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Strain preservation of experimental animals: Vitrification of two-cell stage embryos for multiple mouse strains ☆

Tomoo Eto *, Riichi Takahashi, Tsutomu Kamisako

Central Institute for Experimental Animals, 3-25-12 Tonomachi, Kawasaki-ku, Kawasaki 210-0821, Japan

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ABSTRACT

Strain preservation of experimental animals is crucial for experimental reproducibility. Maintaining complete animal strains, however, is costly and there is a risk for genetic mutations as well as complete loss due to disasters or illness. Therefore, the development of effective vitrification techniques for cryopreservation of multiple experimental animal strains is important. We examined whether a vitrification method using cryoprotectant solutions, P10 and PEPeS, is suitable for preservation of multiple inbred and outbred mouse strains. First, we investigated whether our vitrification method using cryoprotectant solutions was suitable for two-cell stage mouse embryos. In vitro development of embryos exposed to the cryoprotectant solutions was similar to that of fresh controls. Further, the survival rate of the vitrified embryos was extremely high (98.1%). Next, we collected and vitrified two-cell stage embryos of 14 mouse strains. The average number of embryos obtained from one female was 7.3–33.3. The survival rate of vitrified embryos ranged from 92.8% to 99.1%, with no significant differences among mouse strains. In vivo development did not differ significantly between fresh controls and vitrified embryos of each strain. For strain preservation using cryopreserved embryos, two offspring for inbred lines and one offspring for outbred lines must be produced from two-cell stage embryos collected from one female. The expected number of surviving fetuses obtained from embryos collected from one female of either the inbred or outbred strains ranged from 2.9 to 19.5. The findings of the present study indicated that this vitrification method is suitable for strain preservation of multiple mouse strains.

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Introduction

Mice are used as experimental animals in many fields, including genetics, medicine, pharmacy, physiology, and biology [2,24,25], and many inbred and outbred strains have been developed. The development of DNA injection technology in the 1980s [10] followed by gene targeting technology using embryonic stem cells [4] led to a marked increase in the number of genetically modified mouse strains. Genetically modified mice may be backcrossed with mice of other genetic backgrounds to alter the phenotype [5]. Maintaining complete strains of mice, the number of which is continuously increasing, however, is costly [19]. In addition, there is a risk of strain loss due to genetic mutations [15] and disasters [6].

Cryopreservation of early embryos is extremely effective for the preservation of mouse strains [20]. Although several methods are available for cryopreservation of early mouse embryos, including freezing [35] and vitrification [28], the survival rate of early embryos after cryopreservation tends to be higher for the vitrification technique with little difference among strains [18] compared to freezing [29–31,34]. Embryo development of multiple strains after cryopreservation also tends to be better with vitrification than with freezing [7]. For vitrification of early embryos, it is necessary to apply cryoprotectant solution (CPS) and a vitrification technique with high freezing tolerance, low cytotoxicity, prevention of cellular expansion after warming, and no induction of freeze fractures in the vitrification solution [17].

We previously developed a vitrification method with CPS (P10 and PEPeS) that fulfills these criteria [9]. The vitrification method used in this study, however, was designed for rat two-cell stage embryos. In the present study, we investigated whether this vitrification method could be applied for the cryopreservation of two-cell stage embryos of multiple mouse strains. In Experiment 1, we evaluated whether vitrification is suitable for two-cell-stage mouse embryos. In Experiment 2, we examined the efficacy of strain preservation using this method for two-cell stage embryos of 14 mouse strains.

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* Corresponding author.
E-mail address: etoh@ciea.or.jp (T. Eto).

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Materials and methods

Animals

Mouse embryos from 14 strains were used (males 12–16 weeks old and females 8–16 weeks old). Inbred C57BL/10ScN, CBA/N, NOD/Shi, TWY/Jic-ttw, and NOD/Shi-scid mice were obtained from the Central Institute for Experimental Animals (Kawasaki, Japan). Inbred C57BL/6J, C57BL/6N, BALB/cA, BALB/cByJ, DBA/2J, C3H/HeJ, and 129^{+Tar}/Sv mice, and outbred ICR mice were obtained from CLEA Japan, Inc. (Tokyo, Japan). Inbred C57BL/6NCr mice were obtained from Japan SLC, Inc. (Shizuoka, Japan). For embryo transfer, pseudopregnant female Jcl:MCH(ICR) strain mice, 10–16 weeks old (CLEA Japan), were produced by breeding them with vasectomized male mice of the same strain. The mice were reared under the following conditions: room temperature, 22 °C ± 0.5 °C; humidity, 55% ± 5%; lights on 08:00–20:00. Food (CA-1; CLEA Japan Inc.) and water were provided ad libitum. The rearing of the mice and the animal experiments were conducted according to the institutional rules following approval from the Animal Experiment Committee of Central Institute for Experimental Animals in Japan.

Collection, in vitro culture, and in vivo development of two-cell stage embryos

In this experiment, two-cell stage embryos were used. Female mice were injected intra-abdominally with 5IU equine chorionic gonadotropin (Serotropin; ASUKA Pharmaceutical Co., Ltd., Tokyo, Japan), followed by intra-abdominal injection of 5IU human chorionic gonadotropin (Gonadotropin; ASUKA Pharmaceutical Co., Ltd.) 50 h later (17:00–19:00) to induce excessive ovulation. Two-cell stage embryos of BALB/cA, BALB/cByJ, CBA/N, and ICR mice were collected by oviduct flushing after natural mating. Two-cell stage embryos of the other strains were obtained by in vitro fertilization (IVF). We used modified Whitten's medium (mWM) for oviduct flushing and in vitro culture (IVC) of embryos [12,23]. In IVF, we used a modified human tubal fluid (mHTF) in which the calcium concentration was doubled [27]. All reagents used to make the media were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO). The media were added to Petri dishes (351008; Becton Dickinson & Co., Franklin Lakes, NJ) in a dropwise manner (mHTF, 200 μL; mWM, 50 μL) and then covered with mineral oil (26117-45; Nacalai Tesque, Kyoto, Japan). IVF and IVC were performed in an atmosphere of 5% CO_2 and 95% air at 37 °C. Oviduct flushing was conducted according to the method of Horgan et al. [13]. Immediately after administering gonadotropin to female mice, they were mated with males of the same strain. On day 1.5 after mating, the female mice were euthanized by cervical dislocation for collection of two-cell stage embryos. IVF was performed using a modified method of Toyoda et al. [32,33]. Sperm were collected from the cauda epididymis and preincubated for 1.5 h with mHTF. Approximately 16 h after administering gonadotropin to female mice, mHTF containing oocytes was inseminated with the preincubated sperm (100–150 sperm/μL). Approximately 6 h after insemination, the fertilized ova were washed with mWM and IVC was performed with embryos at the two-cell stage or less. The embryos were transferred to the oviducts of pseudopregnant female mice on day 0.5 after mating for examination of in vivo development [14]. Pseudopregnant mice were euthanized on days 17.5–8.5 after embryo transfer and a laparotomy was performed to observe embryo development.

Experiment 1

Cryopreservation of the two-cell stage embryos was performed according to the method of Eto et al. [9]. The solution used for vitrification and warming was prepared by adding cryoprotectant to PB1 [35]. Vitrification was performed with a pre-treatment solution (P10; 10% propylene glycol in PB1) and vitrification solution (PEPeS; 10% propylene glycol, 30% ethylene glycol, 0.3 M sucrose, and 20% Percoll in PB1). SPB1 (0.3 M sucrose in PB1) was used to warm the vitrified embryos. Percoll was obtained from GE Healthcare (Uppsala, Sweden), and other reagents for preparing the solutions were purchased from Sigma–Aldrich Chemical Co. Two-cell stage embryos of the C57BL/6J strain were used in the experiments.

The methods for vitrification and warming of embryos were as follows. The embryos were exposed to P10 at 25 ± 0.5 °C for 5 min. The embryos and 5 μL of P10 were then placed into cryotubes (MS-4501W; Sumitomo Bakelite Co., Ltd., Tokyo, Japan) and cooled to 0 °C for 1 min. We then added 95 μL of precooled (0 °C) vitrification solution to the cryotubes, and 1 min later the cryotubes were plunged into liquid nitrogen for vitrification. The vitrified two-cell stage embryos were stored in liquid nitrogen for at least 1 week.

The vitrified embryos were warmed by shifting the cryotubes from liquid nitrogen to room temperature (25 ± 0.5 °C). Thirty seconds later, 900 μL of SPB1 at 37 °C was added to the cryotubes and the mixture was rapidly stirred 5–6 times. The warmed embryos were placed in PB1 after the addition of SPB1, left undisturbed for 2 min, washed three times with PB1, washed three times with mWM, and then used for IVC.

In this experiment, after the two-cell stage embryos were exposed to the CPS, SPB1 was infused into the cryotubes. The two-cell stage embryos were collected by the same method used for the warming procedures.

The survival rates of the vitrified embryos and fresh embryos exposed to CPS were evaluated under an inverted microscope (OLYMPUS IX70, magnification 200×) after placing the embryos in mWM (Fig. 1). The surviving two-cell-stage embryos underwent IVC for 72 h and development to blastocysts was examined. As a fresh control group, untreated embryos were also subjected to IVC.

Experiment 2

The two-cell stage embryos used in the experiments were collected using the IVF method or oviduct flushing method. Two-cell stage embryos of 14 mouse strains were subjected to vitrification using the same method as described in Experiment 1, and the

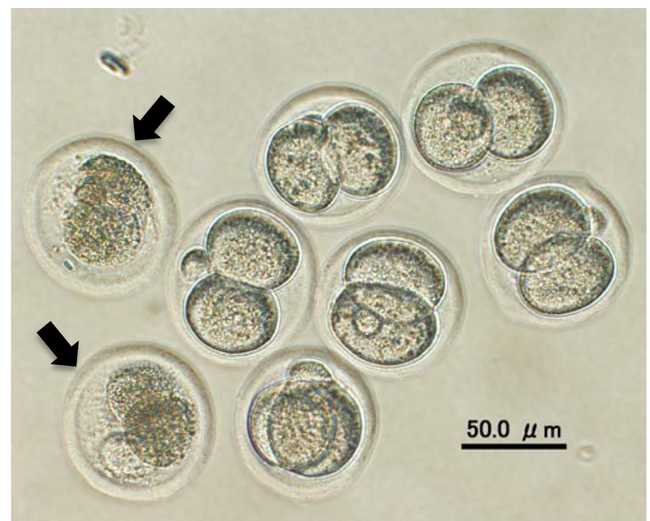

Fig. 1. Cryopreserved C57BL/6J strain 2-cell stage embryos after warming. The arrows show embryos that degenerated.

survival rates of the embryos after warming were evaluated. After warming, the surviving embryos were examined for in vivo development.

Statistical analysis

The experimental results shown in survival rates and development of two-cell stage embryos are expressed as means ± standard error of mean (SEM), and those in other tables are expressed as means. Statistical analysis was conducted with the Student's *t*-test after checking normal distribution. In all analyses, $P < 0.01$ was considered to indicate statistical significance. For analyses of the experimental data, Statcel3 (OMS Publishing, Saitama, Japan), automated analysis software, was used.

Results

In Experiment 1, none of the embryos died, even after exposure of the two-cell stage embryos to CPS (Table 1). There were no significant differences in development to blastocysts in IVC between CPS-exposed embryos (89.9%, 107/109) and fresh embryos (93.3%, 84/90). The survival rate of the vitrified two-cell stage embryos was 98.1% (155/158), which was not significantly different from that of vitrified two-cell stage embryos exposed to CPS ($P > 0.01$). The rate of development to blastocysts in IVC was not significantly different between the vitrified embryos (93.5%, 145/155) and those exposed to CPS or fresh embryos.

In Experiment 2, collection, vitrification, and in vivo development were evaluated in two-cell stage embryos of 14 mouse strains. The fertilization rate (number of two-cell stage embryos/number of embryos examined) was as follows: C57BL/6J 83.2% (830/998); C57BL/6N 95.5% (318/333); C57BL/6NCr 97.3% (253/260); BALB/cA 92.9% (338/364); BALB/cByJ 75.2% (513/682); C3H/HeJ 94.1% (459/488); CBA/N 97.3% (532/547); DBA/2J 48.5% (300/619); C57BL/10ScN 97.5% (349/358); 129^{+Tar}/Sv 64.8% (311/480); NOD/Shi 96.3% (1262/1310); NOD/Shi-scid 96.6% (889/920); TWY/Jic-ttw 80.7% (1388/1720); and ICR 96.1% (518/539) (Table 2). DBA/2J showed a reduced fertilization rate, ranging from 26.7% to 48.1%, compared to other strains, and average collection of two-cell stage embryos dropped from 52.9% to 21.9% compared to the other strains. The average numbers of two-cell stage embryos collected were 16.6, 13.8, 25.3, 16.9, 19.7, 14.8, 33.3, 7.3, 15.9, 19.4, 29.3, 17.4, 20.4, and 22.5 for C57BL/6J, C57BL/6N, C57BL/6NCr, BALB/cA, BALB/cByJ, C3H/HeJ, CBA/N, DBA/2J, C57BL/10ScN, 129^{+Tar}/Sv, NOD/Shi, NOD/Shi-scid, TWY/Jic-ttw, and ICR, respectively (total number of embryos obtained/number of females from which embryos were collected).

The survival rates (number of embryos survived/number of embryos recovered) of vitrified embryos were as follows: C57BL/6J 99.1% (329/332); C57BL/6N 97.5% (195/200); C57BL/6NCr 97.1% (169/174); BALB/cA 96.2% (150/156); BALB/cByJ 98.4% (241/245); C3H/HeJ 96.7% (115/119); CBA/N 94.1% (238/253); DBA/2J 95.0% (114/120); C57BL/10ScN 96.7% (171/177); 129^{+Tar}/Sv 99.2% (129/130); NOD/Shi 92.5% (111/120); NOD/Shi-scid 95.0% (114/120); TWY/Jic-ttw 93.2% (165/177); and ICR 92.8% (142/153); and the differences among strains were not significant ($P > 0.01$; Table 3).

The fetus development rates (number of fetuses/number of embryos transferred) of the fresh embryos and the vitrified embryos, respectively, were as follows: C57BL/6J 60.0% (60/100) and 61.3% (98/160); C57BL/6N 54.8% (91/166) and 47.9% (67/140); C57BL/6NCr 66.7% (40/60) and 60.8% (79/130); BALB/cA 39.4% (67/120) and 31.8% (60/137); BALB/cByJ 38.3% (46/162) and 31.4% (43/194); C3H/HeJ 52.2% (83/159) and 46.4% (51/110); CBA/N 67.9% (91/134) and 63.3% (69/109); DBA/2J 52.2% (60/115) and 42.2% (46/109); C57BL/10ScN 60.0% (72/120) and 56.4%

Table 1
Effect of cryoprotectant solution in vitrification of two-cell stage mouse embryos.

Treatment	Survival of embryos after treated			In vitro development to the blastocyst	
	No. of embryos recovered/treated (% ± SEM)*	No. of embryos survived/recovered (% ± SEM)*	Frequency of experiment	No. of blastocyst/cultured (% ± SEM)*	Frequency of experiment
Fresh control	–	–	–	84/90 (93.3 ± 1.7)	12
Exposure to cryoprotectant solution	199/200 (99.2 ± 0.8)	199/199 (100 ± 0.0)	4	107/119 (89.9 ± 2.1)	12
Vitrification	158/160 (98.8 ± 1.3)	155/158 (98.1 ± 1.9)	4	145/155 (93.5 ± 2.0)	16

* There were no significant differences among any groups ($P > 0.01$).

Table 2
Collection of two-cell stage embryos of multiple mouse strains.

Mouse strain	Classification of genetic background	Method of embryo collection*	Number of females embryo collected	Number of two-cell stage embryos obtained	Rate of fertilized (%)**	Number of collected two-cell stage embryos per female***
C57BL/6J	Inbred	IVF	50	830	83.2	16.6
C57BL/6N	Inbred	IVF	23	318	95.5	13.8
C57BL/6NCr	Inbred	IVF	10	253	97.3	25.3
BALB/cA	Inbred	OF	20	338	92.9	16.9
BALB/cByJ	Inbred	OF	26	513	75.2	19.7
C3H/HeJ	Inbred	IVF	31	459	94.1	14.8
CBA/N	Inbred	OF	16	532	97.3	33.3
DBA/2J	Inbred	IVF	41	300	48.5	7.3
C57BL/10ScN	Inbred	IVF	22	349	97.5	15.9
129^{+Tar}/Sv	Inbred	OF	16	311	64.8	19.4
NOD/Shi	Inbred	IVF	43	1262	96.3	29.3
NOD/Shi-*scid*	Inbred	IVF	51	889	96.6	17.4
TWY/Jic-*ttw*	Inbred	IVF	68	1388	80.7	20.4
ICR	Outbred	OF	23	518	96.1	22.5

* IVF; in vitro fertilization, OF; oviduct flushing of after natural mating.
** Number of two-cell stage embryos obtained/combined number of unfertilized oocytes and two-cell stage embryos.
*** Number of two-cell stage embryos obtained/number of females embryo collected.

Table 3
Survival of vitrified two-cell stage embryos of multiple mouse strains.

Mouse strain	No. of embryos recovered/vitrified (% ± SEM)*	No. of embryos survived/recovered (% ± SEM)*	Frequency of experiment
C57BL/6J	332/336 (98.8 ± 0.6)	329/332 (99.1 ± 0.6)	12
C57BL/6N	200/204 (98.0 ± 1.2)	195/200 (97.5 ± 1.7)	4
C57BL/6NCr	174/174 (100 ± 0)	169/174 (97.1 ± 0.6)	3
BALB/cA	156/156 (100 ± 0)	150/156 (96.2 ± 1.5)	4
BALB/cByJ	245/246 (99.6 ± 0.3)	241/245 (98.4 ± 0.8)	6
C3H/HeJ	119/119 (100 ± 0)	115/119 (96.7 ± 2.8)	3
CBA/N	253/257 (98.4 ± 0.7)	238/253 (94.1 ± 2.1)	6
DBA/2J	120/121 (99.2 ± 0.7)	114/120 (95.0 ± 5.5)	4
C57BL/10ScN	177/178 (99.4 ± 0.7)	171/177 (96.7 ± 1.7)	3
129^{+Tar}/Sv	130/130 (100 ± 0)	129/130 (99.2 ± 0.6)	4
NOD/Shi	120/120 (100 ± 0)	111/120 (92.5 ± 3.8)	3
NOD/Shi-*scid*	120/120 (100 ± 0)	114/120 (95.0 ± 5.0)	3
TWY/Jic-*ttw*	177/177 (100 ± 0)	165/177 (93.2 ± 5.1)	3
ICR	153/157 (97.3 ± 1.5)	142/153 (92.8 ± 3.9)	3

* There were no significant differences among any groups ($P > 0.01$).

Table 4
In vivo development of vitrified two-cell stage embryos of multiple mouse strains.

Mouse strain	Treatment	No. of implantation/transferred (% ± SEM)*	No. of fetuses/transferred (% ± SEM)*	Frequency of experiment
C57BL/6J	Vitrification	143/160 (89.4 ± 4.4)	98/160 (61.3 ± 4.7)	16
	Fresh	82/100 (82.0 ± 4.9)	60/100 (60.0 ± 6.0)	10
C57BL/6N	Vitrification	113/140 (80.7 ± 3.2)	67/140 (47.9 ± 5.0)	14
	Fresh	137/166 (82.5 ± 4.4)	91/166 (54.8 ± 3.8)	17
C57BL/6NCr	Vitrification	104/130 (80.0 ± 5.6)	79/130 (60.8 ± 5.5)	14
	Fresh	46/60 (76.7 ± 14.1)	40/60 (66.7 ± 12.3)	6
BALB/cA	Vitrification	94/137 (69.1 ± 3.7)	60/137 (31.8 ± 3.2)	15
	Fresh	93/120 (77.8 ± 3.0)	67/120 (39.4 ± 3.1)	14
BALB/cByJ	Vitrification	113/194 (59.0 ± 4.2)	43/194 (31.4 ± 3.7)	23
	Fresh	100/162 (61.7 ± 4.9)	46/162 (38.3 ± 4.7)	18
C3H/HeJ	Vitrification	81/110 (73.6 ± 6.5)	51/110 (46.4 ± 6.3)	12
	Fresh	122/159 (76.7 ± 5.9)	83/159 (52.2 ± 5.2)	18
CBA/N	Vitrification	76/109 (69.7 ± 6.9)	69/109 (63.3 ± 6.8)	12
	Fresh	100/134 (74.6 ± 5.4)	91/134 (67.9 ± 4.6)	14
DBA/2J	Vitrification	75/109 (68.8 ± 4.3)	46/109 (42.2 ± 3.9)	14
	Fresh	88/115 (76.5 ± 5.3)	60/115 (52.2 ± 5.7)	14
C57BL/10ScN	Vitrification	113/149 (75.8 ± 6.6)	113/149 (56.4 ± 4.9)	10
	Fresh	91/120 (75.8 ± 5.1)	72/120 (60.0 ± 5.9)	12
129^{+Tar}/Sv	Vitrification	85/129 (65.9 ± 7.4)	81/129 (62.8 ± 7.2)	14
	Fresh	66/91 (72.5 ± 8.5)	59/91 (64.8 ± 8.8)	10
NOD/Shi	Vitrification	94/111 (84.7 ± 3.8)	58/111 (50.9 ± 4.2)	12
	Fresh	115/140 (82.1 ± 6.2)	67/140 (60.9 ± 6.6)	14
NOD/Shi-*scid*	Vitrification	85/114 (74.6 ± 5.6)	59/114 (60.8 ± 5.9)	12
	Fresh	94/110 (85.5 ± 2.1)	76/110 (63.9 ± 3.4)	11
TWY/Jic-*ttw*	Vitrification	69/97 (71.1 ± 6.7)	67/97 (60.4 ± 6.0)	10
	Fresh	96/119 (80.7 ± 6.7)	100/119 (71.4 ± 6.5)	12
ICR	Vitrification	95/118 (80.5 ± 5.2)	88/118 (74.6 ± 4.8)	14
	Fresh	86/100 (86.0 ± 4.8)	81/100 (81.0 ± 5.9)	10

* There were no significant differences among fresh and vitrification groups of same strains ($P > 0.01$).

(113/149); 129^{+Tar}/Sv 64.8% (59/91) and 62.8% (81/129); NOD/Shi 60.9% (67/140) and 50.9% (58/111); NOD/Shi-scid 63.9% (76/110) and 60.8% (59/114); TWY/Jic-ttw 71.4% (100/119) and 60.5% (67/97); and ICR 81.0% (81/100) and 74.6% (88/118). There were no significant differences between vitrified and fresh embryos in each strain ($P > 0.01$; Table 4).

Discussion

Early embryos exposed to CPS may die due to cytotoxicity of the cryoprotectant, depending on the type and concentration of the agents added to the CPS [21]. In Experiment 1, however, the cytotoxic effects of the CPS (P10 and PEPeS) used for vitrification of two-cell stage mouse embryos were low (Table 1). The survival rate of 2-cell stage embryos after vitrification was high and stable (98.1 ± 1.9, Table 1). Propylene glycol added to the CPS enhances the permeation of the cryoprotectant into two-cell stage mouse embryos better than other cell-permeable cryoprotectants [26]. Presumably, freezing tolerance of the embryo was increased by the enhanced permeability of the rapid intracellular propylene glycol. To prevent rapid cell expansion after warming, sucrose was added to PEPeS and SPB1 [9], thus embryo damage due to shock by osmotic pressure was decreased [8]. Depending on the CPS or cooling conditions, freeze fractures may occur in CPS, which could damage the embryos [16]. PEPeS, however, inhibits the occurrence of freeze fractures due to the effect of Percoll [9]. Therefore, destruction of the embryos by freeze fractures is extremely low. There was no significant difference in the in vitro development rate between the vitrified embryos and fresh control. Therefore, mouse 2-cell stage embryos in CPS (P10 and PEPeS) are considered to have adequate freezing tolerance. Based on the findings of Experiment 1, our vitrification method is suitable for cryopreservation of two-cell stage mouse embryos.

There are some reports of significant differences between strains in the survival rate of cryopreserved embryos using the frozen method [31,34]. In Experiment 2, the survival rates for the

Table 5
Expected value for number of live fetuses made from per female used in embryo collection.

Mouse strain	Number of collected two-cell stage embryos per female[A*]	Recovery rate of vitrified embryos (%)[B*]	Survival rate of vitrified embryos (%)[C*]	Developmental rate of live fetuses (%)[D*]	Expected number of live fetuses from per female[**]
C57BL/6J	16.6	98.8	99.1	61.3	10.0
C57BL/6N	13.8	98.0	97.5	47.9	6.3
C57BL/6NCr	25.3	100.0	97.1	60.8	14.9
BALB/cA	16.9	100.0	96.2	31.8	5.2
BALB/cByJ	19.7	99.6	98.4	31.4	6.1
C3H/HeJ	14.8	100.0	96.7	46.4	6.6
CBA/N	33.3	98.4	94.1	63.3	19.5
DBA/2J	7.3	99.2	95.0	42.2	2.9
C57BL/10ScN	15.9	99.4	96.7	56.4	8.6
129^{+Tar}/Sv	19.4	100.0	99.2	62.8	12.1
NOD/Shi	29.3	100.0	92.5	50.9	13.8
NOD/Shi-*scid*	17.4	100.0	95.0	60.8	10.1
TWY/Jic-*ttw*	20.4	100.0	93.2	60.4	11.5
ICR	22.5	97.3	92.8	74.6	15.2

* Extracted from other table (A; Table 2, B and C; Table 3, D; Table 4).
** Results were calculated by the formula, expected number of live fetuses = A/B/C/D.

embryos of the 14 strains examined here ranged from 92.0% to 99.4%, and there were no significant differences between strains. In addition, several studies report that the in vivo development of cryopreserved embryos is significantly lower than that of fresh embryos [3,18]. In our experiments, however, there was no significant difference in the in vivo development between the cryopreserved embryos and fresh embryos in all strains. The findings of Experiment 2 suggested that our vitrification method is effective for the cryopreservation of two-cell stage embryos of multiple strains of mice.

For strain preservation using cryopreserved early embryos, strain maintenance is achieved by producing a breeding pair using offspring produced from cryopreserved embryos. Strain maintenance of inbred strains is performed by sib mating [11]. Sib mating is a method of passaging in which a breeding pair is produced from the offspring born to one male/female pair. Therefore, strain preservation requires at least one male and one female offspring from the two-cell stage embryos that have been cryopreserved after collection from a single female. The results of the present study indicate that strain preservation should be possible if the expected number of fetuses produced from the cryopreserved embryos is more than two. The expected number of surviving fetuses obtained from the embryos collected from one female (average number of two-cell stage embryos collected per female/collection rate of vitrified embryos/survival rate of vitrified embryos/embryo development rate) for the inbred C57BL/6J, C57BL/6N, C57BL/6Cr, BALB/cA, BALB/cByJ, C3H/HeJ, CBA/N, DBA/2J, C57BL/10ScN, 129^{+Tar}/Sv, NOD/Shi, NOD/Shi-scid, and TWY/Jic-ttw strains was 10.0, 6.3, 14.9, 5.2, 6.1, 6.6, 19.5, 2.9, 8.6, 12.1, 13.8. 10.1, and 11.5, respectively (Table 5). In the case of outbred strains, passaging is performed after multiple breeding pairs are produced and the offspring of each breeding pair are mated randomly with those from a different breeding pair [22]. Therefore, strain preservation would be possible if one offspring is obtained from the embryos that have been cryopreserved after collection from a single female following the preparation of cryopreserved embryos from multiple pairs. In the case of the ICR strain, which was the outbred strain used in this experiment, the expected number of surviving fetuses for the embryos collected from a single female was 15.1 (Table 5). Our calculations suggested that breeding pairs can be prepared from any strain, and strain maintenance can be restarted as two or more offspring for inbred strains and one or more offspring for outbred strains can be expected from the eggs collected from a single female of all strains. Thus, the results of the present study suggest that the vitrification method [9] for the two-cell stage embryos developed in our laboratory is effective for strain preservation of multiple strains of mice.

Genetic modifications of existing strains are frequently carried out [1], and the in vivo development rate of cryopreserved early mouse embryos may decrease due to genetic background factors [29,30]. This vitrification method, however, does not significantly decrease the in vivo development rate in multiple inbred or outbred strains. Therefore, our cryopreservation method will be a powerful tool for strain preservation of genetically modified mouse strains. The genetic backgrounds of the majority of experimental animal mouse strains are produced by sib mating or random mating. If the genetic background is not managed, mouse strains cannot be maintained. Our vitrification method is effective for cryopreservation of experimental animal mouse strains. Additional studies are needed, however, to further improve the preservation of multiple mouse strains.

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