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EFFECTS OF VARIOUS CRYOPROTECTANTS ON THE SURVIVAL OF MOUSE EMBRYOS CRYOPRESERVED BY THE QUICK FREEZING METHOD

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The effects of concentrations of glycerol, ethylene glycol or dimethylsulphoxide (DMSO) in the presence of either 0.25M lactose or sucrose on the post-thaw survival of mouse quickly-frozen compacted morulae were studied. In this method, the embryos were directly frozen in liquid nitrogen (LN_2) vapor at approximately $-170°C$ for 2 min before being plunged into LN_2. High survival rates of frozen-thawed embryos were obtained when the freezing medium contained 3M ethylene glycol with either 0.25M lactose or sucrose (76.5 and 70.2%, respectively). When the embryos were frozen in glycerol, significantly high survival was obtained with 3M glycerol+0.25M sucrose (73.5%, $P<0.001$). However, a freezing medium containing DMSO with either sugar gave lower survival rates. At a higher concentration of 4M, ethylene glycol with 0.25M lactose gave significantly higher survival rate than glycerol or DMSO ($P<0.05$). Significantly higher rates were obtained at 2M with all 3 cryoprotectants when the freezing medium contained lactose rather than sucrose ($P<0.05$). This study showed that glycerol and ethylene glycol were effective cryoprotectants in the quick freezing of mouse embryos, while DMSO was less effective. In addition, the protective effects of these cryoprotectants are affected by their concentrations and the type of sugar used.

Key words: embryo, cryoprotectant, lactose, sucrose, quick freezing

INTRODUCTION

Mouse preimplantation embryos can be cryopreserved successfully by various methods involving different procedures and types of cryoprotectants. Intracellular ice formation and solution effects have been shown to be the two main probable causes of cellular death during freezing and thawing.[11] In conventional freezing, these detrimental effects can be minimized by the addition of cryoprotectant and partial

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dehydration of cells by adequate slow freezing rates.[9,29] Fast freezing rates, as in the two-step freezing approach can also be used if the cryoprotectant-permeated embryos are partly predehydrated by sucrose before freezing.[4,15,18] However, these techniques are still time consuming and require sophisticated biological freezers.

Recently, mouse embryos have been rapidly frozen by the so-called quick, rapid or ultra-rapid freezing methods. Another new approach, which allows embryos to be directly plunged into LN_2 is vitrification.[16] In quick freezing, the embryos are directly placed into LN_2 vapor at temperatures between $-50°C$ and $-60°C$[30] or -170 and $-180°C$ before being plunged into LN_2.[21,22,23,24,28] Ultra-rapid freezing by direct plunging into LN_2 has also been successful.[1,6,19,25,27] These freezing methods were made possible because the embryos were initially predehydrated at room temperature in a hypertonic solution containing two cryoprotective agents, one permeating and the other non-permeating. With the exception of TROUNSON *et al.* (1988)[26] who used DMSO, most of the above studies used glycerol and sucrose as the permeating and non-permeating cryoprotectants, respectively. However, little comparative work has been done on the protective effects of other cryoprotectants in the quick freezing of mouse embryos.

This study examines the effects of concentrations of three different cryoprotective agents, namely, glycerol, ethylene glycol and DMSO in combination with a constant concentration of 0.25M lactose or sucrose on the survival of frozen-thawed mouse compacted morulae cryopreserved by the quick freezing method.

MATERIALS AND METHODS

Animals

Female Slc : ddY mice, 4～7 weeks of age and 20～28g in body weight were used in the experiment. They were housed in a room with controlled lighting (14/10 hr of light / dark cycle ; light on at 07 : 30) and given commercial feed and water *ad libitum*.

Superovulation and embryo recovery

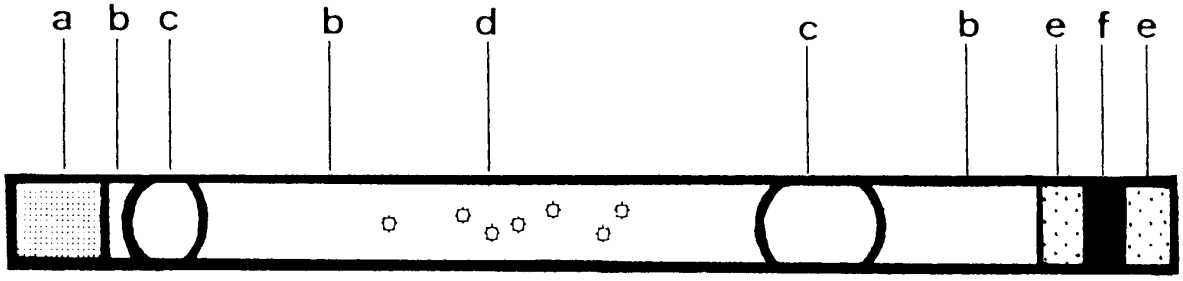
The female mice were induced to superovulate by intraperitoneal injections of 7.5 IU pregnant mare serum gonadotrophin (PMSG ; Serotrophin, Teikoku Zoki, Tokyo, Japan) and 7.5 IU human chorionic gonadotrophin (hCG ; Gonatrophin, Teikoku Zoki, Tokyo, Japan) given 48 hr apart. After the hCG injection, they were paired with males of the same strain and inspected the following morning for the presence of vaginal plugs. The day on which the vaginal plugs were found was designated as Day 1 of pregnancy.

On the night of Day 3 of pregnancy (77～78 hr post hCG) the females were killed by cervical dislocation and compacted morulae were obtained by flushing both the uteri and oviducts with Dulbecco's phosphate buffered saline (PBS [−] ; Nissui Phar. Co., Tokyo, Japan) in which calcium and magnesium salts had been added (PBS) and supplemented with 5% heat-treated calf serum (CS ; Gibco Laboratories, Life Techno-

logy, Inc., New York, U. S. A.). After collection, the embryos were washed three times with fresh PBS+CS, pooled and held at room temperature in sterilized plastic petri dishes (Nunc, Nunclon, Kampstrup, Denmark) until used. Morphologically normal compacted morulae of fair to excellent quality based on the classification by LINDNER and WRIGHT (1983)[10] were used in this experiment.

Freezing procedures

Embryos were randomly assigned to one of the 24 factorial freezing solution combinations of 1, 2, 3 and 4M concentrations of three different cryoprotectants, namely, glycerol, ethylene glycol and DMSO and a constant level of 0.25M sucrose or lactose in PBS+CS. The embryos were placed into the freezing solution directly and equilibrated at room temperature (20~25°C) for 5 min. Seven to 15 embryos were drawn into a 0.25ml plastic French straw (I. M. V., L'Aigle, France) and sealed with straw powder. The volume of freezing medium containing the embryos was 135 μl (7cm) and was separated by an air bubble on each side, with the remainder of the straw filled with similar medium as shown in Text Fig. The sealed straw was then frozen by placing it horizontally on a styrofoam boat with a dimension of 6×15×0.8 cm in the vapor phase of a LN_2 bath in a stainless steel flask, thus giving a position about 0.8 mm above the LN_2, which had a temperature of approximately −170°C (Figs. 1~4). The upper surface of the styrofoam was covered with wire mesh to prevent bending while inside the LN_2 bath (Fig. 2). After 2 min, the straw was plunged into the LN_2 and stored from 1 to 25 days.



Text Fig. Diagrammatic representation of a 0.25ml French straw loaded as indicated: a) straw powder (polyvinylic alcohol; PCV); b) freezing medium; c) air bubbles; d) embryos in freezing medium; e) cotton plug; f) PCV plug

Straws were thawed by gentle agitation in a water bath at 37°C for 20~30 sec. After thawing, the contents of the straw were expelled into a petri dish and the embryos were immediately pipetted into PBS+CS containing 0.5M sugars of the sugar used in the freezing medium. Cryoprotectant dilution was done with a one-step dilution method for 5 min at room temperature. The embryos were then washed

three times in PBS+CS and placed in BMOC-3 microdroplets under paraffin oil for about 10 min. They were then washed again in BMOC-3 and finally cultured by the microdroplet method[3] (Brinster, 1963) in BMOC-3 at 37°C for 48 hr in 5% CO_2 in air. Survival of the embryos was assessed by their ability to develop into expanded, hatching or hatched blastocyst stage after 48 hr of culture. The survival rates of embryos were statistically analysed by Chi-square test.

RESULTS

The effects of concentrations of glycerol, ethylene glycol or DMSO with 0.25M lactose or 0.25M sucrose on the survival of frozen-thawed mouse compacted morulae are shown in Tables 1 and 2. An average of 90% of the embryos were recovered after thawing. Only one out of 106 embryos (in the glycerol group) developed into an expanded blastocyst when the freezing medium contained 1M of any of the cryoprotectants with either sugar.

When the freezing medium contained a mixture of glycerol and 0.25M sucrose, a significantly high survival rate was obtained with 3M glycerol (73.5%, $P<0.01$). There was no significant difference between 1, 2 and 4M glycerol. When in combination with 0.25M lactose, the highest survival rate was obtained with 2M glycerol (64.3%), but there was no significant difference between 2 and 3M glycerol. A lower or higher concentration of glycerol resulted in reduced survival.

Table 1 Effects of type and concentration of cryoprotectant and 0.25M lactose on the post-thaw survival of mouse compacted morulae cryopreserved by quick freezing

Cryoprotectant	Percentages of embryos that developed into expanded blastocysts after culture in BMOC-3			
	Cryoprotectant concentration (M)			
	1	2	3	4
Glycerol	0^{a} (17)	64.3^{bA} (193)	52.8^{bA} (53)	9.1^{aA} (66)
Ethylene glycol	0^{a} (21)	49.6^{bB} (123)	76.5^{cB} (51)	52.4^{bB} (63)
DMSO	0^{a} (21)	41.6^{bB} (77)	32.7^{bC} (52)	0^{aA} (79)

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

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

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

In the sample frozen in the presence of ethylene glycol, significantly higher proportion of embryos developed into expanded blastocysts with higher concentrations (3 or 4M). When the embryos were frozen with 3M ethylene glycol+0.25M lactose or sucrose, high survival rates, 76.5 and 70.1,% respectively were observed. There was a significant difference in the survival rates between 3 and 4M ethylene glycol with both lactose or sucrose ($P<0.01$). A higher proportion of embryos survived quick freezing and thawing with 2M ethylene glycol in combination with lactose than with sucrose (49.6 vs 26.0%, $P<0.01$).

In contrast, survival of embryos frozen with DMSO was lower than of those with either glycerol or ethylene glycol. When sucrose was used, there was no significant difference among the survival rates of embryos frozen with 1, 2, 3 and 4M DMSO. However, 2 and 3M DMSO with lactose gave slightly higher survival rates for frozen-thawed embryos (41.6 and 32.7%, respectively) but no significant difference was observed.

Table 2 Effects of type and concentration of cryoprotectant and 0.25M sucrose on the post-thaw survival of mouse compacted morulae cryopreserved by quick freezing

Cryoprotectant	Percentages of embryos that developed into expanded blastocysts after culture in BMOC-3			
	Cryoprotectant concentration (M)			
	1	2	3	4
Clycerol	6.7^{a} (15)	36.2^{aA} (69)	73.5^{bA} (68)	24.4^{aAB} (41)
Ethylene glycol	0^{a} (15)	26.0^{acAB} (73)	70.2^{bA} (67)	40.5^{cA} (37)
DMSO	0 (17)	14.6^{B} (55)	18.0^{B} (61)	11.6^{B} (43)

Values in parentheses represent the total number of frozen-thawed embryos.
a, b, c : Values with different superscripts in the same line were significantly different ($P<0.05$).
A, B, C : Values with different superscripts in the same column were significantly different ($P<0.05$).

At 2M concentrations of glycerol and ethylene glycol there was no significant difference between these two cryoprotectants with sucrose, but with lactose, glycerol gave a significantly higher survival rate (64.3 vs 49.6%, $P<0.001$). At 3M concentration, both glycerol and ethylene glycol gave high survival rates with sucrose, but

the survival rate in ethylene glycol with lactose was significantly higher than in glycerol with lactose (76.5 vs 52.8%, $P<0.001$). However, at a 4M concentration, ethylene glycol gave a high survival rate especially when in combination with lactose as compared to that with sucrose.

Significantly higher survival rates were obtained at a concentration of 2M in all of the cryoprotectants when lactose was used rather than sucrose ($P<0.01$). However, at 3 or 4M concentration of cryoprotectants, sucrose gave a higher survival rate only in combination with glycerol, and there was no significant difference between the sugars with ethylene glycol and DMSO.

DISCUSSION

Recently, significant progress had been made in embryo preservation using the quick and ultra-rapid freezing methods.[1,21,24,25,27] In this study, the survival rates of embryos frozen in glycerol were similar to the findings of others using comparable methods of predehydration and rapid freezing,[21,24] except for that of WILLIAMS and JOHNSON (1986),[28] in which they reported that 2M glycerol gave optimum post-thaw survival as compared to 3M glycerol with a constant concentration of 0.25M sucrose. The differences in the size of the straw (0.25 vs 0.5ml French straw) and in the volume of freezing medium (135 μl) used in our study probably affected the cooling and warming rates, thus leading to the differences in the survival rates. When glycerol+0.25M lactose was used, the best post-thaw survival was obtained with 2M glycerol, and this finding is in agreement with that of TAKAHASHI and KANAGAWA (1985)[24]. The slightly lower survival rates obtained in our study were probably due to the quality of embryos used in the trial which was from fair to excellent.

The results of this study also showed that a higher concentration of glycerol was necessary to protect the embryos during rapid freezing in LN_2 vapor as compared to conventional freezing.[8] Glycerol decreases the extracellular osmolarity of non-permeating solutes due to its ability to reduce the frozen fraction of water[17] and increases the intracellular osmolarity through permeation. Since, it has been suggested that glycerol protects the embryos during rapid freezing through reducing the osmotic differences between the extra- and intracellular space.[23]

This study showed that ethylene glycol could be an effective cryoprotectant with the quick-freezing method and yielded high survival rates of 76.5 and 70.2% with 3M ethylene glycol containing 0.25M lactose and sucrose, respectively. Ethylene glycol has been reported to be an effective cryoprotective agent for mouse and rat embryos using the conventional method of freezing.[13,14] However, this study also showed that a higher concentration of ethylene glycol was needed to protect the embryos during rapid freezing than in conventional method of freezing. It required an ethylene glycol concentration of about 3M to give the optimum cryoprotective effect, while for

conventional freezing a concentration of 0.9 to 1.5M was found to be the best.[13]

The survival rate of embryos frozen with DMSO was very low. Using a slightly different method of quick freezing, MIYAMOTO, *et al.* (1986),[15] reported that the degree of protection given by DMSO using the quick freezing method was low (27%) as compared to glycerol (78%), propylene glycol (70%), and ethylene glycol (67%). However, 2-cell[27] and 8-cell embryos[26] have been found to survive after ultra-rapid freezing in 3.5M DMSO and 0.25M sucrose, with post-thaw survivals of 71 and 78%, respectively. It has also been reported that for early cell-stage embryos, DMSO is a better cryoprotectant than glycerol,[2] but there are no reports on morulae or later-stage embryos. The low survival rates obtained in our study were probably due to the longer equilibration time used (5 min) since results from the studies done by TROUNSON *et al.* (1987),[26] showed that embryo viability decreased with increasing time of exposure to DMSO, and after 4 to 6 min, embryos showed little developmental capacity *in vitro*. KASAI *et al.* (1981),[5] also showed that at a concentration of 1.5M, DMSO was more harmful than glycerol or ethylene glycol. They observed that morulae stored at 20°C in the presence of 1.5M DMSO completely lost their ability to develop into expanded blastocysts within 24 hr, thus indicating higher toxicity. Therefore, with DMSO, further experiments are required to optimize the conditions for quick freezing.

In this study, predehydration was conducted using either non-permeable sugars such as sucrose and lactose. They exert a significant cryoprotective effect by removing water from the embryos thus causing shrinkage and dehydration. This will reduce detrimental intracellular ice formation during freezing. The optimum concentration of sucrose in the freezing medium was indicated to be between 0.25M and 0.5M. However, no studies regarding the effect of the lactose concentration have been reported yet. In this study, lactose was found to give significantly higher survival rates at a low concentration with all three cryoprotectants (2M). In contrast, sucrose as compared to lactose gave higher survival rates with 3M or 4M glycerol but not with ethylene glycol or DMSO. Therefore, this study suggests that the effects of these two sugars varies with type and concentration of the cryoprotectant. However, the effects of the concentrations of these sugars with the different cryoprotectants cannot be analysed since in this study the concentration of both sugars was held constant at 0.25M. It has been reported that different types of sugars in combination with glycerol gave varying degrees of cryoprotection.[7,24] Therefore, this study indicated that the degree of cryoprotection of a particular cryoprotectant will depend on the type and concentration of the cryoprotectant and sugar used during freezing. Each cryoprotectant has its own properties and characteristics and hence the optimum condition for each cryoprotectant differs.

The quick-freezing method using either glycerol or ethylene glycol allows survival rates comparable to the one obtained with the conventional freezing procedure.[12,20]

However, further investigations are needed to examine other factors such as predehydration and the mechanisms of action of various cryoprotectants during quick freezing. Research to improve the quick-freezing technique should be carried out so that a simple and reliable cryopreservation technique can be developed and applied to the freezing of other mammalian embryos, especially in livestock of economic importance.

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EXPLANATION OF PLATE

PLATE

Fig. 1 Simple apparatus used for quick freezing.

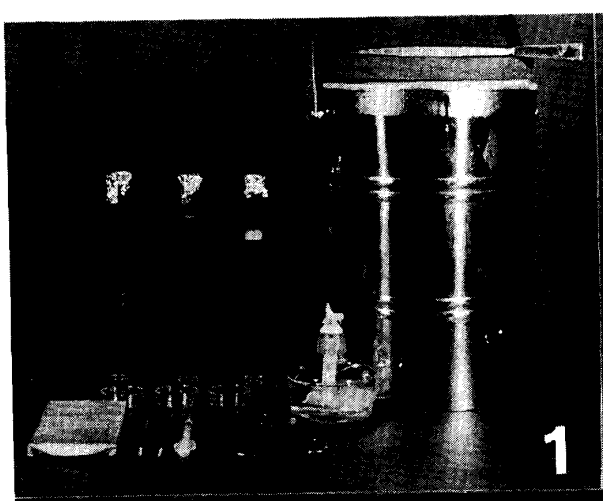
Fig. 2 Styrofoam boat (6×15×0.8cm) with 0.25ml French straw on top of it.

Fig. 3 Styrofoam boat with straw on top of it inside a liquid nitrogen flask.

Fig. 4 A cross-sectional diagram of the liquid nitrogen flask showing the position of the styrofoam boat: a) cover; b) flask; c) straw; d) styrofoam boat; e) wire mesh.

Fig. 5 Mouse compacted morula before equilibration. ×400.

Fig. 6 Mouse compacted morula after 5 min equilibration in freezing medium containing 3M ethylene glycol+0.25M sucrose. Note the shrinkage of the cell mass due to dehydration. ×400.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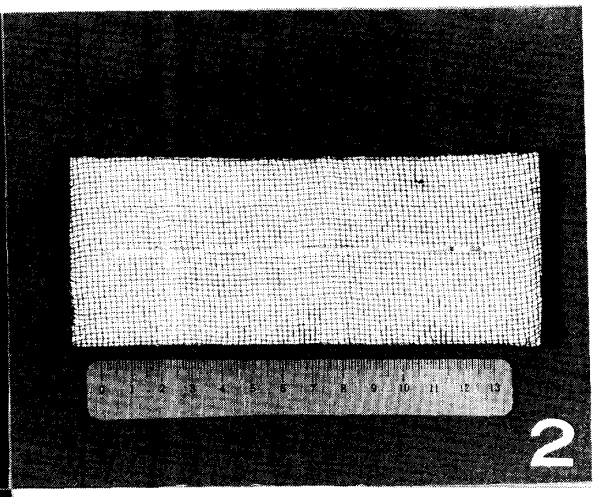
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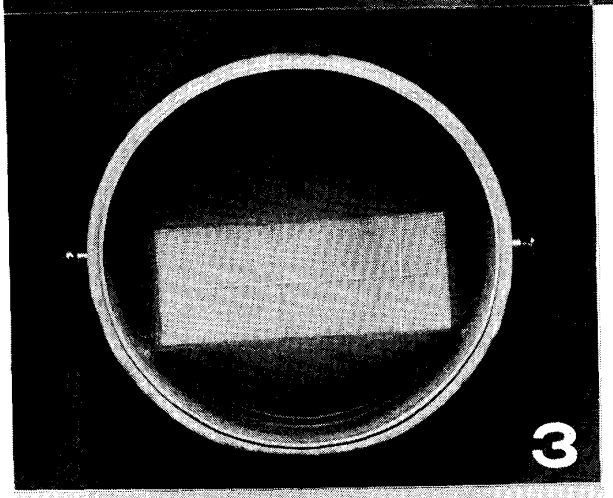
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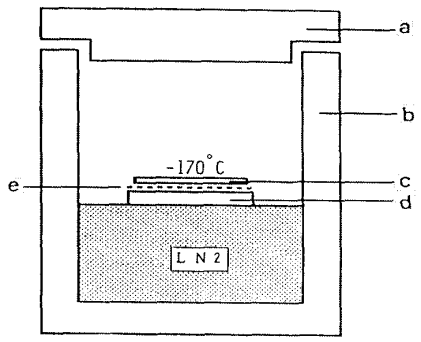
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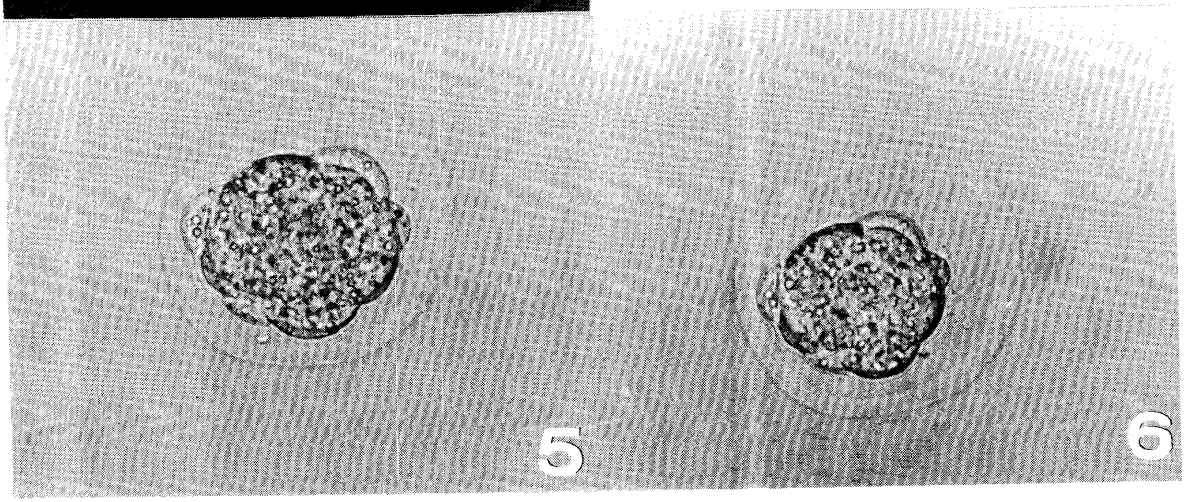
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