The methods used for embryo freezing have relied primarily on the calculations by MAZUR (1977) who demonstrated that injury to the cells cooled at supraoptimal rates was due to intracellular ice formation combined with growth of the intracellular ice by recrystallization during warming. Using the prediction that slow cooling to low temperature and slow warming would enable embryos to survive freezing and thawing, WHITTINGHAM et al. (1972), showed that mouse embryos could survive cryopreservation and develop to young. A major variation on this basic technique was introduced by

WILLADSEN (1977), who showed that, if embryos were cooled to relatively high subzero temperatures of -30 °C to -40 °C before plunging into LN_2, they could survive if thawed rapidly. These techniques require accurately controlled and expensive biological freezer to achieve the long period of slow cooling (0.1 to 0.5 °C/min) and also are time consuming. The slow cooling method was followed by the two-step cooling method, whereby embryos are first prededydrated before rapid cooling (12 °C/min to -30 °C), and rapid plunging into LN_2 (WOOD & FARRANT, 1980; RENARD et al., 1984; BUI-XUAN-NGUYEN et al., 1984).

A radical departure from the above cooling techniques has been the development of vitrification (FAHY et al.,1984) which has been used for the cryopreservation of embryos (RALL & FAHY, 1985; SCHEFFEN et al., 1986; KASAI et al., 1990) and oocytes (NAKAGATA, 1989; KONO et al.,1991; SHAW et al., 1991). To achieve vitrification, the cryoprotectant solution must be increased to 40% (wt/vol) or higher. At the concentration required for vitrification, these solutions are toxic at room temperature and embryos or oocytes can only be exposed to them at low temperature (about 0 °C). Vitrification is rapid because embryos are plunged directly into LN_2 and thawed by transfer from LN_2 to ice water. This method does not also require a biological freezer. However, the toxicity of the cryoprotectants and the need to handle embryos or oocytes at low temperature during exposure and removal of the cryoprotectants are disadvantages of vitrification.

Attempts to simplify cryopreservation techniques without the disadvantages of vitrification led to the development of quick or rapid and ultrarapid freezing methods. Survival of the embryos or oocytes is made possible because, before the temperature has dropped to a level that permits intracellular ice formation, the water content of the embryo or oocyte is reduced to a point at which the subsequent rapid cooling results in the formation of small innocuous intracellular ice crystals (MAZUR, 1990). The embryos or oocytes were initially prededydrated in a hypertonic solution containing two cryoprotectants, one permeating and the other, nonpermeating. Quick or rapid freezing is achieved by placing the embryos in the vapor phase of LN_2 at temperature of -170°C or -180°C (TAKAHASHI & KANAGAWA, 1985, 1990; WILLIAMS & JOHNSON,1986; SZELL & SHELTON, 1986,1987). Successful cryopreservation of embryos have also been reported by direct plunging into LN_2 (ultrarapid or snap freezing) following equilibration in the freezing medium (KRAG et al., 1985 BIERY et al.,1986; TROUNSON et al.,1987,1988).

Successful cryopreservation of mouse embryos by the quick or rapid freezing method was first achieved using glycerol in combination with sucrose (TAKEDA et al.,1984; SZELL & SHELTON, 1986, 1987; WILLIAMS & JOHNSON, 1986; BIERY et al.,1986), trehalose (KRAG et al, 1985), lactose, raffinose, glucose or xylose (TAKAHASHI & KANAGAWA, 1985, 1990). Mouse two- and eight-cell embryos have been cryopreserved

ultrarapidly using dimethyl sulfoxide or propylene glycol with sucrose (TROUNSON et al., 1987,1988; SATHANANTHAN et al.,1988). Using a modified ultrarapid freezing method, SURREY & QUINN (1990) successfully cryopreserved unfertilized mouse oocytes with dimethyl sulfoxide in combination with sucrose.

Recently, ABAS MAZNI et al. (1989,1990) obtained high survival rates with mouse morulae after 5 minutes equilibration in 3 M ethylene glycol with either 0.25 M sucrose or lactose before plunging into LN_2 vapor. Ethylene glycol was first used as a cryoprotectant by MIYAMOTO & ISHIBASHI (1977, 1978) for freezing of mouse and rat embryos using the conventional freezing method. In a similar study, high survival rates were obtained with mouse eight-cell to mid-blastocyst frozen with 1.5 M ethylene glycol compared with other commonly used cryoprotectants such as dimethyl sulfoxide and glycerol (URANO et al.,1986). Ethylene glycol has also been used in the freezing of day-6 to day-8 bovine embryos with survival rate similar to other cryoprotectants (ELSDEN et al.,1982). Most recently, ethylene glycol was successfully used in the cryopreservation of sheep embryos with high in vitro and vivo survival rates (McGINNIS et al.,1993). This cryoprotectant has also been shown to be less toxic to mouse morulae at 20°C than dimethyl sulfoxide, erythritol, dimethyl formamide and sucrose (KASAI et al.,1981, 1990). Because of its low toxicity even at high concentrations, ethylene glycol has recently been used as a main component of newly developed vitrification solutions

(KASAI et al., 1990; ISHIMORI et al., 1992; VALDEZ et al., 1992).

As mentioned above, the use of ethylene glycol as a cryoprotective agent in conventional, quick freezing or vitrification methods has been limited to eight-cell and late-stage embryos (morulae and blastocysts). The only available papers (ABAS MAZNI et al., 1989,1990) reported on quick freezing of mouse compacted morulae using ethylene glycol.

This simple quick freezing method could be used for routine cryopreservation of mouse embryos or embryos of other species, if it could be shown to be effective over a range of embryonic stages. Moreover, the advent of new biotechnologies such as in vitro production of embryos, cloning, gene transfer and sex determination demands for the cryopreservation of unfertilized oocytes and early-stage embryos. It is therefore important that quick freezing method, because of its speed, ease and economy be successfully applied not only to early-stage embryos but also to unfertilized oocytes.

In the quick or rapid freezing methods (MIYAMOTO & ISHIBASHI, 1986; SZELL & SHELTON, 1986; TAKAHASHI & KANAGAWA, 1990) the equilibration of the embryos to a solution containing high concentration of cryoprotectants seems to be of critical importance for embryo viability and the effect of equilibration is due to the permeation of the cryoprotectant into the embryonic cells. However, prolonged equili-

bration in the freezing medium containing high concentration of cryoprotectants results in low embryo survival due to chemical toxicity and/or osmotic shock (RALL, 1987).It has been reported that optimal methods for freezing and thawing differ among embryos at different developmental stages (SCHNEIDER & MAURER, 1983; MASSIP et al., 1984). When quick freezing is applied to early-stage embryos and unfertilized oocytes, longer equilibration in the freezing medium may be necessary to give optimum protection because of their larger surface area (LEIBO et. al., 1974), and their permeability coefficiency to the cryoprotectant is much lower than the more advanced stages such as morula and blastocyst (MAZUR et al., 1976). The use of inherently less toxic cryoprotectants such as ethylene glycol which permits prolonged equilibration even at high concentration could prevent injury due to chemical toxicity or excessive osmotic stress during equilibration or subsequent dilution.

This thesis work was conducted to examine the effectiveness of ethylene glycol in combination with sucrose, lactose or trehalose in the quick freezing of early-stage (one-, two-, four-, and eight-cell) mouse embryos and unfertilized oocytes. In order to determine the optimum survival in vitro and/or in vivo for early-stage mouse embryos and oocytes, the effect of equilibration period was studied in 3 M ethylene glycol with either 0.25 M sucrose, lactose or trehalose before plunging into LN_2 vapor.

PART 1. STUDIES ON THE QUICK FREEZING OF EARLY-STAGE MOUSE EMBRYOS USING ETHYLENE GLYCOL

CHAPTER I

QUICK FREEZING OF MOUSE TWO-, FOUR-, AND EIGHT-CELL EMBRYOS USING ETHYLENE GLYCOL WITH SUCROSE OR LACTOSE: EFFECTS OF DEVELOPMENTAL STAGE AND EQUILIBRATION PERIOD ON SURVIVAL IN VITRO

INTRODUCTION

It has been reported that mouse preimplantation embryos can be successfully cryopreserved after exposure to high concentration of cryoprotectants at room temperature before plunging into LN_2 or LN_2 vapor. Many of these investigations utilized glycerol on mouse embryos with sucrose (TAKEDA et al., 1984; SZELL & SHELTON, 1986, 1987; WILLIAMS & JOHNSON, 1986; BIERY et al., 1986), trehalose (KRAG et al.,1985), lactose, raffinose, glucose, or xylose (TAKAHASHI & KANAGAWA, 1985, 1990). Mouse two-cell and eight-cell embryos have also been successfully cryopreserved by the ultrarapid freezing method using dimethyl sulfoxide or propylene glycol with sucrose (TROUNSON et al., 1987, 1988; Sathananthan et al.,1988).

Recently, ABAS MAZNI et al. (1989,1990) obtained high survival rates with mouse morula after equilibration in 3 M ethylene glycol with either 0.25 M sucrose or lactose for 5 minutes before plunging into LN_2 vapor. This quick freezing method could be used for routine cryopreservation of mouse embryos or other mammalian species, if it could be shown to be effective over a range of embryonic stages. However, no

similar studies have been conducted on mouse early cleavage-stage embryos using the same freezing medium.

Equilibration of the embryos in the freezing medium containing a high concentration of cryoprotectant before quick freezing seems to be of critical importance for embryo survival (MIYAMOTO & ISHIBASHI, 1986; SZELL & SHELTON, 1986; TAKAHASHI & KANAGAWA,1990). This study was therefore conducted to examine the effect of equilibration period in 3 M ethylene glycol with either 0.25 M sucrose or lactose on the viability of mouse two-, four-, and eight-cell embryos cryopreserved by a quick freezing method.

MATERIALS AND METHODS

Animals and embryo recovery

Female ICR mice, 4 to 6 weeks of age and 20 to 25 g in body weight were used as embryo donors. They were maintained on a 14-hour light (5:00 to 19:00 hour): 10-hour dark cycle, and were given pelleted food and water ad libitum.

The female mice were induced to superovulate by intraperitoneal injections of 5 IU pregnant mare serum gonadotropin (PMSG, Serotrophin, Teikoku Zoki, Tokyo, Japan) and 5 IU human chorionic gonadotropin (hCG, Gonatrophin, Teikoku Zoki) given 48 hours apart. After hCG injection, they were paired singly with male of the same strain and inspected the following day for the presence of vaginal plug. The female mice were killed by cervical dislocation and two-, four-, and eight-cell embryos were flushed from the oviduct at 46, 58 and 66 hours after hCG injection using Dulbecco's phos-

phate-buffered saline (PBS, Nissui Pharmaceutical Co.,Tokyo, Japan) in which metal salt solution containing calcium and magnesium (Nissui Pharmaceutical Co.) was added and supplemented with 10% heat-treated calf serum (CS, Gibco Laboratories, Life Technology Inc., New York, USA). After collection, morphologically normal embryos were washed three times with PBS plus 10% CS, then pooled and held at room temperature in sterile plastic culture dish (Nunc, Nunclon, Kamstrup, Denmark) until used.

Freezing procedure

A modified quick freezing method previously described by TAKAHASHI & KANAGAWA (1985,1990) was used in this study. Briefly, the embryos were pipetted into the freezing medium containing 3 M ethylene glycol and a constant level of either 0.25 M sucrose or lactose in PBS plus 10% CS, and equilibrated for 5, 10, 20 and 40 minutes at room temperature. After having settled at the bottom of the culture dish, twelve to fifteen embryos were drawn into 0.25-ml French straw (I.M.V. L' Aigle, France) and the straw was heat-sealed. The volume of the freezing medium containing the embryos was 135 μl and was separated by an air bubble on each side, with the remainder of the straw filled with the freezing medium (Fig. 1). After each equilibration period was attained, the embryos were frozen in LN_2 vapor (approximately - 170°C) by placing the sealed straw horizontally on a styrofoam plate (140 mm X 60 mm X 5 mm) that had a stainless steel mesh on its upper surface and floated in a liquid

nitrogen bath (Figure 2 and 3). Two minutes later, the straw was plunged into the LN_2 and stored for 1 to 60 days.

Thawing, dilution and culture procedure

Thawing was done in a water bath at 37 °C for 20 seconds. Thereafter, the contents of the straw were expelled into a Petri dish, and the embryos were immediately pipetted into PBS plus 10% CS and 0.5 M solution of the same sugar used in the freezing medium. After 5 minutes at room temperature, the embryos were transferred into PBS plus 10% CS for 5 min and then washed three times in microdrops of Whitten's Medium under paraffin oil before culture. The embryos were cultured by the microdroplet method (Brinster, 1963) in Whitten's medium at 37°C in 5% CO_2 in air for 96, 72, and 48 hours for two-, four-, and eight-cell embryos, respectively. Survival of the embryos was assessed by their capacity to develop into expanded blastocysts during culture.

Experiments were replicated four times and the data were analyzed for statistical significance using the Chi-square test.

The effect of exposure to the freezing medium on the survival of mouse two-, four-, and eight-cell embryos without freezing was also examined . Twelve to fifteen embryos were placed in 3 M ethylene glycol with either 0.25 M sucrose or lactose in PBS plus 10% CS for 5, 10, 20 and 40 minutes at room temperature. After the required exposure period was attained, the embryos were transferred into 0.5 M sucrose or lactose solution in PBS plus 10% CS for 5 minutes

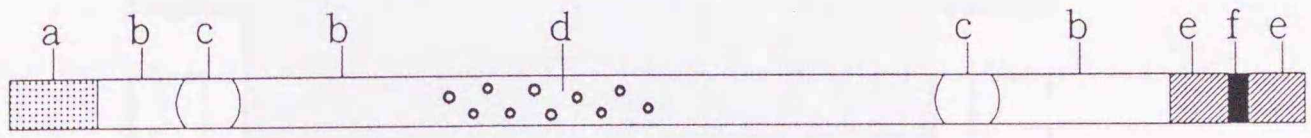

Fig. 1. Diagrammatic representation of a 0.25-ml French straw with 135 μl freezing medium containing the embryos or oocytes a) heat-sealed end b) freezing medium c) air bubble d) embryos or oocytes in freezing medium e) cotton plug f) PCV (polyvinylic alcohol).

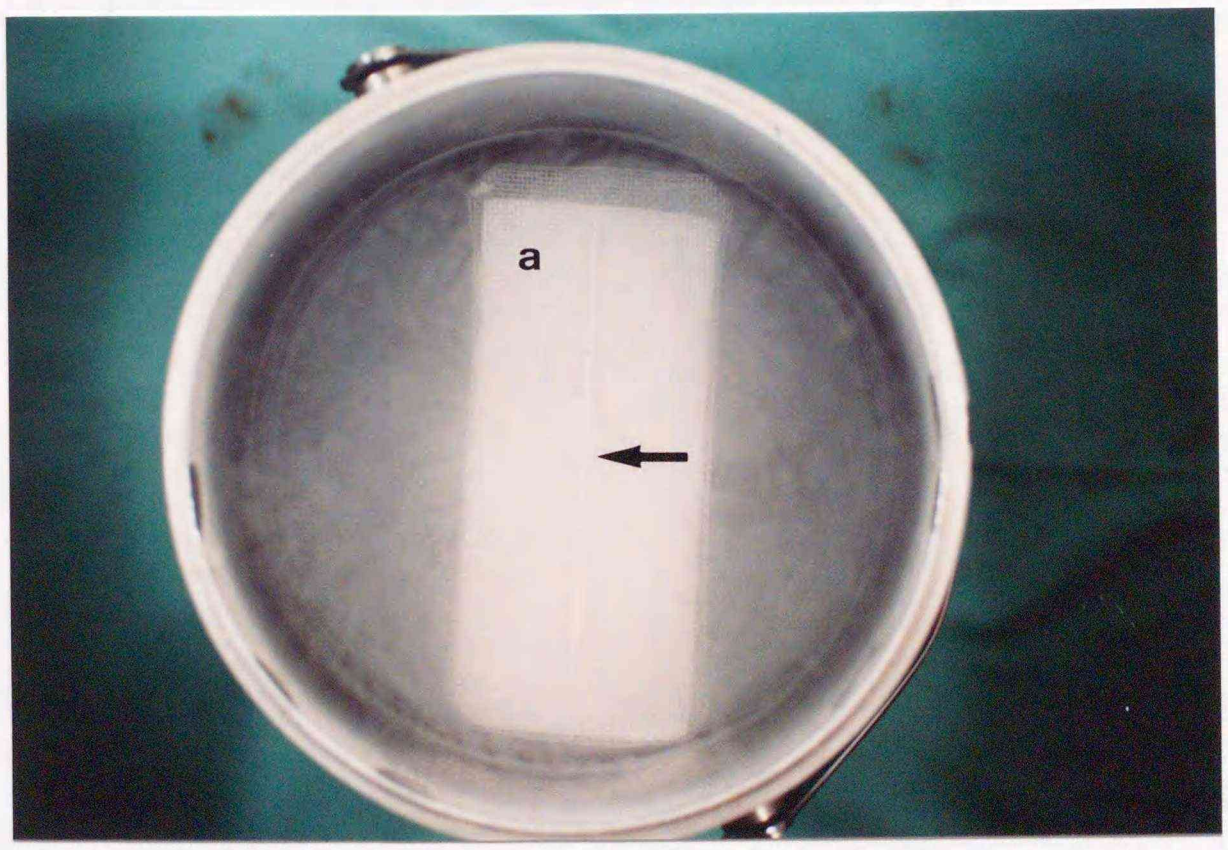

Fig. 2. A 0.25-ml French straw (arrow) loaded with embryos or oocytes and freezing medium placed horizontally on a styrofoam plate (a) floating in a liquid nitrogen bath.

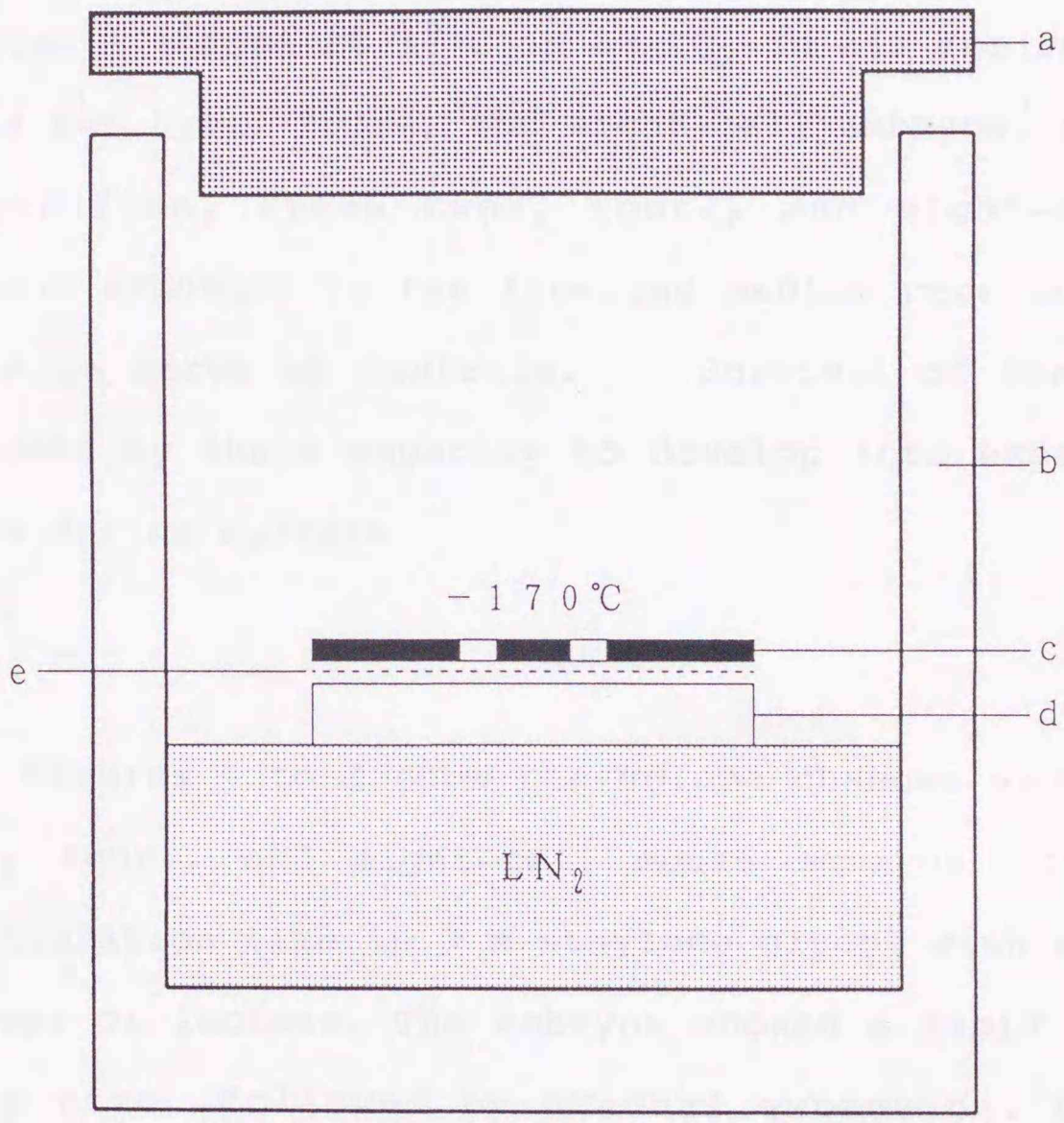

Fig. 3. Diagram of the quick freezing method. A plastic straw loaded with embryos or oocytes and freezing medium is placed horizontally on a styrofoam plate floating in a liquid nitrogen bath a) cover b) flask c) straw d) styrofoam plate e) wire mesh.

to remove the cryoprotectant from the cells. Thereafter, they were transferred in PBS plus 10% CS for 5 minutes, then washed three times with Whitten's medium (Whitten, 1971). Finally, the embryos were cultured in microdrops (100 μl) of Whitten's medium at 37°C in 5% CO_2 in air for 96, 72, and 48 hours for two-, four-, and eight-cell embryos, respectively. In addition, fresh two-, four-, and eight-cell embryos without exposure to the freezing medium were cultured similarly to serve as controls. Survival of the embryos was assessed by their capacity to develop into expanded blastocysts during culture.

RESULTS

Figures 1 to 3 show the volume changes and behavior of two-, four-, and eight-cell mouse embryos to increasing equilibration time in 3 M ethylene glycol with either 0.25 M sucrose or lactose. The embryos showed a rapid reduction in their size followed by gradual expansion. However, the embryos did not regain their original isotonic volumes even after 40 minutes equilibration in the freezing medium.

The effect of equilibration period on the post-thaw survival of early cleavage-stage cryopreserved by the quick freezing method using ethylene glycol with sucrose or lactose is shown in Tables 1 and 2. There was no significant difference on the survival of mouse two-, four-, and eight-cell stage embryos cryopreserved by the quick freezing method using ethylene glycol irrespective of the sugar

(sucrose or lactose) used.

When two- and four-cell embryos were equilibrated in 3 M ethylene glycol with either 0.25 M sucrose or lactose for 5 minutes, the post-thaw viability was significantly lower ($P<0.001$) than when equilibration was prolonged to 10, 20 or 40 minutes. No significant difference was obtained when eight-cell stage mouse embryos were equilibrated for 5, 10, 20 and 40 minutes in the freezing medium before plunging into LN_2 vapor.The highest survival rates were achieved at 10 minutes equilibration for two-, four-, and eight-cell embryos. However, slight but not significant decrease in the viability rate was observed when equilibration was prolonged to 20 and 40 minutes.

Table 3 shows the effect of exposure to 3 M ethylene glycol with either 0.25 M sucrose or lactose on the survival of mouse early cleavage-stage embryos without freezing. Very high percentages (89.2 to 96.4%) of two-, four-, and eight-cell embryos exposed to the freezing medium for 5, 10, 20 and 40 minutes without freezing developed to expanded blastocysts when cultured in vitro. The survival rates were similar to those of the control embryos.

DISCUSSION

The volume changes of two-, four-, and eight-cell mouse embryos in ethylene glycol with sucrose or lactose were characterized by an initial rapid shrinkage and subsequent gradual expansion. These findings strongly suggest that the rapid shrinkage of the embryonic cells (dehydration) was

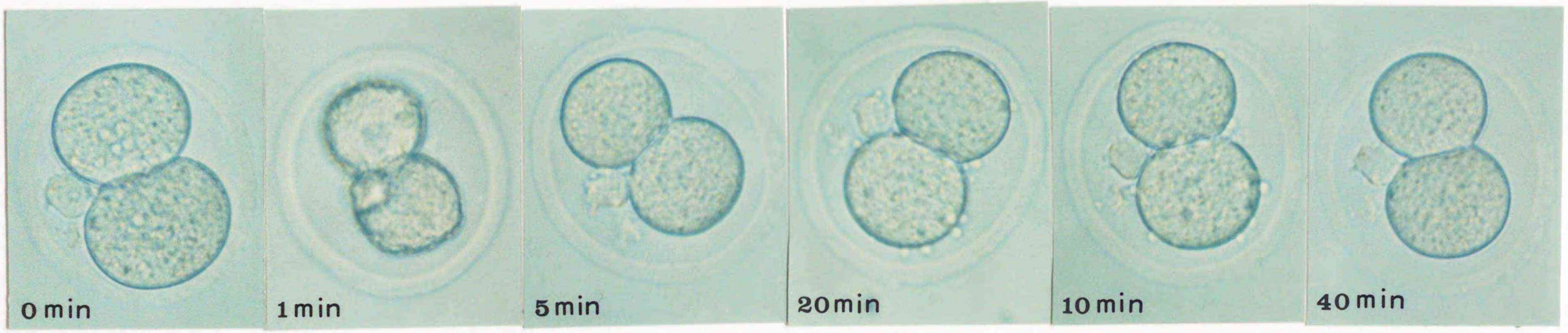

Fig. 4. Light photographs of mouse two-cell embryo during increasing equilibration period in the freezing medium (x 400).

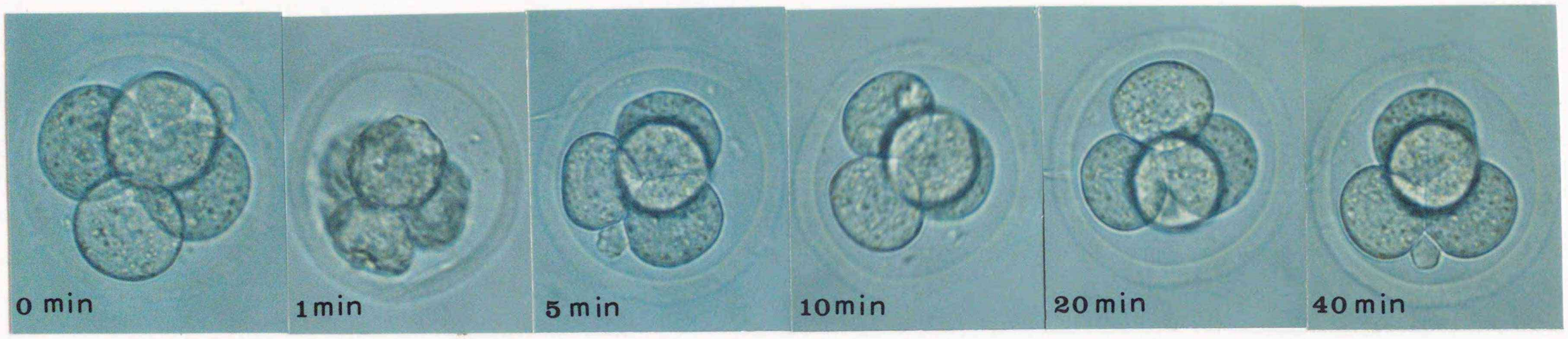

Fig. 5. Light photographs of mouse four-cell embryo during increasing equilibration period in the freezing medium (x 400).

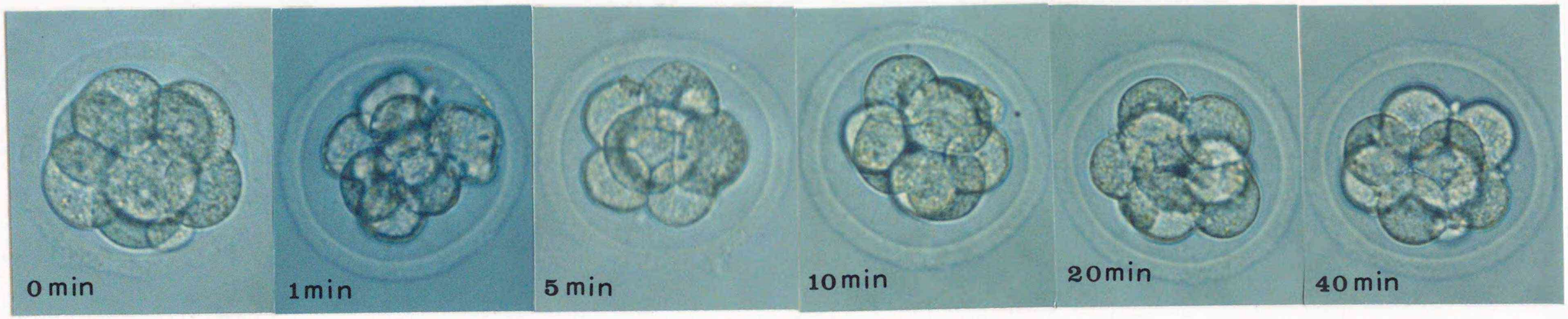

Fig. 6. Light photographs of mouse eight-cell embryo during increasing equilibration period in the freezing medium (x 400).

Table 1. Effect of equilibration period in 3 M ethylene glycol with 0.25 M sucrose on the post-thaw survival of mouse two-, four-, and eight-cell embryos cryopreserved by a quick freezing method

Embryo stage	Equilibration period (minutes)			
	5	10	20	40
2-cell	36.4^{Aa}	73.1^{b}	70.0^{b}	66.6^{b}
	(55)	(52)	(50)	(54)
4-cell	33.3^{Aa}	75.4^{b}	71.7^{b}	67.9^{b}
	(54)	(57)	(53)	(53)
8-cell	68.9^{B}	77.7	73.6	70.3
	(58)	(54)	(53)	(54)

Values represent data from four replicates.

Values in parenthesis represent the total number of frozen-thawed embryos.

Values with different superscripts in the same column are significantly different(A:B, $P<0.001$).

Values with different superscripts in the same line are significantly different (a,b, $P<0.001$).

Table 2. Effect of equilibration period in 3 M ethylene glycol with 0.25 M lactose on the post-thaw survival of mouse two-, four-, and eight-cell embryos cryopreserved by a quick freezing method

Embryo stage	Equilibration period (minutes)			
	5	10	20	40
2-cell	36.2^{Aa}	72.0^{b}	71.7^{b}	65.4^{b}
	(58)	(50)	(53)	(55)
4-cell	35.8^{Aa}	73.2^{b}	69.8^{b}	68.5^{b}
	(53)	(56)	(53)	(54)
8-cell	69.0^{B}	75.0	72.2	69.1
	(55)	(56)	(54)	(55)

Values represent data from four replicates.

Values in parenthesis represent the total number of frozen-thawed embryos.

Values with different superscripts in the same column are significantly different (A:B, $P<0.001$).

Values with different superscripts in the same line are significantly different (a:b, $P<0.001$).

Table 3. The effect of exposure in 3 M ethylene glycol with either 0.25 M sucrose or lactose without freezing on the percentage of mouse early cleavage-stage embryos developing to expanded blastocysts during culture in vitro

Freezing medium	Embryo stage	Exposure period (minutes)				
		0*	5	10	20	40
3 M ethylene glycol with 0.25 sucrose	2-cell	96.0	96.0	92.0	92.0	89.2
	4-cell	96.0	93.1	92.3	93.1	92.8
	8-cell	100.0	96.4	93.1	96.0	93.1
3 M ethylene glycol with 0.25 M lactose	2-cell	96.3	92.6	92.6	92.3	92.3
	4-cell	96.4	92.3	93.1	92.0	92.6
	8-cell	96.2	92.0	96.0	92.8	92.0

* Control (not exposed to the freezing medium nor frozen). Values represent data from two replicates (25 to 29 embryos per treatment group).

followed by gradual permeation of ethylene glycol into the cells. When adequate dehydration and permeation of the cryoprotectant occur, embryos can be frozen with small amount of non-damaging ice crystals (LEIBO, 1977; WHITTINGHAM et al., 1979) while inadequate dehydration may lead to the formation of lethal intracellular ice.

Our results indicate that two-, four-, and eight-cell mouse embryos require at least 10 minutes and 5 minutes equilibration in 3 M ethylene glycol with either 0.25 M sucrose or lactose, respectively before plunging into LN_2 vapor. Two- and four-cell mouse embryos may require longer equilibration time in the freezing medium for adequate permeation of the cryoprotectant because of the bigger size of their blastomeres and lower permeability coefficiency to the cryoprotectant as compared with eight-cell stage embryos (MAZUR et al.,1976). Thus, it can be assumed that the significantly higher ($P<0.001$) survival rate of eight-cell embryos than two- and four-cell embryos at 5 minutes equilibration is due to a higher intracellular concentration of ethylene glycol.

In a quick or rapid freezing method, a combination of dehydration by non-permeating agents such as sucrose or lactose, and permeation of the embryo by the cryoprotectant, enable the cells to supercool to -170 or -196 °C without the formation of lethal intracellular ice (TROUNSON et al.,1987; MAZUR, 1990). However, the necessity for actual permeation with the cryoprotectant has been subject to some debate.

Using a bovine erythrocyte model, MAZUR et al.(1974) showed that actual permeation into the cell by a cryoprotectant is not mandatory for cryoprotection when the cooling process is slow. However, using two-cell mouse embryos, BERNARD & FULLER (1984) reported a better survival rate when sufficient time is allowed for glycerol to permeate into the cells. Moreover, SZELL & SHELTON (1986) showed that embryos dehydrated by sucrose and frozen rapidly require a high extracellular concentration of glycerol, and that an increase in the intracellular glycerol concentration is beneficial for the survival of the embryos. Available evidence suggests that partial permeation by the cryoprotectant as a result of equilibration may be a requisite for protection but complete permeation is not necessary (SZELL & SHELTON, 1986;LEIBO, 1989). Our results indicate that adequate permeation of the embryos by ethylene glycol into the blastomeres before quick freezing is necessary for cryoprotection. This can be attained with equilibration in the freezing medium for 10 minutes for two- and four-cell embryos, and 5 minutes for eight-cell embryos to allow a high concentration of ethylene glycol to permeate into the embryonic cells. Sensitivity of the embryos to quick freezing was only affected by the embryonic stage of development at 5 minutes equilibration, wherein a significantly higher ($P<0.001$) percentage of eight-cell embryos survived than two- and four-cell embryos. At 10, 20, and 40 minutes equilibration periods in the freezing medium, no significant difference on the survival rates of mouse early cleavage-stage embryos was observed.

The highest survival rates in vitro were obtained when mouse two-, four-, and eight-cell embryos were equilibrated in 3 M ethylene glycol with 0.25 M sucrose (73.1%, 75.4% and 77.7%, respectively) or lactose (72.0%, 73.2% and 75.0%, respectively) for 10 minutes before plunging into LN_2 vapor. These are comparable results to those obtained by conventional (URANO et al.,1986) ultrarapid freezing (SATHANANTHAN et al.,1988) and vitrification (RALL et al.,1987; VALDEZ et al.,1990) methods. However, reduction in the viability of the embryos, although not significant, was observed after 20 and 40 minutes equilibration. In the cryopreservation of embryos using quick freezing method, a high concentration of cryoprotectant is required to give optimum protection. A prolonged equilibration could have resulted in a very high intracellular concentration of ethylene glycol, making the embryos more susceptible to osmotic shock caused by the rapid influx of water when diluted out of the cryoprotectant after thawing (LEIBO,1989). The decrease in viability could not have been due to chemical toxicity as this study indicated that exposure of the embryos in 3 M ethylene glycol with either 0.25 M sucrose or lactose for up to 40 minutes without freezing, did not detrimentally affect their capacity to develop in vitro.

Significant difference on the survival of early cleavage-stage mouse embryos cryopreserved by the quick freezing method using ethylene glycol irrespective of the sugar used was not observed. This indicates that sucrose or lactose at

0.25 M concentration can be used effectively in combination with 3 M ethylene glycol in the quick freezing of mouse two-, four-, and eight-cell embryos. This finding is in agreement with previous studies (TAKAHASHI & KANAGAWA, 1985; ABAS MAZNI *et al.* (1990) on mouse compacted morulae cryopreserved by the same quick freezing method.

The results of this study indicate that at 5 minutes equilibration in 3 M ethylene glycol with either 0.25 M sucrose or lactose, the survival rate of early cleavage-stage mouse embryos is stage-dependent. However, a prolonged equilibration period seems to reduce embryonic viability.

Moreover, this study shows that ethylene glycol in combination with either sucrose or lactose can be used effectively in the quick freezing of mouse early cleavage-stage embryos.

SUMMARY

Mouse early cleavage-stage embryos were frozen by direct plunging into liquid nitrogen vapor (LN_2) after equilibration in 3 M ethylene glycol with either 0.25 M sucrose or lactose for 5, 10, 20 and 40 minutes. After thawing, the embryos were cultured *in vitro* and the effect of equilibration period on the viability of the embryos was examined. When two- and four-cell embryos were equilibrated in the freezing medium for 5 minutes, the post-thaw viability was low, but increased when equilibration was prolonged to 10 minutes. Although no significant difference was observed on the post-thaw viability of eight-cell embryos equilibrated from 5 to 40 minutes, the highest survival rate for two-, four-, and eight-cell mouse embryos was at 10 minutes equilibration in the freezing medium before plunging into LN_2 vapor.

The effect of exposure to 3 M ethylene glycol with either 0.25 M sucrose or lactose on the survival of mouse early cleavage-stage embryos without freezing is also described.

CHAPTER II

QUICK FREEZING OF ONE-CELL MOUSE EMBRYOS USING ETHYLENE GLYCOL WITH SUCROSE OR LACTOSE

INTRODUCTION

Cryopreservation of mouse embryos can be achieved by a combined process of dehydration of the embryos with a non-permeating agent (sugar) and permeation with a cryoprotectant before direct plunging into liquid nitrogen (LN_2) vapor (quick or rapid freezing) or into LN_2 (ultrarapid freezing). The above-mentioned freezing methods have utilized glycerol on eight-cell mouse embryos, morulae and blastocysts in combination with sucrose (TAKEDA et al.,1984; BIERY et al.,1986; WILLIAMS & JOHNSON,1986; SZELL & SHELTON,1986, 1987), trehalose (KRAG et al.,1985), lactose, raffinose, glucose or xylose (TAKAHASHI & KANAGAWA, 1985,1990); with dimethyl sulfoxide (TROUNSON et al.,1987, 1988) or propylene glycol (SATHANANTHAN et al., 1988) and sucrose.

ABAS MAZNI et al.(1990) reported the successful use of 3 M ethylene glycol as a cryoprotectant with either 0.25 M lactose or sucrose in the quick freezing of mouse morulae. A similar study (RAYOS et al., 1992a) also yielded high survival rates of two-, four-, and eight-cell mouse embryos using the same freezing medium. With increasing interest in basic research and techniques utilizing early-stage embryos (e.g nuclear transfer, gene injection) it is equally important that the quick freezing techniques be applied success-

fully to one-cell stage embryos.

To date, no study has reported on the quick or ultrarapid freezing of one-cell mouse embryos using ethylene glycol. Equilibration of the embryos in a freezing medium containing high concentration of cryoprotectant before plunging into LN_2 vapor (SZELL & SHELTON, 1986; TAKAHASHI & KANAGAWA, 1990) and dilution of the cryoprotectant after thawing (SCHNEIDER & MAZUR, 1984; LEIBO, 1989) are of critical importance for the survival of the embryos. This study was therefore undertaken to examine the effects of equilibration period on embryos in 3 M ethylene glycol with 0.25 M sucrose or lactose on the survival of one-cell mouse embryos cryopreserved by a quick freezing method.

MATERIALS AND METHODS

Animals and embryo recovery

Female F_1 hybrid (C57BL/6J X CBA) 4 to 8 weeks of age and 18 to 25 g in body weight were used as embryo donors. Female ICR mice, 10 to 12 weeks of age were used as recipients. They were maintained in a room with controlled lighting 14-hour light (5:00 to 19:00 hour): 10-hour dark cycle and given commercial feed and water ad libitum. Female F_1 mice were induced to superovulate with intraperitoneal injections of 5 IU pregnant mare serum gonadotropin (PMSG; Serotrophin, Teikoku Zoki, Tokyo, Japan) and 5 IU human chorionic gonadotropin (hCG; Teikoku Zoki) given 48 h apart. After hCG injection, female mice were paired singly with F_1 males and inspected the following day for the presence of a

vaginal plug. The female mice were killed by cervical dislocation and intact cumulus masses containing the one-cell embryos were excised from oviducts and placed in sterile plastic culture dish (Nunc, Nunclon, Kamstrup, Denmark) with modified phosphate-buffered saline (PB1; WHITTINGHAM, 1971) supplemented with 10% heat-treated calf serum (CS; Gibco Laboratories, Life Technology Inc., New York, USA) and containing hyaluronidase (150 units/ml; Sigma Chemical Co., St. Louis, MO) for 3 to 5 minutes, 20 to 22 hours after hCG injection. When the cumulus cells were detached, the embryos were washed three times in fresh PB1 medium with 10% CS. Only morphologically normal embryos were used in this study. One-cell embryos could be distinguished from unfertilized oocytes by the presence of a second polar body in the perivitelline space and/or by two pronuclei.

Freezing procedure

A modified quick freezing method previously described by TAKAHASHI & KANAGAWA (1985,1990) was used in this study. The embryos were pipetted into the freezing medium consisting of 3 M ethylene glycol with 0.25 M sucrose or lactose in PB1 with 10% CS and equilibrated for 5, 10, 20 and 40 minutes. Ten to 15 embryos were drawn into 0.25-ml French straw (I.M.V. L' Aigle, France) which were heat sealed. After the assigned equilibration period was attained, the embryos were frozen in LN_2 vapor (about -170 °C) by placing the sealed straw horizontally on a styrofoam plate (140 mm X 60 mm X 5 mm) with a stainless steel mesh on its upper sur-

face and floating in a liquid nitrogen bath. Two minutes later, the straw was plunged into LN_2 and stored for 1 to 60 days.

Thawing, dilution and culture procedure

Thawing was done in a water bath at 37 °C for 20 seconds. Thereafter, the contents of the straw were expelled into a Petri dish, and the embryos were immediately pipetted into 0.5 or 1.0 M of the same sugar used in the freezing medium for dilution of the cryoprotectant. After 5 minutes, the embryos were transferred to PB1 with 10% CS. Other embryos were directly diluted into PB1 with 10% CS without first being placed into 0.5 or 1.0 M of sucrose or lactose. Five minutes later, the embryos were washed three times in microdrops of Whitten's medium (WHITTEN, 1971) before being cultured in 100 l microdrops of Whitten's medium at 37°C in 5% CO_2 in air for 96 to 120 hours. All manipulations, unless indicated were performed at room temperature (about 20 °C). Survival in vitro was assessed by the ability of the frozen-thawed one-cell embryos to develop into expanded blastocysts during culture.

Experiments were replicated five to six times, and the data were analyzed for statistical significance using the Student's t-test.

To determine if prolonged exposure to the cryoprotectant affects viability, one-cell mouse embryos were suspended in the freezing medium containing 3 M ethylene glycol with 0.25 M sucrose or lactose without freezing. Ten to 15 embryos were pipetted into 3 M ethylene glycol with 0.25 M su-

crose or lactose for 5, 10, 20 and 40 minutes at room temperature. After the required exposure period was attained, the embryos were transferred into 0.5 M sucrose or lactose for 5 minutes. Thereafter, they were transferred into PB1 with 10% CS for another 5 minutes, then washed three times with Whitten's medium. Finally, the embryos were cultured in microdrops of Whitten's medium at 37°C in 5% CO_2 in air for 96 to 120 hours. In addition, fresh one-cell mouse embryos were cultured similarly to serve as control. Survival of the embryos was assessed by their capacity to develop into expanded blastocysts during culture.

Experiments were replicated four times and data were analyzed by the Chi-square test.

Development in vivo

To assess viability in vivo, blastocysts developing from one-cell mouse embryos, that were frozen after the optimum equilibration period (10 minutes) in the freezing medium and diluted with 0.5 M sucrose or lactose after thawing, were transferred into the uterine horns of female ICR mouse recipients. The recipients were anesthetized with sodium pentobarbital (Nembutal, Abbott Laboratories, Chicago, Illinois, USA) which was diluted ten times with physiological saline solution. The uterine transfer procedure used was previously described by HOGAN et al. (1986) and modified by ABAS MAZNI (1990).

Six to 9 embryos were transferred into each uterine horn of Day 3 pseudopregnant recipient (Day 1= day of find-

ing the vaginal plug) that had been mated with a vasectomized ICR male. The recipients were necropsied on Day 18 of pregnancy, and the numbers of live and resorbing fetuses, as well as implantation sites were examined. Cultured fresh one-cell mouse embryos developing to blastocysts were also transferred into each uterine horn of female recipients to serve as the control.

Experiments were replicated four times and data were analyzed for statistical significance using the Chi-square test.

RESULTS

More than 90% of the embryos were recovered from the freezing straws after thawing. Tables 1 and 2 shows the effects of the equilibration period and dilution method on the percentage of frozen-thawed one-cell embryos developing to the expanded blastocyst stage during culture. Since the *in vitro* survival rate showed a similar pattern when 0.5 or 1.0 M of sucrose or lactose was used for dilution of the cryoprotectant for each equilibration period, the data were pooled from the two groups. Thereafter, the effect of equilibration period was examined. The highest survival rates were attained when the embryos were equilibrated in 3 M ethylene glycol with 0.25 M sucrose (67.2%) or lactose (69.7%) for 10 minutes before plunging into LN_2 vapor, and diluted with either 0.5 or 1.0 M of sucrose or lactose after thawing. Shorter (5 minutes) or prolonged (40 minutes) equilibration yielded significantly lower survival rates.

Dilution by direct transfer of the frozen-thawed embryos into PB1 resulted in significantly lower survival than when 0.5 or 1.0 M of sucrose or lactose was used.

Table 3 shows the effect of exposure to 3 M ethylene glycol with 0.25 M sucrose or lactose on the viability of one-cell mouse embryos without freezing. High percentages (86.1 to 91.2%) of one-cell mouse embryos exposed to the freezing medium for 5, 10, 20 and 40 minutes without freezing developed to expanded blastocysts when cultured *in vitro* at rates similar to the control.

The development rates of one-cell mouse embryos frozen using 3 M ethylene glycol with 0.25 M sucrose or lactose *in vitro and in vivo*, and the nonfrozen control embryos are shown in Table 4. *In vitro* development rates of frozen-thawed one-cell mouse embryos to blastocysts during culture was significantly lower than those of the control embryos (68.0 and 66.7% vs 92.7%). However, when the blastocysts derived from frozen-thawed one-cell mouse embryos were transferred into the uterine horns of the recipients, the rate of development into live fetuses did not significantly differ with the control (47.0 and 45.8% vs 51.6%). Moreover, no significant difference was observed in the implantation rates between frozen-thawed and nonfrozen control embryos (81.6 and 80.0% vs 90.6%).

DISCUSSION

Results from this study indicate that one-cell mouse

Table 1. Effects of equilibration period in 3 M ethylene glycol with 0.25 M sucrose, and the dilution method on the percentage of quickly frozen one-cell mouse embryos developing to expanded blastocysts during culture (mean±SEM)*

Dilution	Equilibration period (minutes)			
method	5	10	20	40
PB1 with sucrose**	43.4±1.6^{Aa}	67.2±1.9^{Ab}	61.3±2.1^{Abc}	58.9±0.8^{Ac}
PB1	32.0±2.8^{Ba}	31.5±0.8^{Ba}	34.0±1.2^{Ba}	26.6±1.1^{Cb}

*Each group had five to six replicates (186 to 200 embryos per group).

**Pooled from 0.5 and 1.0 M sucrose groups.

Values with different superscripts in the same line are significantly different (a:b, $P<0.05$; b:c; a:c, $P<0.01$).

Values with different superscripts in the same column are significantly different (A:B, $P<0.05$; A:C, $P<0.001$).

Table 2. Effects of equilibration period in 3 M ethylene glycol with 0.25 M lactose, and the dilution method on the percentage of quickly frozen one-cell mouse embryos developing to expanded blastocysts during culture (mean±SEM)*

Dilution	Equilibration period (minutes)			
method	5	10	20	40
PB1 with lactose**	41.8±2.7^{Aa}	69.7±1.2^{Ab}	65.4±3.1^{Abc}	60.9±2.1^{Ac}
PB1	35.1±2.2^{Ba}	33.3±2.5^{Ca}	30.7±1.9^{Ca}	28.6±1.7^{Cb}

*Each group had five replicates (122 to 152 embryos per group).

**Pooled from 0.5 and 1.0 M lactose groups.

Values with different superscripts in the same line are significantly different (a:b, $P<0.05$; a:c; b:c, $P<0.01$).

Values with different superscripts in the same column are significantly different (A:B, $P<0.01$; A:C, $P<0.001$).

Table 3. Effect of exposure in 3 M ethylene glycol with 0.25 M sucrose or lactose on the percentage of one-cell mouse embryos developing to expanded blastocysts during culture in vitro

Freezing medium	Exposure period (minutes)				
	0*	5	10	20	40
3 M ethylene glycol with 0.25 M sucrose	92.0 (126)	89.5 (134)	91.2 (154)	89.8 (135)	89.7 (152)
3 M ethylene glycol with 0.25 M lactose	91.2 (110)	89.8 (127)	90.0 (120)	87.2 (109)	86.1 (115)

* Control: not exposed to the freezing medium nor frozen.

Values in parenthesis represent the number of embryos used.

Table 4. In vitro and in vivo development of one-cell mouse embryos quickly frozen using 3 M ethylene glycol with 0.25 M sucrose or lactose

Treatment of one-cell embryos	No.(%)* developing & transferred	No. of recipients	No.(%)** implantation	No.(%)‡‡ live fetus
Frozen-thawed				
Sucrose				
(n=200)	136(68.0)[a]	10	111(81.6)	65(47.0)
Lactose				
(n=180)	120(66.7)[a]	8	96(80.0)	55(45.8)
Nonfrozen control				
(n=69)	64(92.7)[b]	5	58(90.6)	33(51.6)

*No. of embryos developing into blastocysts and transferred to the recipients/No. of embryos cultured for 96 hours.

**No. of live and resorbing fetuses as well as implantation sites/No. of blastocysts transferred.

‡‡No. of live fetuses/No. of blastocysts transferred.

a:b, $P<0.001$.

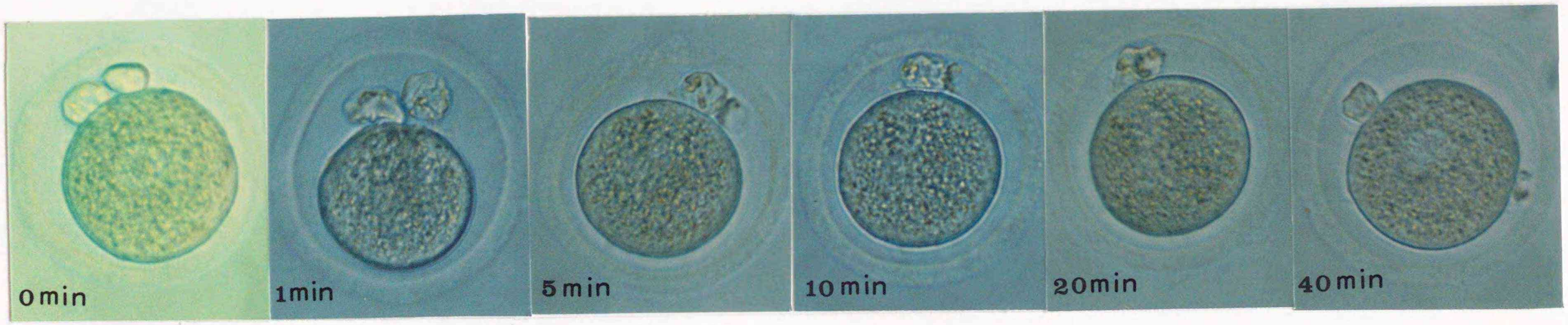

Fig. 1. Light photographs of mouse one-cell embryo during increasing equilibration period in the freezing medium (x 400).

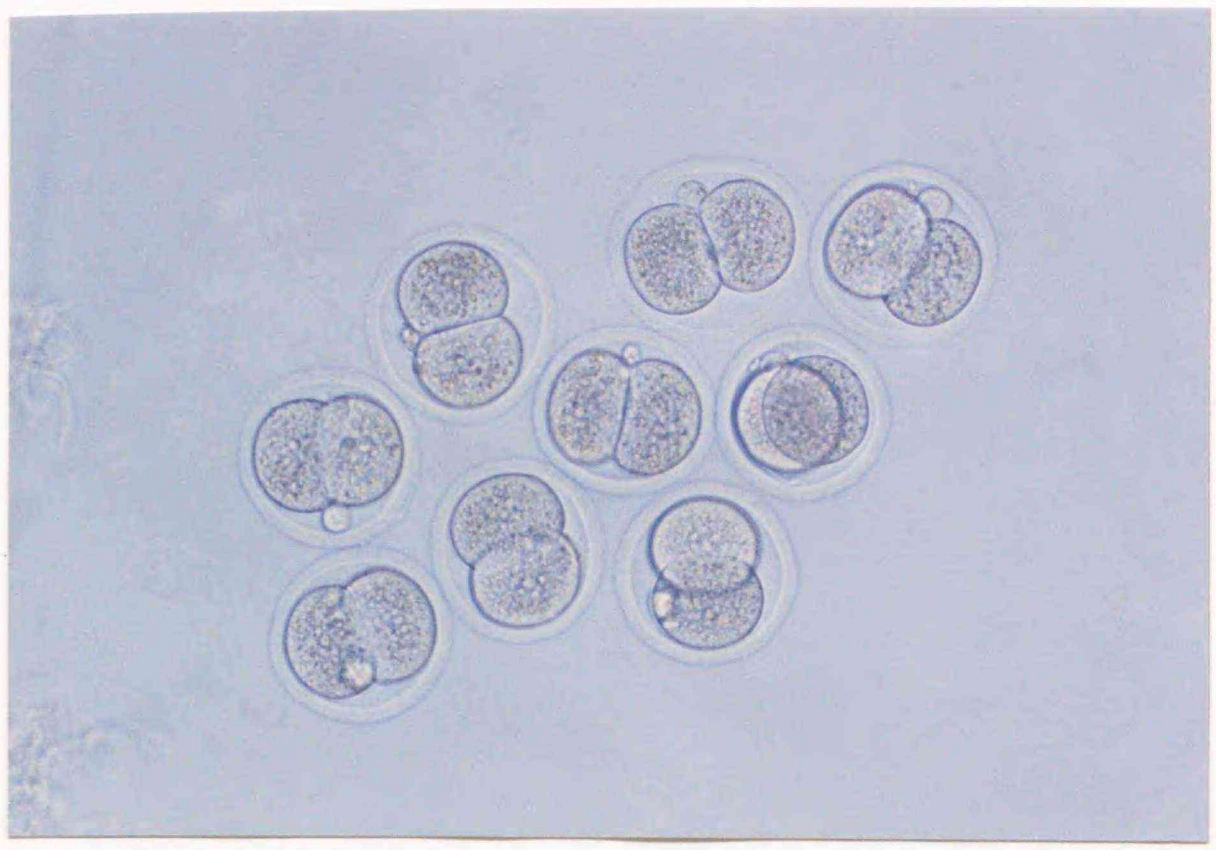

Fig. 2. Two-cell mouse embryos developing from frozen-thawed one-cell embryos 24 hours after culture in vitro (x200).

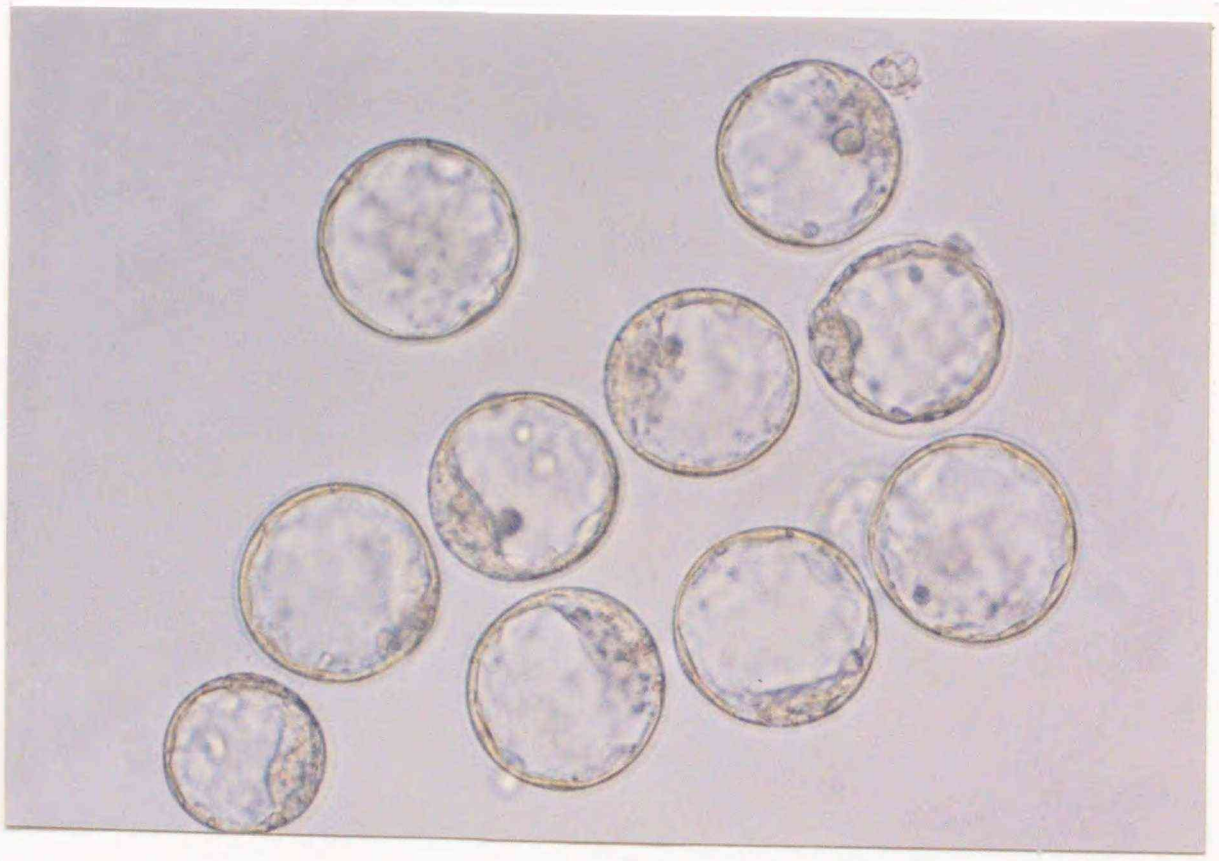

Fig. 3. Expanded blastocysts developing from frozen-thawed one-cell mouse embryos 96 to 120 hours after culture in vitro(x200).

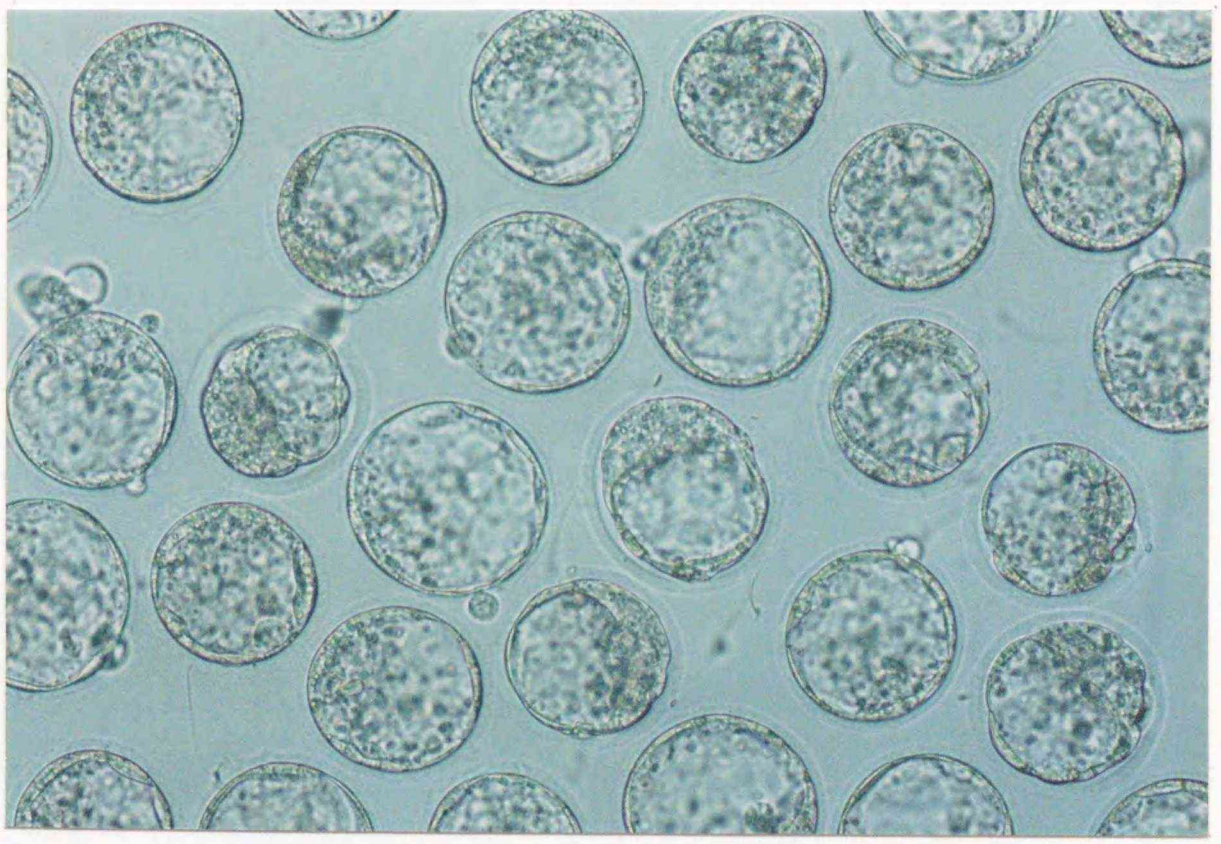

Fig. 4. Blastocysts developing from frozen-thawed one-cell mouse embryos 96 hours after culture *in vitro* before transfer to recipients (x 200).

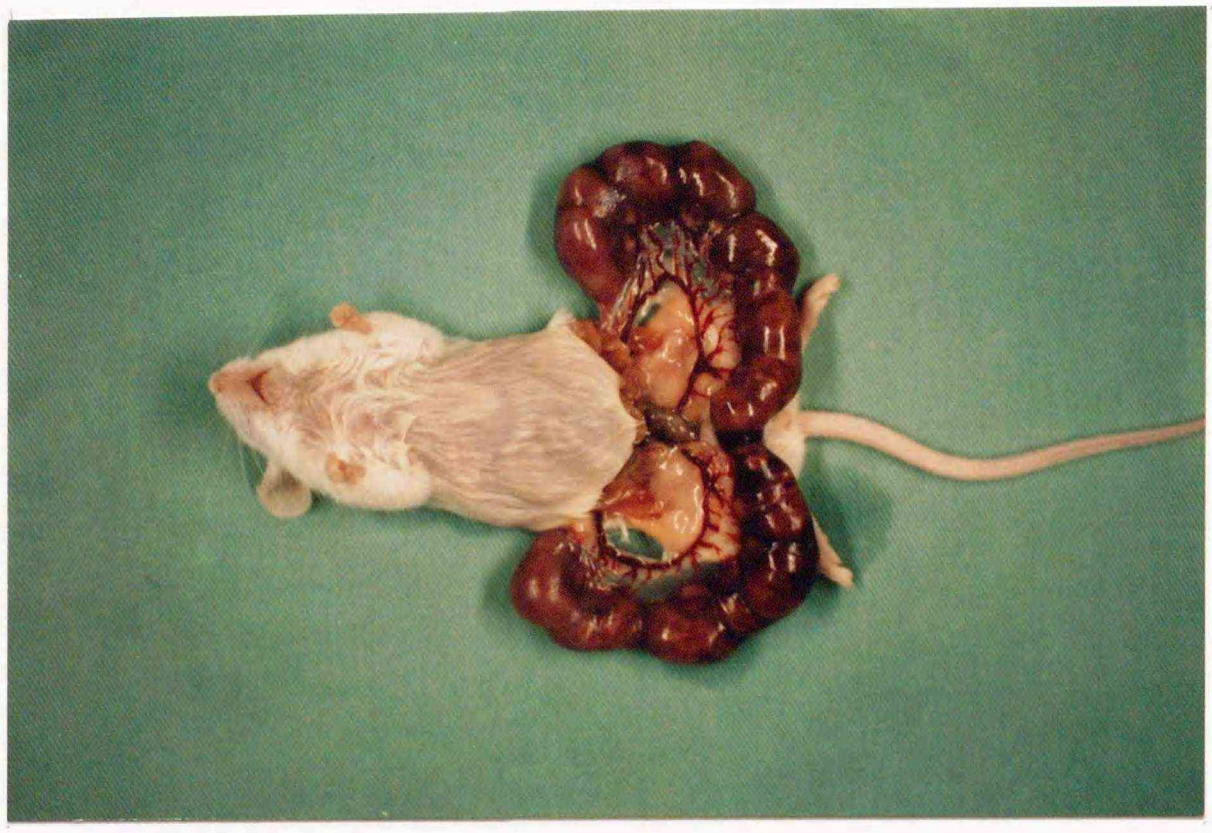

Fig. 5. Recipient necropsied at Day 18 of pregnancy after transfer of blastocysts derived from frozen-thawed one-cell mouse embryos cryopreserved by a quick freezing method.

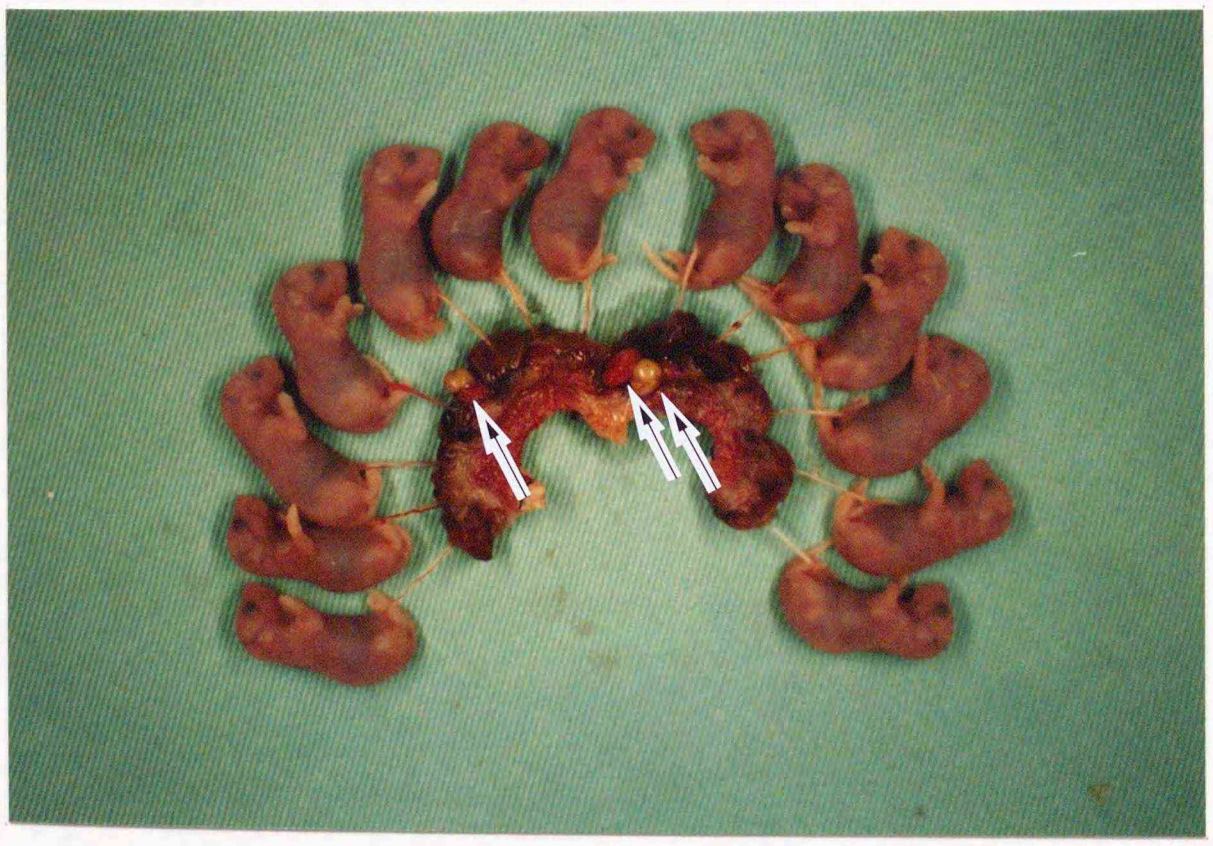

Fig. 6. Late-stage fetuses and implantation sites (arrow) at Day 18 of pregnancy.

embryos require at least 10 minutes of equilibration in 3 M ethylene glycol with 0.25 M sucrose or lactose (freezing medium) before plunging into LN_2 vapor. Shorter equilibration (5 minutes) yielded a significantly lower survival rate, which could have been due to insufficient intracellular concentration of ethylene glycol before freezing. SZELL & SHELTON (1986) suggested that the survival of rapidly frozen embryos depends upon the duration of equilibration, and the effect of equilibration period is due to cryoprotectant permeation into embryonic cells, which is necessary for protection before freezing. As shown in Fig. 1, one-cell mouse embryo initially shrinks when exposed to the freezing medium containing both a permeating and nonpermeating cryoprotectants followed by gradual expansion. This indicates that rapid shrinkage of the embryonic cell (dehydration) was followed by gradual permeation of ethylene glycol into the cell. Longer equilibration period in the freezing solution was necessary for one-cell mouse embryos because of their larger cellular size and volume (LEIBO et al.,1974) and because their permeability coefficiency to the cryoprotectant is much lower than those of more advanced embryonic stages such as the morula or blastocyst (MAZUR et al.,1976). However, reduction in the viability of the embryos was observed after 20 and 40 minutes of equilibration. In the cryopreservation of embryos using a quick freezing method, a high concentration of permeating cryoprotectant is necessary to give optimum protection (ABAS MAZNI et al., 1989,1990).

Prolonged equilibration could have resulted in the permeation of a very high concentration of ethylene glycol into the embryonic cell, making the embryos susceptible to osmotic shock when the cryoprotectant is diluted out after thawing. This reduction in viability could not have been due to chemical toxicity, since our previous study (RAYOS et al., 1992a) showed that exposure of two-, four-, and eight-cell mouse embryos to 3 M ethylene glycol with either 0.25 M sucrose or lactose for up to 40 minutes did not detrimentally affect their capacity to develop into expanded blastocysts when cultures in vitro. This finding was confirmed on one-cell mouse embryos as shown in Table 3.

In vitro development of the frozen-thawed one-cell mouse embryos to the blastocyst stage was significantly lower than the control (68.0 and 66.7% vs 92.7%). This difference could have been due to the presence of intracellular ice in the embryos cryopreserved by the quick freezing method, which could have affected their subsequent development. Moreover, these embryos were permeated with a high concentration of ethylene glycol, and thus, were susceptible to osmotic injury during the dilution of the cryoprotectant.

The use of either 0.5 or 1.0 M sucrose or lactose for the dilution of the cryoprotectant did not show significant difference on the survival of the frozen-thawed one-cell mouse embryos. This indicates that either concentration is effective in the removal of the cryoprotectant from the embryos after thawing. This finding confirms that of a previous study (SZELL & SHELTON, 1986) in which sucrose

concentration of 0.5, 1.0 and 1.5 M in the diluent had similar effect on the rate of development of mouse embryos frozen rapidly using glycerol-sucrose solution. Sucrose or lactose (nonpermeable solutes), when added to the dilution medium controls the amount of swelling the embryo undergoes during the dilution process. They act as osmotic counter-force (SCHNEIDER & MAZUR, 1984) to restrict water movement across the membranes, thus the embryo does not swell but shrinks progressively as the cryoprotectant leaves the embryo. Direct dilution of the frozen-thawed one-cell mouse embryos into isotonic PB1 solution gave low survival rates. This could be due to osmotic shock (SCHNEIDER & MAZUR, 1984; LEIBO, 1989) which occurs if the cryoprotectant that permeated into the embryonic cells can not diffuse out rapidly enough to prevent the sudden influx of water into the cells, leading to excessive swelling and sometimes disintegration during the dilution process.

The highest *in vitro* (67.2 and 69.7%) and *in vivo* survival rates (47.0 and 45.8%) of frozen-thawed one-cell mouse embryos in this study were higher than those of embryos frozen by conventional freezing (WHITTINGHAM *et al.*,1972) or vitrification (KONO & TSUNODA, 1987) methods.

Our present study has shown that ethylene glycol with sucrose or lactose can be used effectively as a cryoprotective agent in the quick freezing of one-cell mouse embryos. Sucrose and lactose at 0.5 or 1.0 M concentration are similarly effective in the dilution of the cryoprotectant after

thawing. Furthermore, blastocysts resulting from frozen-thawed one-cell mouse embryos were found to be capable of developing into late-stage fetuses when transferred to the recipients, with fetal development and implantation rates similar to the control.

SUMMARY

One-cell mouse embryos were frozen by direct plunging into liquid nitrogen (LN_2) vapor after equilibration in 3 M ethylene glycol with 0.25 M sucrose or lactose(freezing medium) for 5 to 40 minutes. After thawing, the embryos were cultured *in vitro* and the effects of the equilibration period and dilution method were examined. No significant difference was observed on the *in vitro* survival of embryos when 0.5 or 1.0 M of the sugar used for dilution of the cryoprotectant for each equilibration period. The highest survival rates (67.2 and 69.7%) were obtained when the embryos were equilibrated in the freezing medium for 10 minutes, and the cryoprotectant was diluted with either 0.5 or 1.0 M of sucrose or lactose for 5 minutes after thawing. Shorter (5 minutes) or prolonged (40 minutes) equilibration of the embryos in the freezing medium before freezing yielded significantly lower ($P<0.01$) survival rates. Dilution by direct transfer of the frozen-thawed embryos into PB1 resulted in lower survival rates ($P<0.05$ or $P<0.001$) than when 0.5 or 1.0 M sucrose or lactose was used. The *in vitro* development to the blastocyst stage of one-cell mouse embryos frozen after 10 minutes equilibration in the freezing medium and diluted after thawing in 0.5 M sucrose or lactose was significantly lower than the control (68.0 and 66.7% vs 92.7%). However, transfer of the blastocysts developing from frozen-thawed one-cell mouse embryos into the uterine horns of the recipients resulted in fetal development and implantation rates similar to the control.

PART 2. STUDIES ON THE QUICK FREEZING OF UNFERTILIZED MOUSE OOCYTES USING ETHYLENE GLYCOL

CHAPTER III

QUICK FREEZING OF UNFERTILIZED MOUSE OOCYTES USING ETHYLENE GLYCOL WITH SUCROSE OR TREHALOSE

INTRODUCTION

Success in the cryopreservation of mouse embryos with development to normal young after transfer to recipients was achieved by WHITTINGHAM et al.(1972). Since then, several studies have been carried out using mouse embryos as experimental models. To date, the embryos of at least 15 mammalian species have been successfully cryopreserved (LEIBO, 1989).

In combination with other reproductive technologies, cryopreservation of oocytes increases the strategies available for the management of human infertility, livestock breeding and conservation of rare genetic stocks. Cryopreservation of unfertilized mouse oocytes by slow cooling have been reported by TSUNODA et al. (1976), PARKENING et al. (1976), WHITTINGHAM (1977), FULLER & BERNARD (1984), GLENISTER et al. (1987) and KO & THRELFALL (1988). However, compared with the success rate obtained with embryos, development rates of frozen-thawed oocytes is still low. Thus, for cryopreservation of oocytes to be acceptable, adequate rates of fertilization, embryo development and viable pregnancy are necessary.

Earlier studies on oocyte cryopreservation utilized the slow or conventional cooling procedures. Most recently, attempts to improve the technique 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 & 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 into liquid nitrogen (LN_2) (ultrarapid freezing). In our previous studies (RAYOS et al., 1992 a,b), one-, two-, four-, and eight-cell mouse embryos were successfully cryopreserved after determining the optimum equilibration period in 3 M ethylene glycol with 0.25 M sucrose or lactose before plunging into LN_2 vapor (quick freezing). Since unfertilized mouse oocytes have been demonstrated to have low permeability to cryoprotectants (MAZUR et al., 1976; JACKOWSKI et al.,1980),longer equilibration in the freezing medium containing high concentration of cryoprotectant may be necessary for survival before quick freezing.

This study was conducted to examine the effect of equilibration period in 3 M ethylene glycol with 0.25 M sucrose or trehalose on the post-thaw, postdilution 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 & CROWE, 1984), has been used as a nonpermeable cryoprotectant in combination with glycerol in rapid

(KRAG et al.,1985) and conventional (HONADEL et al.,1988) freezing of early preimplantation mouse embryos. Compared with sucrose, trehalose has also been reported (KIM et al.,1986) to improve the viability of mouse morula frozen ultrarapidly. Although the use of trehalose was recently mentioned in an abstract (BERNARD et al., 1990) as a cryoprotectant for cryopreservation of unfertilized oocytes, no results were given. This study also compares trehalose with sucrose as a nonpermeable cryoprotectant in the quick freezing of unfertilized mouse oocytes.

MATERIALS AND METHODS

Animals and oocyte recovery

Female F_1 hybrid mice (C57BL/6J X CBA), 6 to 8 weeks of age and 20 to 25 g in weight were used as oocyte donors. Female ICR strain mice 10 to 12 weeks of age and 30 to 40 g in weight were used as recipients. They were maintained in a room with controlled lighting 14-hour light (5:00 to 19:00 hour) : 10-hour dark cycle and given commercial feed and water ad libitum. Female F_1 mice were induced to superovulate with intraperitoneal injections of 5 IU pregnant mare serum gonadotropin (PMSG; Serotrophin, Teikoku Zoki, Tokyo, Japan) and 5 IU human chorionic gonadotropin (hCG; Teikoku Zoki) given 48 hours apart. Fourteen to 15 hours after hCG injection, the mice were killed by cervical dislocation and cumulus cell-enclosed unfertilized oocytes were released from the excised oviducts into modified phosphate-buffered saline (PB1; WHITTINGHAM, 1971) supplemented with 10% fetal

bovine serum (FBS; Life Technologies, Inc., Grand Island, New York, USA) and containing hyaluronidase (150 units/ml; bovine testis type I-S, Sigma Chemical Co.,St. Louis, MO) for 5 to 10 minutes. When the cumulus cells were detached, the oocytes were washed three times in fresh PB1 medium with 10% FBS and pooled in sterile plastic culture dishes (Nunc, Nunclon, Kamstrup, Denmark) until used. Only morphologically normal oocytes were used in this study.

Freezing procedure

A modified quick freezing method previously described by TAKAHASHI & KANAGAWA (1985, 1990) was used in this study. Briefly, the oocytes were pipetted into a plastic culture dish containing the freezing medium (3 M ethylene glycol with either 0.25 M sucrose or trehalose) in PB1 with 10% FBS. After the oocytes have settled at the bottom of the culture dish, 10 to 15 oocytes together with the freezing medium were drawn into a 0.25-ml French straw (I.M.V. L' Aigle, France) which was heat-sealed and equilibrated for 5, 10, 20 and 40 minutes. The equilibration period included the time the oocytes spent in the dish containing the freezing medium. After the assigned equilibration period was attained, the oocytes were frozen in LN_2 vapor (about - 170°C) by placing the sealed straw horizontally on a styrofoam plate (140 x 60 x 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 LN_2 and stored for 1 to 120 days.

Thawing and dilution

Thawing was done in a water bath at 37 °C for 20 seconds. Thereafter, the contents of the straw were expelled into a Petri dish and the oocytes were immediately pipetted into a 0.5 M solution of the same sugar used in the freezing medium for one-step dilution of the cryoprotectant (ethylene glycol). After 5 minutes, the oocytes were washed three times with PB1 with 10% FBS and oocytes with normal morphological appearance (surviving oocytes) were transferred into 0.4 ml of TYH medium (TOYODA et al., 1971) supplemented with 4 mg/ml bovine serum albumin (BSA; Sigma Chemical Co.) under paraffin oil for 30 minutes 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 which appeared morphologically normal after freezing and thawing as well as the nonfrozen control, underwent insemination in vitro. Semen was collected from the cauda epididymis, one from each of two mature F_1 (C57BL/6J x 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 minutes. Thereafter, the sperm concentration was determined by means of a hemocytometer and the sperm was incubated further for another 1 to 1.5 hours. A suitable volume (4 to 10 μl) of sperm was added to the medium containing the oocytes and incubated for 6 hours, providing a final sperm concentration of 150

cells/ μl.

To assess the fertilization rate, frozen-thawed as well as the nonfrozen control oocytes were removed 6 hours after insemination, mounted on a slide, fixed with 10% neutral formalin overnight, washed with 99.5% ethanol, stained with 0.25% aceto-lacmoid, then examined 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 female and male pronuclei and a corresponding sperm tail to the total number of oocytes evaluated.

Development in vitro

In a separate experiment, morphologically normal frozen-thawed and control oocytes also underwent insemination in vitro as described above. The oocytes were then washed three times with Whitten's medium (WHITTEN, 1971) supplemented with 3 mg/ml BSA 6 hours after insemination in vitro before being cultured in 100 l microdrops of Whitten's medium with 100 M EDTA (Kanto Chemical Co., Tokyo, Japan) under paraffin oil at 37 C in 5% CO_2 in air. After 19 hours of incubation (24 hours after insemination), the culture was examined under inverted microscope and 2-cell embryos were recorded. Development to the expanded blastocyst stage was assessed 110 to 120 hours after insemination in vitro.

All manipulations were performed at room temperature (20 to 25 °C). Survival of the frozen-thawed oocytes were

assessed at the following levels: proportion of the oocytes morphologically normal after thawing and dilution, development to 2-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 & TROUNSON (1989) and KONO *et al.* (1991). To determine if oocyte activation occurs after quick freezing, thawing and dilution, oocytes frozen after equilibration in 3 M ethylene glycol with 0.25 M sucrose or trehalose for 5, 10, 20 and 40 minutes were incubated in TYH medium for 6 hours without sperm and cultured in microdrops of Whitten's medium for 4 days.

Development *in vivo*

To assess viability *in vivo*, blastocysts derived from oocytes frozen by the optimum treatment (20 minutes equilibration in 3 M ethylene glycol with either 0.25M sucrose or trehalose) were transferred into the uterine horns of Day 3 pseudopregnant female ICR recipients (6 to 9 embryos per horn). The recipients were necropsied on Day 18 of pregnancy, and the number of live and resorbing fetuses , as well as implantation sites were examined. Blastocysts developing from nonfrozen oocytes after insemination and culture *in vitro* were also transferred into the uterine horns of the recipients to serve as control.

Statistical analysis

Experiments were replicated 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 analyzed by the Chi-square test.

RESULTS

Volume changes and behavior of oocyte

The volume changes and behavior of the oocyte during increasing equilibration period in the freezing medium are shown in Fig. 1. After exposure in ethylene glycol with sucrose or trehalose, the oocyte showed a rapid shrinkage and subsequent gradual expansion. However, the oocyte did not return to its original isotonic volume even after 40 minutes of equilibration.

Morphological normality after thawing and dilution

More than 90% of the frozen oocytes were recovered from the freezing straws after thawing and most of them showed normal morphological appearance (Fig. 2). No significant difference was observed on the post-thaw recovery regardless of the equilibration period. Post-thaw and postdilution morphological normality of oocytes frozen after 10, 20 or 40 minutes equilibration in 3 M ethylene glycol with either 0.25 M sucrose or trehalose was significantly higher ($P<0.01$) than with 5 minutes equilibration as shown in Tables 1 to 4. Significant difference on the survival (morphological normality) of frozen mouse oocytes after thawing and dilution was not observed irrespective of the sugar (sucrose or trehalose) used in combination with ethylene glycol.

Fertilization rate

Significant difference in the fertilization rate between mouse oocytes equilibrated in 3 M ethylene glycol with either 0.25 M sucrose or trehalose before plunging into LN_2 vapor was not observed as shown in Tables 1 and 2. However, higher total fertilization and normal fertilization rates were obtained with 20 and 40 minutes equilibration in the freezing medium than with 5 or 10 minutes. Moreover, the fertilization rates of the oocytes cryopreserved after 20 and 40 minutes equilibration were not significantly different from the control, while oocytes cryopreserved after 5 or 10 minutes in the freezing medium had fertilization rates significantly lower ($P<0.01$) than the control. Most of the frozen-thawed oocytes that had undergone fertilization were normally fertilized, possessing female and male pronuclei with corresponding sperm tail (Fig. 4) but their normal fertilization rate was significantly lower than the control ($P<0.01$ or $P<0.001$). Moreover, the rate of polyspermy of the frozen-thawed oocytes (17.0 to 26.5%) was significantly higher than the control (6.4%; $P<0.01$). The highest overall normal fertilization rate was obtained with 20 minutes equilibration in 3 M ethylene glycol with either 0.25 M sucrose (53.4%) or trehalose (49.5%).

Development *in vitro*

Development rates to 2-cell stage embryos of *in vitro* inseminated frozen-thawed oocytes that were equilibrated in the freezing medium for 20 or 40 minutes were significantly

higher ($P<0.05$) than with 5 minutes equilibration as shown in Tables 3 and 4. However, development to 2-cell embryos of oocytes equilibrated in the freezing medium for 10 minutes before quick freezing was not significantly different compared with 5, 20 or 40 minutes equilibration. Similarly, development to the expanded blastocyst stage of oocytes equilibrated for 20 or 40 minutes in 3 M ethylene glycol with either 0.25 M sucrose ($P<0.01$) or trehalose ($P<0.05$) before quick freezing was significantly higher than with 5 minutes equilibration. However, high proportion (82 to 85%) of 2-cell embryos obtained after insemination *in vitro* of frozen-thawed oocytes subsequently developed to expanded blastocysts regardless of the equilibration period. Development rates of the control oocytes after insemination *in vitro* to 2-cell embryos and expanded blastocysts were significantly higher ($P<0.001$) than the frozen-thawed oocytes. The highest overall development rates to expanded blastocysts were attained when the oocytes were frozen after equilibration for 20 minutes in 3 M ethylene glycol with either 0.25 M sucrose (45.0%) or trehalose (43.2%).

Parthenogenetic activation

Parthenogenetic activation or cleavage of frozen-thawed oocytes irrespective of the sugar (0.25 M sucrose or trehalose) used in combination with 3 M ethylene glycol, and the equilibration period was not observed following 4 days of culture in Whitten's medium.

Development *in vivo*

All the recipients (16 for frozen-thawed and 8 for

Table 1. Effect of equilibration period in 3 M ethylene glycol with 0.25 M sucrose on the fertilization rate of mouse oocytes cryopreserved by a quick freezing method

Equilibration period (Min)	No. of oocytes frozen	No. (%) surviving after thawing and dilution and inseminated *in vitro*	No.(%)† fertilized	No.(%)‡ 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

Values with different superscripts in the same column are significantly different (a:b, $P<0.01$).

Values with asterisks are significantly different from the control (** $P<0.01$; *** $P<0.001$).

†No. fertilized/ No. of oocytes inseminated *in vitro*.

‡No. normally fertilized/ No. of oocytes inseminated *in vitro*.

§No. normally fertilized/ No. of oocytes frozen.

Table 2. Effect of equilibration period in 3 M ethylene glycol with 0.25 M trehalose on the fertilization rate of mouse oocytes cryopreserved by a quick freezing method.

Equilibration period (Min)	No. of oocytes frozen	No.(%) surviving after thawing and dilution and inseminated *in vitro*	No.(%)† fertilized	No.(%)‡ 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	88(71.0)^{b}	75(85.1)	60(68.4)**	48.4^{b}***
Non-frozen control		94	88(93.6)	82(87.2)	87.2

Values with different superscripts in the same column are significantly different (a:b, $P<0.01$).

Values with asterisks are significantly different from the control (** $P<0.01$; *** $P<0.001$).

†No. fertilized/ No. of oocytes inseminated *in vitro*.

‡No. normally fertilized/ No. of oocytes inseminated *in vitro*.

§No. normally fertilized/ No. of oocytes frozen.

Table 3. Effect of equilibration period in 3 M ethylene glycol with 0.25 M sucrose on the *in vitro* development of mouse oocytes cryopreserved by a quick freezing method

Equilibration period (Min)	No. of oocytes frozen	No.(%) surviving after thawing and dilution and inseminated *in vitro*	No.(%)† developed to 2-cell embryo	No.(%)‡ 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***

Values with different superscripts in the same column are significantly different (a:b, $P<0.01$; c:d, $P<0.05$).

Values in the same column without asterisks are significantly different from the control (*** $P<0.001$).

†No. developed to 2-cell embryos/ No. of oocytes inseminated *in vitro*.

‡No. developed to expanded blastocysts/ No. of oocytes inseminated *in vitro*.

§No. developed to expanded blastocysts/ No. of oocytes frozen.

Table 4. Effect of equilibration period in 3 M ethylene glycol with 0.25 M trehalose on the *in vitro* development of mouse oocytes cryopreserved by a quick freezing method

Equilibration period (Min)	No. of oocytes frozen	No. (%) surviving after thawing and dilution and inseminated *in vitro*	No.(%)† developed to 2-cell embryo	No.(%)‡ developed to expanded blastocyst	Overall rate of development to expanded blastocysts(%)§
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***

Values with different superscripts in the same column are significantly different (a:b, $P<0.01$; c:d:e, $P<0.05$).

Values in the same column without asterisks are significantly different from the control (*** $P<0.001$).

†No. developed to 2-cell embryos/ No. of oocytes inseminated *in vitro*.

‡No. developed to expanded blastocysts/ No. of oocytes inseminated *in vitro*.

§No. developed to expanded blastocysts/ No of oocytes frozen.

Table 5. In vivo development of mouse oocytes quickly frozen using 3 M ethylene glycol with 0.25 M sucrose or trehalose.

Group	No. of oocytes frozen	No.(%) surviving after thawing and dilution	No.(%) developed to blastocysts and transferred to recipients†	No.(%) fetus‡	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]

†No. of blastocysts/ No. of surviving oocytes inseminated in vitro.

‡No. of live fetuses/ No. of blastocysts transferred.

§No. of live fetuses/ No. of oocytes frozen.

a:b, $P<0.001$.

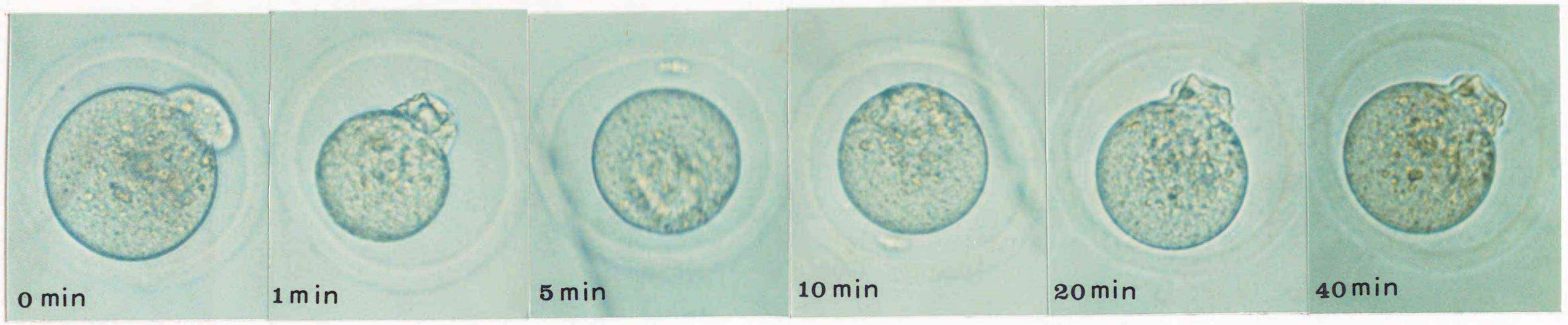

Fig. 1. Light photographs of mouse unfertilized oocyte during increasing equilibration period in the freezing medium (x 400).

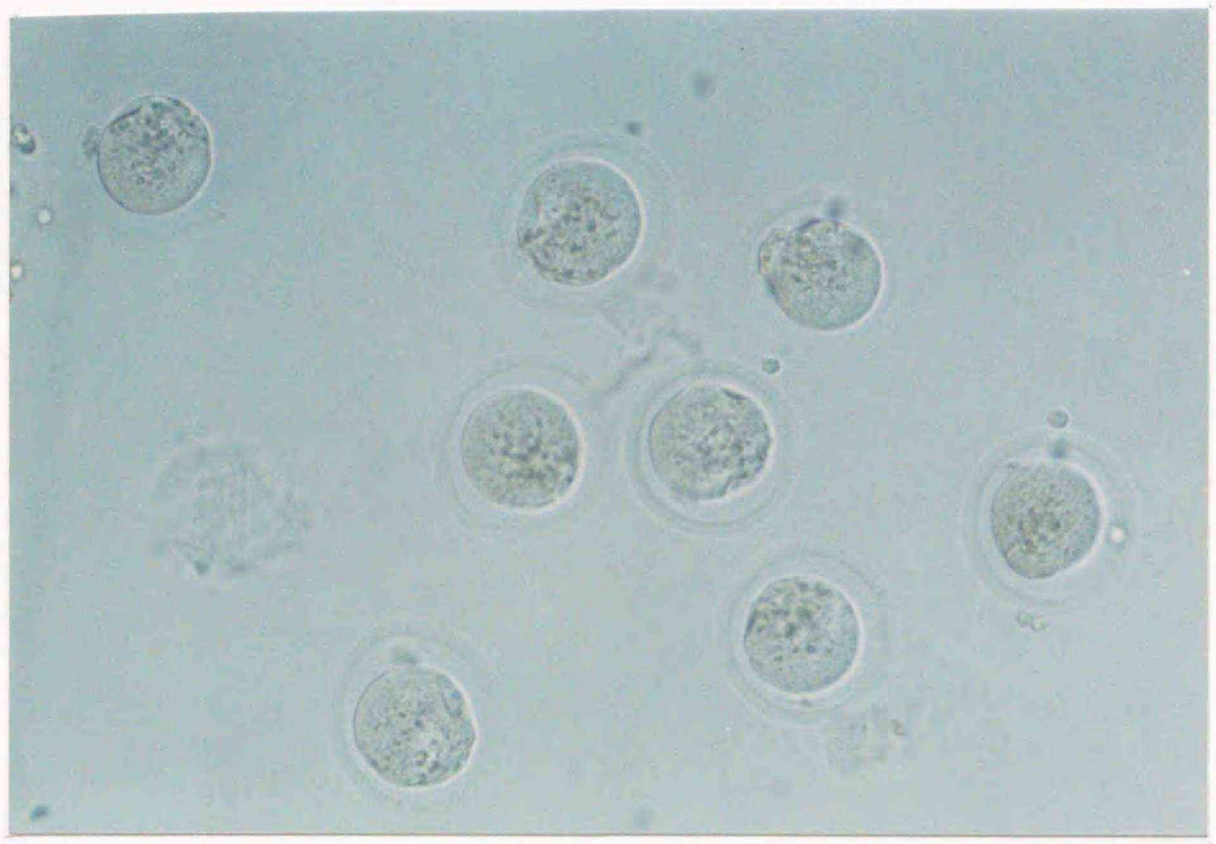

Fig. 2. Morphologically normal mouse oocytes cryopreserved by a quick freezing method after thawing (x200).

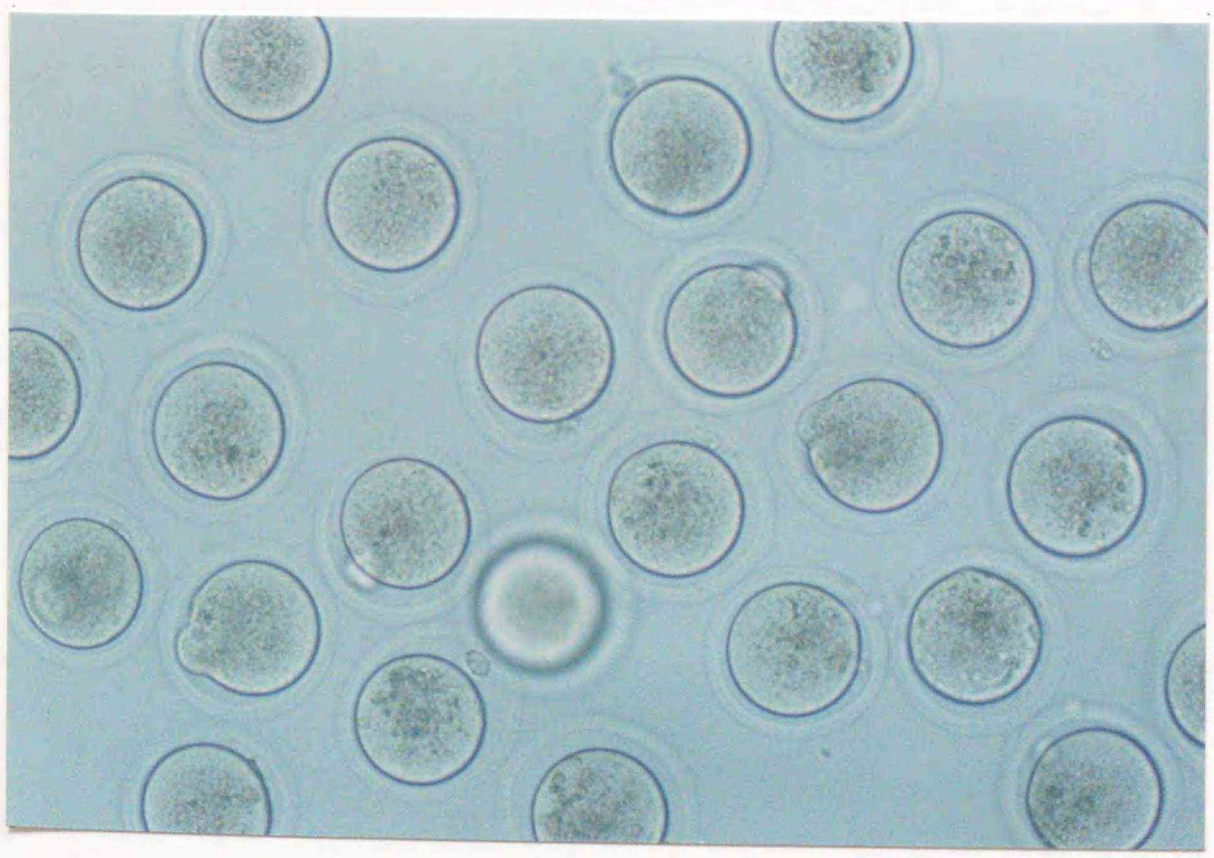

Fig. 3. Surviving (morphologically normal) mouse oocytes after thawing and dilution, before insemination in vitro (x200).

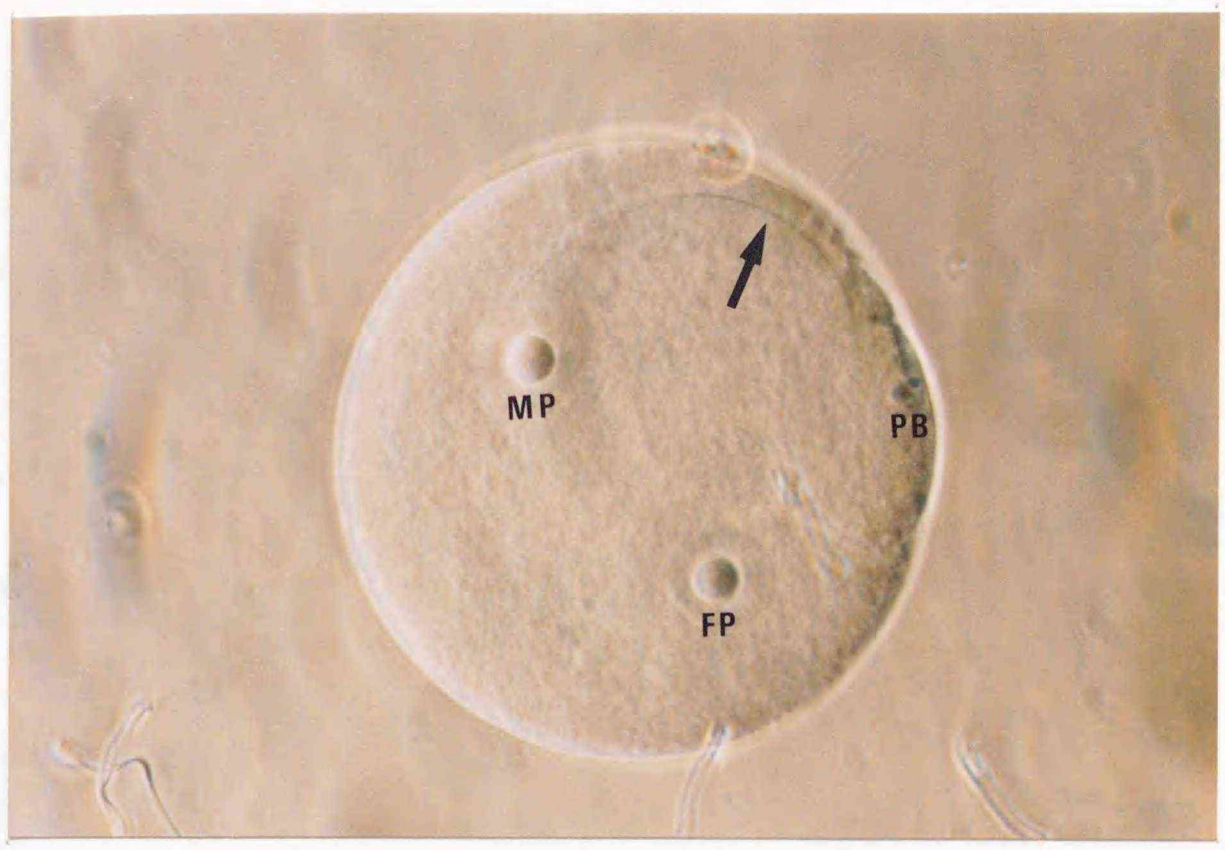

Fig. 4. A normally fertilized frozen-thawed mouse oocyte fixed 6 hours after insemination in vitro showing the female pronucleus (FP) and male pronucleus (MP) with corresponding sperm tail (arrow). PB= polar body. (x 400).

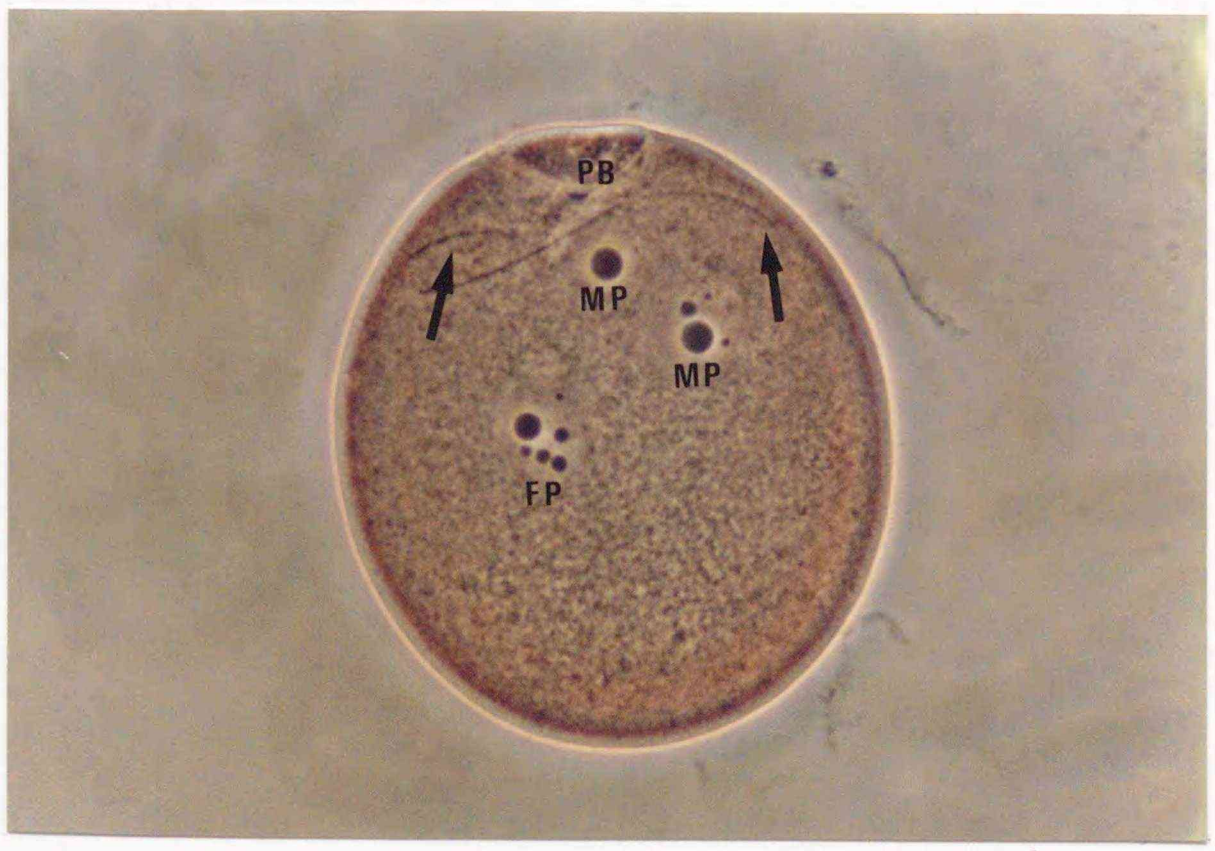

Fig. 5. A polyspermic frozen-thawed mouse oocyte fixed 6 hours after insemination in vitro, showing the female pronucleus (FP) and 2 male pronuclei (MP) with corresponding sperm tails (arrows). PB= polar body. (x 400).

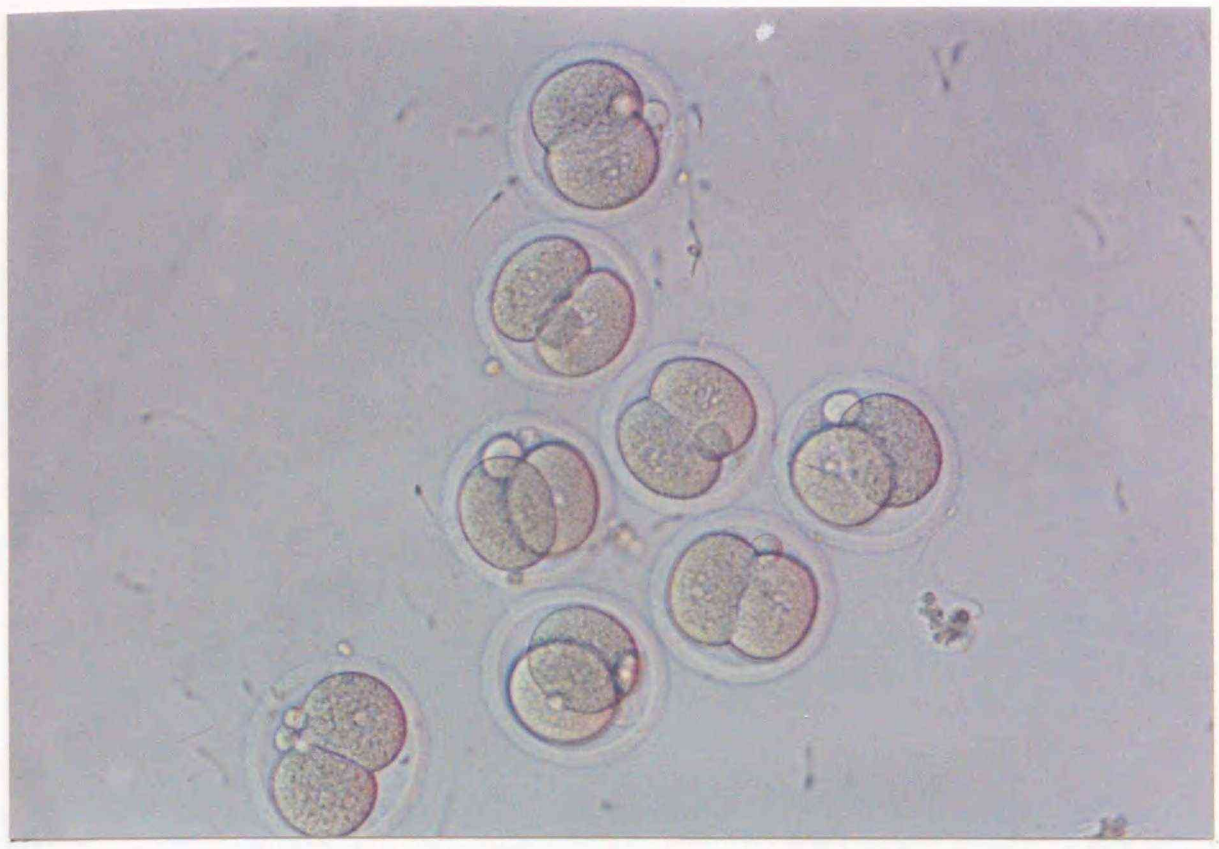

Fig. 6. Two-cell stage embryos derived from frozen-thawed mouse oocytes 24 hours after insemination in vitro (x200).

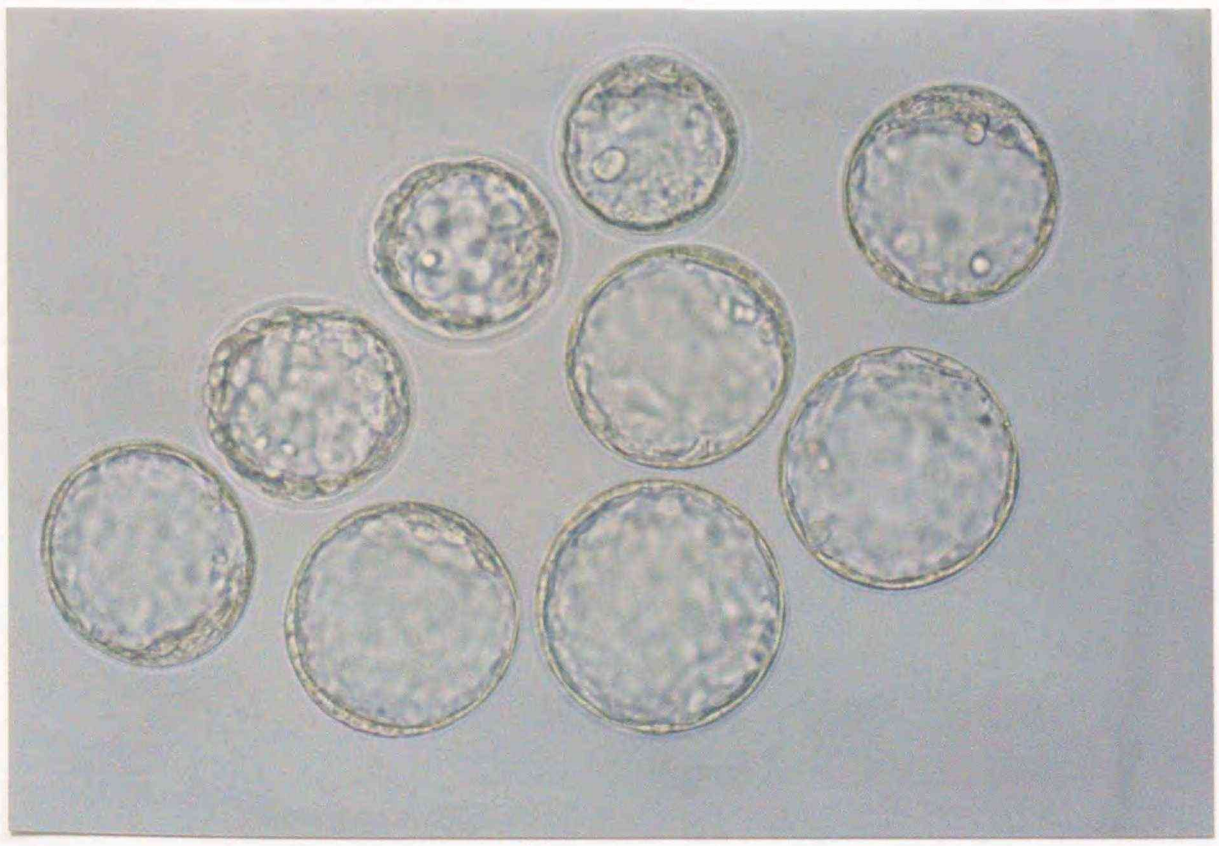

Fig. 7. Expanded blastocysts derived from frozen-thawed mouse oocytes 110 to 120 hours after insemination in vitro (x200).

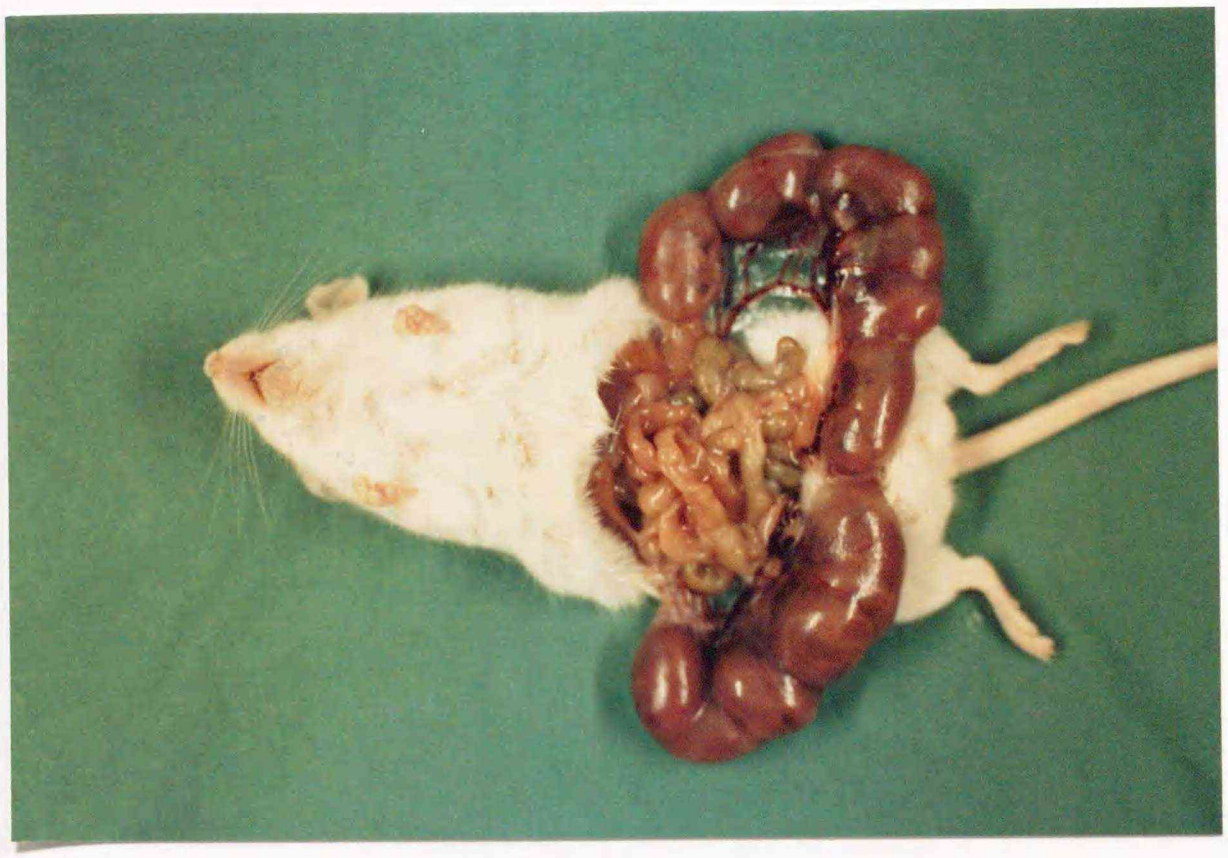

Fig. 8. A recipient necropsied at Day 18 of pregnancy after transfer of blastocysts derived from frozen-thawed mouse oocytes inseminated *in vitro*.

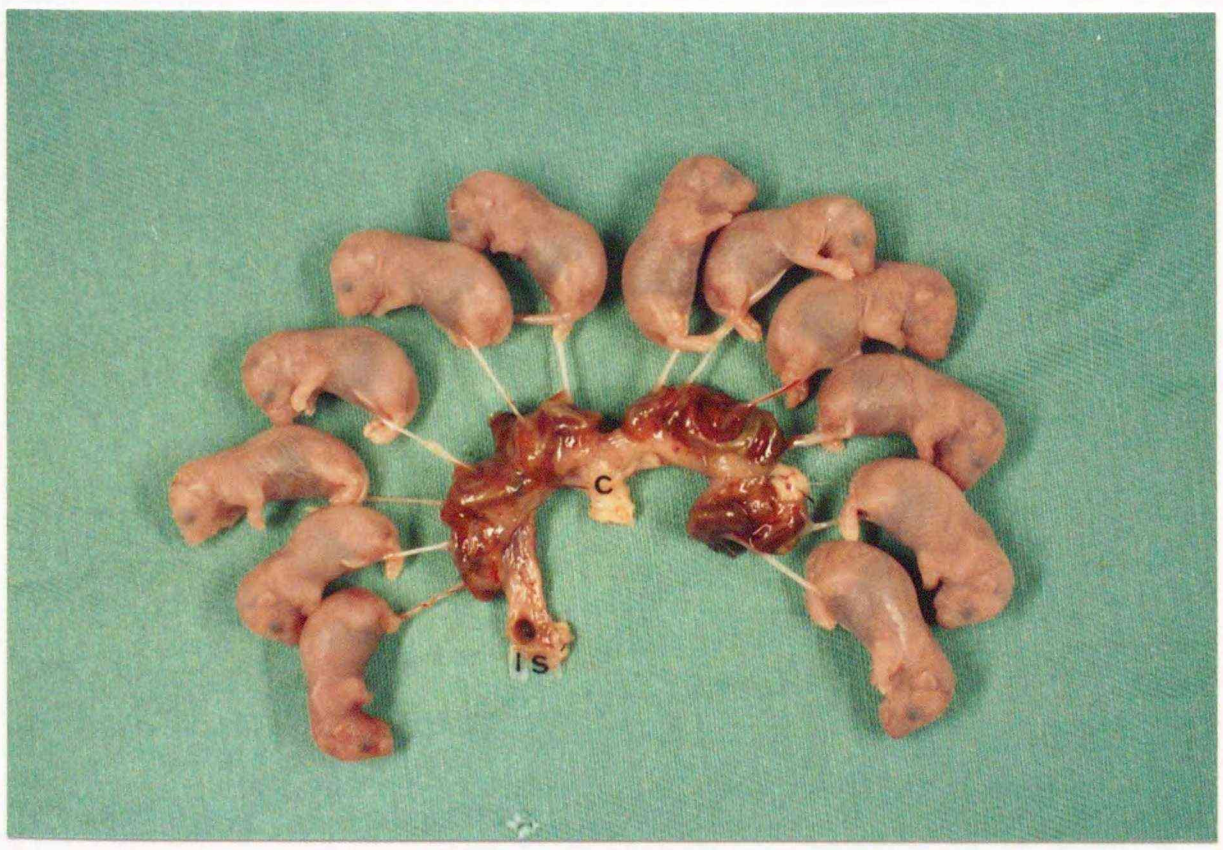

Fig. 9. Late-stage fetuses and implantation site (is) at Day 18 of pregnancy. C= cervix.

control) became pregnant after transfer of blastocysts derived from frozen-thawed and control oocytes.

The *in vivo* development of unfertilized mouse oocytes quickly frozen after 20 minutes equilibration in 3 M ethylene glycol with either 0.25 M sucrose or trehalose is shown in Table 5. Development rates of frozen-thawed oocytes after insemination and culture *in vitro* to the blastocyst stage were significantly lower than the control ($P<0.001$). Blastocysts derived from frozen-thawed and nonfrozen 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 (56.8%). Moreover, the implantation rates between the blastocysts derived from frozen-thawed oocytes were not significantly different with the control (79.5 and 82.1% vs 85.7%). The overall development rates to fetus of blastocysts derived from *in vitro* fertilization of mouse oocytes frozen after 20 minutes equilibration in 3 M ethylene glycol with either 0.25 M sucrose or trehalose 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 into LN_2 vapor.

Our study also confirmed previous reports that equili-

bration in the freezing medium containing high concentration of cryoprotectant is of critical importance for the survival of embryos (MIYAMOTO & ISHIBASHI, 1986; SZELL & SHELTON, 1986; TAKAHASHI & KANAGAWA, 1990) or oocytes (SURREY & QUINN, 1990) frozen at extremely rapid rate than those frozen by the traditional slow freezing. Earlier studies (ABAS MAZNI et al., 1990; RAYOS et al., 1992 a,b) indicate that optimum survival can be obtained with 5 minutes equilibration in 3 M ethylene glycol with 0.25 M sucrose or lactose for mouse morula, and 10 minutes for one-, two-, four-, and eight-cell mouse embryos, thus, allowing sufficient time for the cryoprotectant to permeate into the embryonic cells. Our present study showed that equilibration of unfertilized mouse oocytes for 20 or 40 minutes in the freezing medium before quick freezing results in high post-thaw and post-dilution morphological normality, fertilization, and development to 2-cell embryos and expanded blastocysts. 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 & MAZUR, 1984). Therefore, the length of time that suffices for embryos (5 to 10 minutes) may be insufficient for the permeation of the cryoprotectant into the cell of the oo-

cytes to the extent that the cryoprotective agent can exert its protective effect. Although most of the frozen oocytes were morphologically normal after thawing, more oocytes frozen after short (5 minutes) equilibration may have suffered membrane damage not evident under the stereomicroscope level. Thus, the number of degenerated frozen-thawed oocytes increased after the dilution of the cryoprotectant.

Trehalose has been reported to have a better stabilizing effect on the cell membranes of one- and two-cell rabbit embryos than sucrose, resulting in better viability (SMORAG et al., 1990). Moreover, mouse morula equilibrated in glycerol-trehalose solution before plunging into LN_2 had higher development rate to expanded blastocysts compared with 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 mouse oocytes regardless of the sugar used. This indicates that sucrose or trehalose in combination with ethylene glycol is effective in the quick freezing of unfertilized mouse 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, with viability remaining at the level of sucrose. Thus, it seems that the possible stabilizing effect of trehalose on the cell membranes may be species and cell-stage dependent process.

Unfertilized mouse oocytes cryopreserved after 20 minutes equilibration in the freezing medium had the highest

rate of normal fertilization compared with 5, 10 or 40 minutes of equilibration. This finding was similar to the proportion of surviving frozen-thawed oocytes that developed to 2-cell embryos 24 hours after insemination *in vitro*, respectively. However, subsequent development to expanded blastocysts of the 2-cell embryos derived from frozen-thawed oocytes was similar regardless of the equilibration period. This shows that high proportion of frozen-thawed oocytes which undergo normal fertilization *in vitro* can develop into 2-cell embryos and subsequently into expanded blastocysts.

The fertilization and development rates of frozen-thawed oocytes were significantly lower than that of the control, indicating that some membrane damage may have occurred during freezing because of intracellular ice formation and thawing which could have interfered in the subsequent fertilization and development of the oocytes (WHITTINGHAM, 1977). Membrane damage may result from osmotic forces, including overexpansion of the cell membranes after loss of surface area during dehydration (STEPONKUS & WEIST, 1979) and disruption of membrane integrity by mass movement of water (MULDREW & MCGANN, 1990). Failure of fertilization may also be due to a still undefined modification to the zona pellucida and/or vitelline membrane during freezing and thawing, which inhibits the entry of sperm (CARROLL *et al.*, 1989, 1990; WOOD *et al.*, 1992).

Increased frequency of polyploidy in frozen-thawed oocytes has been reported compared to the nonfrozen control

(CARROLL et al., 1989). Their findings showed that it was caused mainly by the retention of the second polar body and not by polyspermy. This was in contrast with KONO et al. (1991) who reported that increased incidence of polyploidy was due mainly to polyspermic fertilization in vitrified-warmed oocytes compared with the control. This finding was in agreement with our present study in which the incidence of polyspermy, as indicated by the presence of two or more male pronuclei and/or enlarged sperm head (s) was significantly higher than the control.

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 and/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 the zona pellucida during freezing and thawing may enable more than one sperm to enter the egg (WHITTINGHAM & ADAMS, 1976).

There was no indication that any procedure involved in the freezing and thawing in our study stimulated parthenogenetic activation. This finding was in agreement with WHITTINGHAM (1977) and SHAW et al. (1991) who used the conven-

tional and vitrification methods, respectively. However, this was in contrast with KONO et al. (1991) who reported 2 to 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 control 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 which are comparable or superior to oocytes frozen by conventional (TSUNODA et al., 1976; PARKENING et al., 1976, WHITTINGHAM, 1977; FULLER & BERNARD, 1984; GLENISTER et al., 1987; KO & THRELFALL, 1988) or ultrarapid (SURREY & QUINN, 1990) freezing methods. However, our results were lower than those of oocytes cryopreserved by vitrification (NAKAGATA, 1989; KONO et al., 1991; SHAW et al., 1991).

Our study has demonstrated that ethylene glycol in combination with either sucrose or trehalose can be used effectively in the quick freezing of unfertilized mouse oocytes. Quick freezing technique represents an efficient, inexpensive and viable option for the cryopreservation of mouse oocytes and should also be applied successfully to oocytes of other mammals.

SUMMARY

Unfertilized (ovulated) mouse oocytes were frozen by direct plunging into liquid nitrogen (LN_2) vapor after equilibration in a freezing medium containing 3 M ethylene glycol with 0.25 M sucrose or trehalose for 5, 10, 20 and 40 minutes. After thawing and dilution of the cryoprotectant, morphologically normal oocytes 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 normal fertilization rates were obtained with equilibration in 3 M ethylene glycol with 0.25 M sucrose or trehalose for 20 and 40 minutes compared with 5 and 10 minutes equilibration. Development rates to 2-cell embryos and expanded blastocysts of *in vitro* fertilized frozen-thawed oocytes that were equilibrated in the freezing medium for 20 and 40 minutes were significantly higher ($P<0.05$ or $P<0.01$) than with 5 minutes equilibration. To assess *in vivo* development, blastocysts derived from unfertilized mouse oocytes frozen by the optimum treatment (20 minutes equilibration in the freezing medium) were transferred into the uterine horns of Day 3 pseudopregnant female recipients. Development rates of frozen-thawed oocytes to the blastocyst stage after insemination *in vitro* was significantly lower than the 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 the control. The overall development rates to fetus of blastocysts derived from *in vitro* fertilization of mouse oocytes frozen after 20 minutes equilibration in 3 M ethylene glycol with 0.25 M sucrose or trehalose were 20.3 and 22.5%, respectively.

CHAPTER IV

DEVELOPMENTAL CAPACITY OF MOUSE OOCYTES CRYOPRESERVED BY A QUICK FREEZING METHOD BEFORE AND AFTER MATURATION IN VITRO

INTRODUCTION

The methods derived from basic research on in vitro fertilization and embryo transfer have been incorporated into repertoire of available clinical and agricultural technology. Although the methods are extremely useful for treating human infertility and for propagation of valuable animal stocks, they do not take full advantage of the large number of ovarian oocytes, most of which never become available for ovulation and fertilization even when superovulatory treatments are employed (EPPIG et al., 1990). A tremendous number of oocytes having the potential for development and for increasing population is obtainable, if these oocytes could be isolated from their natural environment and mature them in vitro. After maturation in vitro, meiotically competent oocytes can be fertilized, cultured and transferred to recipients or frozen for long-term storage. Moreover, cryopreservation of immature oocytes provides a unique opportunity to store large numbers of female gamete. Improved maturation and freezing procedures may ultimately enable researchers to take advantage of the large number of immature oocytes contained in the mammalian ovary for increasing population of valuable animals and endangered or

rare species.

Despite considerable research during the past 20 years, only modest progress has been made in developing oocyte cryopreservation (PARKS & RUFFING, 1992). Much of the success has been obtained with mature mouse oocytes. There have been few reported attempts to freeze Germinal Vesicle (GV)-stage oocytes (WHITTINGHAM, 1977; HEYMAN *et al.*, 1986; PELLICER *et al.*, 1988; SATHANANTHAN *et al.*, 1988; SCHROEDER *et al.*, 1990) because from the first report of oocyte maturation *in vitro* (PINCUS & ENZMANN, 1935) until recently, efforts to achieve development of *in vitro* matured oocytes have met limited success. Attempts to freeze oocytes after maturation *in vitro* was first reported in mouse (SCHROEDER *et al.*, 1990) and in cattle (LIM *et al.*, 1991, 1992). The above studies used the slow or conventional freezing procedure. Previous studies have shown that two-, four-,eight-, and one-cell mouse embryos (RAYOS *et al.*, 1992 a,b) and unfertilized mouse oocytes (RAYOS *et al.*, in press) could be successfully frozen after equilibration for 10 and 20 min, respectively in a freezing medium containing 3 M ethylene glycol with either 0.25 M sucrose, lactose or trehalose (quick freezing). In the present study, the developmental capacity of mouse oocytes cryopreserved by a quick freezing method before and after maturation *in vitro* was examined.

MATERIALS AND METHODS

Animals

Female F_1 hybrid mice (C57BL/6J x CBA) 4 to 8 weeks of

age and 18 to 25 g in weight were used as oocyte donors. Female ICR strain mice, 10 to 12 weeks of age and 30 to 40 g in weight were used as recipients. They were maintained in a room with controlled lighting 14-hour light (5:00 to 19:00-hour) : 10-hour dark cycle and given pelleted food and water ad libitum. Female F_1 mice were injected intraperitoneally with 5 IU pregnant mare serum gonadotropin (PMSG, Serotrophin, Teikoku Zoki, Tokyo, Japan). Immature (GV-stage) cumulus cell-enclosed oocytes (Fig. 1) were obtained from excised ovaries of the female mice 46 to 48 hours after administration of PMSG and released into modified phosphate-buffered saline (PB1; WHITTINGHAM, 1971) supplemented with 10% fetal bovine serum (FBS, Life Technologies, Inc., Grand Island, New York). The GV-stage oocytes were collected in medium containing 50 μM-1-isobutyl-3-methylxanthine (Aldrich Chemical Co., Milwaukee, WI, USA) to maintain meiotic arrest during processing for freezing. Some of the GV-stage oocytes collected were not frozen but underwent maturation in vitro to serve as control.

Freezing procedure

A modified quick freezing method previously described by TAKAHASHI & KANAGAWA (1985, 1990) was used in this study. The oocytes were pipetted into a culture dish containing the freezing medium (3 M ethylene glycol with 0.25 M sucrose). Ten to 15 oocytes were drawn into a 0.25-ml French straw (I.M.V. L' Aigle, France) which was heat-sealed and equilibrated for 20 minutes. Thereafter, the oocytes were frozen

in liquid nitrogen (LN_2) vapor (about -170°C) by placing the sealed straw horizontally on a styrofoam plate (140 x 60 x 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 LN_2 and stored for 1 to 60 days.

Thawing, dilution and maturation <u>in vitro</u>

Thawing was done in a water bath at 37°C for 20 seconds. Thereafter, the contents of the straw were expelled into a Petri dish and the oocytes were immediately pipetted into 0.5 M sucrose for one-step dilution of the cryoprotectant. After 5 minutes, the oocytes were washed three times with PB1 with 10% FBS and morphologically normal (surviving) oocytes were transferred into 100 µl Waymouth medium (Eppig et al., 1990) supplemented with 5% FBS, 0.23 mM pyruvic acid, 7mM taurine (Sigma Chemical Co., St. Louis, MO, USA), 0.05 mg streptomycin sulfate/ml, 0.075 mg penicillin G potassium salt/ml and 1 µg follicle stimulating hormone (ovine pituitary, Sigma Chemical Co.) under paraffin oil and incubated for 15 to 16 hours at 37°C in an atmosphere of 5% CO_2 in air for maturation <u>in vitro</u>. Thereafter, the oocytes were fixed with 10% neutral formalin overnight, washed with 99.5% ethanol,stained with 0.25% aceto-lacmoid and examined under phase-contrast microscope for assessment of the maturation rate. The oocytes were considered mature when they showed the metaphase II (MII) plate and extruded the first polar body. Nonfrozen GV-stage oocytes were also cultured in microdrops of Waymouth medium for 15 to 16 hours to serve as control.

In a separate experiment, GV-stage cumulus cell-enclosed oocytes were matured *in vitro* before freezing. Fifteen to 16 hours after incubation in the maturation medium, some oocytes were fixed, stained and examined under phase-contrast microscope to assess the maturation rate. The remainder were pipetted into PB1 with 10% FBS and containing 150 units/ml of hyaluronidase (Sigma Chemical Co.) for 5 to 10 minutes. When the cumulus cells were detached, the oocytes were washed three times in PB1 with 10% FBS and pooled in sterile plastic culture dishes until used. The *in vitro* matured oocytes were cryopreserved by a quick freezing method as described previously. Only morphologically normal oocytes were used in this study.

Fertilization *in vitro*

In vitro fertilization was carried out according to the method of TOYODA *et al*. (1971). All surviving oocytes after freezing, thawing and dilution as well as the nonfrozen control underwent insemination *in vitro*. Semen was collected from the cauda epididymis, one each from two mature F_1 (C57BL/6J x CBA) males, allowed to disperse for 30 minutes in 0.4 ml TYH medium under paraffin oil at 37°C in an atmosphere of 5% CO_2 in air before fertilization *in vitro*. Thereafter, the sperm concentration was determined by means of a hemocytometer and the sperm was further incubated for another 1 to 1.5 hours. A suitable volume (4 to 10 μl) of sperm was added to the medium containing the oocytes and incubated for 6 hours, providing a final sperm concentration of 150

cells/μl. To assess the fertilization rate some of the frozen-thawed and nonfrozen control oocytes were removed 6 hours after insemination, fixed and stained as previously described then examined under 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 rate of normal fertilization was determined as the ratio of oocytes with a pair of pronuclei to the total number of oocytes evaluated. The remaining oocytes were cultured in 100 μl microdrops of Whitten's medium (WHITTEN, 1971) supplemented with 3 mg/ml BSA and 100 μM EDTA (Kanto Chemical Co., Tokyo, Japan) under paraffin oil at 37 °C in 5% CO_2 in air. After 19 hours of incubation (24 hours after insemination), the culture was examined under inverted microscope and 2-cell embryos were recorded. Development to the expanded blastocyst stage was assessed 110 to 120 hours after insemination.

Development *in vivo*

In another experiment, blastocysts derived from frozen-thawed and nonfrozen control oocytes after insemination and culture *in vitro* were transferred into the uterine horns of Day 3 pseudopregnant female ICR recipients (6 to 9 embryos per horn). The recipients were necropsied on Day 18 of pregnancy, and the number of live and resorbing fetuses, as well as implantation sites was examined.

Statistical analysis

Experiments were replicated four times and the pooled data on maturation *in vitro*, fertilization, and development *in vitro* and *in vivo* were analyzed by the Chi-square test.

RESULTS

Morphological survival after freezing and thawing

More than 90% of the frozen GV-stage oocytes were recovered after thawing. Morphological normality after thawing and dilution was 68.9% (Table 1). Although some immature oocytes were partially or completely denuded of cumulus cells after thawing and dilution, most of them showed normal morphology (Fig. 2).

Similarly, almost all (95%) of the cryopreserved oocytes matured *in vitro* were recovered from the freezing straws after thawing. After thawing and dilution of the cryoprotectant, 72.7% of the frozen-thawed oocytes showed normal morphology.

Oocyte maturation

Expansion of the cumulus cells of frozen-thawed GV-stage oocytes was not observed 15 to 16 hours after maturation *in vitro* (Fig. 3). Moreover, less than 5% of the oocytes fixed, stained and examined to determine the maturation rate exhibited the MII plate and extruded the first polar body. On the other hand, nonfrozen GV-stage oocytes showed cumulus cell expansion and more than 90% exhibited the MII plate and extruded the first polar body (Figs. 4 and 5).

Fertilization *in vitro*

Because very limited numbers of ovarian oocytes frozen at the GV-stage developed to the MII-stage of meiosis *in vitro* following thawing, no further experiments were conducted on fertilization, development *in vitro* and *in vivo* were.

The total and normal fertilization rates of the frozen-thawed *in vitro* matured mouse oocytes were not significantly different from the control as shown in Table 2. Most of the frozen-thawed oocytes that had undergone fertilization were normally fertilized, as signified by the presence of female and male pronuclei with remnant sperm tail (Fig. 7). Although no significant difference was observed, higher rate of polyspermy (17.2%) was obtained from the frozen-thawed oocytes compared with the control (10.6%).

Development *in vitro*

The rate of development to 2-cell and expanded blastocyst stage embryos of frozen-thawed oocytes after insemination and culture *in vitro* was significantly lower ($P<0.001$ and $P<0.01$, respectively) than the control as shown in Table 3. However, development of 2-cell embryos derived from frozen-thawed oocytes to expanded blastocysts was not significantly different from the control (67.6 vs 72.1%).

Development *in vivo*

A total of 13 out of 16 recipients became pregnant after transfer of blastocysts derived from frozen-thawed and control oocytes matured *in vitro* (Table 4). Pregnancy rates

Table 1. Survival and maturation *in vitro* of cumulus cell-enclosed GV-stage oocytes

Treatment	No. of oocytes	No. (%) of oocytes recovered	No. (%) surviving after thawing and dilution*	No. (%) matured *in vitro*
Frozen	130	119(91.5)	82(68.9)	4(4.9)
Non-frozen	114			109(95.6)

* oocytes with normal morphology.

Values represent data from four replicates.

Table 2. Fertilization rate of mouse oocytes cryopreserved by a quick freezing method after maturation *in vitro*

Treatment group	No. of oocyte examined	No. of oocyte fertilized		
		Total (%)†	Normal(%)‡	Polyspermic(%)§
Frozen-thawed	87	73(83.9)	58(66.7)	15(17.2)
Nonfrozen control	94	84(89.4)	74(78.7)	10(10.6)

Values represent data from four replicates.

†Total no. fertilized/No. of oocytes inseminated *in vitro*.

‡No. normally fertilized/No. of oocytes inseminated *in vitro*.

§No. polyspermy/No. of oocytes inseminated *in vitro*.

Table 3. *In vitro* development of mouse oocytes cryopreserved by a quick freezing method after maturation *in vitro*

Treatment	No. of oocyte examined	No.(%) developed to	
		2-cell embryo†	Expanded Blastocyst‡
Frozen-thawed	102	71(69.6)[a]	48(47.1)[c]
Nonfrozen control	197	165(83.4)[b]	119(60.4)[d]

Values represent data from four replicates.

Values with different superscripts in the same column are significantly different (a:b, $P<0.001$; c:d, $P<0.01$).

†No. developed to 2-cell embryos/No. of oocytes inseminated *in vitro*.

‡No. developed to expanded blastocysts/No. of oocytes inseminated *in vitro*.

Table 4. *In vivo* development of blastocysts derived from quickly frozen mouse oocytes matured, inseminated and cultured *in vitro*

Treatment group	No. of recipients		No. of embryos transferred		No. of implantation(%)†	No. of fetus(%)‡
	used	pregnant	Total	to pregnant recipients		
Frozen-thawed	8	6	118	89	69(77.5)	45(50.6)
Non-frozen control	8	7	120	106	86(81.1)	57(53.8)

Values represent data from four replicates.

†No. of live and resorbing fetuses as well as implantation sites/ No. of blastocysts transferred.

‡No. of live fetuses/ No. of blastocysts transferred.

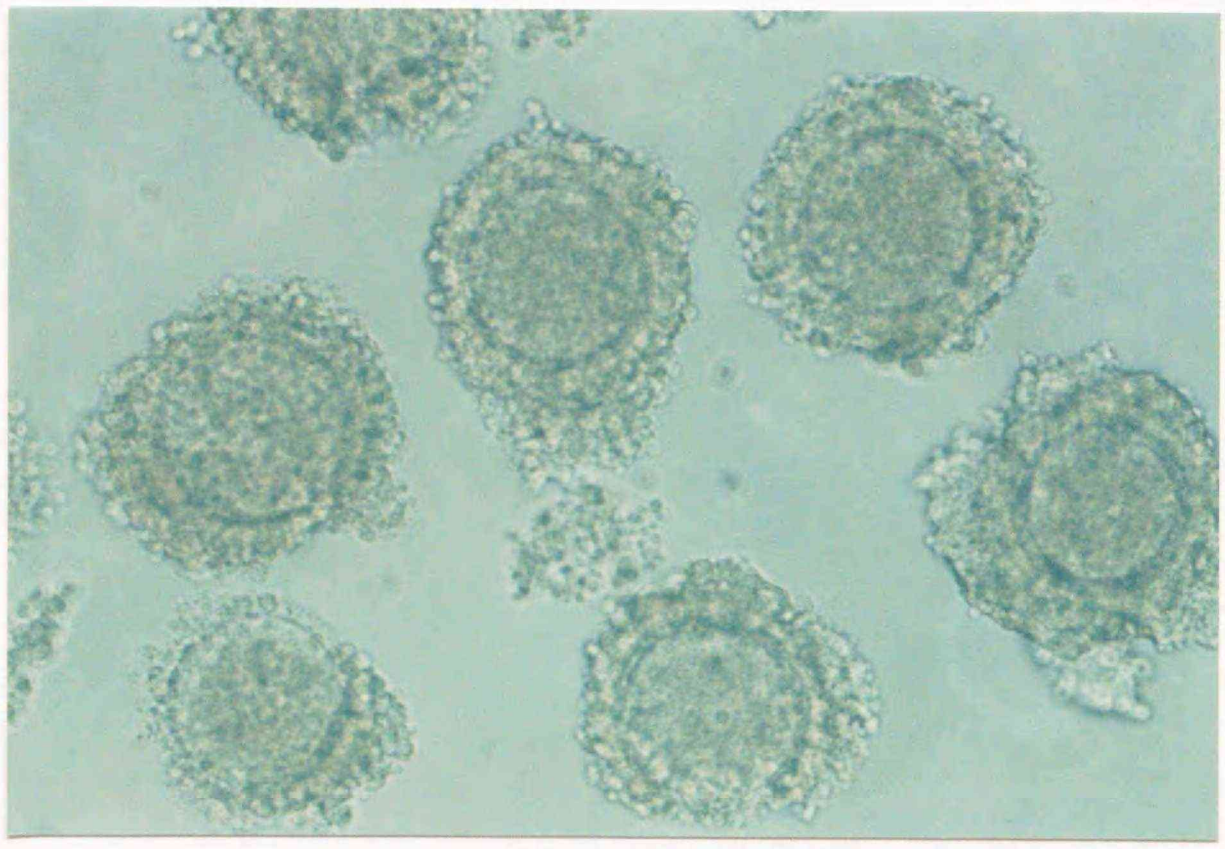

Fig. 1. Cumulus cell-enclosed GV-stage mouse oocytes before freezing or maturation in vitro (x200).

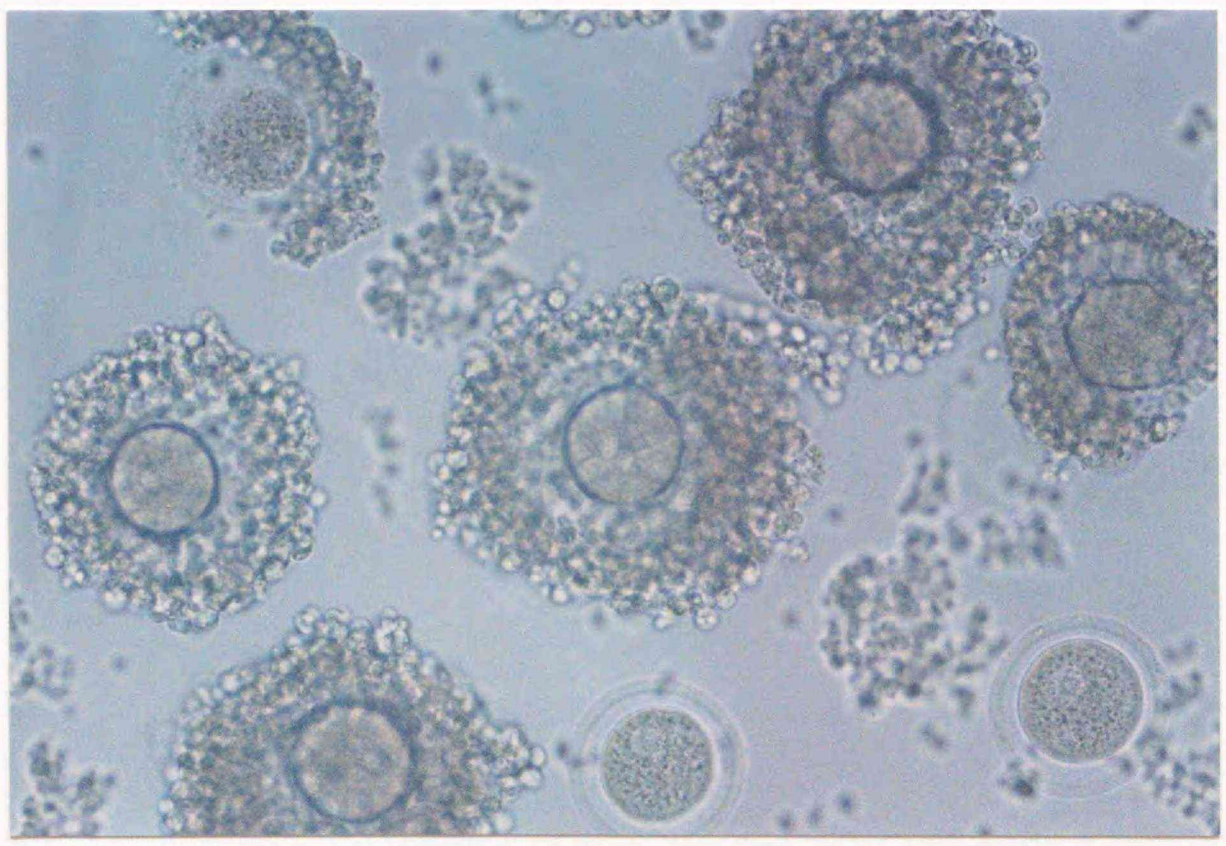

Fig. 2. Frozen-thawed mouse oocytes before maturation in vitro (x200).

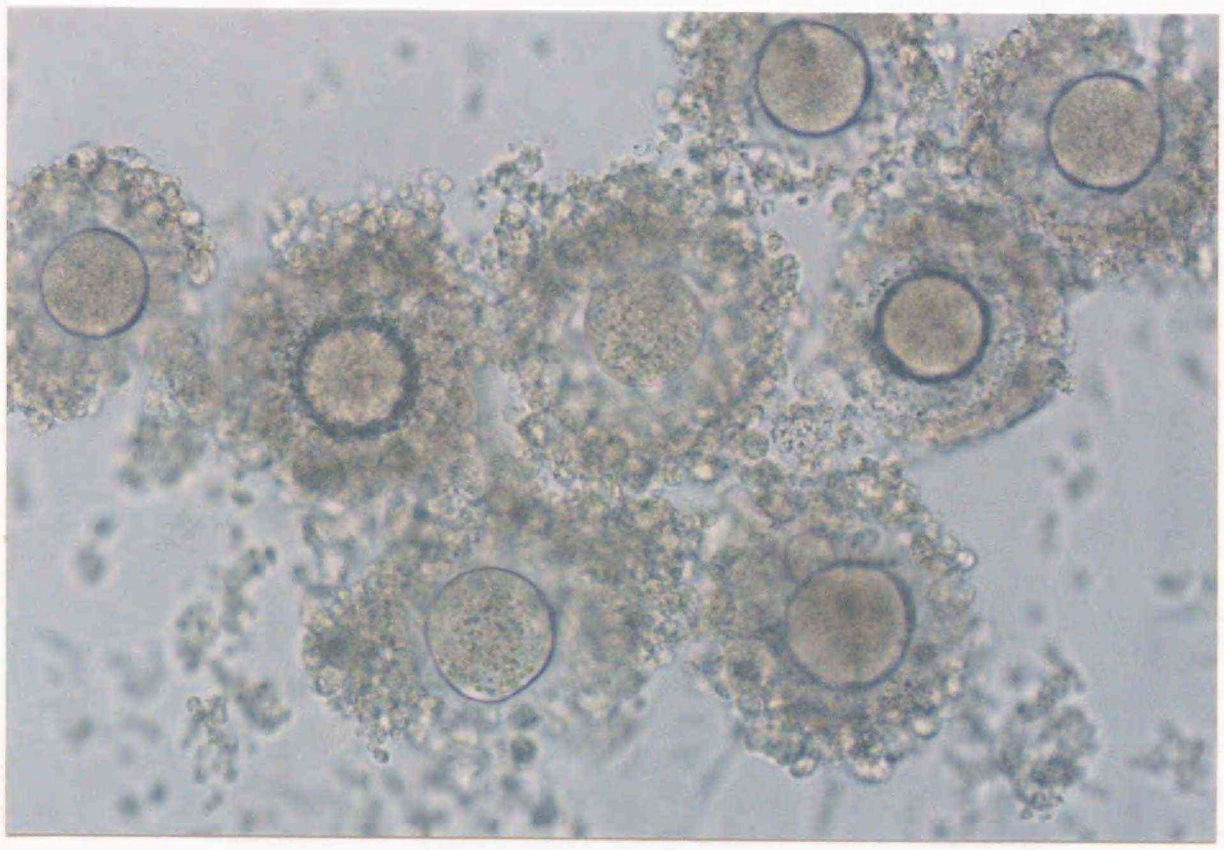

Fig. 3. Frozen-thawed mouse oocyte after maturation in vitro (x200).

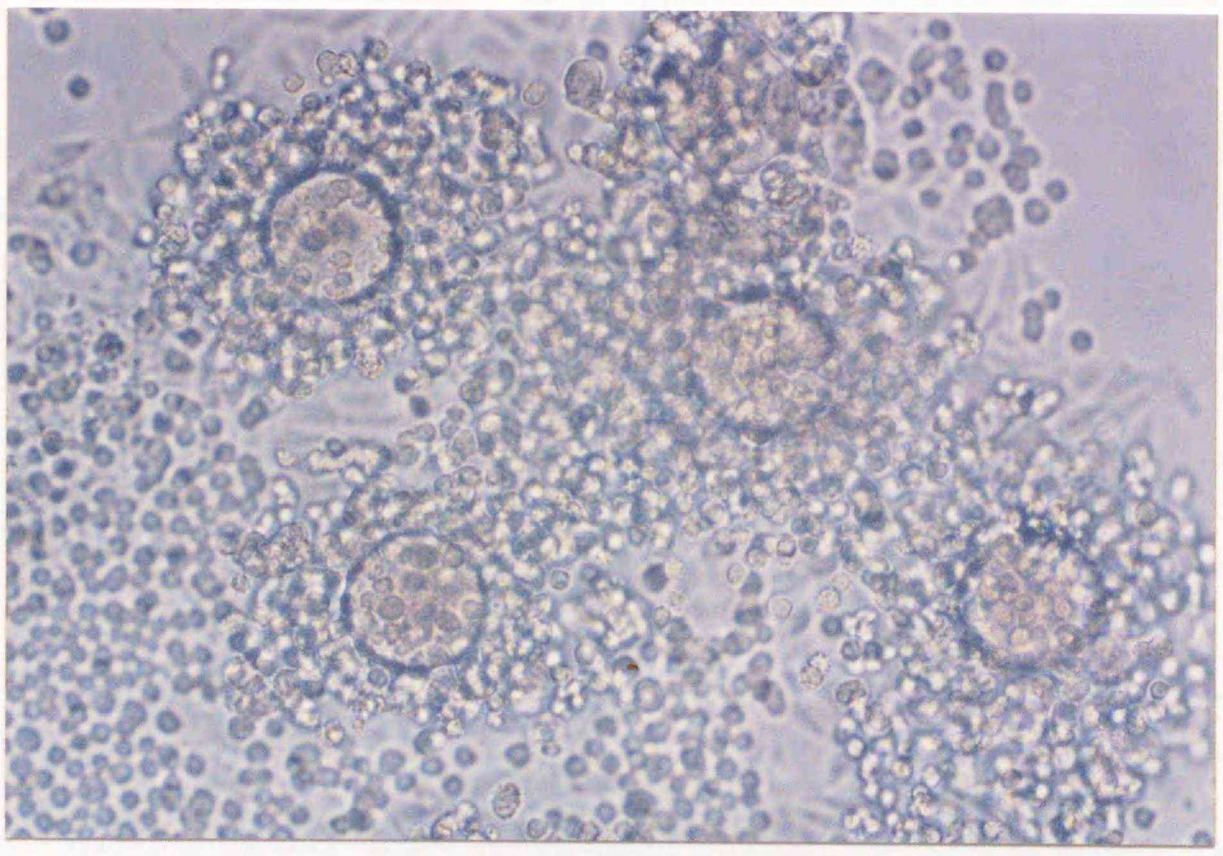

Fig. 4. Nonfrozen mouse oocytes after maturation in vitro (x200).

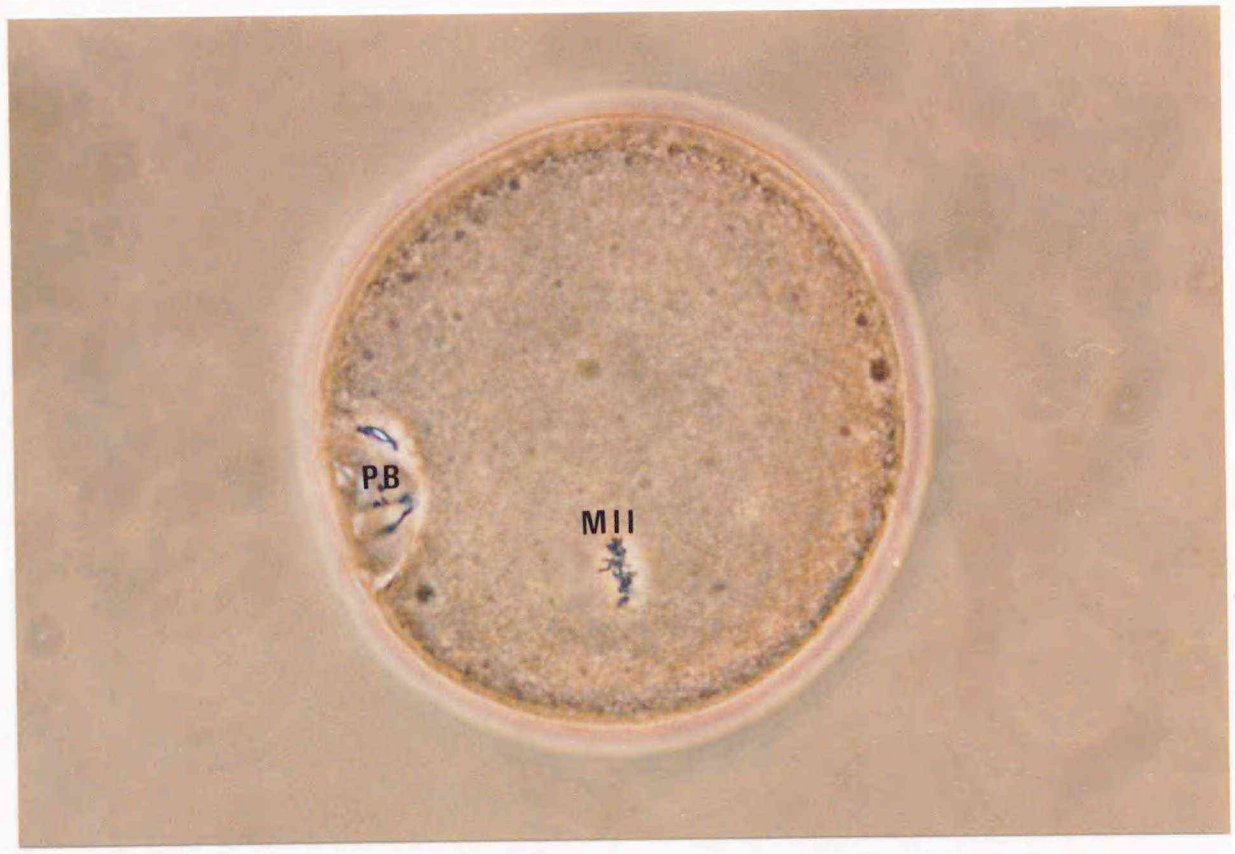

Fig. 5. An oocyte at the metaphase of the second meiotic division fixed 15¼ to 16 hours after maturation in vitro, showing first polar body (PB) and metaphase plate of chromosomes (MII) (x400).

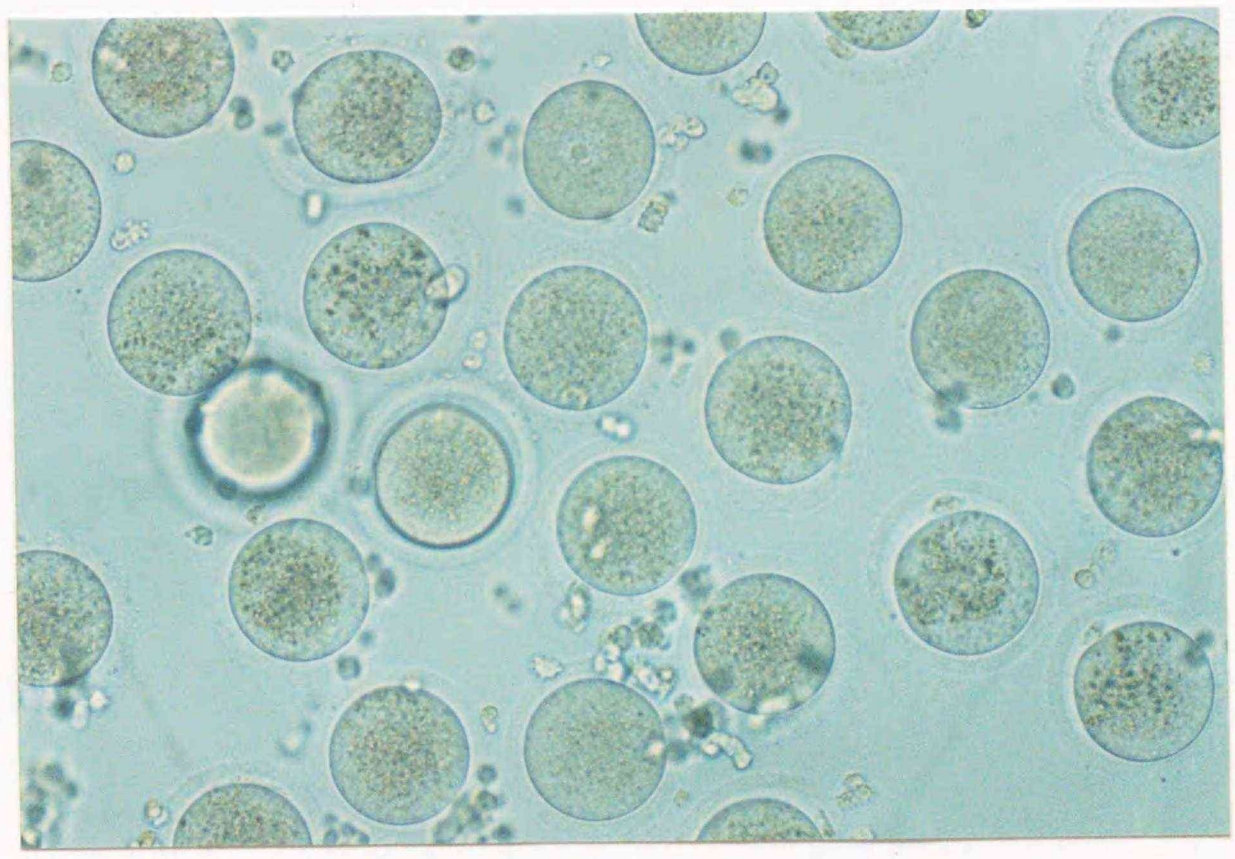

Fig. 6. Frozen-thawed, in vitro matured mouse oocytes before insemination in vitro (x200).

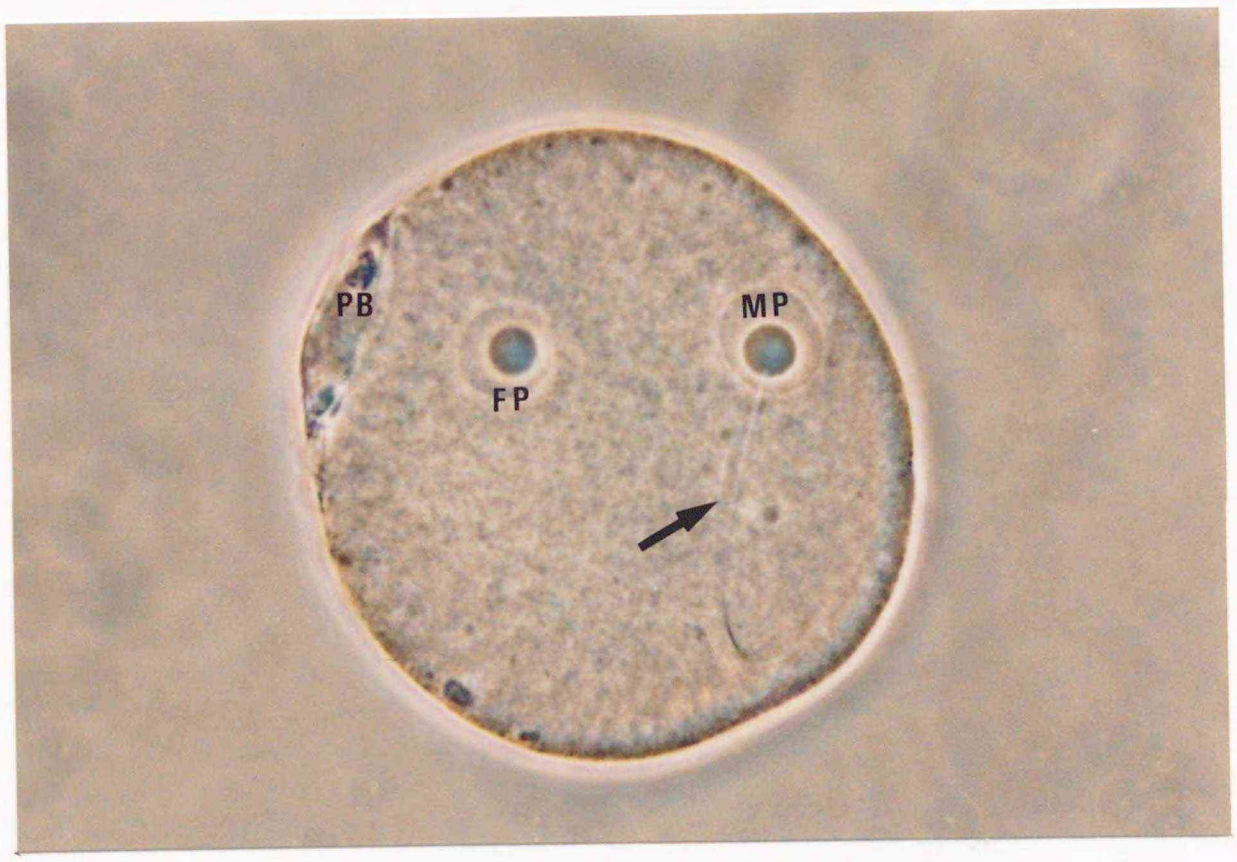

Fig. 7. A normally fertilized frozen-thawed mouse oocyte matured in vitro fixed 6 hours after insemination in vitro, showing a female pronucleus (FP), male pronucleus (MP) and corresponding sperm tail (arrow). PB= polar body. (x400).

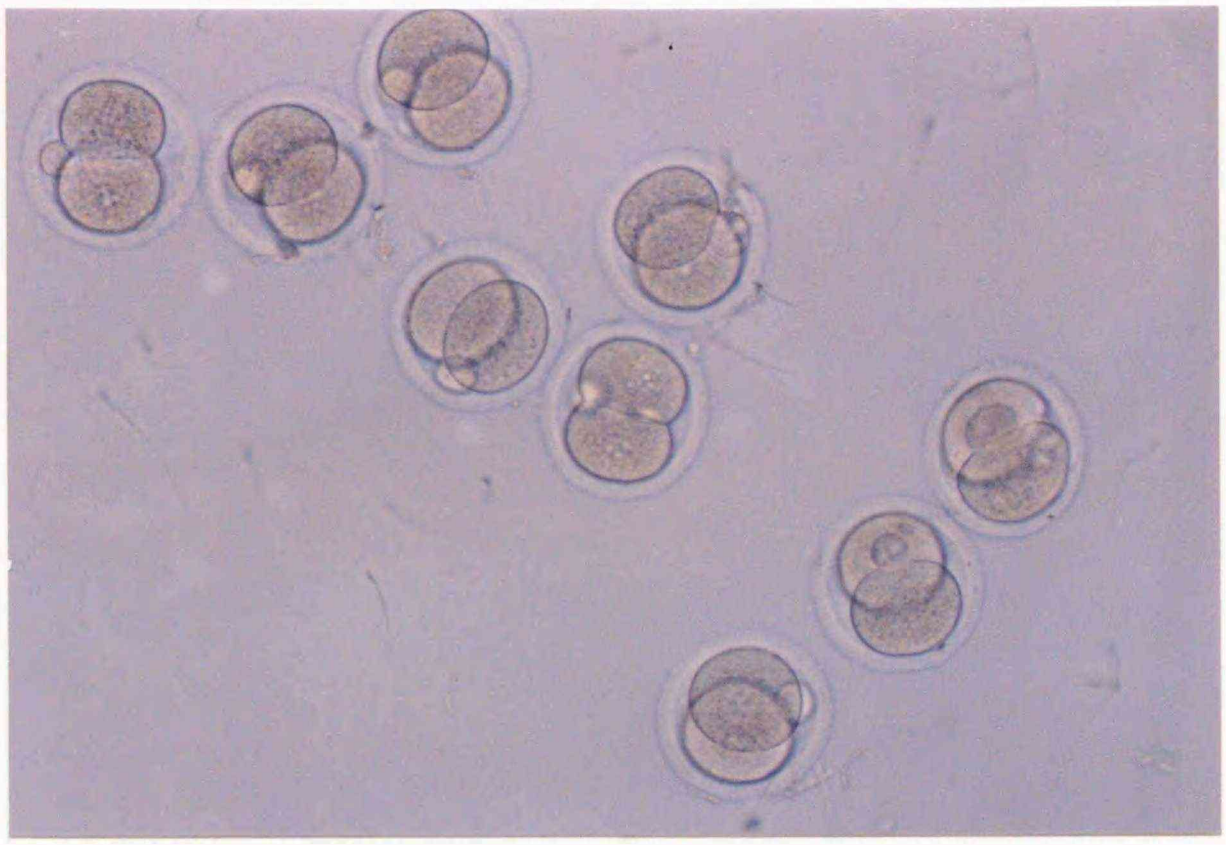

Fig. 8. Two-cell stage embryos derived from frozen-thawed mouse oocytes matured *in vitro* 24 hours after insemination *in vitro* (x 200).

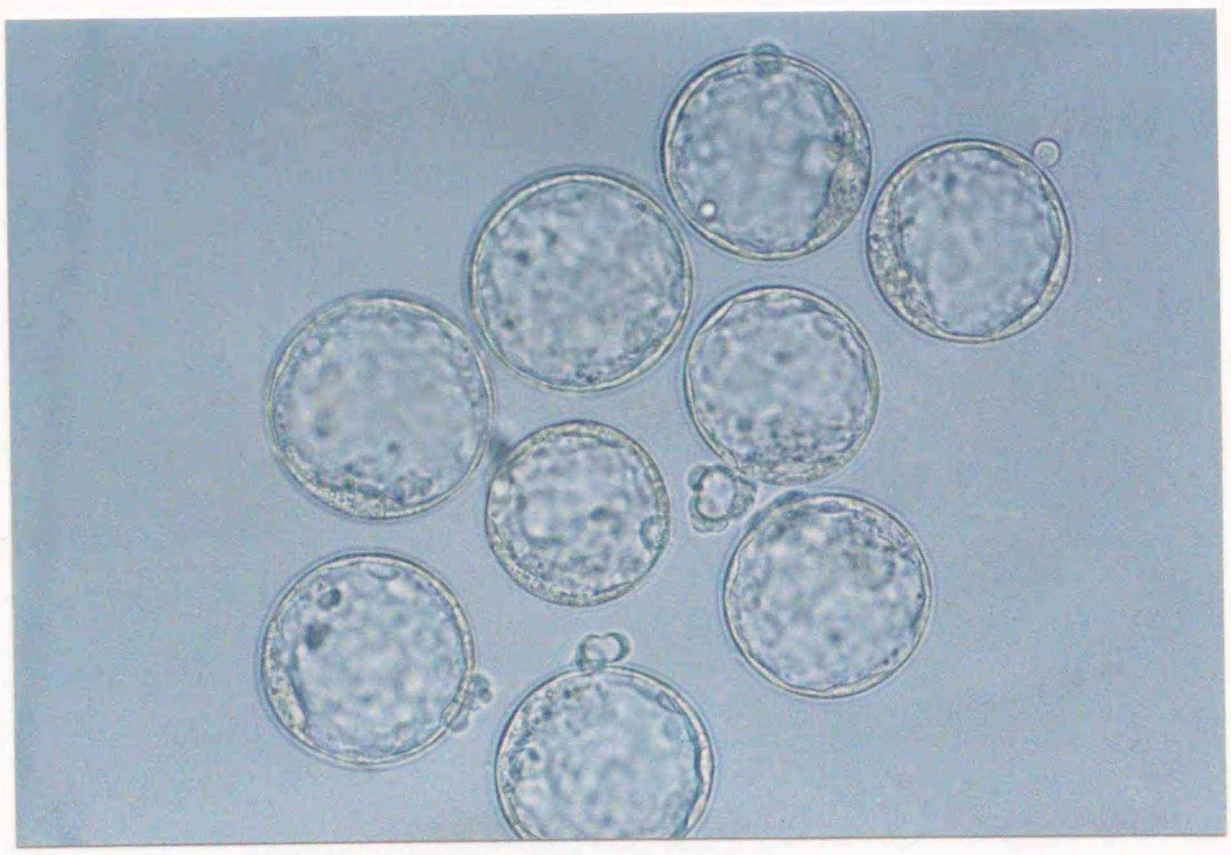

Fig. 9. Expanded blastocysts derived from frozen-thawed mouse oocytes matured *in vitro* 110 to 120 hours after insemination *in vitro* (x 200).

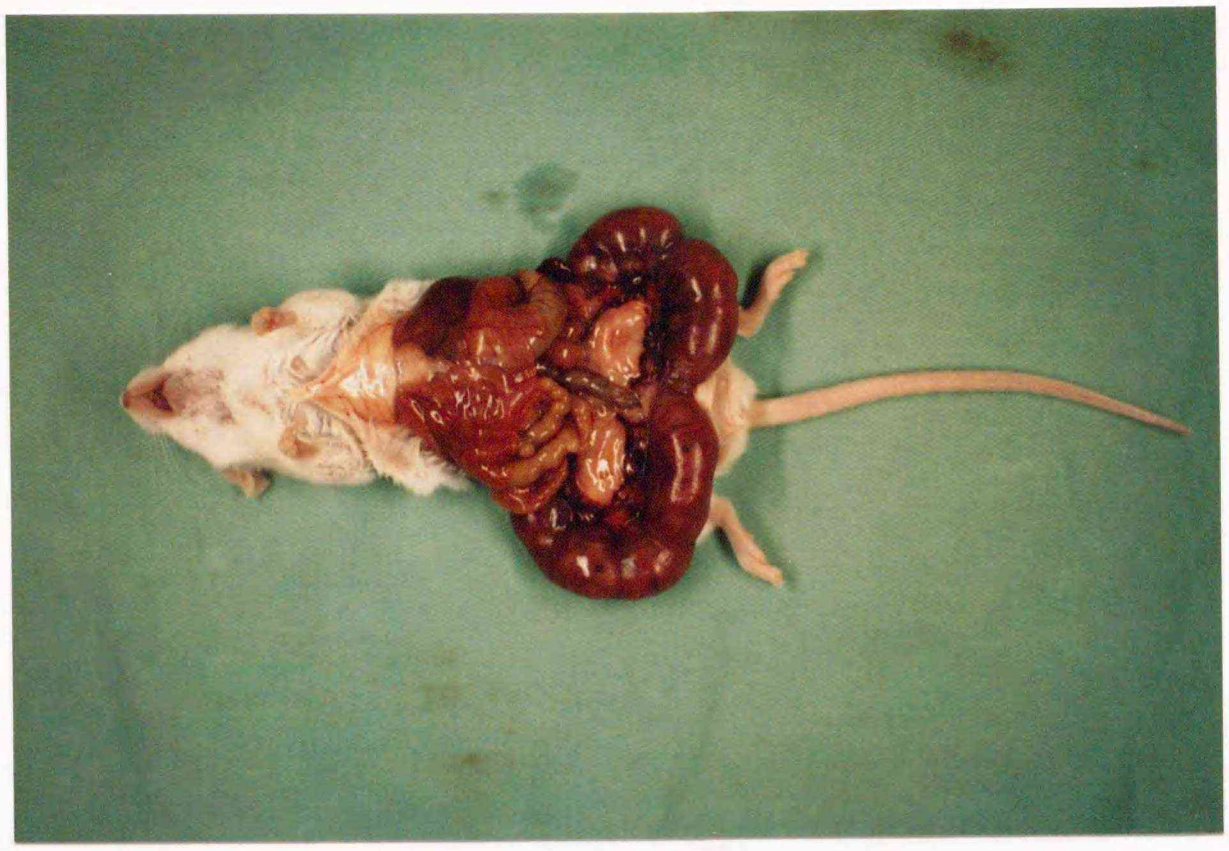

Fig. 10. A recipient at Day 18 of pregnancy after transfer of blastocysts derived from frozen-thawed mouse oocytes matured and inseminated in vitro.

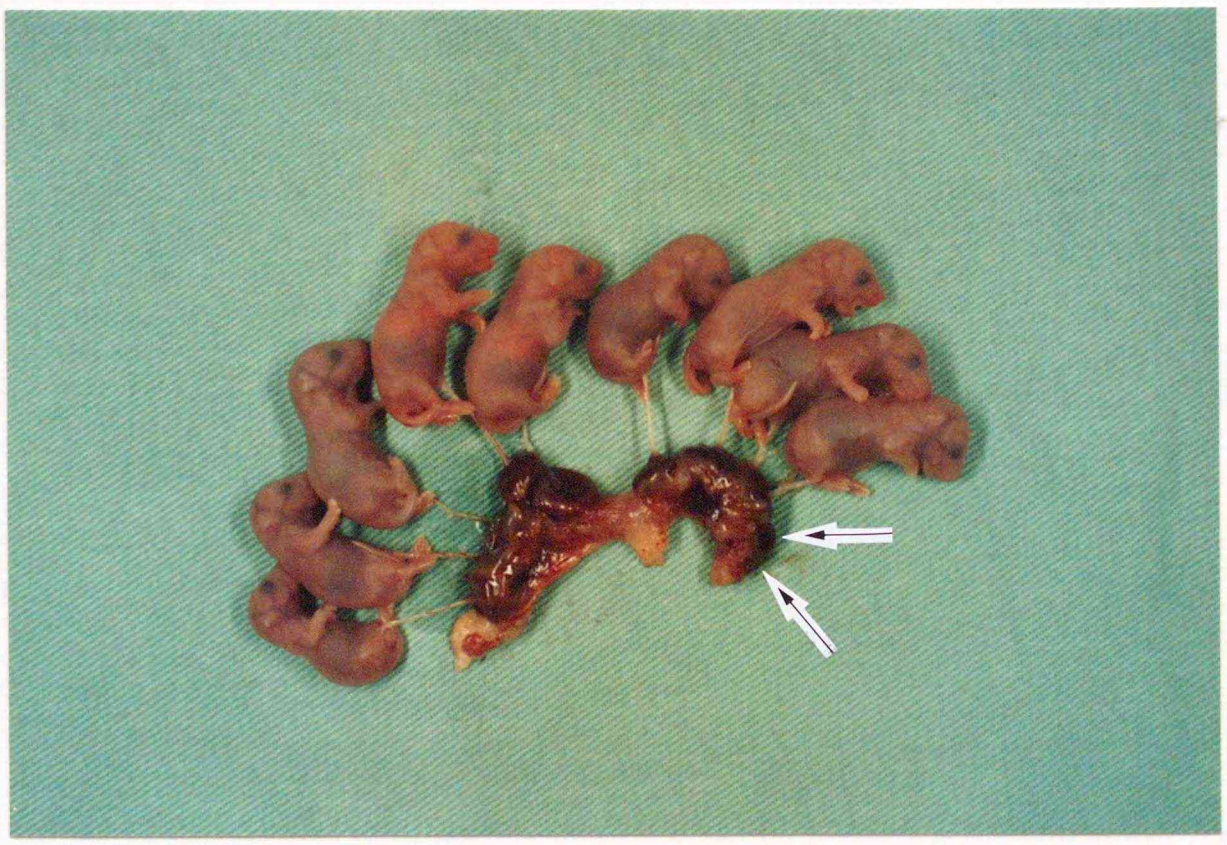

Fig. 11. Late-stage fetuses and implantation sites (arrows) at day 18 of pregnancy.

of 87.5% (7/8) and 75.0% (6/8) were obtained for frozen-thawed and control recipients, respectively. The rates of implantation and development to normal fetuses of blastocysts derived from frozen-thawed oocytes (77.5 and 50.0%) did not significantly differ from the control (81.1 and 53.8%).

DISCUSSION

Results of the present study showed that GV-stage oocytes cryopreserved by the quick freezing method have very low rate (<5%) of maturation *in vitro* compared with the nonfrozen control (>90%) which is in agreement with previous studies (WHITTINGHAM, 1977; SATHANANTHAN *et al.*, 1988). Although SCHROEDER *et al.* (1990) reported high maturation rates (90 to 95%) of oocytes that survived slow freezing, only a few developed to the 2-cell stage and none of the embryos developed to blastocysts. This could be the result of damaged cumulus cells being unable to support maturation fully and/or possible damage to the microtubule organization (SATHANANTHAN et al., 1988; PARKS & RUFFING, 1992).

However, successful cryopreservation of mouse oocytes after maturation *in vitro* using a quick freezing method was obtained in this study. The fertilization and development capacity *in vitro* or *in vivo* between frozen-thawed and nonfrozen *in vitro* matured control oocytes were compared. Our findings showed that frozen-thawed oocytes matured *in vitro* had a lower rate of fertilization and higher rate of

polyspermy than the nonfrozen control which are in agreement with GLENISTER et al. (1987), CARROLL et al. (1989,1990) and our previous study (RAYOS et al., in press). Moreover, development to 2-cell embryos (69.6%) and subsequently to expanded blastocysts (47.1%) of frozen-thawed in vitro matured oocytes were significantly lower than the control (83.4 and 60.4%, respectively). Subsequent development in vitro to expanded blastocysts of 2-cell embryos derived from frozen-thawed oocytes matured in vitro (67.6%) was not significantly different from the control (72.1%) but lower (77.0%) than those of SCHROEDER et al. (1990).

The fertilization and development rates of frozen-thawed oocytes matured in vitro were lower than the control. These could be due to some membrane damage that occurred during freezing from intracellular ice formation (WHITTINGHAM, 1977) or to freeze-thaw-induced changes of the zona pellucida and/or the vitelline membrane which inhibit the entry of sperm (CARROLL et al.,1989, 1990; WOOD et al., 1992). Increased frequency of polyspermy of frozen-thawed oocytes matured in vitro was also observed in our study. This finding agrees with those of GLENISTER et al. (1987), CARROLL et al. (1989) and RAYOS et al. (in press) in frozen-thawed ovulated mouse oocytes. The release of cortical granules during cryoprotectant exposure and freezing appear to cause premature cortical granule reaction leading to either a block to fertilization or an impaired block to polyspermy (PARKS & RUFFING, 1992). Moreover, physical

damage to the zona pellucida or the vitelline membrane during freezing and thawing may allow more than one sperm to penetrate the egg (WHITTINGHAM & ADAMS, 1976).

Forty-five Day-18 normal fetuses were produced from blastocysts that resulted from fertilization and culture of frozen-thawed oocytes matured *in vitro* at a rate similar to the control. Moreover, the rates of implantation between blastocysts derived from frozen-thawed oocytes matured *in vitro* and the control did not significantly differ. Similar result was also obtained from a previous study (RAYOS *et al.*, in press) which utilized ovulated mouse oocytes. The only available report (SCHROEDER *et al.*, 1990) on frozen-thawed mouse oocytes matured and fertilized *in vitro*, yielded only 41 (26%) live young after transfer of 158 2-cell stage embryos to the recipients which was lower than our study.

The study presented here shows that freezing of GV-stage oocytes is difficult using the quick freezing method. However, oocytes matured *in vitro* can be successfully cryopreserved after equilibration for 20 minutes in 3 M ethylene glycol with 0.25 M sucrose before plunging into LN_2 vapor. Although the frozen-thawed oocytes had lower fertilization and *in vitro* development rates compared with the control, blastocysts obtained from frozen-thawed oocytes matured *in vitro* have *in vivo* developmental capacity similar to the control. However, the developmental capacity *in vitro* of quickly frozen-thawed and nonfrozen mouse oocytes isolated

at the GV-stage and matured *in vitro* is lower than that of frozen-thawed and nonfrozen ovulated oocytes (RAYOS et al., in press).

SUMMARY

The survival and developmental capacity of mouse oocytes frozen by a quick freezing method before and after maturation *in vitro* was examined. The survival (morphological normality) rates were 68.9 and 72.7% after thawing and dilution for frozen-thawed GV-stage oocytes and oocytes matured *in vitro*. The maturation of frozen-thawed GV-stage oocytes was very limited, thus further experiments on fertilization, *in vitro* and *in vivo* development were not conducted.

For frozen-thawed oocytes matured *in vitro*, the proportions of fertilized oocytes and 2-cell embryos, 6 and 24 hours after insemination were 83.9% (73/87) and 69.6% (71/102), respectively. Development rate to the expanded blastocyst stage, 110 to 120 hours after insemination *in vitro* was 47.1% (48/102). Transfer of blastocysts obtained from frozen-thawed oocytes matured *in vitro* into the uterine horns of Day 3 pseudopregnant recipients resulted in fetal development and implantation rates similar to the control.

GENERAL CONCLUSIONS

Based on the results of studies on the quick freezing of one-, two-, four- and eight-cell mouse embryos and oocytes using ethylene glycol, the following conclusions were made:

1. Ethylene glycol at 3 M concentration in combination with either 0.25 M sucrose, lactose or trehalose can be used effectively as a cryoprotective agent in the quick freezing of early-stage mouse embryos, ovulated oocytes and oocytes matured *in vitro*. However, cryopreservation of GV-stage oocytes using the quick freezing method is difficult.
2. Equilibration period of early-stage mouse embryos and oocytes in a freezing medium containing high concentration of permeating cryoprotectant (3 M ethylene glycol) before quick freezing is of critical importance for their subsequent post-thaw survival and development *in vitro* and/or *in vivo*. High survival and development rates were obtained with 10 minutes equilibration in the freezing medium for one-, two-, four- and eight-cell mouse embryos and 20 minutes for unfertilized mouse oocytes and oocytes matured *in vitro*.
3. Sucrose and lactose at 0.5 or 1.0 M concentration are equally effective in the dilution of cryoprotectant in one-cell mouse embryos cryopreserved by the quick freezing method.
4. Blastocysts derived from quickly frozen-thawed mouse one-cell embryos, unfertilized oocytes and oocytes matured *in vitro* are capable of implanting and developing into

late-stage fetuses similar to those derived from nonfrozen controls when transferred into the uterine horns of Day 3 pseudopregnant recipients.

SUMMARY

Application of the quick freezing technique is presented in this thesis using ethylene glycol (a permeating cryoprotectant) in combination with sucrose, lactose or trehalose (nonpermeating cryoprotectants) to early-stage mouse embryos, unfertilized oocytes and oocytes matured *in vitro*.

Two-, four-, and eight-cell mouse embryos were frozen by direct plunging into liquid nitrogen (LN_2) vapor after 5 to 40 minutes equilibration in 3 M ethylene glycol with 0.25 M sucrose or lactose (freezing medium). After thawing and dilution of the cryoprotectant, the embryos were cultured *in vitro* and the effects of equilibration period and stage of development were examined. Significant difference on the survival of the embryos cryopreserved by the quick freezing method was not observed irrespective of the sugar used. When two- and four-cell embryos were equilibrated for 5 minutes in the freezing medium, post-thaw viability was low, but increased when equilibration was prolonged to 10 minutes. Although no significant difference was observed on the post-thaw viability of eight-cell embryos equilibrated from 5 to 40 minutes, the highest survival rate for all early cleavage-stage embryos was attained at 10 minutes equilibration before plunging into LN_2 vapor. Exposure of the embryos to the freezing medium up to 40 minutes without freezing did not detrimentally affect their capacity to develop into expanded blastocysts when cultured *in vitro*.

One-cell mouse embryos were frozen after equilibration for 5 to 40 minutes in 3 M ethylene glycol with 0.25 M sucrose or lactose. After thawing and dilution of the cryoprotectant, the effects of equilibration period and the method of dilution were examined. As in the preceding chapter, no significant difference on the survival rate of frozen-thawed one-cell mouse regardless of the sugar used. Significant difference was not also observed on the in vitro survival rate of the embryos with the use of 0.5 or 1.0 M sucrose or lactose for the dilution. However, dilution by direct transfer into isotonic PB1 solution resulted in significantly lower survival rates. The highest survival rates in vitro (67.2 and 69.7 %) were obtained when the embryos were equilibrated for 10 minutes in the freezing medium before quick freezing. Shorter (5 minutes) or prolonged (40 minutes) equilibration yielded significantly low survival rates. To assess survival in vivo, blastocysts derived from one-cell embryos frozen by the optimum treatments (10 minutes equilibration and dilution in 0.5 M sucrose or lactose) were transferred into the uterine horns of Day 3 pseudopregnant recipients. The in vitro development rates to the blastocyst stage of frozen-thawed one-cell mouse embryos were significantly lower than the control (68.0 and 66.7% vs 92.7%). However, the in-vivo development potential of blastocysts derived from frozen-thawed one-cell mouse embryos was similar to the control when transferred to the recipients.

Unfertilized (ovulated) mouse oocytes were cryopre-

served by the quick freezing method after equilibration for 5 to 40 minutes in a freezing medium containing 3 M ethylene glycol with 0.25 M sucrose or trehalose. After thawing and dilution, surviving oocytes were inseminated *in vitro* and the effect of equilibration period on the rates of fertilization and development *in vitro* was examined. No significant difference was observed on the fertilization of frozen-thawed oocytes regardless of the equilibration period nor the sugar used. However, higher total fertilization and normal fertilization rates were attained with 20 and 10 minutes equilibration in the freezing medium than with 5 or 10 minutes. Development rates to 2-cell and expanded blastocysts of frozen-thawed oocytes fertilized *in vitro*, that were equilibrated in the freezing medium for 20 or 40 minutes were significantly higher ($P<0.05$ or $P<0.01$) than with 5 minutes equilibration. To assess development *in vivo*, blastocysts derived from unfertilized oocytes frozen by the optimum equilibration period (20 minutes) in the freezing medium were transferred into the uterine horns of Day 3 pseudopregnant recipients. Although the development rate of frozen-thawed oocytes to blastocysts after insemination *in vitro* was significantly lower than the nonfrozen control, transfer of these blastocysts to the recipients resulted in fetal development and implantation rates similar to the control.

In the last experiment, the survival and developmental capacity of GV-stage oocytes and oocytes matured *in vitro*

frozen by the quick freezing method after the 20 minutes equilibration in 3 M ethylene glycol with 0.25 M sucrose were also examined. The survival rates of frozen GV-stage oocytes and oocytes matured *in vitro* were 68.9 and 72.7%, respectively after thawing and dilution. The maturation *in vitro* of frozen-thawed GV-stage oocytes was very limited, thus no further experiments were conducted on fertilization and development *in vitro* or *in vivo*.

For oocytes matured *in vitro*, the proportions of fertilized oocytes and 2-cell embryos, 6 and 24 hours after insemination were 83.9% (73/87) and 69.6% (71/102), respectively. Development rate to expanded blastocysts 110 to 120 hours after insemination *in vitro* was 47.1% (48/102). Transfer of blastocysts obtained from frozen-thawed and control oocytes matured *in vitro* into the uterine horns of Day 3 pseudopregnant recipients resulted in similar fetal development and implantation rates.

For the first time, mouse early-stage embryos, unfertilized oocytes and oocytes matured *in vitro* were successfully frozen by a combined process of dehydration with sucrose, lactose or trehalose and permeation with ethylene glycol before placing them into LN_2 vapor. The quick freezing technique adopted in this thesis work represents an efficient, inexpensive and viable option for the cryopreservation of mouse early-stage embryos, unfertilized oocytes and oocytes matured *in vitro*, and supplements the initial work on the quick freezing of mouse morula.

要　約

エチレングリコール（細胞膜透過型凍害保護物質）と、スクロース、ラクトースあるいはトレハロース（非透過型凍害保護物質）を組み合わせて用いた急速凍結法の、マウス初期胚、未受精卵子および体外成熟卵子に対する適応性について検討した。

マウスの２、４および８細胞期胚を、３Ｍエチレングリコールと0.25Ｍのスクロースあるいはラクトースを含む凍結媒液に浸漬し、５から40分間平衡した後、液体窒素直上の低温ガス相中に静置して急速凍結を行った。融解後、凍害保護物質を希釈除去し、胚を体外培養することで、平衡時間と胚の発育段階が、凍結・融解後の生存率へ及ぼす影響を調べた。急速凍結によって凍結保存された胚の生存率は、用いた糖類の間で有意な差異は認められなかった。２および４細胞期胚を凍結媒液中で５分間平衡した場合、凍結・融解後の胚の生存性は低かった。しかし、平衡時間を10分間に延長することで生存性は増加した。８細胞期胚においては、５から40分間の平衡時間では、凍結・融解後の生存性に有意な差異は認められなかった。全ての発育段階の初期胚において、液体窒素ガスに投入する前の平衡時間が10分間の場合、最も高い生存率を示した。40分間までの凍結媒液への胚の暴露は、凍結を行わず体外培養をした場合、拡張胚盤胞への発育に抑制的な影響を与えなかった。

マウス１細胞期胚を、３Ｍエチレングリコールと0.25Ｍのスクロースあるいはラクトースを含む凍結媒液中で５から40分間平衡した後、急速凍結を行った。融解後、凍害保護物質を希釈除去して、体外培養を行い、平衡時間と凍害保護物質の希釈除去法の影響について調べた。　前章と同様、　凍結・融解後のマウス１細胞期胚の生存率は、糖類により有意な差異はなかった。凍害保護物質の希釈除去の際に、0.5あるいは１Ｍのスクロースまたはラクトースを用いても、胚の生存性

に差異は認められなかった。しかし、等張ＰＢ１液へ胚を直接浸漬する方法では、生存率は有意に低下した。 急速凍結を行う前に、 胚を凍結媒液中で10分間平衡した場合、最も高い生存率が得られた。平衡時間を短縮（５分間）あるいは延長（40分間）した場合、胚の生存率は有意に低下した。体内での生存性を確かめるために、最適な条件下（10分間の平衡時間と0.5Mのスクロースまたはラクトースによる凍害保護物質の希釈除去）で凍結した１細胞期胚から作出した胚盤胞を偽妊娠３日目の雌マウスの子宮角に移植した。凍結・融解した１細胞期胚の体外培養における胚盤胞への発育率（68.0および66.7％）は対照区（92.7％）に比べ有意に低かった。しかし、移植試験の結果、凍結・融解したマウス１細胞期胚から作出された胚盤胞の体内での発育能は対照区と同程度であった。

マウス排卵卵子を、３Ｍエチレングリコールと0.25Mのスクロースあるいはトレハロースを含む凍結媒液中で５から40分間平衡した後、急速凍結法により凍結を行った。融解後、凍害保護物質を希釈除去し、生存している卵子について体外受精を行い、受精率とその後の体外での発育率に及ぼす平衡時間の影響について調べた。凍結・融解後の体外受精率は、平衡時間および用いた糖類により、有意な差異は認められなかった。しかし、平衡時間が20あるいは10分間の場合の総受精率および正常受精率は、５あるいは10分間平衡を行った場合に比べて高い傾向を示した。体外受精を行った凍結・融解卵子の２細胞期胚あるいは拡張胚盤胞への発育率は、凍結媒液中で５分間平衡した場合に比べて、20あるいは40分間平衡を行った方が有意に高かった（$P<0.05$あるいは$P<0.01$）。体内での発育を調べるために、最適な平衡時間（20分間）を用いて凍結した未受精卵子から作出した胚盤胞を、偽妊娠３日目の雌マウスの子宮角に移植した。体外受精を行った凍結・融解卵子の体外培養における胚盤胞への発育率は、凍結を行わなかった対照区に比べて有意に低かったが、凍結卵子由来の胚盤胞の移植試験では、受胎率および胎子の発育は、対照区と同程度であった。

最後に、３Ｍエチレングリコールと 0.25Mのスクロースを含む凍結媒液中で20分間平衡した後、急速凍結を行った卵核胞期（ＧＶ期）卵子および体外成熟卵子の生存性と発育能について調べた。凍結・融解後のＧＶ期卵子および体外成熟卵子の生存率は、それぞれ68.9および72.7％だった。凍結・融解後のＧＶ期卵子の体外成熟能は非常に低く、その後の体外受精や発育に関する検討は行えなかった。

凍結・融解後の体外成熟卵子において、媒精後６あるいは24時間目における受精胚および２細胞期胚の割合は、それぞれ、83.9あるいは69.6％であった。体外受精後110から120時間における拡張胚盤胞への発育率は 47.1％であった。凍結・融解を行った体外成熟卵子と対照となる非凍結体外成熟卵子のそれぞれから得られた胚盤胞を、偽妊娠３日目の雌マウスの子宮角に移植した結果、受胎率および胎子の発育に差異は認められなかった。

以上の結果より、凍害保護物質として、エチレングリコールと、スクロース、ラクトースあるいはトレハロースを組み合わせて用いることが、マウス初期胚、未受精卵および体外成熟卵子の急速凍結に適していることが示された。

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inches 1 2 3 4 5 6 7 8

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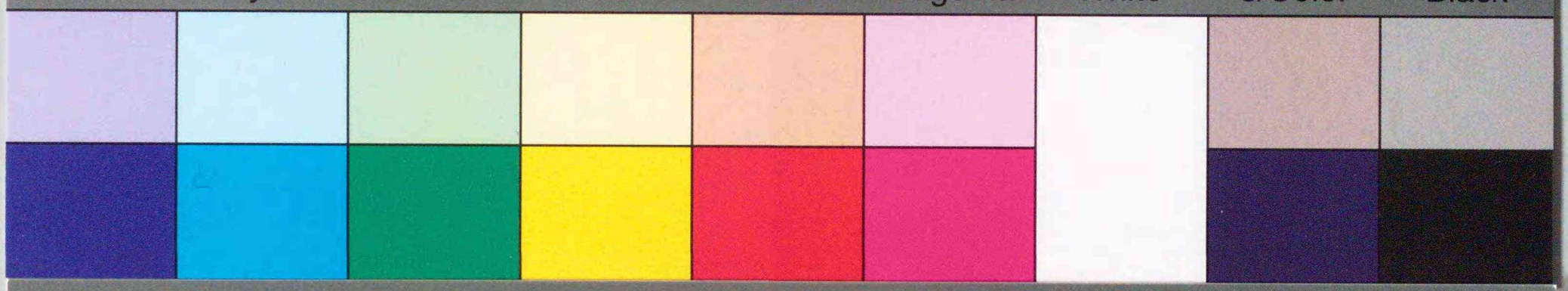
Kodak Color Control Patches

Blue Cyan Green Yellow Red Magenta White 3/Color Black



Kodak Gray Scale

C Y M

A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19