change in their density after exposure to cryoprotectant solutions.

Many embryos at the tail bud stage exposed to filtered seawater containing 9.5% DMSO, 8% ethylene glycol, or 10% propylene glycol solution at 25 °C for 30 min neither floated nor sank in 95% seawater 1 min after being transferred to it but some embryos sank to the bottom (Fig. 4), suggesting that a small amount of these cryoprotectants permeated the embryos at 25 °C during exposure for 30 min. On the other hand, most embryos exposed to filtered seawater containing 5% methanol floated to the top in 95% seawater 1 min after being transferred to it. Thus, although the change in volume of flounder embryos in cryoprotectants in BS2 medium was only slight (Fig. 3), methanol must have permeated the embryos.

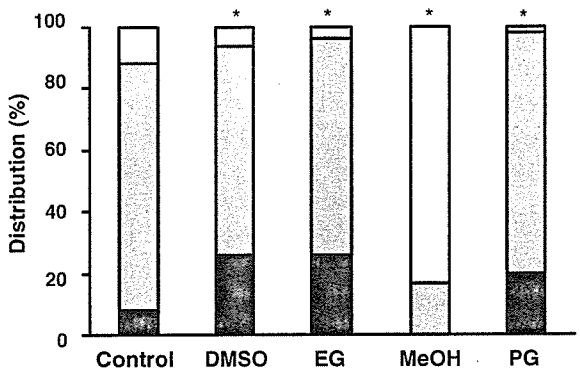

Fig. 4. Permeation by DMSO, ethylene glycol (EG), methanol (MeOH), and propylene glycol (PG) of Japanese flounder embryos assessed by density. Embryos at the tail bud stage were exposed to artificial seawater (control) or artificial seawater containing 9.5% (vol/vol) DMSO, 8% (vol/vol) EG, 5% (vol/vol) MeOH, or 10% (vol/vol) PG at 25 °C for 30 min. Next, 15–25 embryos were poured into 95% seawater in a test tube, mixed by inverting the test tube once, and kept upright for 1 min at 25 °C. Then, the embryos that floated to the surface (open), sank to the bottom (closed), and remained suspended in the middle of the solution (shaded) were enumerated. Data are the sum of triplicate determinants and the total number of embryos in each treatment was 50. *Significantly different from controls ($P < 0.05$).

Sensitivity of embryos to the toxicity of vitrification solutions

Direct exposure of embryos at the tail bud stage to FVS1, containing relatively low concentrations of methanol and propylene glycol, and FVS3, containing relatively high concentrations of the cryoprotectants, for 30 and 60 min was toxic to the embryos (Table 2). Stepwise treatment with FVS1 and FVS3 did not reduce the toxicity of the solutions (Table 2), although Chen and Tian [1] reported that stepwise treatment of embryos with vitrification solution markedly reduced the toxicity [1]. However, a small proportion (4–8%) of embryos survived direct or stepwise exposure (Table 2).

Survival of embryos after vitrification

As preliminary experiments, we cooled and warmed FVS1 and FVS3 in straws and on cryoloops without embryos to see the formation of ice (Table 3). When the solutions in straws were cooled by being plunged into LN_2, FVS1 became opaque partially or entirely but FVS3 remained transparent during cooling, suggesting that ice crystals formed not in FVS3 but in FVS1 during cooling. During warming at 40 °C, FVS3 remained transparent, suggesting that FVS3 remained uncrystallized during warming. When FVS1 and FVS3 on cryoloops were cooled in LN_2 and warmed at 15 °C, not only FVS3

Table 3
Formation of ice crystals in vitrification solution during cooling and warming in straws or cryoloops

Container	Vitrification solution	Cooling	Warming
Straw	FVS1	±	+
	FVS3	–	–
Cryoloop	FVS1	–	+
	FVS3	–	–

– Ice crystals did not form.
± Ice crystals formed partially or entirely in straws and cryoloops.
+ Ice crystals formed entirely in straws and cryoloops.

Table 2
Percent survival of Japanese flounder embryos at the tail bud stage exposed to vitrification solutions at 15 °C

Vitrification solution	Control[a]	After direct exposure		After stepwise exposure	
		30 min	60 min	at treatment[b]	with prolonged exposure[c]
FVS1	89 (25/28)	8 (2/25)	4 (1/25)	4 (1/26)	0 (0/25)
FVS3	76 (19/25)	0 (0/30)	0 (0/30)	5 (1/20)	0 (0/30)

[a] Embryos without treatment.
[b] Embryos were treated stepwise with vitrification solutions (five steps, for 8 min in each step in 40 min).
[c] Embryos were further exposed to vitrification solutions for 8 min after being treated stepwise with the solutions in 40 min.

but also FVS1 remained transparent during cooling. During warming, however, FVS1 became opaque whereas FVS3 remained transparent (Table 3).

Table 4 shows the survival of embryos at the tail bud stage after vitrification in straws together with the formation of extracellular and intracellular ice during cooling and warming. Unfortunately, we could not obtain viable embryos after vitrification in any of conditions examined. When embryos were cooled with LN_2 after being treated with FVS1 in five steps, straws became opaque partially or entirely during cooling, showing the formation of extracellular ice. Thus, in many of the embryos, we could not observe whether ice crystals formed in the embryos in straws during cooling and warming. However, we could observe the appearance of embryos existing in transparent portions of the straws during cooling (about 20–30% of the embryos) and all of the embryos became opaque during cooling, indicating the formation of intracellular ice (data not shown). On the other hand, when embryos treated with FVS3 stepwise were cooled with LN_2 in straws, extracellular FVS3 was transparent but all the embryos became opaque, indicating the intracellular ice was formed during cooling in the embryos treated stepwise even with FVS3. To promote dehydration and/or permeation by the cryoprotectants, we exposed embryos to FVS1 and FVS3 for 60 min after stepwise treatment (Table 4). Again, straws containing embryos exposed to FVS1 became opaque partially and entirely during cooling because of extracellular ice. Therefore, we could not observe whether intracellular ice formed in many of the embryos in straws during cooling and warming. However, we could observe the appearance of embryos existing in transparent portions of the straws during cooling (about 20–30% of the embryos) and all of the embryos became opaque during cooling (data not shown). On the other hand, straws containing embryos exposed to FVS3 remained transparent but all the embryos became opaque during cooling (Table 4), indicating that the intracellular ice formed in the embryos during cooling even after prolonged exposure to FVS1 or FVS3 for 60 min.

Thus, we tried to cool embryos with cryoloops to promote vitrification (Table 5). However, we could not obtain viable embryos after vitrification. FVS1 on cryoloops containing embryos just after stepwise treatment became opaque during cooling. It was quite hard to distinguish by the naked eye whether the formation of ice crystals was limited to the embryos or not. To promote dehydration and/or permeation by cryoprotectants, we exposed embryos to FVS1 for 60 min after stepwise treatment and cooled the embryos on cryoloops. In most cases, FVS1 on cryoloops containing the embryos became opaque during cooling but, in one trial, FVS1 remained transparent during cooling (Table 5). These results strongly suggest that dehydration/permeation by cryoprotectants of embryos was insufficient to cause embryos to vitrify during cooling not only just after stepwise treatment with FVS1 but also after prolonged exposure for 60 min. FVS3 on cryoloops containing embryos just after stepwise treatment also became opaque during cooling but, in some of the trials, FVS3 containing embryos remained transparent during cooling (Table 5). However, the transparent FVS3 became opaque during warming. When we exposed embryos to FVS3 for 60 min after stepwise treatment and cooled them on cryoloops, FVS3 on cryoloops containing the embryos remained transparent during cooling in all of the trials, indicating that extended exposure to

Table 4
Occurrence of crystallization in vitrification solutions and the survival of Japanese flounder embryos at the tail bud stage after vitrification in straws

Vitrification solution	Prolonged[a] exposure	No of embryos vitrified	% of embryos with EIF[b] during		% of embryos with IIF[c] during		% of embryos that survived[d]
			cooling	warming	cooling	warming	
FVS1	–	186	~100[e]	100	–	–	0
	+	28	~100[e]	100	–	–	0
FVS3	–	72	0	0	100	–	0
	+	27	0	0	100	–	0

[a] Embryos were cooled with LN_2 just after stepwise treatment (–) or after prolonged exposure (for 60 min) to vitrification solutions (+).
[b] Extracellular ice formation; a partial or entire whitening of the solution was considered to reflect the formation of extracellular ice.
[c] Intracellular ice formation.
[d] Survival was assessed by the appearance of embryos 60 min after recovery.
[e] Extracellular ice formed partially or entirely in all of the straws but the appearance of 20–30% of embryos, which existed in transparent portions of the straws, could be seen.

Table 5
Occurrence of crystallization in vitrification solutions and the survival of Japanese flounder embryos at the tail bud stage after vitrification on cryoloops

Vitrification solutions	Prolonged[a] exposure	No. embryos vitrified	% of embryos with EIF and/or IIF during[b]		% of embryos that survived[c]
			cooling	Warming	
FVS1	–	125	100	100	0
	+	5	80	100	0
FVS3	–	25	80	100	0
	+	10	0	100	0

[a] Embryos were cooled with LN_2 just after stepwise treatment (–) or after prolonged exposure (for 60 min) to vitrification solutions (+).
[b] % of samples formed intra- and/or extracellular ice; whitening of the drop containing an embryo was considered to reflect the formation of ice.
[c] Survival was assessed by the appearance of embryos 60 min after vitrification.

FVS3 promoted vitrification of the embryos during cooling. However, in all of the trials, the FVS3 became opaque during warming, indicating that, even after the prolonged exposure of embryos to FVS3 for 60 min, dehydration and/or permeation by cryoprotectants of embryos were still insufficient for the embryos to remain uncrystallized during warming.

Discussion

In this study, we have examined the toxicity of individual cryoprotectants, the permeability to cryoprotectants, the toxicity of vitrification solutions, and the survival after vitrification of Japanese flounder embryos.

Japanese flounder embryos were relatively resistant to the toxicity of individual cryoprotectants at lower concentrations, especially, of methanol and propylene glycol (Fig. 1). This result was similar to that reported by Chen and Tian [1]. Among embryos at various developmental stages, those at the tail bud stage were the most resistant to the toxicity of all cryoprotectants examined, whereas embryos at the pre-hatching stage were more sensitive than those at other stages (Fig. 1). From the viewpoint of the sensitivity to the toxicity of cryoprotectants, Japanese flounder embryos may be suitable for vitrification, especially at the tail bud stage using methanol- and/or propylene glycol-based vitrification solutions.

In the next series of experiments, we examined the permeability to water of Japanese flounder embryos from their volume changes in a hypertonic sucrose solution and hypotonic diluted solution. The embryos showed minimal volume changes in the hypertonic and hypotonic solutions (Fig. 2). Although this result suggests that flounder embryos have very low permeability to water, the volume changes were too small to calculate the osmotically inactive fraction and water permeability. Flounder embryos also showed minimal volume changes in cryoprotectant solutions (Fig. 3). Again, these results suggest that the embryos have very low permeability to cryoprotectants, and the volume changes were too small to compare the permeability among cryoprotectants. Such small volume changes in hypertonic and hypotonic solutions and cryoprotectant solutions were reported in zebrafish embryos and mature medaka oocytes [4,8]. We have shown in the medaka that the permeability of the plasma membrane of immature oocytes is high but decreases markedly during only 10 h of the final maturation period [8]. We have speculated that such changes are caused by an acquisition of the ability to tolerate the external non-physiological conditions to which fish embryos are exposed [8]. Thus, minimal volume changes of Japanese flounder embryos in hypertonic and hypotonic solutions and cryoprotectant solutions can be explained by an acquisition of resistance to non-physiological conditions. Although the mechanism is not clear, it is possible that the plasma membrane and/or the chorion become less permeable to water and cryoprotectants. In the present study, we measured the volume change of flounder embryos in hypertonic and hypotonic solutions and cryoprotectant solutions with the intact chorion attached because treatment of embryos with 2 mg/ml pronase (Roche, Mannheim, Germany) or the supernatant of the homogenate of hatching flounder embryos (expected to contain hatching enzymes) [6] failed to remove the chorion. In zebrafish embryos, it has been reported that the chorion of the embryos hinders permeation by DMSO [5]. We already suggested in medaka oocytes that not only the permeability to DMSO of

plasma membrane but also that of the chorion decrease during maturation [8]. Therefore, further studies are needed to clarify whether the chorion of Japanese flounder embryos strongly suppresses the movement of water and/or cryoprotectants.

However, methanol appears to permeate embryos because most embryos that had been exposed to the methanol solution floated in 95% seawater (Fig. 4). On the other hand, the permeation of embryos by other cryoprotectants appears to be slight because many embryos that had been exposed to the cryoprotectants neither floated to the top nor sank to the bottom in 95% seawater (Fig. 4). Since, in zebrafish embryos, the multinucleated yolk syncytial layer, a permeability barrier for cryoprotectants except methanol, is formed at the 50–70% epiboly stage [3], it is possible that a similar permeability barrier is formed in Japanese flounder embryos, but that methanol can easily permeate the barrier. From the viewpoint of membrane permeability, methanol appears to be most suitable for vitrifying Japanese flounder embryos successfully among cryoprotectants commonly used.

Finally, we tried to vitrify Japanese flounder embryos by the method reported by Chen and Tian [1]. We used FVS1 (containing low concentrations of methanol and propylene glycol) and FVS3 (containing high concentrations of methanol and propylene glycol) as vitrification solutions. Chen and Tian [1] reported using FVS1 that a high survival rate (32%) was obtained after vitrification of embryos at the tail bud stage after a five-step treatment [1]. They also obtained viable embryos after vitrification using FVS3. However, we could not obtain viable embryos after vitrification of a total of 478 embryos with either FVS1 or FVS3 (Tables 4 and 5).

There are two factors suspected to be the cause of the failure of successful embryo vitrification.

One is the sensitivity of the embryos to the toxicity of vitrification solutions. As shown in Table 2, only a small number of embryos survived treatment with FVS1 or FVS3 in five steps without cooling, although Chen and Tian [1] reported that the stepwise treatment remarkably reduced the toxicity of vitrification solution [1]. The Japanese flounder embryos we used might be more sensitive to the toxicity of vitrification solutions than those they used.

The other factor is the low permeability of embryos to water and cryoprotectants. As shown in Tables 4 and 5, embryos became opaque during cooling with LN_2, when they were cooled just after being treated with FVS3. Prolonged 60-min exposure to FVS3 after stepwise treatment of embryos with FVS3 was effective in keeping embryos transparent during cooling but did not prevent intracellular ice from forming during warming (Table 5). These results suggest that the permeability of embryos to methanol and propylene glycol is not high enough for cryopreservation, and that even a prolonged 60-min exposure is not sufficient for keeping embryos uncrystallized during cooling and warming. Thus, just after treatment of embryos with FVS1 or FVS3 in five steps, the extent of dehydration and/or permeation by cryoprotectants must be far from that required for vitrification during cooling and warming, and additional time for equilibration with vitrification solutions is needed to vitrify the embryos. However, as shown in Table 2, prolonged exposure to FVS 1 or FVS3 for only 8 min after treatment without cooling resulted in no viable embryos. Therefore, we conclude that it is quite difficult to vitrify the Japanese flounder embryos used in the present study by the method reported by Chen and Tian [1], and thus their cryopreservation method lacks general application to Japanese flounder embryos at least to those from around the coast of the Pacific Ocean in Western Japan. The reason for the conflicting results is not known.

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Brief communication

The permeability to water and cryoprotectants of immature and mature oocytes in the zebrafish (*Danio rerio*) ☆

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Abstract

To identify a stage feasible for the cryopreservation of zebrafish oocytes, we investigated the permeability to water and cryoprotectants of immature (stage III) and mature (stage V) oocytes. The permeability to water (μm/min/atm) of immature oocytes at 25 °C (0.37) was significantly higher than that of mature oocytes (0.10). The permeability ($\times 10^{-3}$ cm/min) of immature oocytes to ethylene glycol, propylene glycol, and Me_2SO (1.49–3.03) at 25 °C was substantially higher than that of mature oocytes ~0. The permeability of immature oocytes to glycerol was also high (1.75), although the permeability could not be measured in mature oocytes. Immature oocytes would be more suitable than mature oocytes for conservation of the zebrafish.

Keywords: Zebrafish; Oocyte; Permeability; Water; Cryoprotectant

Among teleosts, the zebrafish (*Danio rerio*) is an important experimental animal in developmental biology, genetics, and physiology. However, it has been maintained by mating because its embryos are unable to be cryopreserved. Because fish embryos have a large size (resulting in a low surface area/volume ratio), a large amount of egg-yolk, and a thick chorion, and form a complex structure during development, it is difficult to cryopreserve them. However, zebrafish oocytes constitute a single compartment and do not have barriers to permeability like the multinucleated yolk syncytial layer found in zebrafish embryos [4]. These features would be advantageous for cryopreservation. Since zebrafish sperm can be cryopreserved easily [1], the long-term preservation of various stocks of zebrafish could be realized if their oocytes could be cryopreserved. To cryopreserve zebrafish oocytes successfully, it is important to know the stage of maturation most feasible for cryopreservation. In the medaka, another small freshwater fish, we have found that the permeability of immature oocytes to water and cryoprotectants is remarkably higher than that of mature oocytes [8]. Considering their huge embryos/oocytes, rapid permeation of cryoprotectants is favored for successful cryopreservation. Very recently, Zhang et al. reported that the permeability of immature zebrafish oocytes to propylene glycol and Me_2SO was high [11]. A similar change might be observed in zebrafish oocytes.

In this study, we tried to determine the permeability to water and four major cryoprotectants (ethylene glycol, glycerol, propylene glycol, and Me_2SO) of immature and mature zebrafish oocytes to find the most feasible stage of maturation and suitable cryoprotectants for cryopreservation.

About 20–40 mature female zebrafish, purchased from a local fish dealer, were maintained in 60-l aquaria under 14-h light and 10-h dark periods at 28 °C. To obtain immature oocytes at the stage III of maturation, mature female zebrafish were killed by decapitation under anesthesia with 0.2 mg/ml of tricaine in distilled water (tricaine solution).

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The ovaries were recovered and placed in a 75% Leibovitz L-15 medium (pH 7.5) containing 100 μg/ml of gentamycin sulfate at 25 °C, and follicles were separated manually with forceps and scissors. The size of the follicles was measured under a microscope and follicles 0.60–0.69 mm in diameter were collected and used as immature oocytes. Mature oocytes at the stage V were obtained by squeezing the belly of mature females under anesthesia with the tricaine solution at about 30 min before the end of the dark period. The mature oocytes were placed in 4 ml of the 75% Leibovitz L-15 medium at 25 °C until used for experiments.

In this study, we assumed that the osmolality of the oocyte cytoplasm was equilibrated with the 75% Leibovitz L-15 medium (0.24 Osm/kg), because, until used for experiments, immature and mature oocytes were incubated in this medium for at least 60 min; it is sufficient for the oocytes to be equilibrated with the 75% Leibovitz L-15 medium. In fact, there is no volume change in immature and mature oocytes after equilibration for 60 min (data not shown). Then, the oocytes were transferred to the same medium containing 0.15, 0.48, or 0.74 M sucrose (total osmolality being 0.40, 0.80, and 1.20 Osm/kg, respectively), or in a 50% or 60% Leibovitz L-15 medium (0.16 and 0.19 Osm/kg, respectively) diluted with distilled water, with a minimal amount of the 75% Leibovitz L-15 medium using a pipette, and kept at 25 ± 0.5 °C for 60 min. The osmolality contributed by sucrose was calculated from published data on the colligative properties of sucrose in aqueous solutions [9]. The osmolality of the 50, 60, and 75% Leibovitz L-15 media was measured with a freezing point depression osmometer (OM801; Vogel, Giessen, Germany). Microscopic images of the oocytes were recorded using a time-lapse video recorder (ETV-820, Sony, Tokyo, Japan) for 60 min during exposure. The cross-sectional area of the oocytes was measured using an image analyzer (VM-50, Olympus, Tokyo, Japan). Relative cross-sectional area, S, was expressed by dividing the cross-sectional area by the initial area of the same oocyte. The relative volume (V) was obtained from $V = S^{3/2}$. Osmotically inactive fractions (V_b) were obtained from Boyle–van't Hoff plots of the relative volumes of oocytes after exposure to solutions with various osmolalities for 60 min.

To determine the permeability to water and cryoprotectants from the volume changes, an oocyte equilibrated with the 75% Leibovitz L-15 medium at 25 °C for at least 60 min was introduced into the same medium containing sucrose or one of the cryoprotectants (200 μl) covered with paraffin oil in a Petri dish (35 × 10 mm), and kept at 25 ± 0.5 °C for 60 min. Microscopic images of the oocyte were recorded using the time-lapse video recorder for 60 min. The cross-sectional area of the oocyte was measured and the relative volume at various time points of exposure was calculated as described above. Water-permeability (Lp) and cryoprotectant-permeability (Ps) were determined by fitting water and solute movements using a two-parameter formalism [7,2,8]. The Lp of immature and mature oocytes was determined from volume changes in the 75% Leibovitz L-15 medium containing 0.15 M sucrose (total osmolality being 0.40 Osm/kg). The Ps of oocytes for each cryoprotectant was calculated from their volume changes in a 75% Leibovitz L-15 media containing 8% (v/v) ethylene glycol, 10% (v/v) glycerol, 10% (v/v) propylene glycol, or 9.5% (v/v) Me_2SO at 25 ± 0.5 °C for 60 min. The total osmolality of the 75% Leibovitz L-15 medium containing 8% ethylene glycol, 10% glycerol, 10% propylene glycol, and 9.5% Me_2SO was 1.85, 1.83, 1.80, and 1.79 Osm/kg, respectively. The osmolality of the 75% Leibovitz L-15 medium was 0.24 Osm/kg. Different concentrations of cryoprotectants were used to give cryoprotectant solutions a similar osmolality (about 1.8 Osm/kg). The osmolality of ethylene glycol, glycerol, and propylene glycol was calculated from published data on the colligative properties of each cryoprotectant in aqueous solutions [9]. The osmolality of 9.5% Me_2SO in an aqueous solution was measured with a vapor pressure osmometer (Vapro 5520, Wescor, Logan, UT, USA). Other constants were as follows; gas constant (R), 8.206 10^{-2} liter atm/K/mol; absolute temperature (T), 298 K; partial molar volume of water (V_W), ethylene glycol (V_{EG}), glycerol (V_{GLY}), propylene glycol (V_{PG}), and Me_2SO (V_{Me2SO}), 0.018, 0.054, 0.071, 0.070, and 0.069 L/mol, respectively [9,6].

To determine the permeability to water and cryoprotectants, the V_b values of immature oocytes and mature oocytes were calculated. Fig. 1 shows the Boyle–van't Hoff plots of immature and mature oocytes. Immature oocytes shrunk or swelled according to the osmolality of solutions (0.16 to 0.40 Osm/kg), but the plasma membrane of immature oocytes was ruptured during the exposure in solutions with an osmolality of 0.80 Osm/kg or more. A linear Boyle–van't Hoff relationship was observed in immature oocytes ($r^2 = 0.99$). Mature oocytes also shrunk in hypertonic

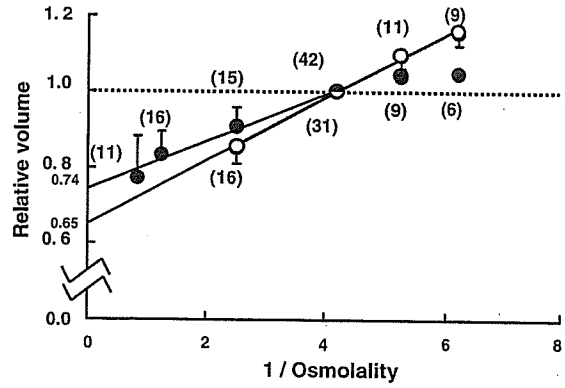

Fig. 1. Boyle–van't Hoff plots of relative volumes of immature (open) and mature (closed) zebrafish oocytes. Both immature and mature oocytes were equilibrated with a 75% Leibovitz L-15 medium. Then, immature oocytes were equilibrated with a 75% Leibovitz L-15 medium containing 0.15 M sucrose (0.40 Osm/kg) or with a 50% or 60% Leibovitz L-15 medium (0.16 and 0.19 Osm/kg, respectively) diluted with distilled water at 25 °C for 60 min. Mature oocytes were equilibrated with a 75% Leibovitz L-15 medium containing 0.48 M or 0.74 M sucrose (0.80 and 1.20 Osm/kg, respectively) or with a 50% or 60% Leibovitz L-15 medium diluted with distilled water at 25 °C for 60 min. Data are indicated as means of relative volumes ± SD. Numbers in parentheses indicate the number of oocytes used.

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sucrose solutions in response to the osmolality of the solutions, but mature oocytes did not swell in hypotonic solutions. Similar phenomenon in hypotonic solutions have been reported in zebrafish embryos [5]. Since freshwater fish spawn in freshwater where the oocytes are then fertilized and develop, mature oocytes and embryos must acquire the resistance to hypotonic conditions [8]. Fortunately, mature oocytes shrunk in hypertonic solutions in response to the osmolality of solutions. Moreover, the membrane-permeability in hypertonic solutions is important, because for cryopreservation, the cells are exposed to a cryoprotectant solution having very high osmolality. Thus, we determined the V_b of mature oocytes from relative volume in hypertonic solutions (0.24 to 1.20 Osm/kg, $r^2 = 0.97$). The V_b value was 0.65 for immature oocytes and 0.74 for mature oocytes. Zhang and colleagues estimated the V_b of immature and mature oocytes to be 0.70 and 0.64, respectively, from their relative volumes in 'hypertonic conditions' [10,11]. These values are similar to the ones obtained in the present study. We used our values to determine the permeability to water and cryoprotectants of oocytes.

Immature oocytes shrunk rapidly in the 0.15 M sucrose solution (0.40 Osm/kg), but mature oocytes shrunk slowly (Fig. 2A). The Lp (μm/min/atm) value of immature oocytes (0.37) calculated from the data was markedly higher than that of mature oocytes (0.10) (Table 1). This shows that water permeates immature oocytes much faster than mature oocytes. It has been shown in zebrafish embryos that the Lp is low; 0.02–0.05 μm/min/atm, assuming that the initial internal osmolality is 0.04 Osm/kg, and 0.04–0.10 μm/min/atm, assuming that the initial internal osmolality is 0.30 Osm/kg at the six-somite stage [3]. Therefore, their higher permeability to water makes immature oocytes more attractive than mature oocytes and embryos for cryopreservation.

Figs. 2B–E shows the changes in volume of immature oocytes and mature oocytes at 25 °C in the 75% Leibovitz L-15 medium containing ethylene glycol, glycerol, propylene glycol, or Me_2SO. In all of these solutions, immature oocytes shrunk and regained their volume within 60 min, suggesting that the cryoprotectants permeated immature oocytes efficiently. On the other hand, mature oocytes shrunk very slowly and did not regain their volume during 60 min of exposure in the solution with ethylene glycol, propylene glycol, or Me_2SO, suggesting that these cryoprotectants permeate mature oocytes quite slowly. In the glycerol solution (Fig. 2C), mature oocytes shrunk and ruptured within 15 min of exposure.

The L_p (μm/min/atm) of immature oocytes in various cryoprotectants (0.17–0.28) was markedly higher than that of mature oocytes (0.03–0.05), and no marked difference in the Lp values of immature oocytes was observed among cryoprotectant solutions (Table 1). The values were slightly lower than the value in hypertonic sucrose solutions (0.37). Ps values ($\times 10^{-3}$ cm/min) of immature oocytes for ethylene glycol, glycerol, propylene glycol, and Me_2SO were 2.80, 1.75, 1.49, and 3.03, respectively (Table 1). As far as we

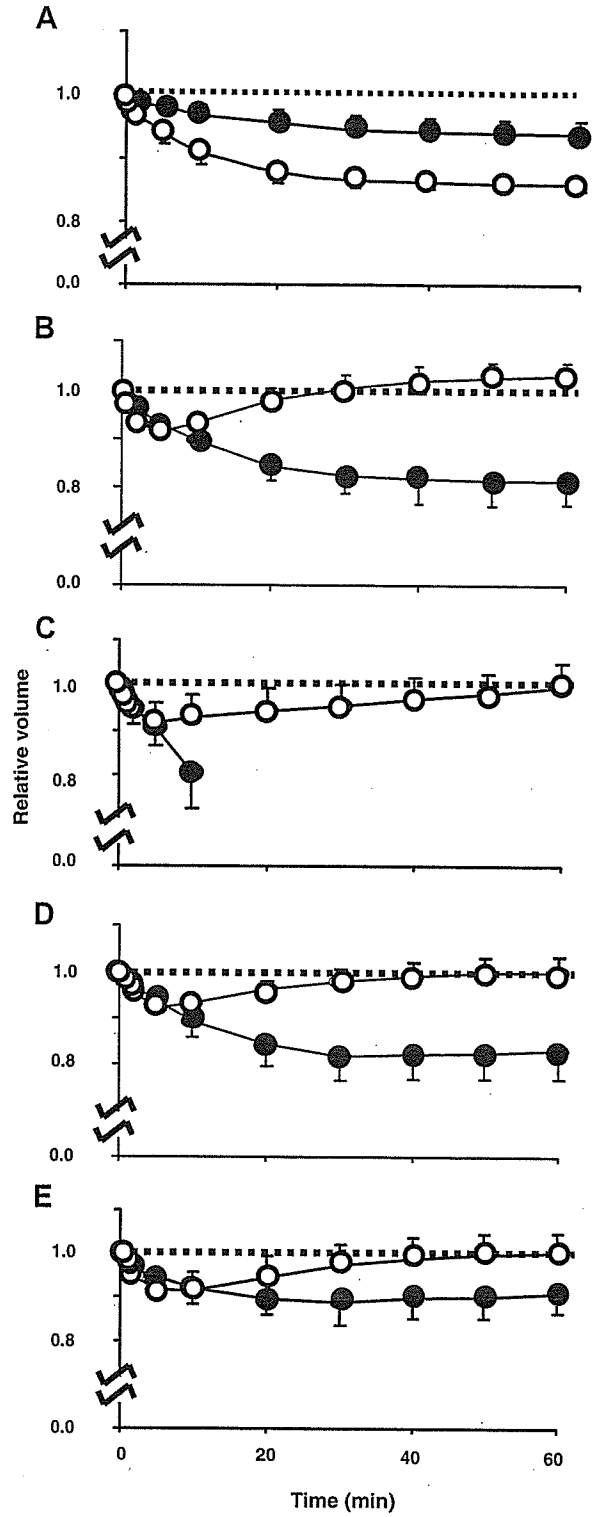

Fig. 2. Changes in cell volume of immature (open) and mature (closed) zebrafish oocytes in a 75% Leibovitz L-15 medium containing 0.15 M sucrose (A), 8% (v/v) ethylene glycol (B), 10% (v/v) glycerol (C), 10% (v/v) propylene glycol (D), or 9.5% (v/v) Me_2SO (E) at 25 °C for 60 min. Data are indicated as means of relative volumes ± SD. Data for immature oocytes were from 8 to 10 oocytes, and those for mature oocytes were from 4 to 12 oocytes.

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Table 1
The permeability of zebrafish oocytes to water (Lp, μm/min/atm) and cryoprotectants (Ps, $\times 10^{-3}$ cm/min)

Oocytes	Permeability	Permeabilities of oocytes in solutions with				
		0.15 M Suc[a]	8%EG[b]	10%Gly[c]	10%PG[d]	9.5%Me_2SO
Immature (stage III)	Lp	0.37 ± 0.08	0.27 ± 0.10	0.17 ± 0.07	0.17 ± 0.06	0.28 ± 0.08
	Ps	–	2.80 ± 1.38	1.75 ± 1.67	1.49 ± 0.67	3.03 ± 1.92
Mature (stage V)	Lp	0.10 ± 0.09	0.03 ± 0.01[e]	nd[f]	0.03 ± 0.01[e]	0.05 ± 0.01[e]
	Ps	—	~0	nd[f]	~0	~0

[a] Sucrose.
[b] Ethylene glycol.
[c] Glycerol.
[d] Propylene glycol.
[e] Lp values were estimated, assuming that Ps values were 0.
[f] Not determined.

know, this is the first report to determine the permeability of zebrafish oocytes to ethylene glycol and glycerol. On the other hand, Ps values of mature oocytes were too low to be determined from the volume changes (Fig. 2, Table 1). Similar changes in permeability to cryoprotectants were observed in medaka oocytes [8]. Therefore, during maturation, oocytes of freshwater fish must become less permeable to water and to small neutral solutes, probably by acquiring resistance to hypotonic conditions before being spawned in freshwater. Hagedorn et al. reported that the permeability of zebrafish embryos to propylene glycol and to Me_2SO was quite low [4]. Thus, not only mature oocytes but also embryos are less permeable to cryoprotectants than immature oocytes. The higher cryoprotectant-permeability of immature oocytes would also be an advantage over mature oocytes and embryos.

In this study, we showed that ethylene glycol and Me_2SO permeate immature oocytes more efficiently than other cryoprotectants. Therefore, in terms of permeability, ethylene glycol and Me_2SO would be more suitable for cryopreservation than glycerol and propylene glycol. Very recently, Zhang et al. reported the permeability of immature zebrafish oocytes to water (μm/min/atm) and to cryoprotectants ($\times 10^{-3}$ cm/min) in solutions with propylene glycol (Lp, 0.20; P_{PG}, 0.93) and Me_2SO (Lp, 0.17; P_{Me2SO}, 0.95) at 22 °C [11]. The Lp values in the propylene glycol solution and Me_2SO solution in the present study are similar to those in their report, but their values were lower (P_{PG}, 1.49; P_{Me2SO}, 3.03) (Table 1). This might be caused by the difference in temperature; Ps was measured at 25 °C in the present study but versus 22 °C in Zhang et al.'s study.

In this study, we determined the permeability of mature oocytes, although Zhang et al. reported that they could not determine the permeability, because the change in volume was only slight and untheoretical in solutions with 2 M propylene glycol or 2 M Me_2SO [11]. When we examined the volume changes of mature oocytes under the same conditions as in their study (not described), we often observed a similar phenomenon, but this phenomenon occurred only in about 10–20% of mature oocytes both in the additional study and in the present study. Most mature oocytes usually shrunk and swelled in cryoprotectant solutions, and the volume changes fitted well with the simulated curves for the oocytes. The difference in the strain of zebrafish might be involved in this discrepancy.

In conclusion, as observed in medaka oocytes, immature oocytes would be more suitable for cryopreservation than mature oocytes and embryos in the zebrafish. In addition, ethylene glycol and Me_2SO would be suitable cryoprotectants for cryopreservation, from the viewpoint of permeability.

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Original Article

The improvement in fertilizing ability of cryopreserved mouse spermatozoa using laser-microdissected oocytes

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Aim: The C57BL/6 mouse strain is now commonly used for producing transgenic/knockout strains. However, the fertilizing ability of these spermatozoa decreases as a result of cryopreservaion. Although the micromanipulation technique has been established to increase their fertilizing ability, it requires a considerable degree of technical skill. In the present report, we investigate the simple microdissection of zona pellucida by laser to increase the fertilizing ability of cryopreserved spermatozoa.

Methods: C57BL/6J spermatozoa were cryopreserved using a solution consisting of 18% raffinose/3% skim milk. Oocytes of the same strain were placed in PB1 medium containing 0, 0.25, 0.50 or 0.75 mol sucrose. The zona pellucida of oocytes was microdissected by laser with different pulse lengths selected from 0.45 to 0.65 ms. Microdissected oocytes were then fertilized with cryopreserved spermatozoa, and the subsequent development of embryos was assessed.

Results: When oocytes were microdissected in PB1 medium without sucrose, 81.5% of the oocytes were fertilized. The fertilization rates increased significantly as the pulse length was lengthened when compared with oocytes with intact zona pellucida. Furthermore, normal offspring were obtained in all experiments.

Conclusion: The fertilizing ability of cryopreserved spermatozoa is improved when oocytes with their zona pellucida microdissected by laser were used. (Reprod Med Biol 2006; 5: 249–253)

Key words: C57BL/6 mouse, cryopreservation, *in vitro* fertilization, laser-microdissection, spermatozoa.

INTRODUCTION

SUCCESSFUL REPORTS OF mouse sperm cryopreservation have been published since the 1990s.[1–6] Subsequently, an improved cryopreservation method using a solution consisting of 18% (w/v) raffinose and 3% (w/v) skim milk was described by Nakagata and Takeshima.[7] This cryopreservation method was also fully applied to various strains, including wild mice and transgenic mice.[8–10] In recent years, large mouse sperm/embryo cryobanks have been established in several countries around the world. Sperm cryopreservation techniques have been contributing to the efficient maintenance of strains, and to transportation that is safer and cheaper than that with live mice. Furthermore, sperm preservation solved the serious problem of a lack in breeding space by the mass production of new mouse strains, such as transgenic, knockout and mutant.[11]

The C57BL/6 mouse strain is now commonly used for producing these transgenic and knockout strains. Unfortunately, several reports have been published that C57BL/6 mouse spermatozoa that had been cryopreserved using a raffinose/skim milk solution showed a decrease in fertilizing ability.[2,8,12–14] In order to increase fertilizing ability, micromanipulation techniques such as partial zona pellucida dissection (PZD),[15] partial zona pellucida incision by using a piezo-micromanipulator (ZIP)[16,17] and intracytoplasmic sperm injection (ICSI)[18,19] have been established. However, these micromanipulation techniques require a considerable degree of technical skill.

It is known that partial zona pellucida dissection by laser beam has been used for assisted hatching to increase the implantation rate of human embryos.[20,21]

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In the present report, the simple microdissection of zona pellucida by laser to increase the fertilizing ability of cryopreserved mouse spermatozoa was investigated.

MATERIALS AND METHODS

Animals

ALL ANIMALS WERE purchased from CLEA Japan (Tokyo, Japan). C57BL/6 J males and females for sperm and oocyte donors were aged over 12 weeks and 9–10 weeks, respectively. ICR females, recipients for the transfer of 2-cell embryos, were aged 12–15 weeks. All animals were maintained in an air-conditioned (temperature: 22 ± 2°C, humidity: 60 ± 10%) and light-controlled room (light on 07.00–19.00 hours). The Animal Care and Use Committee of the Kumamoto University School of Medicine approved all procedures carried out in the present study.

Sperm cryopreservation

Spermatozoa were cryopreserved using the method described previously.[22] Briefly, two cauda epididymides were removed from males, and were then transferred to 100 μL of the cryopreservation solution consisting of 18% (w/v) raffinose and 3% (w/v) skim milk in a 4-well multi dish (Nunc A/S). The cauda epididymides were minced well by micro spring scissors, and the spermatozoa were then dispersed by gently shaking the dish. Sperm suspension was loaded into 10 μL aliquots in 0.25 mL sampling straws (IMV Technologies, L'Aigle Cedex, France). Ten straws were heat-sealed at both ends and cooled into liquid nitrogen vapor for 15 min. After this, these straws were plunged directly into the liquid nitrogen.

Oocytes collection

Oocytes were collected from females induced to superovulate by an intraperitoneal injection of 7.5 IU PMSG (Teikokuzoki Co., Tokyo, Japan), followed by an injection of 7.5 IU hCG (Teikokuzoki Co.) 48 h later. Cumulus-oocyte complexes were collected from the oviducts at 15–16 h after hCG injection. Oocytes were freed from cumulus cells by treatment with HTF medium[23] containing 0.1% hyaluronidase (H3506, Sigma-Aldrich, St Louis, MO, USA). Cumulus-freed oocytes were rinsed and kept in fresh HTF medium at 37°C in 5% CO_2 and 95% air until laser-microdissection.

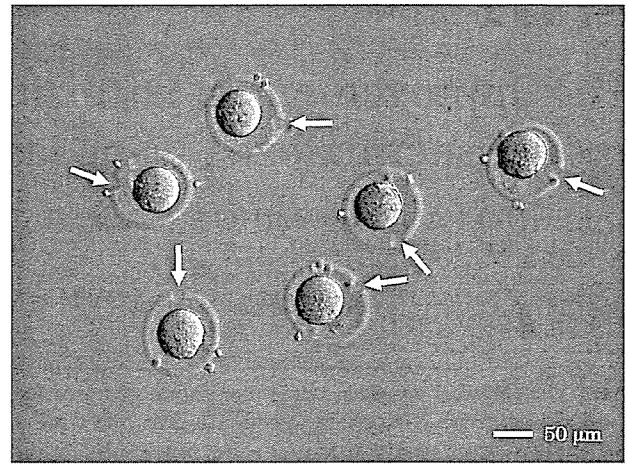

Figure 1 Oocytes with their zona pellucida microdissected by laser. The arrow shows a hole microdissected by laser.

Laser-microdissection of zona pellucida

The Saturn 3 laser system (Research Instruments Ltd, Cornwall, UK) was used for the microdissection of zona pellucida. The laser system was coupled with an inverted microscope (IX71, Olympus Co., Tokyo, Japan). An image of the oocytes was projected onto a PC/video monitor using a video camera. Each of the 50 oocytes were placed on the bottom of a 100 μL drop of PB1 medium[24] containing 0, 0.25, 0.50 or 0.75 mol sucrose covered with paraffin oil in a culture dish. The zona pellucida were focused sharply and dissected individually by laser beam (output wavelength: 1480 nm, power output from patch lead: 350 mW) with different pulse lengths selected from 0.45 to 0.65 ms (Fig. 1). After this, oocytes were transferred to 90 μL of fresh HTF medium.

In vitro fertilization

A straw with spermatozoa was left at 37°C in a water bath for 15 min. The sperm suspension was pushed out in the 90 μL of HTF medium, after which it was kept for 30 min at 37°C in 5% CO_2 and 95% air. After this, the sperm suspension was introduced into 10 μL aliquots to 90 μL of HTF medium containing microdissected oocytes. Sperm and oocytes were then cultured at 37°C in 5% CO_2 and 95% air.

Embryo transfer

Oocytes were observed two pronuclei 5 h after insemination and embryos that developed to the 2-cell stage

were selected 24 h after insemination. Fertilization rates were calculated by the number of 2-cell embryos from oocytes microdissected. These 2-cell embryos were transferred into the oviducts of pseudopregnant females that had been mated with vasectomized males of the same strain on the day before embryo transfer. The number of offspring was counted on 19.5 days of gestation by cesarean section. The rates of offspring were calculated by number of offspring from oocytes transferred or microdissected.

Analysis of data

Each experiment was repeated over three times. The χ^2-test using Yates correction for continuity was used for the analysis of all data obtained from the present study.

RESULTS

IN EACH EXPERIMENT, over 200 oocytes were used for laser-microdissection and the development of over one hundred embryos was assessed after transfer into pseudopregnant females. No oocytes damaged by laser beam and with polyspermy were observed in the present study. As shown in Figure 2, a high fertilization rate was obtained when oocytes microdissected in PB1 medium without sucrose were used. In contrast, no significant difference was obtained in the proportion of development to offspring of oocytes microdissected in PB1 medium containing a different concentration of sucrose used in the present study. All offspring obtained in the present experiment were apparently normal.

When oocytes were microdissected by laser with different pulse lengths selected from 0.45 to 0.65 ms in PB1 medium without sucrose, fertilization rates increased significantly as the pulse length was lengthened (Fig. 3) ($P < 0.05$). Although, the rate of offspring to the number of oocytes microdissected also increased significantly as the pulse length was lengthened, it decreased significantly when oocytes were microdissected by laser with pulse length of 0.65 ms ($P < 0.05$). All offspring obtained in the present experiment were also apparently normal.

DISCUSSION

IN THE PRESENT study, it was shown that the fertilizing ability of cryopreserved mouse spermatozoa was improved when oocytes with their zona pellucida microdissected by laser were used. Furthermore,

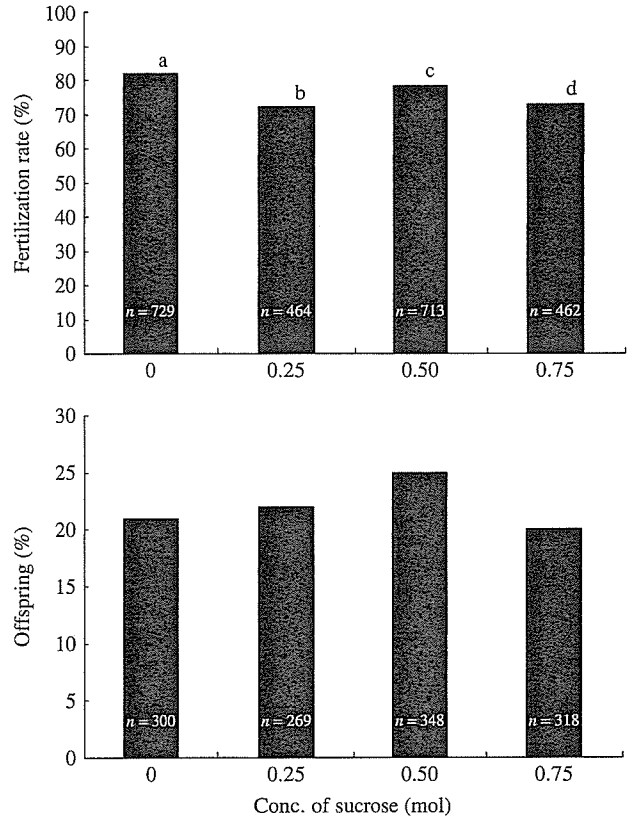

Figure 2 The fertilization rate and the development to offspring of oocytes microdissected in PB1 medium containing 0, 0.25, 0.50 and 0.75 mol sucrose. Fertilization rate = number of 2-cell embryos/number of oocytes microdissected. Percentages of offspring = number of offspring/number of 2-cell embryos transferred. Significant difference, a *vs* b, d; b *vs* c, $P < 0.05$.

laser-microdissected oocytes fertilized with spermatozoa finally developed into normal offspring.

Mouse spermatozoa, especially C57BL/6, are known to be sensitive to freezing, resulting in reduced motility and fertility.[2,8,12–14] Although, the reason for this phenomenon has been unknown, fertility could be increased by microdissection of the zona pellucida using a steel needle[15] and piezo-micromanipulater[16,17] or injection of spermatozoa intracytoplasmically.[18,19] Presently, technical modification of the freezing protocol[25] and freeze-drying preservation that takes the place of cryopreservation[26–29] is being actively studied for further efficient sperm preservation. The laser-microdissection showed in the present study also demonstrated an improvement in the fertilizing ability of cryopreserved mouse spermatozoa, as well as various micromanipulation techniques described previously (Figs 2 and 3).

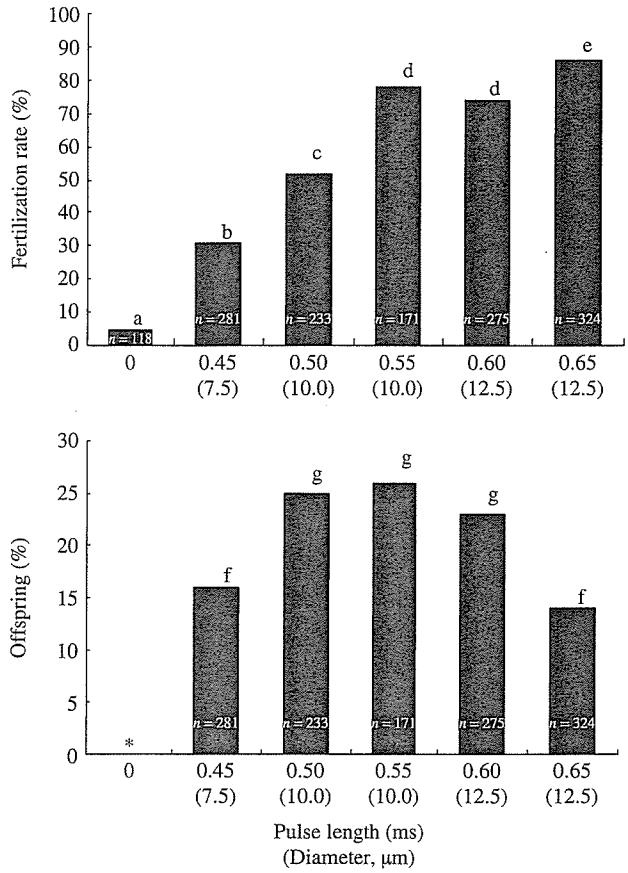

Figure 3 The fertilization rate and the development to offspring of oocytes microdissected by laser with a different pulse length selected from 0.45 to 0.65 ms in PB1 medium without sucrose. Fertilization rate = number of 2-cell embryos/number of oocytes microdissected. Percentages of offspring = number of offspring/number of oocytes microdissected. Percentages with different lowercase letters are significantly different ($P < 0.05$). *Means that embryos were not transferred.

In the present study, the perivitelline space of oocytes was widened using a hypertonic solution containing a high concentration of sucrose to protect the oolemma from damage by the laser. However, a high proportion of oocytes were fertilized with cryopreserved spermatozoa and these embryos developed into normal offspring, even when oocytes were microdissected without the widening of perivitelline space in PB1 medium without sucrose (Fig. 2). This result suggested that microdissection by laser caused no damage to the oolemma near the hole and the subsequent development of embryos.

When oocytes were microdissected by laser with different pulse lengths selected from 0.45 to 0.65 ms in PB1 medium without sucrose, as shown in Figure 3, fertilization rates increased significantly as the pulse length was lengthened. Although the rate of offspring to the number of oocytes microdissected also increased as the pulse length was lengthened, it decreased significantly when oocytes were microdissected by laser with a pulse length of 0.65 ms. It is possible that blastomeres might escape from the hole of zona pellucida microdissected by laser in the oviduct of pseudopregnant females.[16] The results in the present study showed that the hole size of the zona pellucida microdissected by laser is important for the development and implantation of embryos after transfer into pseudopregnant females. Although a high fertilization rate was obtained by enlarging the hole size of the zona pellucida, optimal settings need to be defined. In the present study, we established a pulse length selected from 0.55 to 0.60 ms as the optimum setting of laser-microdissection for the efficient production of embryos.

Intact zona pellucida works to prevent the transmission of pathogens to the oocytes.[30] In order to examine the risk of pathogen infection to embryos, we fertilized laser-microdissected oocytes with cryopreserved spermatozoa derived from mice infected with the mouse hepatitis virus (MHV), and the offspring obtained from these embryos were tested for MHV infection. All offspring had negative test results (data not shown). It is thought that the risk of pathogen transmission by the microdissection of zona pellucida is considerably low,[31] but further study is necessary for other pathogens.

The C57BL/6 mouse strain is now commonly used for producing transgenic and knockout strains. However, the fertilizing ability of these spermatozoa is still low after cryopreservation. The microdissection of zona pellucida by laser is a simple technique in which special skills are unnecessary. This technique will become a powerful tool for the mass production of embryos or offspring from cryopreserved mouse spermatozoa that show low fertilizing ability.

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—Full Paper—

Application of Laser-Assisted Zona Drilling to *In Vitro* Fertilization of Cryopreserved Mouse Oocytes with Spermatozoa from a Subfertile Transgenic Mouse

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Abstract. Development of assisted reproductive technologies is necessary to obtain fertilized oocytes in a subfertile transgenic mouse strain. Here, we showed the application of laser-assisted drilling of the zona pellucida to *in vitro* fertilization of cryopreserved mouse oocytes with sperm from subfertile transgenic mice (C57BL/6N-Tg(UCP/FAD2)U8 strain). After cryopreservation by vitrification, the recovery and survival rates of the zona-drilled mouse oocytes were 97% (97/100) and 94% (91/97), respectively. *In vitro* fertilization of the cryopreserved zona-drilled mouse oocytes with sperm from the subfertile transgenic mice was greatly facilitated (60%, 55/91) compared to that of the cryopreserved zona-intact mouse oocytes (11%, 81/768). *In vitro* fertilized embryos that developed to the 2-cell stage were again cryopreserved by vitrification, and after warming they were transferred into recipient females. Subsequently, six viable offspring were delivered, and all were confirmed to be transgenic mice. These results indicate that laser-assisted zona drilling of oocytes combined with cryopreservation by vitrification may be a useful approach for large-scale production of *in vitro* fertilized embryos for managing transgenic mouse strains with reproductive disabilities such as subfertile sperm.

Key words: *In vitro* fertilization, Subfertile, Transgenic mouse, Vitrification

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Cryopreservation of embryos or gametes has been successfully used to maintain genetically manipulated mouse strains. However, some disease models show specific characteristics of weak reproductive ability and/or sterility, which often make it difficult to maintain mouse strains owing to the inability to produce further generations [1, 2]. Especially, the C57BL/6 strain has generally been used for producing transgenic mice, but the sperm of this strain genetically shows low motility and low fertility after cryopreservation or upon aging of the males. As a result, it is not only difficult to cryopreserve sperm from mice with this genetic background, but it is also difficult to produce early stage embryos on a large scale, even

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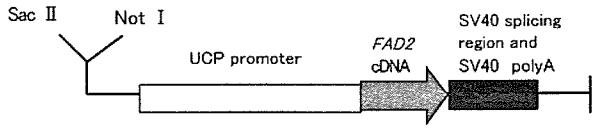

Fig. 1. Construction of the *UCP/FAD2* transgene. The 9.8 kb fragment contained a 7.5 kb mouse *UCP* promoter fused to a 1.6 kb cDNA for *Spinacia FAD2*, a fragment of the SV40 splicing region, and an SV40 polyadenylation site. *UCP*: uncoupling protein. *FAD2*: fatty acid desaturase 2.

by *in vitro* fertilization [3]. For subfertile sperm, assisted reproductive technologies such as intracytoplasmic sperm injection or partial zona dissection have been applied for the production of fertilized embryos. However, it is very difficult to treat numerous oocytes manually using such methods [4–6]. Therefore, development of a simple and efficient procedure for large-scale preparation of fertilized embryos using subfertile sperm is important for managing strains with reproductive disabilities. Recently, a laser-assisted zona drilling method, which is an approach to facilitate *in vitro* fertilization, has been developed [7].

In this study, in order to establish a basic system for large-scale preparation of fertilized oocytes in the subfertile transgenic mouse, we used vitrified zona-drilled mouse oocytes that were produced using the laser-assisted zona drilling method and cryopreserved by vitrification. After warming, the vitrified zona-drilled oocytes were fertilized *in vitro* with sperm from subfertile transgenic mice. The ability of the resultant embryos to develop to full term was then investigated.

Materials and Methods

Animals

Transgenic mice with the Δ12 fatty acid desaturase gene (*FAD2*, GenBank accession no. AB094415) regulated by the UCP promoter (C57BL/6N-Tg (UCP/FAD2) mice, abbreviated as U/FAD2-Tg mice) were produced by a previously described procedure [8]. In brief, *FAD2* cDNA was cloned from spinach [9], and the transgene constructed as shown in Fig. 1 was injected into the male pronuclei of zygotes collected from B6D2F1 mice (Slc: B6D2F1; Japan SLC, Inc., Shizuoka, Japan). Twenty-four h after DNA injection, morphologically normal zygotes that had developed to the 2-cell stage were transferred into the oviducts of pseudopregnant female mice. A vaginal plug was recognized on this day in the mice used for this procedure (Day 1) (Scl: ICR; Japan SLC, Inc.). Each subline of the heterozygous transgenic mice was crossed with C57BL/6 mice (Jcl: C57BL/6N; Japan CLEA Inc., Tokyo, Japan) for either 8 or 15 generations before use in these studies, and then homozygous sublines of U/FAD2-Tg mice were established. U/FAD2-TgU8 mice, one of the sublines of the U/FAD2-Tg mice, were used in the experiments described below. All animal procedures conformed to the Guidelines of Kinki University for the Care and Use of Laboratory Animals.

Production of zona-drilled mouse oocytes

Mature C57BL/6 female mice were superovulated by intraperitoneal injection of 7.5 IU pregnant mare's serum gonadotropin (PMSG, Serotropin; TEIZO Pharmaceutical, Tokyo, Japan) and 7.5 IU human chorionic gonadotropin (hCG, Gonatropin; TEIZO Pharmaceutical) 48 h later. Mature MII oocytes surrounded by cumulus cells were collected from the ampulla of the oviducts 15 h after hCG injection and placed in HTF medium (ARK Resource Co. Ltd., Kumamoto, Japan) [10]. The cumulus cells were removed by treatment with 0.1% hyaluronidase in HTF medium for 3 min at 37 C in 5% CO_2 in air, followed by washing in HTF medium 3 times. Laser-assisted drilling of the zona pellucida of oocytes was performed as described elsewhere [7]. In brief, denuded oocytes were used in zona drilling with laser equipment as described below (Saturn 3 laser system; Tokyo Instruments, Inc., Tokyo, Japan). Oocytes were put into 0.5 M sucrose solution to shrink the cytoplasm to avoid laser-induced cytoplasmic damage. The laser beam was applied to the point on the zona pellucida that showed the widest perivitelline space, and a hole (ϕ=12 μm) was drilled in each zona pellucida (wave length: 1.480 nm, output: 165–200 mW, pulse width: 0.1–7.0 msec), as demonstrated in Fig. 2. Using this method, 500 oocytes could be treated in 1 h without any damage. After zona drilling, oocytes were washed with HTF medium 3 times and cryopreserved by vitrification.

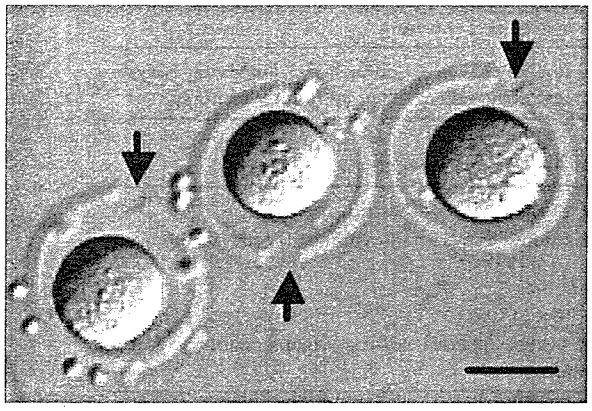

Fig. 2. Zona-drilled oocyte with a hole of equal size in the zona pellucida (in this case, ϕ=12 μm) produced by a laser beam. The scale bar presents 50 μm.

Cryopreservation of zona-drilled mouse oocytes by vitrification

Vitrification of the zona-drilled oocytes was performed in accordance with the procedure described by Nakao *et al.* [11]. After washing 3 times in HTF medium, the zona-drilled oocytes were moved into 1 M dimethyl sulfoxide (DMSO) in PB1 medium. Fifty oocytes in 5 μl of 1 M DMSO in PB1 medium were put into each freezing tube, placed in a precooled (0 C) metal block cooler (CHILL HEAT, CHT-101; IWAKI, Tokyo, Japan), and left to stand for 5 min. Then, 45 μl of precooled DAP213 preservation solution (2 M DMSO, 1 M acetamide, and 3 M propylene glycol in PB1 medium) was added to each freezing tube, and the mixture was left to stand for a further 5 min, after which the cooled freezing tube was plunged into liquid nitrogen (LN_2). For warming of vitrified zona-drilled oocytes, the freezing tubes were taken out of the LN_2 container and 900 μl of prewarmed (37 C) 0.25 M sucrose in PB1 medium was added to each tube to dilute the preservation solution. After pipetting several times, the oocytes were recovered and washed 3 times in TYH medium. The morphology of the oocytes was evaluated under a stereomicroscope. The Surviving and morphologically normal oocytes were cultured in 200 μl drops of TYH medium under mineral oil at 37 C in 5% CO_2 in air.

In vitro fertilization

In vitro fertilization of the vitrified zona-drilled oocytes with sperm from U/FAD2-TgU8 mice that had previously been shown to have low fertility both in the case of natural mating and *in vitro* fertilization was performed as described by Toyoda *et al.* [12]. In brief, sperm was collected from the cauda epididymis of the U/FAD2-TgU8 mice and resuspended in 200 μl drops of TYH medium (Mitsubishi Kagaku Iatron Inc., Tokyo, Japan) under mineral oil. The sperm were capacitated in an atmosphere of 5% CO_2 in air at 37 C for 1.5 h and then inseminated at a concentration of 150 spermatozoa/μl in 200 μl drops containing oocytes. Oocytes and sperm were incubated in an atmosphere of 5% CO_2 in air at 37 C for 6 h, after which oocytes with a second polar body and both male and female pronuclei were washed in fresh TYH medium and cultured for 18 h in an atmosphere of 5% CO_2 in air at 37 C. After 24 h of insemination, embryonic development to the 2-cell stage was evaluated by morphological examination under a stereomicroscope. As controls, oocytes vitrified using the same procedure except for zona drilling were examined for *in vitro* fertilization.

Cryopreservation of 2-cell stage embryos by vitrification

Two-cell stage embryos derived from *in vitro* fertilization were cryopreserved by vitrification, as described above. After about 1 month, the embryos were warmed as described above and were then cultured in 50 μl drops of modified Whitten's medium under mineral oil at 37 C in 5% CO_2 in air. Morphologically normal 2-cell stage embryos were immediately transferred into the oviducts of Day 1 pseudopregnant female mice (Jcl: MCH (ICR); Japan CLEA) for further development to full term. As controls, embryos produced using the same procedure except for zona drilling were examined for cryopreservation, *in vitro* fertilization, and ability to develop to full term. Autopsies of the recipient animals were performed on Day 19.5. The number of implantation sites and fetuses were examined.

Statistical analysis

Statistical significance was assessed between groups using Student's *t*-test, and differences of $P<0.05$ were considered significant.

Table 1. Survival rate of vitrified zona-drilled mouse oocytes after warming

Oocytes	No. of oocytes vitrified	No. (%)[a] of oocytes recovered	No. (%)[b] of surviving oocytes after warming
Zona-drilled oocytes	100	97 (97)	91 (94)
Zona-intact oocytes	160	144 (90)	141 (98)

[a]Percentages of recovered oocytes were calculated based on the number of vitrified oocytes.
[b]Percentages of surviving oocytes after warming were calculated based on the number of recovered oocytes.

Table 2. *In vitro* fertilization of vitrified zona-drilled mouse oocytes with spermatozoa derived from C57BL/6N-Tg(UCP/FAD2)U8 homozygous male mice

Oocytes	No. of oocytes used	No. (%)[a] of oocytes fertilized	No. (%)[b] of embryos that developed to 2-cell stage
Zona-drilled oocyte	91	55 (60)[c]	48 (87)
Zona-intact oocyte	768	81 (11)[d]	70 (86)

[a]Percentages of fertilized oocytes were calculated based on the number of oocytes used.
[b]Percentages of oocytes that developed to the 2-cell stage were calculated based on the number of fertilized oocytes.
[c, d]Values with different superscript letters are significantly different ($P<0.01$).

Results

The survival rate of the vitrified zona-drilled oocytes after warming is shown in Table 1. After warming, the recovery rate from the freezing tube was 97% (97/100), and 91 of the 97 recovered oocytes (94%) showed normal morphology and no significant difference from vitrified zona-intact oocytes (98%, 141/144).

The fertilization rate of the vitrified zona-drilled oocytes with U/FAD2-TgU8 mouse sperm is shown in Table 2. The zona-drilled oocytes were fertilized at a rate of 60% (55/91), which was a significantly higher rate ($P<0.01$) than that of the zona-intact oocytes (11%, 81/768). The fertilized embryos successfully developed to the 2-cell stage with no difference in development rate between the zona-drilled oocytes and zona-intact oocytes [87% (48/55) vs. 86% (70/81)].

Two-cell stage embryos derived from *in vitro* fertilization of warmed and vitrified zona-drilled oocytes with sperm from subfertile U/FAD2-TgU8 mice were cryopreserved by vitrification and warmed about 1 month later. As shown in Table 3, after warming, most of the 2-cell stage embryos derived from the zona-drilled oocytes showed normal morphology, the same as that of the zona-intact oocytes. There was no difference in survival rate between embryos derived from zona-drilled oocytes and those derived from zona-intact oocytes [94%(45/48) vs. 95%(38/40)]. The cryopreserved 2-cell stage embryos were then transferred to the recipient mice. As a result, regardless of embryo source, all recipients became pregnant and normal offspring were born after full-term development (Table 4).

Discussion

In the present study, we showed that early mouse embryos could be successfully produced by *in vitro* fertilization of laser-assisted zona-drilled oocytes with subfertile transgenic mouse sperm, and the embryos were able to develop to full-term after transfer to recipient mice. In the investigation of the fertilization ability of cryopreserved sperm using several strains, sperm from the C57BL/6 strain shows comparatively low potential for *in vitro* fertilization [13]. Consistent with this study, the U/FAD2-Tg mice in the current study, which are of C57BL/6 genetic background, showed relatively low fertilization ability (11%, 81/768), as shown in Table 1. Partial dissection of the zona pellucida has been reported to result in improvement of *in vitro* fertilization with subfertile

Table 3. Survival rate of vitrified 2-cell stage embryos derived from *in vitro* fertilization of vitrified zona-drilled oocytes with C57BL/6N-Tg(UCP/FAD)U8 homozygous spermatozoa after warming

Oocytes	No. (%) of 2-cell stage embryos			
	Vitrified	Warmed	Recovered	Surviving[a]
Zona-drilled oocytes	48	48	48 (100)	45 (94)
Zona-intact oocytes	70	40	40 (100)	38 (95)

[a]Percentages of surviving 2-cell stage embryos were calculated based on the number of recovered embryos.

Table 4. Full-term development of vitrified and warmed 2-cell stage embryos derived from *in vitro* fertilization of vitrified zona-drilled oocytes with C57BL/6N-Tg(UCP/FAD2)U8 homozygous spermatozoa after transfer to recipient mice

Origin of embryos	No. of 2-cell stage embryos transferred	No. (%) of recipients		No. (%)[a] of embryos implanted	No. (%)[b] of offspring
		Used	Pregnant		
Zona-drilled oocytes	45	2	2 (100)	16 (36)	6 (13)
Zona-intact oocytes	38	2	2 (100)	17 (47)	6 (16)

[a]Percentages of embryos implanted were calculated based on the number of embryos transferred.
[b]Percentages of offspring were calculated based on the number of embryos transferred.

sperm [4]; however, in our laboratory, partial dissection of the zona pellucida only slightly improved the rate of *in vitro* fertilization using U/FAD2-TgU8 sperm (27%, 33/122, unpublished data). Alternatively, we found that drilling of the zona pellucida greatly facilitated *in vitro* fertilization of oocytes with subfertile sperm, without occurrence of polyspermy (unpublished observation). Thus, these results suggest that laser-assisted zona drilling of oocytes is an easy and effective procedure for producing fertilized oocytes with subfertile transgenic sperm.

In addition to improvement of *in vitro* fertilization using zona-drilled oocytes, we demonstrated the utility of vitrification for cryopreservation of mouse oocytes and early embryos. Unfertilized oocytes have specific characteristics, such as a huge cell volume and low cell-membrane permeability, compared with preimplantation embryos and other somatic cells [14]. Due to these characteristics, unfertilized oocytes are damaged easily by changes in osmotic pressure and physical stress, and therefore it is difficult to cryopreserve unfertilized oocytes by the conventional slow-freezing method, which involves addition of cryoprotectant into the ooplasm and subsequent dehydration of the ooplasm through slow cooling [15]. In the current study, we used a vitrification method for cryopreservation of zona-drilled oocytes, since vitrification theoretically avoids ice formation both inside and outside oocytes and practically involves simple steps. Therefore, vitrification facilitates cryopreservation of mouse oocytes using a procedure in which oocytes are first put into 1 M DMSO, treated with DAP213 preservation solution (a mixture of cryoprotectants at high concentration), and finally plunged into and preserved in LN_2. This vitrification procedure is effective not only for cryopreservation of early embryos [11, 16–19], but also for mouse and rat unfertilized oocytes [20, 21], mouse pronuclear oocytes [22, 23], and transgenic rat pronuclear oocytes [24]. Our results showed that live offspring were successfully delivered after transfer of *in vitro* fertilized embryos, which were cryopreserved twice by vitrification at the oocyte and 2-cell stages. Taken together, we suggest that the vitrification procedure is also very effective for cryopreservation of unfertilized oocytes from transgenic mice.

In conclusion, we have demonstrated that zona drilling of oocytes combined with cryopreservation by vitrification provides a useful approach for producing certain transgenic mouse strains, especially those with subfertile transgenic sperm. The zona-drilling method uses laser equipment, which makes it possible to produce a large amount of zona-drilled oocytes in a relatively short amount of time and to adjust the pore size of the zona pellucida. Therefore, these procedures are likely to be applicable to managing various transgenic mouse strains in combination with banking and shipment of cryopreserved transgenic oocytes.

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