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Successful production of offspring derived from mouse zygotes vitrified with carboxylated ε-poly-L-lysine and polyvinyl alcohol without serum

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Abstract. The vitrification of zygotes is important for their use as donors for generating genome-edited mice. We previously reported the successful vitrification of mouse zygotes using carboxylated ε-poly-L-lysine (COOH-PLL). However, this vitrification solution contains fetal calf serum (FCS), which contains unknown factors and presents risks of pathogenic viral and microbial contamination. In this study, we examined whether polyvinyl alcohol (PVA) can be used as an alternative to FCS in vitrification solutions for mouse zygotes. When COOH-PLL was added to the vitrification solutions, zygotes vitrified with solutions containing 0.01% PVA (PV0.01) and those vitrified in a control solution containing FCS (75.6%) developed into blastocysts (78.4%). In addition, there were no significant differences in the ability to develop to term between the control solution (46.6%) and PV0.01 (44.1%) groups. In conclusion, we clearly demonstrated that PVA can replace FCS in our vitrification solution supplemented with COOH-PLL for mouse zygotes.
Key words: Carboxylated ε-poly-L-lysine, COOH-PLL, Mouse, Vitrification, Zygote

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Cryopreservation of mammalian zygotes is valuable for generating and maintaining genetically modified and genome-edited animals. Vitrification using a minimal volume cooling method is an efficient cryopreservation method for assisted reproductive technology (ART) and experimental purposes [1]. In this method, ethylene glycol (EG) and dimethyl sulfoxide (DMSO) were used as cryoprotective agents (CPAs) (ED solution). In a previous study, we improved the developmental ability of vitrified zygotes using a novel CPA, carboxylated ε-poly-L-lysine (COOH-PLL), in mice [2] and pigs [3]. In our modified protocol, EG and COOH-PLL were used as CPAs in the cryopreservation (EP) solution. However, the solutions used for cryopreservation (wash, equilibration, vitrification, and warming solutions) contained fetal calf serum (FCS), which is widely used for cryopreservation [4]. FCS contains unknown factors and presents risks of pathogenic viral and microbial contamination [5], thus there is a wide demand for their replacement with non-animal alternatives [6]. Polyvinyl alcohol (PVA) has been employed as a substitute for FCS in vitrification/warming solutions for bovine embryos [7] and oocytes [8], porcine embryos [9], and ovine embryos [6]. PVA can effectively inhibit ice formation and growth in solution during vitrification and warming procedures [10], and is therefore a good substitute for serum in vitrification. In this study, we further examined the effect of using PVA instead of FCS in ED and EP solutions to improve the vitrification protocol for mouse zygotes.

As shown in Table 2, there were no significant differences in rates of survival (Control: 80.2 ± 7.4%; PV0.01: 84.2 ± 5.8%; PV0.1: 78.6 ± 8.1%; PV1: 77.9 ± 12.7%), development to 2-cells (Control: 63.4 ± 4.4%; PV0.01: 74.2 ± 5.0%; PV0.1: 69.1 ± 6.3%; PV1: 61.2 ± 12.6%), or development to the blastocyst (Control: 50.9 ± 6.7%; PV0.01: 63.3 ± 3.4%; PV0.1: 53.7 ± 10.7%; PV1 = 53.2 ± 12.3%) among the PVA groups ($P > 0.05$). Next, we examined whether PVA was also effective for vitrification using the EP solution (Table 2). We used 0.01 and 0.1% PVA because the survival and developmental rates were not significantly different, whereas PV1 was slightly lower in the results for the ED solution. When PV0 (EP solution without FCS and PVA) was used for vitrification, the survival (71.8 ± 6.9%) and developmental rates (2-cell: 53.2 ± 7.7%; blastocyst: 26.6 ± 6.1%) were lower than that of the other groups (Table 2). In EP solution, PV0.01 showed higher rates of development to the blastocyst stage (78.4 ± 4.2%) than those of PV0.1 (51.9 ± 10.6%) ($P < 0.01$), and this rate was similar to that of the control group (75.6 ± 6.5%) ($P > 0.05$) (Table 2). Although PVA can inhibit ice formation and growth in vitrification/warming solutions [10], it also has a negative effect on protein synthesis after vitrification [6]. For ovine blastocyst vitrification, 0.1% PVA was used instead of 20% FCS [6]. In contrast, the concentration of PVA (0–1%) did not affect the survival or developmental ability of *in vivo*-derived porcine morulae and blastocysts [9]. In the present study, the addition of 0.01% PVA to the EP solution yielded higher developmental rates than did the addition of 0.1% PVA (Table 2). Even though there were no statistically significant differences, PV0.01 in the ED solution also showed higher survival and developmental rates than the other PVA concentrations (Table 2). Therefore, we believe that 0.01%

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PVA is effective not only in EP solutions but also in ED solutions. Taken together, these results strongly suggest that optimization of the PVA concentration in the vitrification solution is essential and is dependent on the species and probably the developmental stage. Similar to PVA, COOH-PLL has been found to reduce the risk of damage due to ice recrystallization during freezing and thawing. An experiment conducted by Matsumura and Hyon showed that there were high survival rates in various cell lines after cryopreservation using a solution without serum but supplemented with COOH-PLL [11, 12]. More recently, they discovered the mechanism of COOH-PLL as a CPA [12]. During the cooling process, the signals of water, sodium ions, and polymer chains in the COOH-PLL solution spread, resulting in an increasingly restricted mobility and increased solution viscosity. Furthermore, strong intermolecular interactions promote the glass transition of COOH-PLL, trapping water and salt in the gaps of the reversible matrix and preventing intracellular ice formation and osmotic shock during freezing, which leads to reduced cellular stress by cryopreservation [12]. In addition, macromolecules such as bovine serum albumin (BSA; a component of FCS) and PVA can prevent the attachment of embryos onto glass pipettes, plastic dishes, and plastic sheets of Cryotop during vitrification and warming procedures. We attempted zygote vitrification with 0% PVA in the ED solution; however, most zygotes were lost due to their attachment onto glass pipettes, and we could not use 0% PVA in the ED solution (data not shown). COOH-PLL is also a macromolecule; therefore, we evaluated 0% PVA in an EP solution (PV0 in Table 2) to investigate whether COOH-PLL could complement FCS or PVA in mouse zygotes. However, our results showed the lowest survival and developmental rates in the PV0 group (Table 2), suggesting that COOH-PLL may require other macromolecules or serum with at least 15% COOH-PLL solution for the vitrification of mouse zygotes. We evaluated the developmental ability to term by transferring 2-cell embryos delivered from vitrified zygotes using control (20% FCS) and PV0.01 in EP solutions, which showed higher rates of blastocyst formation. Our results showed no significant differences between the control (46.6%) and PV0.01 (44.1%) groups in the EP solution (Table 3).

In conclusion, our results indicate that PVA can replace FCS in vitrification solutions supplemented with COOH-PLL in the vitrification of mouse zygotes. Our current protocol can be widely used for vitrification of mouse zygotes as donors for generating genome-edited mice.

Table 1. Components of the modified PB1 as a base solution of vitrification solution

Compound	M.W.	mM	mg/100 ml
$CaCl_2 \cdot 2H_2O$	147	0.9	13.2
$MgCl_2 \cdot 6H_2O$	203.3	0.49	10.0
Dulbecco's PBS (–) *	–	–	960
Glucose	178.6	5.56	100
Sodium pyruvate	110	0.33	3.6
Penicillin G	–	–	6.3

* Dulbecco's PBS (–) "Nissui" (Nissui Pharmaceutical, Tokyo, Japan).

Methods

All chemicals and reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless stated otherwise. All procedures for handling and treatment of animals were conducted according to the guidelines established by the Ethical Committee for Vertebrate Experiments at Azabu University (ID#170324-9). All the mice were purchased from Charles River Laboratories (Yokohama, Japan). Crlj:C57BL/6J females (4–5 weeks old) were used for metaphase II (MII) oocyte collection and Crlj:C57BL/6J males (12–24 weeks old) were used for sperm collection. Mature female Crlj:ICR mice (12–14 weeks old) were used as recipients of the embryo transfer. Vasectomized male Crlj:ICR mice (20–30 weeks old) were used to induce pseudo-pregnancy. The mice were housed in an environmentally controlled room with a 12 h dark/12 h light cycle at a temperature of 23 ± 2°C and a humidity of 55 ± 5% with free access to a laboratory diet and filtered water. Following the protocols of previous studies [2, 13], zygotes were obtained via in vitro fertilization (IVF) using frozen or thawed sperm. Zygotes were vitrified 6 h after insemination as previously described, [2] with some modifications. In brief, 5–20 zygotes were placed in an equilibrium solution composed of 7.5% (v/v) EG (Kanto Chemical, Tokyo, Japan) + 7.5% (v/v) DMSO + 20% FCS (Life Technologies, CA, USA) or PVA (0.01, 0.1, 1%) in modified Dulbecco's phosphate buffered medium (mPB1) [14] without bovine serum albumin (BSA) (Table 1) (ED solution) for 3 min at 25°C. The zygotes were then transferred into vitrification solution composed of 15% (v/v) EG + 15% (v/v) DMSO + 0.5 M sucrose + 20% FCS or PVA (0.01, 0.1, 1%) in mPB1 (ED solution) for 1 min at 25°C and vitrified using Cryotop [1]. To prepare equilibrium and vitrification solutions using COOH-PLL, 7.5% (w/v) and 15% (w/v) COOH-PLL were added, respectively, instead of DMSO (EP solution). The zygotes were warmed by immersing the Cryotop into a warming solution composed of 0.5 M sucrose + 20% FCS or PVA (0.01, 0.1, 1%) in mPB1 at 37°C for 3 min, and then placed

Table 2. Rates of survival and *in vitro* development of zygotes vitrified using indicated solutions

	Solution	No. of zygotes	No. of survival (%) *	No. of 2-cells (%) *	No. of blastocysts (%) *
	Control	112	87 (80.2 ± 7.4)	71 (63.4 ± 4.4)	57 (50.9 ± 6.7)
	PV0.01	120	99 (84.2 ± 5.8)	89 (74.2 ± 5.0)	76 (63.3 ± 3.4)
ED	PV0.1	136	107 (78.6 ± 8.1)	94 (69.1 ± 6.3)	73 (53.7 ± 10.7)
	PV1	139	108 (77.9 ± 12.7)	85 (61.2 ± 12.6)	74 (53.2 ± 12.3)
	Control	164	150 (92.3 ± 4.9) ^{a}	144 (87.8 ± 5.5) ^{c}	124 (75.6 ± 6.5) ^{e}
	PV0	124	84 (71.8 ± 6.9) ^{b}	66 (53.2 ± 7.7) ^{d}	33 (26.6 ± 6.1) ^{f}
EP	PV0.01	104	102 (95.7 ± 2.3) ^{a}	96 (89.7 ± 3.7) ^{c}	54 (78.4 ± 4.2) ^{e}
	PV0.1	116	112 (98.4 ± 1.1) ^{a}	104 (92.3 ± 3.0) ^{c}	91 (51.9 ± 10.6) ^{ef}

ED, 15% ethylene glycol + 15% dimethyl sulfoxide; EP, 15% ethylene glycol + 15% carboxylated ε-poly-L-lysine; Control, 20% fetal calf serum; PV, polyvinyl alcohol. All percentages are expressed as mean ± standard error of the mean (SEMs). Different superscripts denote significant differences (a *vs.* b, c *vs.* d, e *vs.* f; $P < 0.01$).

Table 3. *In vivo* development of mouse 2-cells derived from zygotes vitrified using ethylene glycol and carboxylated ε-poly L-lysine-supplemented (EP) solutions

FCS or PVA	No. of 2-cells transferred	Pregnant/ Recipients	No. of pups (%)
FCS 20%	88	6/6	41 (46.6%)
PVA 0.01%	102	6/6	45 (44.1%)

FCS, fetal calf serum; PVA, polyvinyl alcohol.

in mPB1 with 20% FCS or PVA (0.01, 0.1, 1%) at 37°C for 5 min. After washing three times with potassium simplex optimization medium containing amino acids (KSOMaa)[15], the zygotes were cultured in a 100-µL drop of KSOMaa covered in paraffin oil, and their survival was evaluated. Development to the 2-cell and blastocyst stages was examined at 24 and 96 h, respectively. To evaluate the *in vivo* development of vitrified zygotes, 2-cell embryos derived from vitrified/warmed zygotes were transferred into the oviducts of recipient mice, as previously reported [2]. Each experiment had at least three replicates. More than 100 zygotes were used for each treatment group to evaluate the survival and developmental rates (Table 2). All percentage data were subjected to arcsine transformation before statistical analysis. The data were analysed by one-way analysis of variance (ANOVA) and Tukey's test to quantify survival and developmental rates (Table 2) and by a two-tailed Student's *t*-test to quantify development rates *in vivo*. A value of $P < 0.05$ (Table 3) was considered significant. Percentages are expressed as the mean ± standard error of the mean (SEMs).

Conflict of interests: The authors declare the following financial interests/personal relationships that may be considered potential competing interests: Kazuaki MATSUMURA, Suong-Hyu HYON has patent #JP WO2009/157209 A1 2009.12.30 issued to Bioverde Inc.

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