



## The Effect of Cryopreservation on the Mouse Embryos at Various-pronuclear Stages

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**ABSTRACT :** This study was carried out to establish an appropriate condition for the efficient cryopreservation of the mouse pronuclear embryo. *In vitro* cryopreservation of pronuclear embryos was carried out by slow freezing or vitrification methods and development rate of 2-cell, blastocyst and hatched blastocyst was measured as well as survival rate of the thawed pronuclear embryo. After slow freezing, vitrification and thawing of mouse pronuclear embryos, the survival rate and blastocyst development rate for the vitrification group was 97.3 and 53.4%, respectively, which was significantly higher as compared to the slow freezing group with 88.6 and 23.9%, respectively ( $p < 0.05$ ). Blastocyst developmental rate in each experimental group was significantly higher for 21 h in the post-hCG group at 40.5-57.0% than the 24 h post-hCG group at 40.5% ( $p < 0.05$ ). ICM (Inner cell mass) cell numbers of blastocyst-stage embryos during the different stages of mouse pronuclear embryos, slow freezing and vitrification period in the control and vitrification groups were  $22.1 \pm 2.7$  and  $17.0 \pm 3.1 \sim 22.0 \pm 3.2$ , respectively; hence, the slow freezing group ( $10.2 \pm 2.0$ ) had significantly higher cell numbers than those of the other two groups ( $p < 0.05$ ). Trophoblast (TE) cell number in the control group,  $65.8 \pm 12.6$ , was significantly higher than in the slow freezing group,  $41.6 \pm 11.1$  ( $p < 0.05$ ). The total cell numbers in the control group and 21 h post hCG group were  $87.9 \pm 13.6$  and  $81.8 \pm 14.1$ , respectively, and were significantly higher than for the slow freezing group ( $51.8 \pm 12.6$ ;  $p < 0.05$ ). (**Key Words :** Inner Cell Mass, Mouse, Pronuclear Embryo, Slow Freezing, Vitrification)

### INTRODUCTION

Since the first report on the production of first offspring (Whittingham et al., 1972) from the mammalian cryopreserved embryo transfer, the studies are continuously carrying out to optimize the condition for freezing and thawing and also various mammals are producing offspring from cryopreserved embryo transfer. Lately, development of assisted reproductive technology for infertility couple and the technology requirement of an appropriate period for embryo freezing and thawing are gradually increasing.

In the past, the cryopreservation with slow freezing

method was commonly used where expensive freezer and time consuming disorders were necessary. However, lately vitrification method with high concentration of cryoprotectant is most commonly used method for simple cryopreservation (Whittingham et al., 1972). Rall and Fashy (1985) reported the high survival rate of oocyte after vitrification and thawing using a cryoprotectant VS1. Subsequently, many studies have been carried out in this regard and lately it is commonly used technique in the cryopreservation of various animal oocytes (Kong et al., 2000; Huang et al., 2002; Lee et al., 2003; Misumi et al., 2003; Mukaida et al., 2006). In vitrification of oocyte, the oocytes are exposed at a high concentration of cryoprotectant solution to prevent the primary cause of oocyte damage from the formation of intra and extra cellular ice crystal and also, possible rapid freezing of oocyte. Furthermore, this method can overcome the problems such as the disadvantage of slow freezing and ultra rapid freezing, that is, an increase of intracellular electrolyte concentration due to loss of intracellular dehydration and formation of intracellular ice crystal. Cryoprotectant in vitrification includes sub-unit molecules of cryoprotectant such as glycerol, propanediol, ethylene

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glycol, DMSO that penetrate into an intracellular of oocyte and macromolecule of non penetrating agent such as ficoll, sucrose, trehalose, xylose, glucose and so on. For cryopreservation of oocyte, Martino et al. (1996), Vajta et al. (1998), Lane et al. (2001) and Hashimoto et al. (2007) used grid (electron microscope), OPS (open filled straw), nylon loop and fine polypropylene strips, respectively and published improved experimental results.

Oocyte cryopreservation is carried out at various developmental stages with unfertilized oocytes. At the time of immature oocytes freezing, damage to cytoplasm, destruction and change of molecular structure in spindle fiber, vitelline membrane, zonapellucida and cortical granules along with formation of abnormal aster have decreased the number of spindle fibers; consequently, these would be able to produce a decrease in embryo developmental rate when they are thawed and fertilized. At the time of embryos freezing, damage to spindle fiber and cortical granules are observed in Metaphase II stage during the exposure of cryoprotectant and freezing (Stachecki et al., 1998; Van Blerkom et al., 1989). The current problem during immature oocyte cryopresearvation is low embryo developmental rate and birth rate; this is explained as being due to the premature release of cortical granules in cytoplasm, structural damage to zonapellucida and hardening after cryopreservation. Recently, it is known as being due to cytoskeleton disorder such as chromosome non-disjunction by the microtubule damage, pronucleus migration from microfilament damage and polar body extrusion disorders (Sterzik et al., 1992; Van der Elst, 1993).

Generally, various survival and developmental rates are produced according to oocyte developmental stages (Mandelbaum et al., 1998). Veek et al. (1993) and Damario et al. (1999) showed the highest pregnancy rate in cryopreserved pronuclear embryo and obtained a high implantation rate with an absence of 1-cell and spindle fiber after thawing. The survival rate of embryo can be precisely verified after thawing (Testart et al., 1986; Cohen et al., 1988; Fugger et al., 1988), even if, the cells are in an unstable stage due to pronuclear migration and fusion. Also, it is reported that the reproduction of cytoskeleton in cytoplasm has grown prosperously. Therefore, this study has been carried out to establish the efficient pronuclear embryo cryopreservation with an investigation of survivability, embryo developmental rate and ICM (Inner Cell Mass) and TE (Trophectoderm) cell numbers by slow freezing and vitrification of mouse pronuclear embryo during culture period.

## MATERIAL AND METHODS

### Mouse embryo collection

In the present study, B6CBAF1 mice (hybrid cross

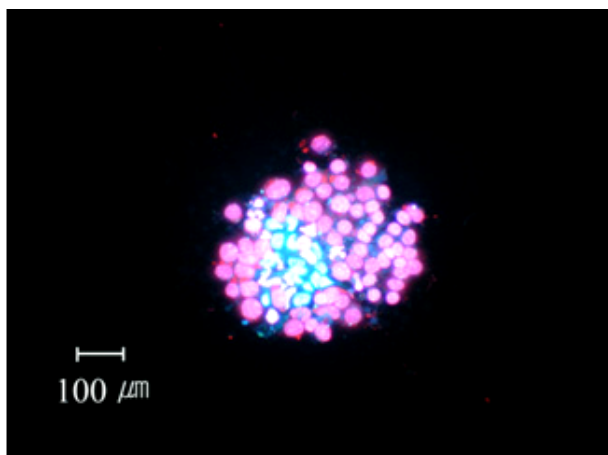
between C57BL and CBA; 6-8 weeks) was used. For the experimental design the light and dark phase conditions were regulated for 12 h with 40-60% humidity and the temperature ranging between 22-25°C. 5 IU of pregnant mare serum gonadotrophin (PMSG; Sigma-Aldrich Chemical, St Louis, Mo) was injected into the abdominal cavity of mice. Post-PMSG injection, 5 IU of human choliconic gonadotrophin (hCG; Pregnyl, Serono, Anbunne, Switzerland) was injected for superovulation. The females were mated with same breed males for fertilization and after 21 h, both oviducts were collected by cervical dislocation of mice (Wang et al., 2007). Then, oviduct flushing was carried out by using sterilized PBS (Gibco BRL, Grand Island, NY) solution for aspiration of pronuclear embryos (Jang et al., 2008). The collected embryos were washed three times with culture media and treated with culture medium containing 0.1% (w/v) hyaluronidase (cat. No. H-3759, Sigma) for 5 min to remove cumulus cells. The selected normal pronuclear embryos were cultured with G1 (G1-5, Vitrolife, Sweden), culture medium 37°C supplemented with 5% CO<sub>2</sub> (Care and treatment of all animals in this study was approved by the Ethical Committee of medical center of daegu CHA general hospital: Animal Ethical Care is essential to accept this article).

### Embryo culture

For this study, the embryos were cultured in microdrops (5/10 µl) of culture medium under Paraffin oil (G-OIL, Vitrolife, Sweden) in a sterile Petri dish. And then, according to the experimental conditions, further, they were cultured with control group after slow freezing and vitrification thawing. All the embryos were cultured in G1 culture media for 48 h followed by replacing with G2 (G2-5, Vitrolife) culture media for 96 h.

### Slow freezing and thawing

Slow freezing of mouse pronuclear embryos was carried out at 21 h after hCG injection, using a culture media CJ1, according to the method of Stachecki et al. (1998). As a cyroprotectant, the culture media containing 0.05% (w/v) BSA (cat. No. A-6003, Sigma) was added to PROH (cat. No. P-1009, Sigma). During slow freezing, the embryos were exposed to media containing 1.5 M PROH for 10 min, and then transferred to culture media containing 1.5 M PROH and 0.1 M sucrose (cat. No. S-9378, Sigma) for 15 min. From there, each 15 embryos were loaded into a 0.25 ml French straw (IMV International, Mineapolis, MN) with freezing media. Programmable freezer (Planer, UK) (-2°C /min to -7°C) was used for slow freezing. The seeding was performed by touching the side of a straw with forceps that was plunged into liquid nitrogen (LN<sub>2</sub>) and the straw was held for 10 min. After 10 min hold at -7°C, gradually the



**Figure 1.** Differentially stained mouse blastocysts with blue nuclei representing the inner cell mass (ICM) and pink to red nuclei representing outer cells (TE).

temperature was lowered at a rate of  $-0.3^{\circ}\text{C}/\text{min}$  to  $-30^{\circ}\text{C}$ , then the straw was plunged into  $\text{LN}_2$  and subjected to store for 2 weeks. For thawing, the straw stored at  $-196^{\circ}\text{C}$  was exposed to room temperature for approximately 30 sec. and then submerged it in a water bath at  $30^{\circ}\text{C}$ . Thawed-embryos were washed with culture media (6 steps) for 10 sec., i) 0.2 M sucrose+1.0 M PROH, ii) 0.2 M sucrose+0.5 M PROH, iii) 0.2 M sucrose, iv) 0.1 M sucrose, v) CJ1 medium, respectively to remove cryoprotectant. Then, the culture was carried out after the removal of PROH and sucrose from the embryos and washed for more than three times with fresh medium.

### Vitrification

Vitrification of mouse pronuclear embryos was performed at 21, 24, 27 and 30 h after hCG injection, using a basic culture medium CJ1 containing 0.05% (w/v) BSA for 5 min. The embryos were exposed to freezing medium containing 8% (v/v) demethyl sulfoxide (cat. No. D-2650, Sigma) and 8% (v/v) ethylene glycol (cat. No. E-9129, Sigma) for 1 min and 45 sec followed by transferring to a freezing media containing 16% (v/v) DMSO, 16% (v/v) EG, 10 mg/ml ficoll (cat. No. F-2878, Sigma) and 0.65 M sucrose for 30 sec. Embryos (5-8) were loaded onto a cryoloop (cat. No. HR4-963, Hampton research, USA) and plunged directly into  $\text{LN}_2$  for vitrification. The vitrified embryos were washed with 0.25 M sucrose solution for 2

min and then transferred to 0.125 M sucrose solution for 3 min. The warmed embryos were remained in a basic media for 5 min. for equilibrium and removal of cryoprotectant from the embryos. The embryos then were washed three times and cultured in each fresh culture medium.

### Inner cell mass and trophectoderm cell count

After thawing, the thawed embryos were cultured to blastocyst and the differential staining was carried out using propidium iodide (cat. No. P-4170, Sigma) and bisBenzimide (cat. No. B-2261, Sigma) of embryo grade. The zona pellucida of mouse blastocysts was or were processed in 0.5% (w/v) protease (cat. No. P-6911, Sigma) solution for 5 min, and then washed for 3 to 5 times with PBS solution containing 0.1% (v/v) PVA (cat. No. P-8136, Sigma). Then, they were cultured for 1 h in anti-bovine whole serum (cat. No. B-8270, Sigma) followed by staining after processing with guinea pig complement (cat. No. S-1639, Sigma) containing 10  $\mu\text{g}/\text{ml}$  PI and 10  $\mu\text{g}/\text{ml}$  bisBenzimide for 1 h. The stained blastocysts were washed 3 to 5 times with PBS solution; whole mount of stained blastocysts was attached onto a slide glass and each number of inner cell mass (ICM) and trophectoderm (TE) were counted under the fluorescent microscope ( $\times 100$ -200, IX71; Olympus, Tokyo, Japan) (Kim et al., 2007; Figure 2). The bisbenzimidide stained ICM nuclei fluoresced blue, while TE nuclei, which stained with both bisbenzimidide and PI, fluoresced red or pink (Figure 1).

### Statistics

The data were statistically analyzed by  $\chi^2$ -test with SPSS program for its significance. p value of less than 0.05 was considered not significant.

## RESULTS

### Experiment 1

Survival and development rate of pronuclear embryo after slow freezing and vitrification. After slow freezing, vitrification and thawing of pronuclear mouse embryos, their survival and development rates of hatched blastocyst were studied and data are represented in Table 1. The samples for control and experimental groups were taken after sorting of pronuclear embryos at 21 h post hCG injection. The survival rate of control, vitrification and slow

**Table 1.** Survival and development rates of pronuclear embryos after slow freezing and vitrification

Group	No. of examined zygotes	No. of Survival (%)	No. (%) of embryos development to		
			$\geq 2$ -cell	BL <sup>1</sup>	Hatched/BL <sup>1</sup>
Control <sup>2</sup>	80	80 (100.0) <sup>a</sup>	78 (97.5) <sup>a</sup>	64 (80.0) <sup>a</sup>	46 (57.5) <sup>a</sup>
Vitrification	75	73 (97.3) <sup>a</sup>	56 (76.7) <sup>b</sup>	39 (53.4) <sup>b</sup>	15 (20.5) <sup>b</sup>
Slow freezing	80	71 (88.6) <sup>b</sup>	64 (90.1) <sup>a</sup>	17 (23.9) <sup>c</sup>	7 (9.9) <sup>b</sup>

<sup>a, b, c</sup> With the same columns, values with different superscripts differ significantly ( $p < 0.05$ ).

<sup>1</sup> blastocyst. <sup>2</sup> At 21 h post hCG.

**Table 2.** Survival and development rates of various-pronuclear stage embryos during at vitrification

Time (h)	No. of examined zygotes	No. of Survival (%)	No. (%) of embryos development to		
			≥2-cell	BL <sup>1</sup>	Hatched/BL <sup>1</sup>
Control <sup>2</sup>	120	120 (100.0) <sup>a</sup>	115 (95.8) <sup>a</sup>	85 (70.8) <sup>a</sup>	55 (45.8) <sup>a</sup>
21 h	124	121 (97.6) <sup>ab</sup>	113 (93.4) <sup>a</sup>	69 (57.0) <sup>b</sup>	28 (23.1) <sup>b</sup>
24 h	125	116 (92.8) <sup>bc</sup>	94 (81.0) <sup>b</sup>	47 (40.5) <sup>c</sup>	22 (18.9) <sup>b</sup>
27 h	106	98 (92.5) <sup>bc</sup>	84 (85.7) <sup>bc</sup>	44 (44.9) <sup>bc</sup>	17 (17.3) <sup>b</sup>
30 h	112	106 (94.6) <sup>bc</sup>	99 (93.4) <sup>ac</sup>	55 (51.9) <sup>bc</sup>	18 (16.9) <sup>b</sup>

<sup>a,b,c</sup> With the same columns, values with different superscripts differ significantly (p<0.05).

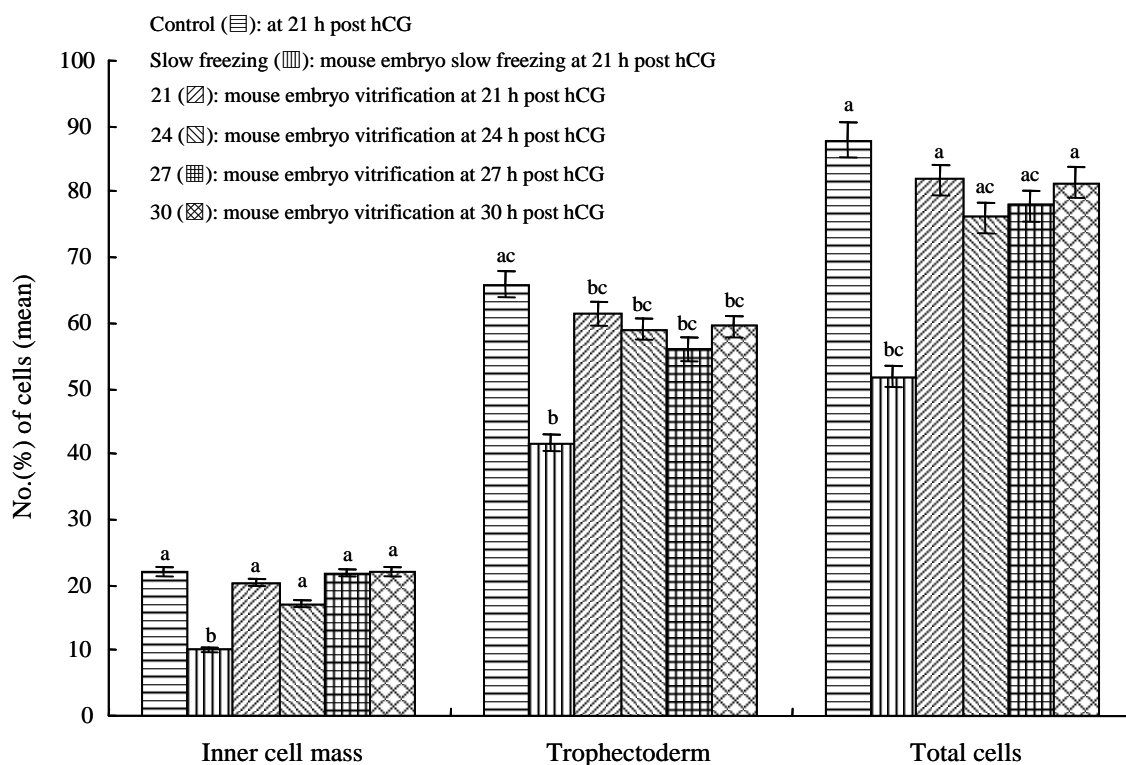
<sup>1</sup>blastocyst. <sup>2</sup> At 21 h post hCG.

freezing groups were noted to be 100, 97.3 and 88.6%, respectively. Hence, the survival rate of control and vitrification groups were significantly higher than those of slow freezing group (p<0.05). The developmental rate of 2-cell embryos in control and slow freezing groups were found to be 97.5 and 90.1%, respectively, which were significantly higher than that of vitrification group with about 76.7% (p<0.05). The rate of blastocyst development in the control group was found to be highest with about 80% and vitrification group with 53.4%, which was significantly higher than that of slow freezing group with about 23.9% (p<0.05). The rate of hatched blastocyst development in the control, vitrification and slow freezing groups were found to be 57.5, 20.5 and 9.9%, respectively. Thus, the results obtained for control group were found significantly higher as compared to vitrification and slow

freezing groups. Although the percentage of development rate of vitrification group was found higher than that of slow freezing group, there was no significant difference.

**Experiment 2**

Survival and development rates of various-pronuclear stage embryos at vitrification. The effects of various-pronuclear stage embryos at vitrification on survival and development rates of hatched blastocyst are shown in Table 2. The pronuclear embryos of 21 h post hCG injection were taken as a control group, for the treatment groups, the post injected mice were taken at 21, 24, 27 and 30 h as experimental groups. The survival rate in the control group was noted as 100%, whereas in 21 h post hCG injection group it was highest with 92.5 to 97.6% as compared to other experimental groups with no significant differences.



**Figure 2.** The effect of slow freezing and vitrification periods at pronuclear stage on cell numbers of blastocysts stage embryos in mouse. <sup>a,b,c</sup> With the same columns, values with different superscripts differ significantly (p<0.05).

The developmental rates of 2-cell embryos in the control, 21 and 30 h post hCG groups were found to be 95.8, 93.4 and 93.4%, respectively, which were significantly higher than 24 h post hCG group ( $p < 0.05$ ). The blastocyst developmental rate in the control group was noted to be 70.8%, whereas in experimental group it was found to be 40.5 to 57.0%. 21 h post hCG group had a highest rate among the tested experiment groups, and it was significantly higher than that of 24 h post hCG group with about 40.5% ( $p < 0.05$ ). The hatched blastocyst development in the control group was 45.8% whereas it was noted to be 16.9 to 23.1% in experimental groups. Among the experimental groups, 21 h post hCG group had a highest rate with no significant differences.

### Experiment 3

The effect of mouse pronuclear stage embryos during slow freezing and vitrification on cell numbers of blastocysts stage embryos is shown in Figure 2. ICM cell numbers in control and vitrification groups were found to be  $22.1 \pm 2.7$  and  $17.0 \pm 3.1$  to  $22.0 \pm 3.2$ , respectively. ICM cell number in control and vitrification groups were significantly higher than those of slow freezing group ( $10.2 \pm 2.0$ ;  $p < 0.05$ ). And, TE cell number in the control group, which was noted to be  $65.8 \pm 12.6$  was found significantly higher than slow freezing group as  $41.6 \pm 11.1$  ( $p < 0.05$ ). Although, the vitrification group had a higher cell numbers ( $56.0 \pm 13.9$  to  $61.5 \pm 13.2$ ) than the slow freezing group, yet was considered to be not significantly different. The total cell numbers in the control group and 21 h post hCG group were found to be  $87.9 \pm 13.6$  and  $81.8 \pm 14.1$ , respectively and were significantly higher than slow freezing group as  $51.8 \pm 12.6$  ( $p < 0.05$ ).

## DISCUSSION

After the study on cryopreservation of mouse embryos by Whittingham et al. (1972), many researchers used a variety of species embryos to study the components of cryoprotectant and freezing method for optimization of freezing condition and to increase the survival rate of embryos after cryopreservation. Production of ice crystal formation during the freezing process gives a fatal damage to embryos cell membrane which consequently affects in the survival rate of embryo after thawing. Hence, the researchers used a cryoprotectant as a method to reduce the ice crystal formation; but, a cryoprotectant itself contains a strong toxicity, thus it is very important to select optimal condition for cryoprotectant and regulation of their processing time or concentration.

In 1983, Trounson and More achieved a successful pregnancy after cryopreservation and thawing of human

embryo in slow freezing using DMSO (Dimethylsulfoxide). DMSO is a suitable cryoprotectant without production of toxicity in 2, 4 and 8 cells of early stage embryos but it is reported that there appears to be a toxic effects in the later stage embryos (Kasai et al., 1990). Since then, Cohen et al. (1986) developed a less toxic cryoprotectant using glycerol and recently, the least toxic cryoprotectant, ethylene glycol is universally used (Bautista et al., 1998).

Initially, slow freezing using an expensive freezer was commonly used for cryopreservation but lately vitrification is more widely used due to its easy method. There are disadvantages in slow freezing such as an ice crystal formation in cytoplasm, toxicity of cryoprotectant and osmotic pressure change that may affect the survivability of oocyte and time consumption for oocyte freezing. Recently, to overcome these disadvantages, the embryos were exposed to a high concentration of cryoprotectant for a short time and processed a rapid dehydration to minimized the ice crystal formation in cytoplasm and achieved a high survival rate in vitrification than in slow freezing (Kasai et al., 1990; Rall and Wood, 1994; Hu et al., 2006; Joshi et al., 2006). Moreover, Lane and Gardner reported that in comparison between vitrification and slow freezing, pyruvate uptake and embryo cell numbers in slow freezing were substantially lower than the control group (Chen, 1986). Also, in this study, the survival rate of embryos and blastocyst production in vitrification (97.3 and 53.4%, respectively) was significantly higher than in slow freezing (88.6 and 23.9%, respectively) ( $p < 0.05$ ), as well as the total cell number of blastocyst in vitrification group ( $76.0 \pm 13.7 \sim 87.9 \pm 13.6$ ) was significantly higher than the slow freezing group ( $51.8 \pm 12.6$ ) ( $p < 0.05$ ).

During vitrification process, a cryopreservation container is an important factor with a cooling rate in freezing of oocytes. Plastic straw (Tachikawa et al., 1993), Open pulled straw (OPS) (Schattn et al., 1985; Shaw et al., 1995), glass micropipette (Kong et al., 2000), EM grid (Martino et al., 1996), cryoloop (Courtney et al., 2006), fine polypropylene strips (Hashimoto et al., 2007) and others are used to load embryos by researchers. Especially, cryoloop is used among these methods for vitrification of embryos because of its various advantages such as easy to use and also safe to store during freezing and thawing. Using electron microscope grid (EM grid) and straw for vitrification, there were difficulties with micromanipulation and collection rate; hence, Lane and Gardner (2001) used cryoloop as a vitrification container and achieved similar results in the comparison and control groups with respect to embryo developmental rate, hatched blastocyst rate, implantation rate, embryo growth rate and blastocyst cell numbers by using vitrified-thawed mouse embryos. With a similar method, human embryos were vitrified and there

were no differences between comparison and control groups with respect to embryo developmental rate and blastocyst cell numbers. This may be due to decrease of cell damage by using a cryoloop with a minimal amount of cryoprotectant (<1 µl) for vitrification and reduced impacts of heat change from extremely rapid cryopreservation. Also, at a vitrification process, exposure time to cryoprotectant is decreased; hence decreased toxicity exposure reported that cryoprotectant with buffer solution decreased the cell damage from pH change (Lane and Gardner, 2001).

Since the birth of normal baby after cryopreservation of human embryo by Chen (1986), various methods are tried out for embryo cryopreservation; however, after thawing, embryo survival rate, developmental rate and birth rate are still at a standstill. At embryo cryopreservation, generally, embryos in a higher developmental stage result in a higher survival rate of embryo after freezing and thawing; hence, cryopreserve embryos at the blastocyst stage. But, Veeks et al. (1993) and Damario et al. (1999) showed the highest pregnancy rate in pronuclear embryo cryopreservation and survivability of embryos was able to identify more accurately during thawing, as well as high implantation rate after thawing due to the absence of spindle fiber. Consequently, non-disjunction of chromosome and chromosome abnormality were caused by spindle fiber damage during the matured oocyte cryopreservation. As an alternative, cryopreservation of immature oocyte with less chromosome abnormality was suggested; however, it has been reported that there would be a high oocyte loss rate during *in vitro* maturation after thawing process (Cooper et al., 1998). Kang et al. (1999) demonstrated the high survival rate of rabbit pronuclear embryo in vitrification and slow freezing (63.3 and 57.7%, respectively) but developmental rate of hatched blastocyst in both methods was substantially lower after 2-cell death and over (6.1 and 11.5%, respectively). Gajda and Smorag (1999) also stated that the developmental rate of rabbit pronuclear embryo to blastocyst was low after vitrification and thawing. However, Isachenko et al. (2005) and Shaw et al. (1995) showed a high developmental rate of human and mouse pronuclear embryo in cryopreservation.

In this study, slow freezing and vitrification were carried out for mouse pronuclear embryo cryopreservation to establish the effectiveness of pronuclear embryo cryopreservation by analysis of embryo survival, developmental rates and ICM and TE cell number. As a result, vitrification showed the higher survival rate and embryo development rate than that of slow freezing and there were no significant differences in embryo developmental rate and cell numbers during culture period of pronuclear embryo vitrification. Further study regarding molecular structure and production of offspring using

blastocyst produced by according to vitrification period is needed to achieve more accurate pronuclear blastocyst cryopreservation.

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