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반복적인 생쥐 배아 동결이

배아의 생존, 발달 및 동결 손상에

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Embryonic survival, development and  
cryoinjury of repeatedly frozen mouse  
preimplantation embryos

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Embryonic survival, development and  
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preimplantation embryos

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## ABSTRACT

# Embryonic survival, development and cryoinjury of repeatedly frozen mouse preimplantation embryos

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### Objective

Although guidelines about the number of embryos to be transferred differ among each country or individual IVF center, efforts to reduce the number of transferred embryo in a single cycle have been continuously made. If embryos were frozen in the past cycle and one or more embryos are transferred in the present cycle, surplus embryos should be re-frozen. It has been concerned that re-frozen embryos would have great cryoinjury, low survival, and subsequent poor

pregnancy success. The aim of this study is to investigate the embryonic survival, development, and expressions of cryoinjury-related genes in once-frozen, twice-frozen, and three-time-frozen mouse preimplantation embryos using indirect vitrification methods.

## **Methods**

Experimental animal study. Six hundred 8-cell stage embryos were obtained from 60 female mice and randomly assigned to control and three experimental groups (150 embryos per each group). 8-cell stage embryos were vitrified by indirect vitrification methods and warmed once, twice, and three times, respectively. We analyzed the developmental outcome including survival rate, blastocyst formation rate, the percentage of hatching/hatched blastocyst including the cell number differences. And we used DAPI staining in hatching or hatched blastocysts for cell count. And a part of hatching or hatched blastocysts were subjected to real-time quantitative RT-PCR (qRT-PCR) for quantification of cryoinjury-related genes.

We examined the expressions of cryoinjury related genes (Casp3, Cirbp, Sod 1, Gpx 3, Cat). And we also evaluated three anti-oxidant enzymes against ROS production on mouse embryo (Sod 1, Gpx 3, Cat). Data were analyzed by the comparative cycle threshold method in all experiments using Histone H2A.Z (H2afz) as endogenous reference genes. All mRNA expressions were normalized to that of H2afz mRNA. The experiments were repeated 6 times using different sets of embryos.

## Results

In three frozen groups, overall survival rate, blastocyst–formation rates, the proportions of hatching/hatched blastocyst, and the cell count in hatching/hatched blastocyst were all similar compared to non–frozen control group.

The mRNA expression of Casp 3, Cirbp showed no significant differences between 4 groups. And the mRNA expression of Sod 1, Gpx 3, Cat was significantly elevated in the experimental group.

## Conclusion

This is the first study to evaluate the efficacy of three times of repetitive freezing–warming of mouse 8–cell embryos, and also the first study using indirect (closed) vitrification method. Repeatedly frozen mouse preimplantation 8–cell embryos even up to three times could preserve good survival and embryonic development. Similar expression of mRNAs for cryoinjury–related markers (Cirbp, Casp 3) or elevated anti–oxidant related enzymes (Sod 1, Gpx 3, Cat) indicates the safety of repeatedly frozen embryos at a molecular level. We demonstrated that closed (indirect) vitrification method also have efficacy and safety in frozen technologies.

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**Keywords:** embryo re–freezing, indirect vitrification, in vitro fertilization, cryoinjury, frozen embryo transfer

**Student number:** 2014–25039

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## Introduction

Embryo freezing is regarded as an important part of infertility treatment. Embryo freezing technology lowers the risk of ovarian hyperstimulation syndrome and multifetal pregnancies and increases cumulative pregnancy rate (Ferraretti, et al., 1999) (MacKay, et al., 2006) (Reddy, et al., 2007). Although guidelines about the number of embryos to be transferred differ among each country or individual IVF center, efforts to reduce the number of transferred embryo in a single cycle have been continuously made regarding to these guidelines (Practice Committee of American Society for Reproductive and Practice Committee of Society for Assisted Reproductive, 2013, Practice Committee of Society for Assisted Reproductive and Practice Committee of American Society for Reproductive, 2012). Elective single embryo transfer (eSET) is now widely used in many IVF centers (Yokota, et al., 2001) (Maheshwari, et al., 2011, Veleva, et al., 2009). Therefore, the need for embryo freezing is ever increasing.

Along with the wide use of embryo freezing, technology about re-freezing of embryos has been commonly encountered in the IVF laboratory. If three embryos were frozen in the past cycle and two embryos are transferred in the present cycle, one embryo should be re-frozen. It has been concerned that re-frozen embryos would have great cryoinjury, low survival, and subsequent poor pregnancy success. The clinical outcomes after use of re-frozen embryos have been reported in several studies. There are three case reports with regards successful pregnancy or delivery after transfer of twice-frozen embryos in human (Farhat, et al., 2001) (Yokota, et al., 2001) (Smith, et al., 2005). In three retrospective comparative studies, pregnancy and live birth rate was similar between once-frozen and twice-frozen embryos (Kumasako, et al., 2009) (Murakami, et al., 2011) (Koch, et al.,

2011). However, studies evaluating the outcomes after transfer of three-time-frozen embryos are currently not available. In a mouse model, similar outcomes between once-frozen and twice-frozen embryos have been reported, but study evaluating the outcome of three-times-frozen embryo, subsequently analyzing of gene expression using real time PCR is lacking (Snabes, et al., 1993) (Nowshari and Brem, 2000, Sheehan, et al., 2006).

In a global trend of fewer embryo transfers, researches on survival and embryonic development after repeatedly frozen embryos are mandatory to ensure its safety. The present study is aimed to investigate the embryonic survival, development, and expressions of cryoinjury-related genes in once-frozen, twice-frozen, and three-time-frozen mouse preimplantation embryos.

# Materials and Methods

## Animals

Total number of 60 five-to-six-week old BDF-1 female mice (Orient Co., Seoul, Korea) were cared under a 12 hours light : 12 hours dark cycle at 23° C and fed ad libitum and used in this study in accordance with the institutional guidelines established by the Animal Care and Use Committee (IACUC) of the Seoul National University of Bundang Hospital.

## Retrieval of mature oocytes and in vitro fertilization

The mice were treated with intraperitoneal (ip) injection of 5 IU pregnant mare' s serum gonadotropins (PMSG; Daesung Microbiological Labs, Uiwang, Korea) followed by ip injection of 5 IU human chorionic gonadotrophin (hCG; Daesung Microbiological Labs) 48 hours later. After 16 to 20 hours following hCG administration, the mice were killed by cervical dislocation. Then the oviducts were dissected and placed in 1-mL of washing medium (modified mouse tubal fluid, mMTF) supplemented with 0.8% (w/v) bovine serum albumin (BSA; Sigma-Aldrich, St Louis, MO, USA). Cumulus oocyte complexes were released by tearing the ampulla of the oviducts. The cumulus cells were removed enzymatically using 85 IU/mL hyaluronidase (Cook, Brisbane, Australia) and by mechanical dissociation using a glass pipette. Only morphologically normal mature MII oocytes, as judged by the presence of a first polar body, were used in our study.

The epididymal sperms were retrieved from the cauda epididymis 12 number of 8-12-week-old BDF-1 male mice. The sperm suspensions were pre-incubated for

1.5 hours in capacitation medium (mMTF supplemented with 0.8% BSA). Then the oocytes were inseminated with sperms at a final concentration of 2 million/mL at 37° C in humidified 5% CO<sub>2</sub> in air. After 6 hours, the inseminated oocytes were washed twice by pipetting and transferred to the embryo culture medium (Global medium, Life Global, Guilford, CT, USA) supplemented with 0.4% BSA. Formation of 2-cell embryos was identified at 24 hours after insemination and considered as normal fertilization. Those cleaved embryos were transferred to new embryo culture medium, and their development up to 8-cell stage was evaluated at 48 hours after insemination. At this time, 8-cell stage embryos were randomly allocated to control and three experimental groups

### **Vitrification/warming and culture up to blastocyst**

Six hundred 8-cell stage embryos were obtained from 60 female mice and randomly assigned to control and three experimental groups (150 embryos per each group). 8-cell stage embryos were vitrified and warmed once, twice, and three times, respectively. Non-vitrified group served as control. In 'once vitrified' group, warming was performed one week later from the vitrification. In the present study, we designed the interval between vitrification and warming was one week for considering the time to experiment, as illustrated in Fig. 1. In other mouse embryo cryopreservation studies, the interval between vitrification and warming is various from several hours to several years (Nowshari and Brem, 2000, Snabes, Cota and Hughes, 1993). And also there are several studies about the outcomes of human embryo refreezing, the interval of storing embryos was from several months to years (Check, et al., 2001, Kumasako, Otsu, Utsunomiya and Araki, 2009)

Vitrification was performed by indirect vitrification method (Rapid-I kit, Vitrolife,

Goeteborg, Sweden). 8-cell stage embryos were suspended in equilibrium solution composed of basic medium (HEPES-buffered TCM-199 supplemented with 20% FBS), 7.5% ethylene glycol (EG) and 7.5% dimethyl sulfoxide (DMSO) for 5 minutes and moved to vitrification solution containing basic medium, 15% EG, 15% DMSO and 0.5 mol/L sucrose (Sigma-Aldrich) for 45 to 60 seconds at room temperature (RT). 8-cell embryos were placed onto a cryo-container (five embryos per each) and then plunged immediately into liquid nitrogen for storage. For warming, the cryo-containers were immersed directly in a 37° C warming solution (containing 1.0 mol/L sucrose in basic medium) for 1 minute. The warmed 8-cell embryos were transferred to 0.5 mol/L and 0.25 mol/L sucrose in basic medium for 3 minutes, respectively, and then washed twice with washing medium in basic medium.

Warmed embryos were transferred to culture medium at 37° C and maintained with 5% CO<sub>2</sub> in humidified air. Survival rate of the 8-cell embryos was assessed 1 hour after incubation on the basis of morphologic appearance of membrane integrity and discoloration of the blastomere. The surviving embryos were further cultured for 2 days in Global medium (Life Global) supplemented with 0.4% BSA. The obtained blastocysts were classified as early, mid, hatching, or hatched. In control group, the 8-cell stage embryos were not vitrified and continuously cultured up to 4 days after insemination.

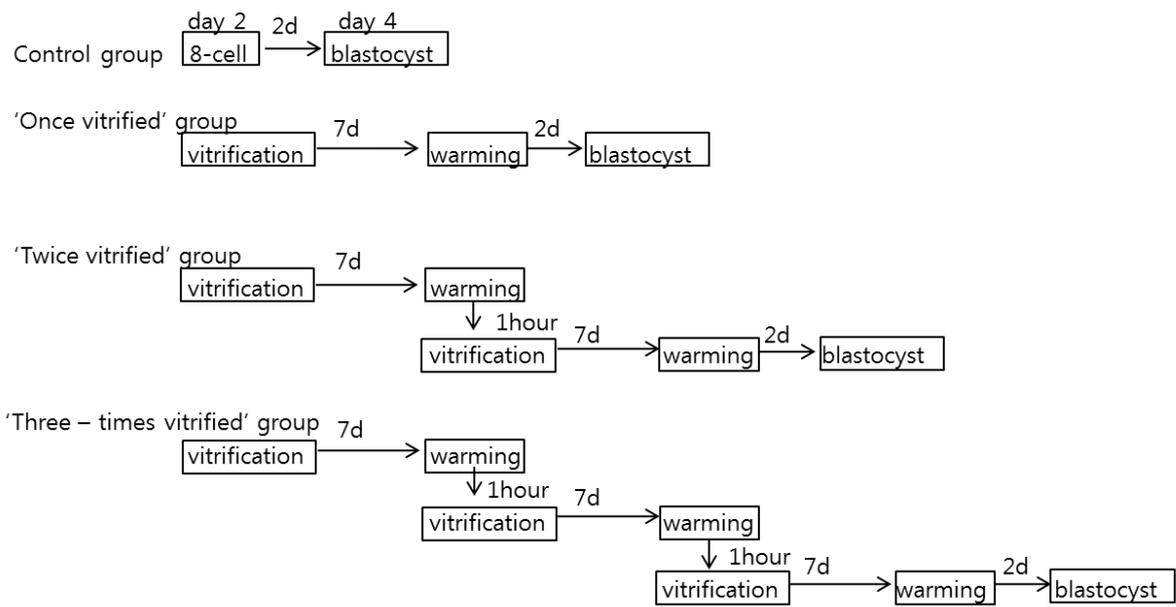


Figure 1. Experimental design

† d : day

## Blastocyst Cell Counts

Only hatching or hatched blastocysts were subjected to DAPI staining for cell count. And we assigned for 24 of hatching/hatched blastocyst to examine cell number in each group (Table 2).

The blastocysts were mounted onto slides under a coverslip in Vectorshield mounting medium (Vector laboratories, Burlingame, CA, USA) containing 0.5  $\mu\text{g}$  of DAPI. The localization of chromatin revealed by DAPI fluorescence was observed under 400X magnification with the use of a fluorescence microscope (Carl Zeiss AG; Oberkochen, Germany) with a Hamamatsu digital camera imaging system.

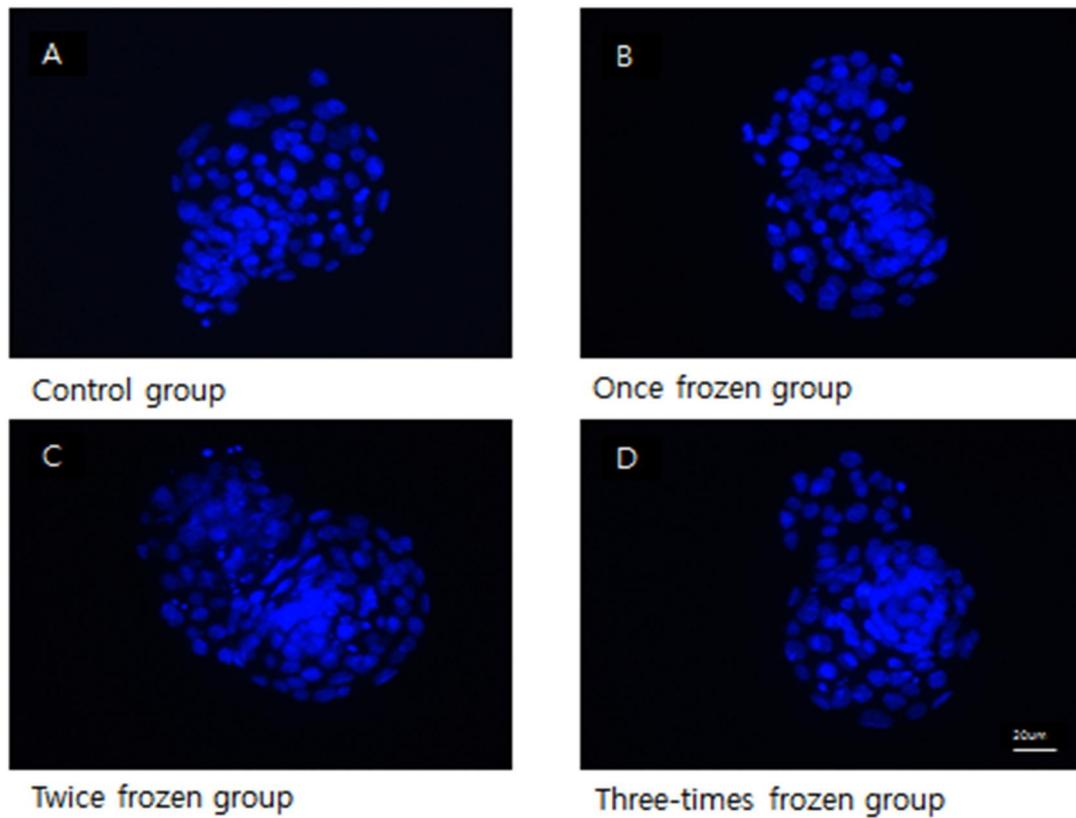


Figure 2. Microphotographs showing fluorescent staining of DAPI (4',6-diamidino-2-phenylindole staining) for cell count from repetitive vitrification – warming process (400X)

- (A) blastocyst from from control group
- (B) blastocyst from once frozen group
- (C) blastocyst from twice frozen group
- (D) blastocyst from three-times frozen group

## Real-time RT-PCR analysis

A part of hatching or hatched blastocysts were subjected to real-time quantitative RT-PCR (qRT-PCR) for quantification of cryoinjury-related genes. The number of hatching/hatched blastocyst submitted to real time qRT-PCR was 65–70 in each group. And the number of times of repeat was 6 in each group, respectively (Table 2). All samples were analyzed twice in each group, respectively.

All procedures were performed after pooling of ten blastocysts per each experimental set. Total RNA was extracted using the Trizol method and cDNAs were synthesized by the Suprime script RT premix (GeNet Bio, Daejeon, Korea) according to the manufacturer's instructions. Real-time qRT-PCR was performed with a 7500 Real Time PCR system with SYBR Green (Bioneer, Daejeon, Korea). Real-time qRT-PCR was carried out in a 20 mL reaction volume containing 10 mL Accupower 2X Greenstar qPCR Master Mix, 3 mL cDNA, and 5 mL distilled water. Specific primers were purchased from IDTDNA (Coraville, IO, USA) as listed in Table 1; cold-inducible RNA-binding protein (Cirbp) as cold-shock protein; caspase (Casp) 3 as an apoptosis marker; superoxide dismutase (Sod) 1, glutathione peroxidase (Gpx) 3, and catalase (Cat) as oxidative stress markers. Sequences for specific primers and the conditions are listed in Table 1. Real-time qRT-PCR was repeated two times and the values were averaged.

Data were analyzed by the comparative cycle threshold method in all experiments using Histone H2A.Z (H2afz) as endogenous reference genes (Lee, et al., 2016). And the RNA level of each target gene relative to reference gene was estimated for each sample presented as fold change as shown in previous study (Shirazi, et al., 2016) We performed quantitation of gene amplification to determine the cycle threshold ( $C_T$ ) that was based on fluorescence detected within the geometric region of the semilog amplification plot. All mRNA expressions were normalized to that of H2afz mRNA. The experiments were repeated at least 6 times using different sets of embryos.

**Table 1. Primer sequences and their condition for real-time quantitative RT-PCR**

Gene	Primer sequence	Product size (bp)	Accession number	Annealing temperature (° C)	Cycles
Cirbp	F: GTTGGTGTCTGAAGCTGAGT R: TTAGGAAGCTTGGGTGTGTG	127	NM_007705	60	45
Casp 3	F: GGACTGGATGAACCACGAC R: GACTGATGAGGAGATGGCTTG	124	NM_009810	60	45
Sod 1	F: GTCCTTTCCAGCAGTCACAT R: GGTTCACGTCCATCAGTATG	146	NM_011434	60	45
Gpx 3	F: CCCAGAATGACCAAGCCAA R: GCAGTATGCAGGCAAATATATCC	123	NM_008161	60	45
Cat	F: ATCTTCCTGAGCAAGCCTTC R: CAAGTTGGTTAATGCAGATGGAG	112	NM_009804	60	45
H2afz	F: CACTCTGGTGGATAAGCTCAA R: GACAATGTACTTGTTGCTGAGTG	105	NM_016750	60	45

Cirbp; cold-inducible RNA-binding protein, Casp 3; caspase 3, Sod 1; superoxide dismutase 1, Gpx 3; glutathione peroxidase 3, Cat; catalase, H2afz; Histone H2A.Z

## Statistical Analyses

To compare the proportion of developmental outcome, a Chi square test was used. Numeric data were presented as median [95% confidence interval] and the data in each experimental group were compared with the data in non-frozen control group by the Wilcoxon test. The data of relative expression of mRNA of five genes were analyzed by student t-test. All data were analyzed using statistical package for social science version 22.0 (SPSS, Chicago, IL, USA), and  $p < 0.05$  was considered statistically significant.

## Results

Data were prospectively collected from total of 600 8-cell stage embryo (150 embryos for each group). All embryos retrieved from the mouse were equally divided into four groups in accordance with the number of freezing – warming, using indirect vitrification methods.

### 1. Survival and development to blastocyst of frozen–thawed 8–cell embryos

The survival rates of embryo was 98.7%, 96.0%, 96.0%, respectively in once, twice, three–time frozen group. A total of 134/150 (89.3%) embryos were cultured to blastocyst as controls. And the blastocyst forming rate was 89.1, 90.3, 89.6%, respectively in the once, twice, three–time frozen experimental group. And the number of hatching/hatched blastocyst per blastocyst was 100/134 (74.6%), 95/132(72.0%), 99/130(76.2%), 95/129(73.6%) in the control, once, twice, three–time frozen group, respectively.

There was no significantly differences between the 3 experimental group compared to control group on embryo developmental outcomes. Ant the cell number of hatching/hatched blastocyst was not significantly different between 4 groups (86.0, 90.5, 81.5, 76.0/cell, respectively). Of those embryos surviving, the number of re–freezing and warming up to 3 times had no deleterious effect on survival rate, blastocyst formation rate, the percentage of hatching/hatched blastocyst including the cell number differences.

## 2. The gene expression of target genes.

In the current study, we analyzed cryoinjury related genes (Cirbp, Casp3, Sod1, Gpx3, Cat) using real time qPCR. We examined the expression of Cirbp, and Casp3 did not show significantly differences in the 3 experimental group compared to control group. In this study, we also analyzed the superoxide dismutase (Sod) 1, glutathione peroxidase (Gpx) 3, and catalase (Cat) as oxidative stress markers as antioxidant enzymes against ROS on mouse embryos. The Cat expression was significantly higher in the once vitrified group compared to control group ( $p < 0.05$ ). And the expressions of Sod 1 was significantly higher in the once frozen group ( $p < 0.01$ ) and in the three-time frozen group ( $p < 0.05$ ) than in the control group. And the expression of Gpx 3 was significantly different in the once, twice, and three-time frozen group compared to control group ( $p < 0.005$ ).

**Table 2. Developmental outcomes**

	Non-frozen control	Once frozen	Twice frozen	Three-time frozen	p-value
No. of 8-cell stage embryo	150	150	150	150	–
No. of survived embryo (survival rate)	–	148 (98.7%)	144 (96.0%)	144 (96.0%)	NS
No. of blastocyst (% per survived embryo)	134 (89.3%)	132 (89.1%)	130 (90.3%)	129 (89.6%)	NS
No. of hatching/hatched blastocyst (% per total blastocyst)	100 (74.6%)	95 (72.0%)	99 (76.2%)	95 (73.6%)	NS
No. of hatching/hatched blastocyst submitted to cell number	24	24	24	24	–
No. of cell in hatching/hatched blastocyst*	86.0 [74.8 – 96]	90.5 [80.2 – 110.8]	81.5 [75.2 – 91.8]	76.0 [71.2 – 96.6]	NS
No. of hatching/hatched blastocyst submitted to real time qRT-PCR	65	65	70	65	–

† P <0.05 when compared with the data in non-frozen control.

\*Median [95% confidence interval].

Table 3. The relative expression of mRNA of five genes in warmed 8-cell mouse embryos. Related expression levels are expressed as median.

	control	Once frozen	Twice frozen	Three-time frozen	p-value
Cirbp	4.55 [4.42-4.84]	4.38 [4.19-4.83]	4.48 [4.27-4.98]	4.55 [4.44-4.95]	NS
Casp3	5.77 [4.98-6.61]	6.49 [4.97-7.35.]	6.62 [4.90-9.33]	6.31 [4.39-7.12]	NS
Sod1	1.09 [0.97-1.77]	0.99** [0.74-1.52]	1.33 [1.10-1.79]	0.91* [0.80-1.69]	**, p < 0.01 *, p < 0.05
Gpx3	5.69 [5.48-5.91]	2.52*** [2.49-3.04]	3.16*** [2.94-3.48]	2.30*** [2.23-2.64]	***, p < 0.005
Cat	8.18 [6.91-8.54]	6.32* [7.75-8.05]	8.91 [6.47-10.61]	8.13 [6.36-9.77]	*, p < 0.05

All values are mean  $\pm$  SD.

† P < 0.05 when compared with the data in non-frozen control.

\*\*\*, p < 0.005

\*\* , p < 0.01

\* , p < 0.05

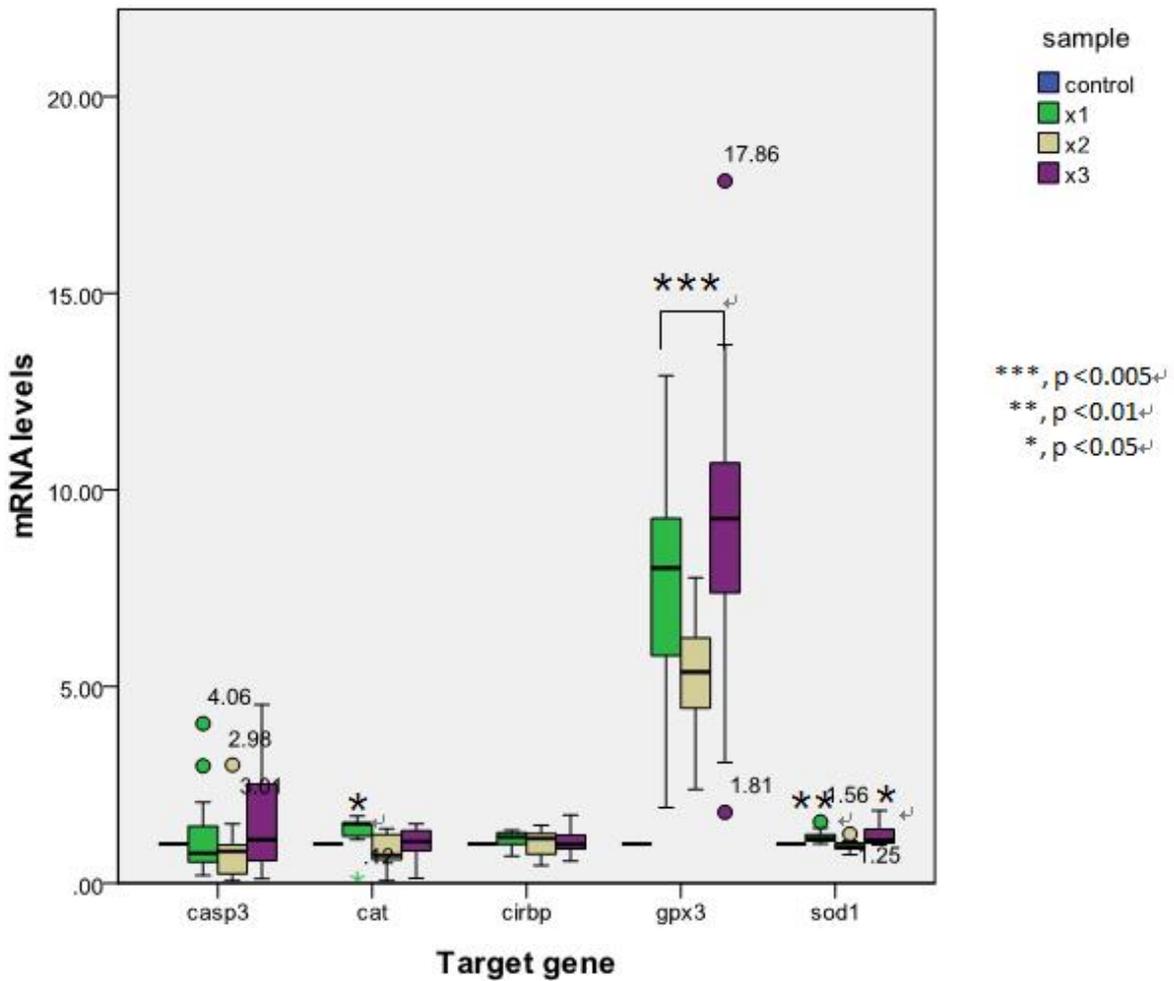


Figure 3. Box-and-Whisker plot showing the distribution of mRNA levels of target genes. In the Box-and-Whisker plot, the central box represents the values from the lower to upper quartile (25 to 75 percentile). The middle line represents the median. A line extends from the minimum to the maximum value, excluding extreme values (outliers) which are displayed as separate points. An extreme value is defined as a value that is smaller than the lower quartile minus 1.5 times the interquartile range, or larger than the upper quartile plus 1.5 times the interquartile range.

## Discussion

In previous mouse study, once frozen and twice frozen group showed a similar survival rate of warmed 8-cell embryo implantation rate, and number of live fetuses (Nowshari and Brem, 2000). However, there was no animal study to evaluate the developmental outcomes of three-time-frozen mouse embryos.

In the present study, once, twice, or three-times frozen 8-cell mouse embryos showed unequivocally high survival rate (96% – 99%). In three frozen groups, overall blastocyst-formation rates, the proportions of hatching/hatched blastocyst, and the blastomere numbers in hatching/hatched blastocyst were all similar when compared with non-frozen control. Therefore, repeated vitrification of 8-cell embryos even up to three times appears to be efficient to preserve their survival and developmental potential. But, it may need further experiment to validate the implantation rate following pregnancy or live birth rate.

The expressions of cryoinjury related genes were evaluated to investigate whether there is damage of repetitive freezing-warming process in 8-cell stage embryos. The cold injury-related genes, *Cirbp* is evolutionarily conserved RNA-binding proteins and expressed in response to temperature change. The mRNA level of *Cirbp* is elevated after mild cold stress (32 – 34° C) in mouse or human somatic cells including sertoli cells or germ cells (Danno, et al., 2000) (Zhu, et al., 2016). Thus the mRNA expressions of *Cirbp* in certain cells may be used as a surrogate marker for cryoinjury. If the mRNA expressions of *Cirbp* is elevated in certain frozen-thawed cells, it can be said that the cells are insulted by cryoinjury. We previously demonstrated that supplementation of necrostatin 1, an anti-necrosis protein, in in vitro maturation media could enhance survival rate after vitrification of subsequent in vitro matured mouse oocytes (Jo, et al., 2015).

And we evaluated the apoptosis-related genes Casp 3. Caspases are involved in the apoptotic cell death and expression of Caspase 3 are commonly examined for the detection of apoptosis of mouse cleavage stage embryo or blastocyst (Shin, et al., 2011), although caspase 8 and 9 are also commonly examined (Schulte, et al., 2015).

In aerobic organisms, reactive oxygen species (ROS) may originate from embryo metabolism and/or embryo surroundings as an early marker for toxicity evaluation , later inducing apoptosis (Dai, et al., 2015). ROS can alter cellular molecules or also induce developmental block and retardation. There are complimentary actions against ROS; the external protection is achieved by non-enzymatic antioxidants, and the internal protection mainly composed of antioxidant enzymes (Guerin, et al., 2001). The most important antioxidant enzymes are manganese SOD (MnSOD), copper zinc SOD (CuZnSOD), Catalase, and GPX (Salleh and Giribabu, 2014). Sod 1 plays a crucial role in protecting cells against ROS-induced oxidative damage (Blomberg, et al., 2005).

In assisted reproduction technology, many oocytes, sperm, or embryos are cryopreserved and many ROS can be generated during the process. The level of antioxidant enzymes may be used a marker for protection from ROS (Guerin, El Mouatassim and Menezo, 2001). Loss of antioxidant enzyme activity strongly suggest that oxidative stress occurred during and/or after cryopreservation, thus low level of Sod 1 may affect deleterious effect of cryopreservation on embryo viability or development.

In the present study, we can find that the significance of change of mRNA expression was found in Gpx 3. In previous study, Xiao et al., did experimental study using melamine as oxidative stress inducing material in mice. The expression of GPX was higher in low concentration of melamine than in the higher

concentration group in contrast to mRNA expression of SOD. In other study, Cheng et al., did experimental study focusing on ROS related injury on embryo development of deer. They analyzed of anti-oxidative enzymes (Sod, Gpx, Cat) mRNA expression and developmental outcome of embryos. They described that Sod are the first catalysts involved in the conversion of  $O_2$  into  $H_2O_2$ , and  $H_2O_2$  requires Gpx and Cat detoxification. They demonstrated that these enzymes have defense mechanism against damage involved by ROS (Cheng, et al., 2014)

Although there are various results depending on studies, we can assume that the changes of anti-oxidant enzymes reflect defense mechanism, and the significant elevation of Gpx 3 expression is also related with protective function of cryoprotectant agent compared to Cirbp.

Since no differences were found in the developmental outcomes, and the level of mRNA expression compared to housekeeping gene was higher (significantly or not) in the experimental group, it can be assumed that repeated vitrification of 8-cell embryos did not involved significant oxidative stress related damage harming the developmental outcomes.

The mRNA expression for Cirbp, Casp 3 were all similar among non-frozen control and frozen groups. It can be interpreted as that repeated vitrification of 8-cell embryos even up to three times does not cause significant cryoinjury. Since the mRNA expression levels of Casp 3 in three frozen groups were similar to non-frozen control, repeated vitrification of 8-cell embryos did not increase apoptotic events.

This is the first study to evaluate the efficacy of 3 times of repetitive freezing-warming of mouse 8-cell embryos, and also the first study using indirect (closed) vitrification method. There are concerns that there is slower cooling rates than direct (open) system, and it may be critical on embryo survival after freezing-

warming (Vajta, et al., 2015). The recent guideline of the American Society for Reproductive Medicine regarding human oocytes or embryos (2013) states that 'there also are theoretic infectious disease concerns with the use of open vitrification methods. And the guidelines of European Parliament (2004, 2006) has impelled scientist to look for solutions that would maintain vitrification in an aseptic status.

Mazur et al., demonstrated that cell death during vitrification procedure is not the ice crystal formation during cooling but the recrystallization during warming (devitrification). They also proved that warming rates are of equal or higher important as the cooling rates (Mazur and Seki, 2011, Seki and Mazur, 2009, Seki and Mazur, 2012). And there is previous studies indirect (closed) vitrification system is an efficient and safe method for both oocytes and embryo vitrification from potential harm outside environment, minimizing the potent risk of contamination although there is few reports of contamination related problem in open system, showing clinical efficiency compared to open system (Gook, et al., 2016, Jo, et al., 2013, Papatheodorou, et al., 2016)

In conclusion, repeatedly frozen mouse preimplantation embryos even up to three times could preserve good survival and embryonic development. The expression of mRNAs for cryoinjury-related markers indicates the safety of repeatedly frozen embryos at a molecular level. It may be a better study if we had the experiment proven long term outcomes, following the clinical pregnancy rate or live birth rate. More studies are needed to prove the efficacy of repetitive embryo re-freezing.

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국 문 초 록

## 반복적인 생쥐 배아 동결이

배아의 생존, 발달 및 동결 손상에

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### 목적

체외수정 시술 시 다태임신 등의 합병증을 예방하고자 2009년 개정된 미국 생식의학회/보조생식의학회의 가이드라인에 따라 이식배아의 개수를 최소한으로 제한하는 것이 추세이며 최근에는 단일배아 이식 및 남은 배아를 동결 보존하여 추후 이식하는 냉동배아 이식이 증가 추세이다.

과거에 동결 보존한 배아를 대상으로 동결보존배아 이식주기를 진행할 때는 한 vial 에 통상 3개씩의 배아가 보관되어 있어 3개의 배아를 한꺼번에 해동한 후 만일 1개나 2개의 배아만을 이식할 때는 나머지 배아를 재 동결해야 하는 상황이 발생한다. 이렇게 배아를 두 번 얼리게 되면 동결손상이 가중되어 배아의 생존능이나 향후 임신 성공율에 지장을 초래할 가능성이 있으나 이에 대한 국내 연구는 전무하다. 또한 재동결한 배아의 생존능이나 임신 예후에 관한 국외 보고로 증례보고, 후향적 비교 연구, 마우스 배아를 이용한 실험적 연구 등이 소수 보고되기는 하였지만 3회 이상의 동결 및 해동 배아를 대상으로 한 국내,외 보고 및 연구는 전무한 실정이다. 이 연구의 목적은 3회까지의 동결 손상이 생쥐의 배아에 미치는 영향에 관한 연구로, 생존능과 예후 및 안전성에 관련성을 살펴 보고자 함이다.

## 실험 방법

전향적 동물 실험 연구로, 본 연구에서는 생쥐의 8세포기 배아를 대상으로 1회 동결군, 2회 동결군 및 3회 동결군으로 나누어 배아 생존능과 발달능 및 동결손상 정도를 파악하고자, 생쥐는 BDF1 종을 이용하며 얼리는 단계에서 8-cell 배아 기준 배아수는 각 군 150개로 대조군을 포함한 총 4군으로 무작위 분배한다. 마우스 난자 및 정자 획득과 수정 및 이후 체외수정시술의 제반 과정은 본 연구진이 기 발표한 논문의 프로토콜을 따른다. 동결법은 널리 쓰이는 유리화 동결법을 적용하고, 간접 동결법인 Rapid-i kit 기구를 이용한다. 배아 생존능과 발달능은 포배기 배아 형성율, 세포수 및 caspase3 염색을 통한 apoptosis rate으로 평가하고 동결손상 정도는 동결손상과 연관된 mRNA 발현을 qRT-PCR 로 측정하여 평가한다. 타겟 유전자는 cold shock 의 지표인 Cirbp, apoptosis 의 지표인 Casp 3, 항산화효소로 알려진 Sod 1, Gpx 3, Cat 를

comparative cycle threshold method 를 이용하여 상대적 발현량을 분석하였다. Housekeeping gene 으로 H2afz 를 사용하였고, 각 실험은 6번씩 반복하였다.

## 결과

대조군 과 비교하여 1회, 2회, 3회 동결 실험군에서 survival rate 는 100% 에 가까운 생존율을 보였고, blastocyst 의 형성율(89.3, 89.1, 90.3, 89.6%, 각각), hatching/hatched blastocyst 의 비율(74.6, 72.0, 76.2, 73.6%, 각각), cell count (86.0, 90.5, 81.5, 76.0개/cell, 각각) 냉동의 횟수가 증가하여도 유의한 차이를 보이지 않았다.

Real-time qPCR 에서, cold injury 관련 유전자인 Cirbp 와 apoptosis 관련 유전자 Casp 3 의 mRNA 상대적 발현량은 실험군에서 대조군과 비교하여 유의한 차이나 변화를 보이지 않았다.

ROS 산화 관련 스트레스 관련 gene 인 Cat, Gpx, Sod1 은 실험군에서 대조군에 비하여 유의하게 상승되는 변화가 관찰되었다.

## 결론

8 - cell mouse embryo 를 세 번 얼리는 데 cryo-injury 를 받지 않았는지를 확인하기 위하여, 배아를 1회에서 3회까지 동결 보존하여 해동하여 developmental outcome 과 cryo-injury related gene 의 발현을 관찰하였다.

먼저, embryo survival rate, blastocyst formation rate, cell count 등은 결과적으로 동결 과정을 거쳐도 유의한 차이가 관찰되지 않았다. 또한 cryo-injury 를 반영하는 gene 의 발현과 관련하여서는, 유의한 변화가 관찰되지 않았거나, 배아 발달에 손상을 주지 않기 위하여 자체적인 보호 작용을 할 것으로 추정되는 항산화 작용과 관련한 gene 의 변화가 있었음을 관찰하였다

단일 배아 이식- 배아 동결 보존 기술의 향상으로 향후 다태 임신으로 야기되는 많은 합병증을 줄이면서도 건강한 단태아를 분만하는 것은 거스를 수 없는 세계적인 트렌드 이다. 이러한 여건에서 필수 불가결 적인 배아 동결 및 해동, 재동결 과정에서 손상이 유의하게 없다면 앞으로 난자 채취로 인한 난소 과자극 증후군 등의 문제를 줄이는 데에도 기여할 것으로 생각한다.

하지만 추가적으로 blastocyst 를 이식하여 추후 임신율과 분만의 outcome 과 관련한 실험을 추가적으로 진행하였다면 장기적인 영향을 보다 명확히 알 수 있었을 것으로 생각한다. 본 연구 결과는 향후 난임 분야에서 배아 이식 관련 가이드라인을 준수하는데 있어 의미 있는 정보를 제공하리라 생각된다.

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**주요어:** 배아 재동결, 간접 유리화동결법, 체외수정 기술, 동결 손상, 냉동 배아 이식

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