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—Full Paper—

Effect of the Polyvinylpyrrolidone Concentration of Cryoprotectant on Mouse Embryo Development and Production of Pups: 7.5% of PVP is Beneficial for *In Vitro* and *In Vivo* Development of Frozen-Thawed Mouse Embryos

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Abstract. In this study, we investigated the effect of polyvinylpyrrolidone (PVP) concentration on *in vitro* and *in vivo* development of 2 cell stage, vitrified ICR mouse embryos using a cryoprotectant consisting of ethylene glycol (EG) and sucrose. M2 was selected as the basic medium for vitrification and thawing. After equilibration with 4% (v/v) EG at 37 C for 15 min, the embryos were vitrified with 35% EG, 5, 6 or 7.5% (w/v) PVP and 0.4 M sucrose at 37 C for 30 sec. One week later, the cryotubes of cryopreserved embryos in liquid nitrogen were directly immersed into a 37 C water bath for 1 min and transferred serially into 300 μl of 0.5 or 0.3 M sucrose at room temperature for 5 min and M2 medium at 37 C for 10 min. The surviving embryos were cultured in KSOM (potassium simplex optimized medium) for 96–120 h in an atmosphere of 5% CO_2 in humidified air. Survival was evaluated by morphological appearance, including membrane integrity and presence of apoptotic blastomeres after thawing. For *in vivo* evaluation, blastocysts were transferred to the uteri of pseudopregnant mice. The survival rates of the 5 and 7.5% PVP concentration groups showed a significantly higher difference compared with that of the 6% PVP group (85.5 and 86.5 *vs.* 71.2%), respectively. Each pup in the of 5 and 6% groups was cannibalized immediately after parturition. A litter of live pups was obtained from only the 7.5% PVP groups. Our study indicated that supplementation of EG and sucrose cryoprotectant solution with 7.5% PVP is optimal for successful vitrification of 2-cell stage ICR mouse embryos.

Key words: Cryopreservation, Embryo, Mouse, Polyvinylpyrrolidone (PVP), Vitrification

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Since the first report of mouse embryo cryopreservation was published in 1972 [1, 2], there have been many technical improvements in cryoprotectant (CPAs) preparation. Factors known to affect the cryopreservation of mouse embryos involve the effectiveness of different CPAs, equilibration times of CPAs, cooling and warming rates, osmotic pressures of CPAs, temperatures for various cooling conditions and dilution process [3–9].

The use of vitrification protocols is a way to avoid cell damage caused by intracellular ice formation, osmotic and chilling injury and zona and blastomere fracture. Vitrification as an ultrarapid cooling technique has been used in cryopreservation of cells, tissues and organs at low temperatures by creating a completely vitreous (glass-like) state [10]. At a sufficiently low temperature, cooling solutions become highly viscous, and solidification occurs without ice formation. Ice formation is usually observed when the CPA concentration is higher than 40% (w/v) [11]. Improvements in ice formation have been made 1) by selecting the most appropriate CPAs, 2) by using a mixture of two or more CPAs, macromolecules and nonpermeating agents and 3) by using stepwise equilibration (two or three steps) in vitrification solutions of intermediate concentration at room temperature or after freezing to 4 C [12].

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Macromolecules used in embryo cryopreservation are used to increase the viscosity of vitrification solution and to avoid extracellular ice formation during the cooling step. Non-permeating macromolecules are less toxic than low molecular weight (Mw) permeating and low Mw non-permeating CPAs and can replace them partially, and can also make the vitrification solution less toxic [13]. Moreover, macromolecules are known to reduce damage to the zona pellucida [14].

Polyvinylpyrrolidone (PVP) is one of the many macromolecules widely used for cryopreservation of normal cells, oocytes and embryos in the hamster [15, 16], human [17], bovine [18–20] and mouse [1, 2, 13, 21–23].

Bergers *et al*. [24] and Dumoulin *et al*. [14] showed that PVP cannot prevent zona and cell damage as efficiently as the other macromolecules (i.e., ficoll, dextran) tested and that relatively short exposure of 2-cell stage mouse embryos to PVP is clearly deleterious to their subsequent development. However, Titterington *et al*. [25] described that PVP provides a lower rate of zona disruption and avoids the need for screening for pathogenic contaminants, such as human immunodeficiency virus and hepatitis B and C. They also showed that inclusion of PVP-Percoll in the freezing medium for 2-cell stage mouse embryos reduced the incidence of zona damage and increased the survival rate of embryos following *in vitro* culture after warming [26]. The usefulness of supplementing PVP into cryoprotectants has been controversial, and the

concentration of PVP needs to be optimized through *in vivo* and *in vitro* examinations.

The aim of this study was to investigate the effect of PVP concentration on cryopreservation of 2-cell stage mouse embryos and both *in vitro* and *in vivo* development of frozen-thawed embryos.

Materials and Methods

Animals and chemicals

For collection of embryos and embryo transfer, ICR mice were purchased from Orient Bio (Namyangju, Korea) and maintained at the SPF animal facility of the Dental Research Institute, Seoul National University. They were housed under controlled 12-h day-night cycles and were provided with pelleted rodent diet and water *ad libitum*. All animals were used for experiments according to the guidelines of the Dental Research Institute. All chemicals were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA) unless otherwise stated.

Embryo collection

Embryos were recovered from 6–8 week-old female mice that had been intraperitoneally injected with 7.5 IU of equine chorionic gonadotrophin (eCG) and 7.5 IU of human chorionic gonadotrophin (hCG) 48 h later. Successful mating of females with same strain male mice was identified by the presence of vaginal plug the following morning. Two-cell stage embryos were obtained by flushing the oviducts 44–46 h after the hCG injection. The embryos were washed three times and stored in M2 medium at 37 C prior to use.

Cooling and warming of embryos

All solutions used in the vitrification and warming procedures were prepared using M2 medium. The vitrification in this study was performed by the method of Tada *et al*. [27, 28] and Bagis *et al*. [29], with slight modifications. Embryos were suspended in an equilibration solution consisting of 4% (v/v) EG at 37 C for 15 min. After equilibration, groups of 15–20 embryos were rinsed three times in 20–30 μl drops of vitrification solution consisting of 35% (v/v) EG, 5, 6 or 7.5% (w/v) PVP and 0.4 M sucrose. Embryos were exposed to vitrification solution for 30 sec. Embryos were transferred into 1.8 ml cryotubes (Nunc, Roskilde, Denmark) containing 20–30 μl of vitrification solution and then directly plunged into liquid nitrogen (LN_2) and stored for 1 week. For warming, the cryotubes were immersed into a 37 C water bath for 1 min and then transferred serially into 300 μl of 0.5 M sucrose at room temperature for 5 min, 0.3 M sucrose at room temperature for 5 min and M2 medium at 37 C for 10 min. The embryos were washed three times and then cultured in KSOM medium (Millipore, Benford, MA, USA) under mineral oil at 37 C in a humidified atmosphere of 5% CO_2 in air.

Assessment of embryo survival and development after warming

Right after warming, survival of embryos was assessed by observing the intactness of blastomeres and zona pellucida. Embryos with an abnormal appearance, like apoptotic blastomere and blebs in the intracellular zone, were considered to be damaged during vitrification. *In vitro* development to the balstocyst stage was examined every 12 h after warming for 96–120 h.

Embryo transfer

Blastocysts developed from vitrified and warmed 2-cell stage embryos were transferred into the uterine of each pseudopregnant female ICR mouse from 8 to 10 weeks of age. The pseudopregnant mice to be used as embryo transfer recipients were bred with vasectomized ICR males, and the day a vaginal plug was observed was considered to be 0.5 days pc. The pseudopregnant females were used at 3.5 dpc for uterine transfers. Live pups after uterine transfer were born by natural delivery.

Statistical analysis

All percentage data and data sets obtained from this study were expressed as means ± SD. Differences in developmental rates and cell numbers of embryos between experimental groups were analyzed by a Duncan multiple range test using SPSS version 12.0 for Windows (SPSS, Chicago, IL, USA). Differences were considered to be significant if the P-value was less than 0.05.

Results

Optimal concentration of PVP determined by in vitro development after vitrification

In our preliminary experiments, when PVP was not involved in the vitrification solution (0% PVP concentration), the survival rate and development of mouse embryos were 27.3% (12/44) and 25% (3/12), respectively.

After thawing, we excluded cryo-injured embryos showing dark cytoplasm or a wrinkled membrane in one or two of the blastomeres from counting for surviving embryos. Even after this, a few embryos with apoptotic blastomeres appeared in the middle of development, and they were also not counted as surviving embryos. As shown in Table 1, the survival rates of the 5 and 7.5% PVP groups (85.5 and 86.5%) were significantly higher than 6% PVP group (71.2%; $P<0.05$). However, development into blastocysts showing a blastocele was not significantly different between the groups (Fig. 1). Before embryo transfer, a few of the blastocysts in 5 and 6% groups contained blastoceles that failed to expand and that exhibited shrinkage and thick ZPs (data not shown).

Production of pups after uterine transfer of blastocysts

One of the 5 recipients in both the 5 and 6% PVP groups gave birth to a pup two days later than expected (Table 2). However, right after parturition, the pups were cannibalized by the recipient mice. In the 7.5% PVP group, five healthy pups were delivered on Day 21 (Fig. 2).

Discussion

This study shows that PVP, a constituent of cryoprotectant consisting of EG and sucrose, could be used to prevent cryoinjury in vitrified-warmed embryos. Among the different concentrations of PVP, 7.5% PVP was proved to be excellent for *in vitro* and *in vivo* development of ICR mouse embryos.

Table 1. Effect of different concentrations of PVP on *in vitro* development of vitrified 2-cell stage mouse embryos

PVP concentration (%)	Embryos treated (no. expt.)	No. surviving (%)	No. of embryos developed to			
			4-cell (%)	8-cell (%)	Morula (%)	Blastocyst (%)
5	138 (4)	118 [a] (85.5 ± 4.5)	111 (94.1 ± 14.8)	107 (90.7 ± 13.8)	107 (90.7 ± 13.8)	75 (63.6 ± 23.3)
6	139 (4)	99 [b] (71.2 ± 5.3)	96 (97.0 ± 16.4)	93 (93.9 ± 15.4)	93 (93.9 ± 15.4)	74 (74.7 ± 24.7)
7.5	111 (4)	96 [a] (86.5 ± 7.5)	94 (97.9 ± 4.3)	92 (95.8 ± 8.3)	92 (95.8 ± 8.3)	80 (83.3 ± 4.4)

Values in the same column with different superscripts are significantly different ($P<0.05$).

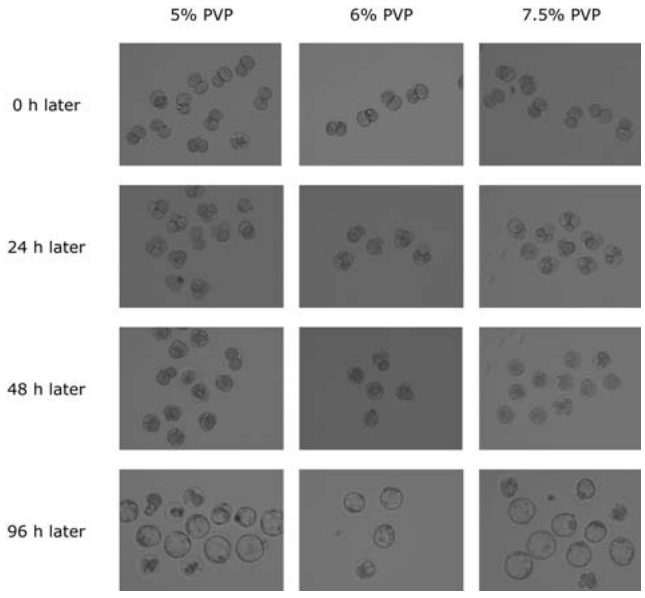

Fig. 1. Development of vitrified mouse embryos at different concentrations of polyvinylpyrrolidone (PVP) 0 h later: 2-cell stage, 24 h later: 4-cell stage, 48 h later: 8-cell stage, 96 h later blastocyst stage. Magnification × 200.

Table 2. Production of pups after uterine transfer of vitrified blastocysts derived from normal fertilization

PVP concentration (%)	No. of embryos transferred (No. of transfers)	No. of pups
5	69 (5)	1
6	70 (5)	1
7	80 (5)	5

Fig. 2. Two-day-old young developed from vitrified blastocysts (7.5% PVP group). The pups appeared normal, and were active after delivery.

Mouse embryos have been successfully cryopreserved using numerous permeating and non-permeating compounds. When embryos are frozen slowly, they remain in equilibrium with a highly concentrated extracellular solution, and their intracellular regions do not freeze. When embryos are frozen rapidly, their survival rate depends on the concentration of cryoprotectants and the duration of exposure to the vitrification solution.

Vitrification provides various potential benefits and advantages over freezing procedures, such as decreaed osmotic and chilling injury. Schneider [31] reported that cooling injury occurs by two mechanisms: intracellular ice-formation and vitrification solution effects. Both of these mechanisms can lead to membrane damage, disruption of the zona pellucida and subsequent cell death. Rapid vitrification of early stage mouse embryos can result in chromosomal aberrations, and this is associated with the freezing properties of the cryoprotectants [32]. However, vitrification procedures need much higher concentrations of cryoprotectants than conventional slow freezing. Due to their toxicity, vitrification solutions must be composed in such a way as to reduce these effects.

Since Rall and Fahy [10] first reported embryo cryopreservation using a vitrification procedure, much experimentation has been concerned with improving vitrification solutions, and creating a solution with reduced toxicity. Toxicity of cryoprotectants may be reduced through 1) a shorter exposure time [33], 2) pre-equilibration in the vitrification solution to be used [10], 3) reducing the temperature at which equilibration is performed and 4) rapid cooling rates [33].

Other investigations into improvement of vitrification have focused on the combination of macromolecules in vitrification solutions. Macromolecules have been found to stop ice formation, to facilitate vitrification [33] and to protect the zona pellucida against physiological damage [24]. The few blastocysts that did not have a reduced zona thickness in the 5 and 6% groups could have resulted from the vitrification procedure, and this might have affected the litter size. Our results confirm that PVP reduces damage caused by osmotic swelling, prevents disruption of the zona pellucida and facilitates the vitrification procedure.

Whittingham [34] reported successful use of PVP for cryopreservation of 8-cell stage mouse embryos and early blastocysts. When embryos were frozen to –79 C in a 7.5% PVP solution, the survival rate was 55–65%. However, the frozen-thawed embryos did not survive, and 2-cell stage embryos did not survive at all. Wilmut [2] investigated the toxicity and effectiveness of various CPAs, including PVP. The toxicity of 7.5% PVP in vitrification solutions was assessed by incubation for 4 h at 20 C. He found that using an undialysed PVP reduced the survival rate significantly.

Leibo and Oda [35] investigated whether 7.5 or 15% PVP enhanced the cryoprotective properties of EG at various concentrations. They reported survival rates of up to 95% for mouse zygotes and 8-cell stage embryos, frozen either slowly or rapidly. They also reported that PVP should be used directly and purified by dialysis. On the other hand, Bergers *et al*. [24] reported about the effects of macromolecules on cryopreservation of 2-cell stage mouse embryos. They found that addition of macromolecules such as dextran, Ficoll and PVP significantly reduced the incidence of zona and cellular damage, but that exposure of embryos to PVP was deleterious to their subsequent development.

In the use of PVP for bovine oocytes, Suzuki *et al*. [36] reported that 5% PVP had a beneficial effect compared with 10 or 20% PVP for conventional slow freezing of germinal vesicle stage bovine oocytes. However, Checura and Seidel [20] reported that 20 and 6% PVP solutions were effective for vitrification of mature bovine oocytes.

Five live pups were delivered after uterine transfer, but only in the 7.5% PVP group. The small litter size of this study might indicate that morphologically invisible damage could have occurred to the zona pellucida that obstructed the hatching of early and expanding blastocysts in the recipients' uteri.

Our present results show that a combination of EG and 7.5% PVP in a vitrification protocol is advantageous to the survival and development of 2-cell stage mouse embryos.

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