—Original Article—

Equilibrium vitrification of mouse embryos at various developmental stages using low concentrations of cryoprotectants

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Abstract. We previously developed a new vitrification method (equilibrium vitrification) by which two-cell mouse embryos can be vitrified in liquid nitrogen in a highly dehydrated/concentrated state using low concentrations of cryoprotectants. In the present study, we examined whether this method is effective for mouse embryos at multiple developmental stages. Four-cell embryos, eight-cell embryos, morulae, and blastocysts were vitrified with EDFS10/10a, 10% (v/v) ethylene glycol and 10% (v/v) DMSO in FSa solution. The FSa solution was PB1 medium containing 30% (w/v) Ficoll PM-70 plus 0.5 M sucrose. The state of dehydration/concentration was assessed by examining the survival of vitrified embryos after storage at –80°C. When four-cell embryos and eight-cell embryos were vitrified with EDFS10/10a in liquid nitrogen and then stored at –80°C, the survival rate was high, even after 28 days, with relatively high developmental ability. On the other hand, the survival of morulae and blastocysts vitrified in liquid nitrogen and stored at –80°C for four days was low. Therefore, morulae and blastocysts cannot be vitrified in a highly dehydrated/concentrated state using the same method as with two-cell embryos. However, when blastocysts were shrunken artificially before vitrification, survival was high after storage at –80°C for four days with high developmental ability. In conclusion, the equilibrium vitrification method using low concentrations of cryoprotectants, which is effective for two-cell mouse embryos, is also useful for embryos at multiple stages. This method enables the convenient transportation of vitrified embryos using dry ice.

Key words: Cryoprotectant, Dehydration, Embryos, Osmolality, Vitrification

With the expansion of genetically engineered mouse models, cryopreservation technology of mouse embryos is useful for reducing the costs of maintaining a large number of mouse variants. The protocol for cryopreserving embryos can be divided into two categories: slow freezing and vitrification.

With the original slow-freezing protocol, embryos are cooled at a slow rate to –70ºC to maintain the chemical potential of their intracellular water close to that of water in the partially frozen extracellular solution. Therefore, it is referred to as equilibrium slow-freezing, and embryos are frozen in 'a near-equilibrium state' [1]. The embryos at –70ºC were dehydrated sufficiently and intracellular solutes and cell permeating cryoprotectants were concentrated. Therefore, they can survive rapid cooling in liquid nitrogen and rapid/slow warming after cryopreservation.

Vitrification is a common strategy for the cryopreservation of mammalian embryos and has replaced the slow-freeze method because it does not require a programmed freezer, and the protocol is simple and instant [2, 3]. Moreover, higher survival is expected because extracellular ice is not formed [4]. Vitrification is the ultimate non-equilibrium cryopreservation process because embryos are cooled

under conditions in which ice forms neither in the cell nor in the surrounding medium [1]. However, vitrification has several disadvantages. One of them is that embryos are vitrified in an insufficiently dehydrated/concentrated state compared to those cryopreserved with original slow freezing. Therefore, samples are much more delicate and must be stored below –130°C (glass transition temperature) and they must be handled carefully to prevent spontaneous crystallization while also warmed rapidly to prevent intracellular ice from forming [1]. Another disadvantage is that high concentrations of permeating cryoprotectants must be used as cryopreservation solutions [5]. Therefore, embryos may be damaged by the toxicity of permeating cryoprotectants in the cryopreservation solution [6].

To overcome the first disadvantage of the vitrification method, we developed an equilibrium vitrification method using EFS35c, where mouse embryos at multiple developmental stages can be vitrified in a highly dehydrated/concentrated state as those cryopreserved with the original slow-freezing method and can be transported with dry ice [7, 8]. We called this vitrification method 'equilibrium vitrification'. However, EFS35c contains high concentrations of permeating and non-permeating cryoprotectants (35% (v/v) ethylene glycol (EG) and 0.98 M sucrose, respectively) with high osmolality (23.3 moles/kg) to promote dehydration of the cytoplasm. Therefore, this method cannot be applied to embryos with high sensitivity to the toxicity of cryoprotectants or osmolality.

To overcome the second disadvantage of vitrification, we developed a novel equilibrium vitrification method using EDFS10/10a solution, by which two-cell mouse embryos can be cryopreserved using lower concentrations of permeating cryoprotectants (10% (v/v) ethylene

glycol (EG) and 10% (v/v) dimethyl sulfoxide (DMSO)) and a non-permeating cryoprotectant (0.4 M sucrose) [9]. The solution has a relatively low chemical toxicity and osmolality (6.43 moles/kg). With this solution, vitrified two-cell mouse embryos were stored at –80°C for 4–28 days, suggesting that the embryos were vitrified in a highly dehydrated/concentrated state as those cryopreserved in a near-equilibrium state with the original slow-freezing technique.

Novel equilibrium vitrification using EDFS10/10a has advantages over conventional vitrification: 1) the vitrification solution is less toxic and has a lower osmolality, resulting in less damage to embryos; 2) vitrified embryos can be stored in a freezer at –80°C for a certain period if the liquid nitrogen (LN_2) tank is not functioning or for arrangement of samples; and 3) vitrified embryos can be transported short distances using dry ice.

The availability of this method will increase if it can be extended to mouse embryos at other developmental stages.

In the present study, we examined whether equilibrium vitrification using EDFS10/10a could be applied to mouse embryos at other developmental stages.

Materials and Methods

Animals

Female ICR mice (CLEA Japan Inc., Tokyo, Japan) (8–12-weeks old) were induced to superovulate by intraperitoneal injection of 5 IU of equine chorionic gonadotropin (Serotropin; Teikokuzoki, Tokyo, Japan) and 5 IU of human chorionic gonadotropin (hCG; Puberogen; Sankyozoki, Tokyo, Japan) 48 h apart. After hCG injection, females were placed with males of the same strain, and the following morning, the presence of a vaginal plug was considered to indicate mating. All animals were housed under a 14 h light-10 h dark photoperiod with food and water available *ad libitum* at 23 ± 1.5°C. The Animal Care and Use Committee of Kochi University approved the protocols for animal experiments.

Media

Modified M16 medium [10] was used to culture the embryos. Embryo cryopreservation was performed using 10% (v/v) EG and 10% (v/v) DMSO (Nacalai Tesque, Kyoto, Japan) in FSa solution (EDFS10/10a) (Table 1). The FSa solution was PB1 medium [11] containing 30% (w/v) Ficoll PM-70 (GE Healthcare, Chicago, USA) and 0.5 M sucrose. The pretreatment solution was PB1 medium containing 5% (v/v) EG and 5% (v/v) DMSO (ED5/5). Permeated cryoprotectants in embryos were removed in PB1 medium containing 0.5 M sucrose (S-PB1). Unless otherwise noted, chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Collection of mouse embryos and culture

Four- and eight-cell mouse embryos were flushed with PB1 medium from the oviducts at 53–55 and 67–68 h after hCG injection, respectively. Morulae were flushed from the uterine horn at 76–78 h after hCG injection. To obtain blastocysts, collected morulae were cultured in modified M16 medium under paraffin oil in a culture dish in a CO_2 incubator at 37°C (5% CO_2 and 95% air) for 12–16 h. Only blastocysts with blastocoel formation and cavity expansion but no expanded zona were used. In some experiments, the blastocoel was shrunken artificially by puncturing it with a microneedle (Fig. 1).

Embryos were washed twice with PB1 medium, and those with normal morphology were used for the experiments.

Vitrification of embryos

Embryos were vitrified using a two-step method. Five to eight embryos were first suspended in ED5/5 solution at 25°C for 2 min. They were then transferred into a drop of EDFS10/10a, vitrification solution, washed three times, and then transferred into a larger column of EDFS10/10a solution in a 0.25-ml plastic insemination straw (IMV, L'Aigle, Normandy, France) at 25°C with a minimal vitrification solution volume. The configuration of the straw has been described previously [12]. After the embryos were exposed to the vitrification solution for 60 sec, the straws were immediately plunged into the LN_2. Certain vitrified samples were removed from the LN_2 and immersed in ethanol in a Dewar flask precooled in a freezer at –80°C for 4, 7, or 28 days.

Warming of vitrified embryos

When warmed from –80°C, the straw samples were directly immersed in water at 25°C. When warmed from LN_2, the straw
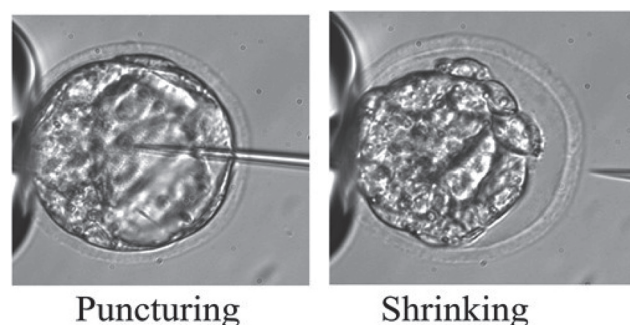
Fig. 1. Artificial shrinkage by puncturing the blastocoel before vitrification.

Table 1. Composition of vitrification solution

	EG (% v/v)	DMSO (% v/v)	Sucrose (M)	Ficoll PM-70 (% w/v)	PB1 (% v/v)	Molarity (mol/L)	Molality (moles/kg)
ED5/5	5	5	-	-	90	1.6	2
EDFS10/10a	10	10	0.4	24	56.2	3.2	6.43

EG, ethylene glycol; DMSO, dimethyl sulfoxide.

sample was kept in air at room temperature (25°C) for 10 s to allow it to pass through the glass transition temperature of the cytoplasm slowly to prevent fracture damage and then immersed in water at 25°C [13]. Upon melting the S-PB1 medium in the straw, the contents of the straws were expelled into a watch glass containing 1 ml of S-PB1 medium at 25°C and mixed gently. Embryos were collected and transferred into fresh S-PB1 medium under paraffin oil at 25°C. Five minutes after warming, the embryos were transferred into fresh PB1 medium under paraffin oil at 25°C. Upon transfer to fresh PB1 medium, embryos were observed to determine whether they swelled because swelling is a consequence of the formation of intracellular ice [14]. After being warmed, the embryos were transferred to modified M16 medium and cultured in a CO_2 incubator (5% CO_2 and 95% air) at 37°C.

Assessment of in vitro survival

Vitrified embryos were cultured in modified M16 medium. As a control, embryos were pretreated with ED5/5 solution for 2 min at 25°C, treated with EDFS10/10a solution for 1 min at 25°C, and then recovered in S-PB1 medium at 25°C without cooling. As the fresh control, fresh embryos were cultured without treatment.

The survival of four-cell embryos, eight-cell embryos, and morulae was assessed by their morphology after 1 h of culture and by their ability to develop into morulae and blastocysts during 48–72 h of culture. The survival of blastocysts was assessed at 1 h and 12–48 h post-culture by re-expansion and hatching, respectively.

Statistical analysis

Comparisons between groups were carried out by one-way analysis of variance (ANOVA) using GraphPad Instat software, v.7.04 (GraphPad Software Inc., La Jolla, USA) with the Tukey-Kramer multiple comparison test. Results are expressed as the mean ± standard deviation (SD); $P < 0.05$, considered significant. All experiments were independently performed three or more times under similar conditions.

Results

The viability of four-cell embryos vitrified at –196°C and stored at –80°C

When four-cell embryos were vitrified and stored at –80°C for 4–28 days, survival immediately after warming was quite high (Table 2). The proportion was not significantly different from vitrified and fresh controls. Therefore, four-cell embryos were vitrified in a highly dehydrated/concentrated state using this method.

The ability of four-cell embryos vitrified/stored at –80°C for 4–28 days to develop into morulae was high and not significantly different from that of the vitrified and fresh controls. However, blastocyst formation slightly decreased as the storage period at –80°C increased, and was significantly lower than that of the fresh control.

The viability of eight-cell embryos vitrified at –196 °C and stored at –80°C

When eight-cell embryos were vitrified and stored at –80°C for 4–28 days, almost all embryos survived morphologically after warming (Table 3). Therefore, eight-cell embryos were vitrified in a highly dehydrated/concentrated state using this method.

The ability of embryos vitrified/stored at –80°C for 4–28 days to develop into morulae was high and not significantly different from that of the vitrified or fresh controls. However, blastocyst formation slightly decreased as the storage period increased at –80°C.

The viability of morulae vitrified at –196°C and stored at –80°C

When morulae were vitrified and warmed directly, survival after warming was high, as was the ability to develop into blastocysts (Table 4). The proportions were not significantly different from those of the fresh controls.

However, when vitrified morulae were stored at –80°C for 4–28 days, survival markedly decreased as the storage period at –80°C increased. As the proportion of swelled blastomeres increased as the storage period increased, intracellular ice would be formed during storage at –80°C; swelled blastomeres immediately after warming suggested intracellular ice formation [14]. Therefore, morulae were

Table 2. The viability of four-cell embryos vitrified with EDFS10/10a solution at –196°C and then stored at –80°C for varying periods before being warmed

Treatment [1]	Time kept at –80°C (day)	No. of embryos		No. of blastomeres [2]		No. alive [3]		No. of morulae [4]		No. of blastocysts [5]	
		Vitrified	Recovered	Swelled (%) [6]	Morphologically normal (%) [6]	Num (%) [6]	± SD	Num (%) [6]	± SD	Num (%) [6]	± SD
Fresh control	–	–	60	0 (0)	240 (100)	60 (100)	0	60 (100)	0	57 (95) [a]	5
Solution control	–	–	60	0 (0)	240 (100)	60 (100)	0	59 (98)	4	55 (92) [ab]	6
Vitrified	0 (control) [7]	60	60	0 (0)	240 (100)	60 (100)	0	57 (95)	5	55 (92) [ab]	6
	4	60	59	0 (0)	234 (99)	59 (100)	0	55 (93)	6	46 (78) [bc]	5
	7	60	60	4 (2)	231 (98)	58 (98)	4	55 (93)	6	42 (71) [c]	10
	28	60	60	6 (3)	228 (95)	58 (97)	5	55 (92)	8	41 (68) [c]	9

[1] Five to eight embryos were loaded in each straw or treated for the solution control or fresh control, and three to four straws were prepared for each treatment in each experiment. Each datum is a total of three replicates. [2] Embryos were observed just after being transferred into PB1 medium. [3] Embryos were observed after 1 h of culture in modified M16 medium. [4] Embryos were examined after 48 h of culture in modified M16 medium. [5] Embryos were examined after 72 h of culture in modified M16 medium. [6] Percentage of recovered embryos; values with different superscripts are significantly different (P < 0.05). [7] Warmed in water at 25°C without being stored at –80°C.

Table 3. The viability of eight-cell embryos vitrified with EDFS10/10a solution at –196°C and then stored at –80°C for varying periods before being warmed

| Treatment[1] | Time kept at –80°C (day) | No. of embryos | | No. of blastomeres [2] | | No. alive [3] | | No. of morulae [4] | | No. of blastocysts [5] | |
		Vitrified	Recovered	Swelled (%) [6]	Morphologically normal (%) [6]	Num (%) [6]	± SD	Num (%) [6]	± SD	Num (%) [6]	± SD
Fresh control	–	–	62	0 (0)	496 (100)	62 (100)	0	62 (100)	0	60 (97) [a]	3
Solution control	–	–	61	0 (0)	488 (100)	61 (100)	0	61 (100)	0	59 (97) [a]	4
Vitrified	0 (control) [7]	60	60	0 (0)	480 (100)	60 (100)	0	60 (100)	0	56 (93) [ab]	5
	4	61	61	0 (0)	488 (100)	60 (98)	3	59 (97)	4	54 (89) [bc]	7
	7	60	60	0 (0)	480 (100)	59 (98)	3	59 (98)	3	53 (88) [bc]	5
	28	62	61	0 (0)	488 (100)	60 (97)	4	58 (95)	4	53 (87) [c]	5

[1] Five to eight embryos were loaded in each straw or treated for the solution control or fresh control, and three to four straws were prepared for each treatment in each experiment. Each datum is a total of three replicates. [2] Embryos were observed just after being transferred into PB1 medium. [3] Embryos were observed after 1 h of culture in modified M16 medium. [4] Embryos were examined after 24 h of culture in modified M16 medium. [5] Embryos were examined after 48 h of culture in modified M16 medium. [6] Percentage of recovered embryos; values with different superscripts are significantly different (P < 0.05). [7] Warmed in water at 25°C without being stored at –80°C.

Table 4. The viability of morulae vitrified with EDFS10/10a solution at –196°C and then stored at –80°C for varying periods before being warmed

| Treatment [1] | Time kept at –80°C (day) | No. of embryos | | No. of embryos [2] | | No. alive [3] | | No. of blastocysts [4] | |
		Vitrified	Recovered	Swelled (%) [5]	Morphologically normal (%) [5]	Num (%) [5]	± SD	Num (%) [5]	± SD
Fresh control	–	–	60	0 (0) [a]	60 (100) [a]	60 (100) [a]	0	58 (97) [a]	4
Solution control	–	–	60	0 (0) [a]	60 (100) [a]	60 (100) [a]	0	57 (95) [a]	5
Vitrified	0 (control) [6]	60	60	0 (0) [a]	60 (100) [a]	60 (100) [a]	0	54 (90) [a]	7
	4	66	65	41 (63) [b]	28 (43) [b]	36 (55) [b]	10	27 (42) [b]	10
	7	64	64	41 (64) [b]	27 (42) [b]	29 (45) [b]	8	25 (39) [b]	10
	28	67	67	67 (100) [c]	0 (0) [c]	13 (19) [c]	16	8 (12) [c]	6

[1] Five to eight embryos were loaded in each straw or treated for the solution control or fresh control, and three to four straws were prepared for each treatment in each experiment. Each datum is a total of three replicates. [2] Embryos were observed just after being transferred into PB1 medium. [3] Embryos were observed after 1 h of culture in modified M16 medium. [4] Embryos were examined after 24 h of culture in modified M16 medium. [5] Percentage of recovered embryos; values with different superscripts are significantly different (P < 0.05). [6] Warmed in water at 25°C without being stored at –80°C.

able to vitrify with this protocol, but were vitrified in an insufficient dehydrated/concentrated state.

Viability of intact blastocysts vitrified at –196°C and stored at –80°C

When blastocysts were vitrified and warmed directly from LN₂, the ability to re-expand and hatch was low, though the ability of the solution controls and fresh controls was high (Table 5).

No blastocysts re-expanded after being vitrified and stored at –80°C for four days.

Therefore, blastocysts were not dehydrated and/or concentrated sufficiently for vitrification.

Viability of shrunken blastocysts vitrified at –196°C and stored at –80°C

When blastocysts were artificially shrunk by puncturing the blastocoel and vitrified, the ability to re-expand and hatch increased markedly (Table 6). Moreover, their ability after storage at –80°C for four days was observed as high, although it gradually decreased as the storage period increased.

Therefore, blastocysts that were artificially shrunken were vitrified in a highly dehydrated/concentrated state using this method.

Discussion

In the present study, we demonstrated that mouse embryos at different developmental stages can be vitrified in a highly dehydrated/concentrated state using EDFS10/10a, similar to original slow freezing.

In a previous study, a high proportion of two-cell embryos vitrified with EDFS10/10a survived even after storage at –80°C for four to 28 days [9]. In the present study, the survival of four- and eight-cell embryos was as high as that of two-cell embryos and was not significantly different from that of fresh embryos, suggesting that four- and eight-cell embryos were vitrified in a highly dehydrated/concentrated state. Although the surface area of four- and eight-cell embryos is larger than that of two-cell embryos, the permeability of the plasma membrane of the embryos to water and cryoprotectants is as low as that of two-cell embryos [15, 16]. Therefore, four- and eight-cell embryos were sufficiently dehydrated and concentrated using the vitrification protocol for two-cell embryos.

Table 5. The viability of intact blastocysts vitrified with EDFS10/10a solution at –196°C and then stored at –80°C for four days before being warmed

Treatment [1]	Time kept at –80°C (day)	No. of embryos		No. of re-expanded blastocysts [2]		No. hatched [3]	
		Vitrified	Recovered	Num (%) [4]	± SD	Num (%) [4]	± SD
Fresh control	–	–	60	60 (100) [a]	0	59 (98) [a]	3
Solution control	–	–	60	60 (100) [a]	0	55 (92) [a]	6
Vitrified	0 (control) [5]	70	70	40 (57) [b]	8	9 (13) [b]	4
	4	60	60	0 (0) [c]	0	0 (0) [c]	0

[1] Five to eight embryos were loaded in each straw or treated for the solution control or fresh control, and three to four straws were prepared for each treatment in each experiment. Each datum is a total of three replicates. [2] Embryos were observed after 1 h of culture in modified M16 medium. [3] Embryos were examined after 48 h of culture in modified M16 medium. [4] Percentage of recovered embryos; values with different superscripts are significantly different ($P < 0.05$). [5] Warmed in water at 25°C without being stored at –80°C.

Table 6. The viability of shrunken blastocysts vitrified with EDFS10/10a solution at –196°C and then stored at –80°C for varying periods before being warmed

Treatment [1]	Time kept at –80°C (day)	No. of embryos		No. of re-expanded blastocysts [2]		No. hatched [3]	
		Vitrified	Recovered	Num (%) [4]	± SD	Num (%) [4]	± SD
Fresh control	–	–	50	50 (100) [a]	0	48 (96) [a]	5
Solution control	–	–	50	49 (98) [ab]	0	44 (88) [ab]	6
Vitrified	0 (control) [5]	50	50	44 (88) [ab]	8	41 (82) [ab]	5
	4	52	52	47 (90) [ab]	6	37 (71) [bc]	10
	7	50	50	43 (86) [b]	10	28 (56) [c]	13
	28	51	51	33 (65) [c]	16	18 (35) [d]	16

[1] Five to eight embryos were loaded in each straw or treated for the solution control or fresh control, and three straws were prepared for each treatment in each experiment. Each datum is a total of three replicates. [2] Embryos were observed after 1 h of culture in modified M16 medium. [3] Embryos were examined after 48 h of culture in modified M16 medium. [4] Percentage of recovered embryos; values with different superscripts are significantly different ($P < 0.05$). [5] Warmed in water at 25°C without being stored at –80°C.

On the other hand, the survival of morulae after vitrification and storage at –80°C was low, although survival after being directly warmed from LN_2 was high. As the number of swollen morulae increased when they were stored at –80°C, dehydration and concentration of morulae were poor. For morulae, the permeability of the plasma membrane to water and cryoprotectants is markedly high because of the expression of aquaporins [15, 17, 18]. Therefore, as cryoprotectants permeated into the cytoplasm rapidly, water re-permeated into the cytoplasm during treatment with EDFS10/10a because the osmolality of the cytoplasm was equilibrated with that of EDFS10/10a. As a result, embryos were vitrified in an insufficient dehydrated/concentrated state [1]. Another possibility is gap junctions between blastomeres. It was previously reported that ice can pass from one cell to the supercooled content of an adjoining cell through the pores in the gap junctions between the two cells [19, 20]. As gap junctions are formed among blastomeres when embryos are compacted and become morulae, intracellular ice can propagate from one blastomere to another through gap junctions.

The survival of intact blastocysts after warming decreased. It was low even when they were directly warmed from LN_2, and no embryos survived after storage at –80°C for four days. Artificial shrinkage

of human blastocysts before vitrification increases survival and pregnancy outcomes and does not damage the blastocyst [21–24]. Therefore, blastocysts were shrunk by puncturing the blastocoel before vitrification. As most shrunken blastocysts survived vitrification, the low survival of intact blastocysts may have been because of ice formation in the blastocoel. Notably, the survival of vitrified blastocysts after storage at –80°C also markedly improved. This indicates that artificially shrunken blastocysts were vitrified in a highly dehydrated/concentrated state, although the permeability of the plasma membrane of blastocysts to water and cryoprotectants was as high as that of morulae [15]. Further studies are needed to clarify why shrunken blastocysts can be vitrified in a highly dehydrated/concentrated state using this method.

Despite the developmental ability of four- and eight-cell embryos as well as shrunken blastocysts vitrified with EDFS10/10a and stored at –80°C for four days was high, developmental ability was significantly reduced as the storage period at –80°C increased to seven or 28 days. Similar results were obtained for two-cell mouse embryos in a previous study [9]. This suggests that EDFS10/10a damaged embryos based on toxicity during long-term storage at –80ºC. Seki and Mazur reported that mouse oocytes can be cryopreserved

using EAFS10/10a (containing 10% (v/v) EG, 10% (v/v) acetamide, 24% (w/v) Ficoll PM-70, and 0.4 M sucrose) in LN_2 and then stored at –80°C for one to three months, but developmental ability decreased gradually during storage [25]. Therefore, with storage at –80°C, water in the embryos was devitrified without recrystallization because of the high degree of dehydration. However, as the chemical reaction in the cytoplasm and plasma membrane would proceed because of the cytoplasm and EDFS10/10a transitioning from the glass phase to the liquid phase, the embryos were damaged by the toxicity of permeating cryoprotectants during long-term storage at –80ºC.

In conclusion, equilibrium vitrification using low concentrations of cryoprotectants can be performed for mouse embryos at multiple stages. The proposed method has several advantages: 1) The vitrification medium (EDFS10/10a) contains lower concentrations of permeating and non-permeating cryoprotectants, and is thus less toxic to embryos; 2) As this method does not require ultra-rapid cooling/warming, skilled techniques or minute devices (for ultra-rapid vitrification) are not required; 3) The process is instant and quick; 4) The procedure can be performed at room temperature without a programmable freezer; 5) This method is useful for two-, four-, and eight-cell embryos and shrunken blastocysts; 6) Vitrified embryos can be transported on dry ice; and 7) This method can be easily applied to "closed systems" because the embryos are vitrified in a highly dehydrated/concentrated state and do not require rapid warming [26].

We believe that this method can be applied to routine embryo cryopreservation and transportation in mouse facilities and biomedical laboratories. Although our results are restricted to mouse embryos, this method is expected to be useful for other mammalian species, including humans, and for oocytes and embryos that are highly sensitive to the toxicity of cryoprotectants and high osmolality.

Acknowledgements

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