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Vitrification of one-cell mouse embryos in cryotubes

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ABSTRACT

Preventing intracellular ice formation is essential to cryopreserve cells. Prevention can be achieved by converting cell water into a non-crystalline glass, that is, to vitrify. The prevailing belief is that to achieve vitrification, cells must be suspended in a solution containing a high concentration of glass-inducing solutes and cooled rapidly. In this study, we vitrified 1-cell mouse embryos and examined the effect of the cooling rate, the warming rate, and the concentration of cryoprotectant on cell survival. Embryos were vitrified in cryotubes. The vitrification solutions used were EFS20, EFS30, and EFS40, which contained ethylene glycol (20, 30 and 40% v/v, respectively), Ficoll (24%, 21%, and 18% w/v, respectively) and sucrose (0.4 0.35, and 0.3 M, respectively). A 5-μl EFS solution suspended with 1-cell embryos was placed in a cryotube. After 2 min in an EFS solution at 23 °C, embryos were vitrified by direct immersion into liquid nitrogen. The sample was warmed at 34 °C/min, 4,600 °C/min and 6,600 °C/min. With EFS40, the survival was low regardless of the warming rate. With EFS30 and EFS20, survival was also low when the warming rate was low, but increased with higher warming rates, likely due to prevention of intracellular ice formation. When 1-cell embryos were vitrified with EFS20 and warmed rapidly, almost all of the embryos developed to blastocysts *in vitro*. Moreover, when vitrified 1-cell embryos were transferred to recipients at the 2-cell stage, 43% of them developed to term. In conclusion, we developed a vitrification method for 1-cell mouse embryos by rapid warming using cryotubes.

1. Introduction

Interest in the cryopreservation of mammalian oocytes and embryos has accelerated in two major areas. One is in assisted reproduction for women, and the other is in gene banks as a cost-effective method for maintaining mutant mouse lines and other laboratory mammals. In the cryopreservation of oocytes/embryos, the formation of more than a trace amount of ice in the cell is lethal. Strategies developed to prevent intracellular ice formation (IIF) are slow freezing [20] and vitrification [13]. Nowadays, vitrification has become more widely used because of its simplicity and high survivability. When cells are vitrified with a minimum degree of supercooling, i.e., at near equilibrium, no ice should form in the cell during cooling and warming. In most cases, however, the degree of supercooling is not minimal, and thus invisible minute ice crystals will form in cells during cooling. These minute ice crystals are innocuous as long as they remain minute. During warming, however, they can recrystallize into larger lethal crystals, as a result of differences in surface free energy [14]. Recrystallization results in cellular damage.

With a few exceptions [1,3], the literature on vitrification has emphasized the role of the cooling rate on IIF prevention. As a strategy to increase the cooling rate, ultra-rapid vitrification was developed, in which small tools such as electron microscope grids [7], open pulled straws [19], Cryoloops [6], and Cryotops [5] are used. By using these devices, it is possible to achieve a very high cooling rate (> 10,000 °C/min) by manipulating oocytes/embryos with a very small volume (about 0.1 μl) of vitrification solution. Given the higher cooling/warming rate, cells are able to survive greater supercooling. Ultra-rapid vitrification was first developed for the cryopreservation of bovine oocytes to circumvent chilling injury, but is now mainly used to prevent IIF in cells like oocytes and blastocysts, which are more difficult to cryopreserve [7].

In addition to rapid cooling, it is also believed that the vitrification solution in which cells are suspended must have a high concentration of cryoprotective solutes, especially cell-permeating cryoprotectants. Therefore, it is believed that vitrification requires both the cooling rate and the concentration of a cell-permeating cryoprotectant be high to prevent IIF. However, our findings in the vitrification of mouse oocytes and 8-cell embryos are not consistent with these previous studies [15–18]. We have shown that rapid warming has a more dominant effect over both rapid cooling and a high concentration of cryoprotectant on the survival of vitrified mouse oocytes and 8-cell embryos. Our

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hypothesis is that the high survival of vitrified mouse oocytes/embryos is derived more from rapid warming than rapid cooling and a high concentration of cryoprotectants.

Genome editing research using microinjection of CRISPR/Cas9 nucleases into 1-cell mouse embryos is ongoing. For this research, 1-cell embryos must be stably supplied. In cleaved mouse embryos, the membrane permeability to water and cryoprotectant is higher than that in 1-cell embryos, and the size of the cell is smaller. Especially in morulae, the permeability is quite high due to expression of aqua-glyceroporin in the plasma membrane [2]. Therefore, mouse morulae can be vitrified efficiently in handy cryotubes [10]. However, it is potentially more difficult to vitrify 1-cell mouse embryos in cryotubes, as the permeability is low and cell size is larger than that of cleaved embryos. Vitrification and rapid warming could be an effective method to cryopreserve 1-cell embryos. When 1-cell embryos were cryopreserved with a minute device (hollow fiber), survival was high and stable [8]. However, cryotubes or plastic straws are widely used as containers for cryobanking. In cryotubes, survival is not stable in some cases, but handling is easy, the tools are cost-effective, and many embryos can be contained. Considering the dominant effect of the warming rate over the cooling rate and the concentration of permeating cryoprotectant, we aimed to vitrify 1-cell mouse embryos with rapid warming using cryotubes.

2. Materials and methods

2.1. Animals

Male and female ICR and C57BL/6J mice (purchased from Japan SLC, Inc.) were used in the study. All animals were housed under a 12-h dark 12-h light cycle (light 07:00 to 19:00). All animal experiments were approved by the Animal Experimentation Committee at Akita University and were performed in accordance with the committee's guiding principles (ID: a-1-2812).

2.2. Collection of embryos and embryo transfer

In addition to 1-cell embryos, embryos at later stages were also used. Female ICR mice (8–12 weeks old) were induced to superovulate with intraperitoneal injections of 5 IU pregnant mare serum gonadotropin (PMSG) and 5 IU human chorionic gonadotropin (hCG) (ASKA Pharmaceutical Co. Ltd., Tokyo, Japan) given 48 h apart. Females were mated with mature males of the same strain. For the collection of 1-cell embryos and 2-cell embryos, oviducts of mated animals were flushed with M2 medium (Millipore Corporate Headquaters, Billerica, MA, USA) 25 h and 44 h after injection of hCG, respectively. One-cell embryos were freed from cumulus cells by suspending them in M2 medium containing 0.5 mg/ml hyaluronidase followed by washing with fresh M2 medium. Morulae and blastocysts were collected by flushing the uterine horn 78 h and 92 h after hCG injection, respectively.

In one experiment, 1-cell embryos of C57BL/6J mice were produced by *in vitro* fertilization. Spermatozoa from cauda epididymis of C57BL/6J male mice (12–16 weeks old) were suspended in 100 µl of human tubal fluid (HTF) medium (Ark Resource, Kumamoto, Japan) [12]. They were preincubated at 37 °C under 5% CO_2 in air for 1–2 h. Female mice of the same strain were induced to superovulate with injections of 7.5 IU PMSG and 7.5 IU hCG given 48 h apart. After 13 h of hCG injection, cumulus-oocyte complexes were collected from the oviduct and suspended in a 20 µl drop of HTF medium. Oocytes were inseminated by adding a small drop of sperm suspension to the drop of HTF medium (final sperm concentration being 2–3 x 10^5 cells/ml). Three to 6 h after insemination, oocytes were removed from the fertilization drop, washed, and 1-cell embryos with two pronuclei were used for vitrification experiments.

Table 1
The composition of vitrification solutions used in this study.

Solution	Ethylene glycol		Ficoll		Sucrose (M)	Osmolality (mol/kg water)
	(%, v/v)	(M)	(%, w/v)	(M)		
EFS20	20	3.30	24	0.0034	0.40	7.40
EFS30	30	5.02	21	0.0030	0.35	12.0
EFS40	40	6.29	18	0.0026	0.30	18.0

2.3. Composition of vitrification solution

Embryos were vitrified with an EFS Solution, which was developed by Drs. Kasai and Edashige's group [4,9,11] for cryopreservation of mouse embryos. The EFS solution was a mixture of ethylene glycol and FS solution. The FS solution was a modified phosphate buffered saline (PB1) containing 30% (w/v) Ficoll (Ficoll PM-70, GE Healthcare, Bio-Science AB, Uppsala, Sweden) and 0.5 M sucrose (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The EFS20, EFS30 and EFS40 solutions consisted of 20%, 30% and 40% ethylene glycol, respectively, and 80%, 70% and 60% FS solution, respectively. The final concentration of Ficoll and sucrose is shown in Table 1.

2.4. Vitrification procedure

One-cell embryos were suspended in a 30 µl drop of EFS20, EFS30 and EFS40 at 23 °C. After 1 min and 30 s, 10–15 embryos in a 5-µl drop of medium were placed in a cryotube with a fine pipette (Gilson, Middleton, WI, USA). Thirty seconds later, the embryos were cooled by being placed in liquid nitrogen (LN_2) gas or by direct immersion of the cryotube into LN_2 (Table 2). Because the samples were stored in liquid nitrogen, they were in direct contact with liquid nitrogen. Thus, 2.0 min elapsed from the first exposure of the embryos to the vitrification solution to the initiation of cooling.

2.5. Achieving various cooling rates and warming rates

To examine the effect of the cooling rate, 1-cell embryos were vitrified using 2 different protocols, as described above (Table 2). To determine the cooling rate, the temperature of 5 µl EFS20 in a cryotube was monitored each second for 5 min using a digital thermometer (Fig. 1, Center SE-309, Satoshoji Co., Kawasaki, Japan). The cooling rate was determined based on the time taken to traverse the range between 20 °C and −100 °C. The 2 cooling rates were 35 °C/min, and 4,100 °C/min (Table 2). Samples were rapidly warmed (6,600 °C/min), as described below.

To examine the effect of the warming rate, 1-cell embryos were rapidly cooled (4,100 °C/min). For warming, the cap of the cryotube was removed and LN_2 was discarded. First, the sample was moderately warmed by holding it in air at 23 °C for 1 min to prevent fracture damage at around −110 °C. Then, the sample was subjected to 3 warming protocols. Warming protocol 1 consisted of exposure to air at 23 °C for 2 more min. In warming protocols 2 and 3, 1 ml of PB1 medium containing 0.5 M sucrose (sucrose solution) at 23 °C and 37 °C, respectively, was added into the cryotube. The resulting warming rates were 34 °C/min, 4620 °C/min, and 6570 °C/min, respectively (Table 3).

Table 2
Protocol of cooling procedure.

Protocol	Cooled by	Cooling rate ± SE (°C/min)[a]
1	LN_2 vapor	35 ± 2
2	LN_2	4098 ± 594

[a] The cooling rates were calculated between 20 and -100 °C.