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## BRIEF COMMUNICATION

# QUICK FREEZING OF MOUSE EMBRYOS BY DIRECT PLUNGE INTO LIQUID NITROGEN VAPOR: EFFECTS OF SUGARS

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Conventionally, cryopreservation of mammalian embryo has been accomplished by suitable slow cooling in the presence of a cryoprotectant to very low temperatures before storage in liquid nitrogen (LN<sub>2</sub>). However, it has been demonstrated in mouse,<sup>7,13)</sup> cattle<sup>1,4)</sup> and rabbit<sup>10)</sup> that embryos can be frozen by using a more rapid technique with the two-step freezing method: i. e., rapid cooling to a subzero holding temperature (-20° to -79°C), followed by rapid cooling into LN<sub>2</sub>. Moreover, it was reported recently that mouse embryos can endure quick freezing by being plunged directly into LN<sub>2</sub>.<sup>5,6,9,11,12)</sup> In these studies, embryos were frozen with glycerol and nonpermeable sugars such as sucrose<sup>5,6,11,12)</sup> and trehalose,<sup>6)</sup> or with the vitrification solution.<sup>9)</sup> Up to now, there have been no reports mentioning the effect of other sugars and the viability of embryos upon transfer to the recipient animals after the quick freezing. In the present study, the effects of several types of sugars under various glycerol concentrations on the viability of frozen-thawed embryos were examined, and normal young were obtained after the transfer of embryos frozen by the quick freezing procedure.

Female ddY mice, aged 4 to 5 weeks, were induced to superovulate by intraperitoneal injections of 5 IU of pregnant mare's gonadotrophin and 5 IU of human chorionic gonadotrophin (hCG) given 48 hours apart. The mice were mated with males of the same strain after the injection of hCG. Embryos at the morula stage were flushed from the uteri with Dulbecco's phosphate-buffered saline supplemented with 5% heat-treated calf serum (PBS+CS) at 76 to 78 hours after the injection of hCG. The embryos were washed with fresh PBS+CS before transfer to the freezing medium, and then pipetted into different freezing media: PBS+CS containing 1, 2, 3 or 4 M-glycerol and 0.25 M-sugar (raffinose, lactose, sucrose, glucose or xylose). In the

control group, no sugar was added to the freezing medium. Eight to fourteen embryos were drawn into a 0.25ml plastic straw. Five minutes after exposure to the freezing medium, embryos were frozen by plunging the sealed straw directly into LN<sub>2</sub> vapor (approximately -170°C). Two minutes later, the straw was immersed in LN<sub>2</sub> and stored for 1 to 57 days. After thawing in a 37°C water bath, embryos were expelled from the straw, and pipetted directly into the PBS+CS containing 0.5 M of sugar, which was the same as the sugar used in the freezing medium. Embryos in the control group were placed into sugar-free PBS+CS. After 5 minutes at room temperature, all embryos were washed in Brinster's modified Krebs-Ringer bicarbonate-buffered medium (BMOC-3)<sup>3</sup>, and then cultured by the microdroplet method<sup>2</sup> in BMOC-3 at 37°C for 48 hours in 5% CO<sub>2</sub> in air. Survival of the embryos was assessed by their ability to develop into expanded blastocysts during the culture. Experiments were repeated 4 to 9 times for each treatment, and the data were analyzed for statistical significance using the  $\chi^2$ -test.

Effects of glycerol and sugars on the survival of frozenthawed embryos were shown in the table. Viabilities of the embryos frozen with sugar-free media (control

TABLE *Effects of glycerol and sugars on the survival of mouse morulae after quick freezing by direct plunging into liquid nitrogen vapor*

GLYCEROL CONCENTRATION IN THE FREEZING MEDIUM (M)	PERCENTAGES OF EMBRYOS THAT DEVELOPED INTO EXPANDED BLASTOCYSTS AFTER CULTURE IN BMOC-3 FOR 48 HOURS					
	Type of sugar added to the freezing medium					Sugar-Free (Control)
	Raffinose	Lactose	Sucrose	Glucose	Xylose	
1	0.0 <sup>a,A</sup> (4,43)	14.3 <sup>a,B</sup> (4,42)	7.7 <sup>a,AB</sup> (4,39)	2.6 <sup>a,AB</sup> (4,39)	15.9 <sup>a,B</sup> (7,44)	2.6 <sup>a,AB</sup> (4,38)
2	64.0 <sup>b,AB</sup> (5,50)	80.4 <sup>b,B</sup> (5,51)	66.7 <sup>b,AB</sup> (6,57)	68.8 <sup>b,AB</sup> (5,48)	60.8 <sup>b,A</sup> (5,51)	5.3 <sup>a,C</sup> (4,38)
3	62.9 <sup>b,A</sup> (7,62)	71.8 <sup>b,A</sup> (9,85)	72.2 <sup>b,A</sup> (7,72)	66.0 <sup>b,A</sup> (5,50)	36.0 <sup>c,B</sup> (5,50)	7.3 <sup>a,C</sup> (4,41)
4	14.3 <sup>c,A</sup> (4,42)	63.3 <sup>b,B</sup> (5,49)	29.4 <sup>c,AC</sup> (5,51)	38.1 <sup>c,C</sup> (4,42)	13.0 <sup>a,A</sup> (5,46)	0.0 <sup>a,D</sup> (4,41)

Values in parentheses represent the number of replicates and the total number of embryos used.

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

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

Effects of glycerol and sugars on the survival of frozen-thawed embryos were shown in the table. Viabilities of the embryos frozen with sugar-free media (control group) were much lower than those of the embryos frozen with glycerol and sugar. The highest survival rates were obtained by freezing with 2 or 3 M-glycerol and sugar. When raffinose, sucrose or glucose were added to the freezing medium, survival rates of embryos in the groups with 2 and 3 M-glycerol were significantly higher than those in the groups with 1 and 4 M-glycerol. However, when lactose was used, there was no significant difference among the survival rates of the embryos frozen with 2, 3 and 4 M-glycerol. In the presence of 2 M-glycerol, the survival rate in the group with lactose was higher than those in the groups with the other sugars. When 3 M-glycerol was used, the survival in the group with xylose was significantly lower than those in the groups with the other sugars.

To examine the *in vivo* development of embryos frozen by the quick freezing procedure, frozen embryos were transferred to recipient mice. In this experiment, morulae were frozen with 2 M-glycerol plus 0.25 M-lactose and stored in LN<sub>2</sub> for 1 to 39 days. After thawing, glycerol was removed in the medium containing 0.5 M-lactose, and the embryos were cultured for 4 hours in BMOC-3. Eighty-six normal morulae were transferred to the uterine horns of five ICR females on Day-3 of pseudopregnancy (7 to 10 embryos per horn, 15 to 20 embryos per animal). The recipients were allowed to litter, and 26 normal young (30.2%) were born from the five recipients. Sixty-four (74.4%) implantation sites were found.

In the present quick freezing procedure, it appeared that all the types of sugar used in this study may act as a cryoprotectant in the presence of glycerol. In the freezing of bull spermatozoa, NAGASE et al.<sup>8)</sup> suggested that disaccharides (lactose, sucrose, maltose) and trisaccharide (raffinose) provided a better protective effect than pentose (xylose) and hexoses (galactose, fructose) after semi-rapid and rapid freezing. This difference in the protective effect among the sugars on the motility of spermatozoa was similar to that on the viability of embryos. However, the survival of the embryos was affected by glycerol concentration in the freezing medium. Further investigations are needed to examine the mechanism by which these sugars exert favorable effects on the viability of embryos during the quick freezing. Satisfactory results on the viability of embryos were obtained both *in vitro* and *in vivo*. If successfully applied to other mammalian embryos, especially those of livestock animals, the quick freezing procedure described herein could be a simple technique for the preservation of embryos.

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