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Effect of droplet vitrification on mitochondrial membrane potential and developmental competence in two-cell mouse embryos

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The accelerated cooling rate associated with vitrification reduces injuries attributed to cryopreservation and improves the post-freezing developmental competence of vitrified embryos. In this study, embryos were vitrified and warmed and morphologically evaluated for their development to blastocysts. Survival rates between the fresh (96.7% ± 3.8%) and vitrified embryos (90.7% ± 5.1%) did not differ significantly ($P > 0.05$). The mitochondrial membrane potential of fresh control cells measured by 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanide iodide staining was similar to that of cryoprotected and vitrified embryos. Mitochondrial staining with rhodamine 123 did not differ among the fresh, cryoprotected, and vitrified embryos. Moreover, the distribution of H_2O_2, assessed by 2′,7′-dichlorodihydrofluorescein diacetate staining, did not differ among the groups. The results showed that the developmental rate did not differ significantly among the fresh (87.8% ± 11.3%), cryoprotected (83.2% ± 7.6%), and vitrified 2-cell embryos (75.8% ± 14.2%). The mean number of the inner cell mass (ICM), trophectoderm (TE), and apoptotic cells was counted and statistically compared, and although the number of ICM and TE was decreased in the cryoprotected and vitrified embryos, there were no significant differences among the groups ($P > 0.05$). During the cultivation period, randomly selected blastocysts from each group were stained using either 4′,6-diamidino-2-phenylindole and bisbenzimide or the terminal deoxynucleotidyl transferase mediated dUTP nick-end labeling technique. The incidence of apoptosis appeared to be almost identical in all the groups. Droplet vitrification could subsequently lead to high survival and developmental rates of cryopreserved mouse embryos.

Keywords: apoptosis; mitochondrial membrane potential; vitrification

Introduction

Vitrification is a process by which a liquid is solidified into a non-crystalline (glassy) phase by lowering the temperature and greatly increasing the viscosity. Vitrification relies on a fast cooling rate that does not result in the formation of ice crystals. The greatest obstacle to successful vitrification is that high concentrations of cryoprotectants are toxic to embryos at room temperature.

Vitrification depends on variables such as the type and concentration of cryoprotectants, the temperature of the vitrification solution at exposure, the duration of exposure, the developmental stage of the embryos, and the type of vitrification device (Liebermann et al. 2002), which can decrease the toxic effects of the cryoprotectant and improve the survival rate of embryos. Vitrification devices have been designed to ensure that small volumes of the sample are in an intimate contact with liquid nitrogen, thereby enabling the samples to achieve greater cooling rates and reach the glass state. The likelihood of fracture damage is lower in the case of flexible containers, such as a plastic straw or a polypropylene cryotube, than in the case of rigid containers (Rall 1987). A reduction in cryoprotectant volume (1–2 μl) has been achieved using the straw method (Selman and El-Danasouri 2002). This system has been successfully used to vitrify human embryonic stem cells (Reubinoff et al. 2001) and inner cell mass (ICM) cells of blastocyst-stage embryos (Desai et al. 2011). Leem et al. (2009) reported that the development-related gene group showed both up- and down–regulation expression patterns in mouse embryonic stem cells. Nevertheless, cryopreservation of embryos still causes significant morphological, and biochemical problems, including membrane damage (Pfaff et al. 2000), cellular organelle dysfunction (Saunders and Parks 1999), and cytoplasmic disorganization (Marti et al. 1997).

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Mitochondria, by virtue of their ability to generate adenosine triphosphate (ATP), have been known to play a central role in maintaining the normality of early mammalian development (Van Blerkom and Motta 1979). In early embryos of mice and humans, high- and low-polarized mitochondria have a distinct spatial distribution in the pericortical/subplasmalemmal and perinuclear domains, respectively (Van Blerkom et al. 2002). Changes in spatial distribution or polarity detected in thawed embryos (Ahn et al. 2002) have been associated with developmental incompetence or abnormalities. Differences in mitochondrial membrane potential ($\triangle\Psi$) detected within and between cultured cells have largely been thought to reflect corresponding differences in mitochondrial function or levels of activity (Dedov and Roufogalis 1999).

Oxidative stress induces mitochondrial damage (Kowaltowski and Vercesi 1999). Reactive oxygen species (ROS) have been implicated in the impaired development of mammalian embryos *in vitro* (Johnson and Nasr-Esfahani 1994), and an increase in ROS has been associated with the two-cell embryo block observed in mouse embryos only after *in vitro* culture (Noda et al. 1991). H_2O_2 is a mediator of apoptosis in blastocysts (Pierce et al. 1991), which appears to be related to the appearance of cytoplasmic fragments in blastocysts (Yang and Rajamahendran 1999). In assisted reproduction technology, the freeze-thaw process sensitizes cells to ROS. ROS-associated modification of membrane lipids and the resulting spatial modifications of membrane structures may lead to cryodamage.

Various methods of ultra-rapid vitrification of mammalian embryos have been used, including vitrification in droplets (Riha et al. 1991), in open pulled straws (Vajta et al. 1998), on solid surfaces (Dinnyes et al. 2000), and on cryotops (Zhao et al. 2007). We evaluated the effect of the droplet vitrification method on mitochondrial membrane potential, apoptosis, and developmental competence in two-cell-stage mouse embryos.

Materials and methods

Two-cell embryo collection

All experiments involving animals were approved by and performed in strict accordance with the guidelines of the appropriate institutional animal care and use committees. Superovulation was induced in female imprinting control region (ICR) mice aged 6–8 weeks by injecting 7.5 IU pregnant mare serum gonadotropin (PMSG; Dae Sung Microbiological Labs Co., Ltd., Korea), followed by an injection of 7.5 IU human chorionic gonadotropin (hCG; Dae Sung Microbiological Labs Co., Ltd.) after 48 h. These females were then mated with males of the same strain and examined for the presence of a vaginal plug on the following day. The females with vaginal plugs were considered pregnant and were killed 48 h post-hCG injection by cervical dislocation. Two-cell embryos were flushed from the oviducts by using Quinn's medium (Sage In-Vitro Fertilization Inc., Trumbull, CT, USA). Two-cell embryos that were considered normal under microscopic observation were chosen for vitrification.

The embryos were washed with Quinn's medium supplemented with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and cultured in 1 ml of Quinn's medium containing 10% serum protein substitute (SPS) (Sage In-Vitro Fertilization Inc.) in a humidified incubator with 5% CO_2 at 37°C. Embryos were examined after 24, 48, and 72 h to monitor development and grade embryos, and those showing signs of fragmentation or delayed development were excluded from the study.

Preparation of pull and cut straws

Pull and cut (PNC) straws were made by making some modifications to the method described by Vajta et al. (1997). Briefly, the 0.25-ml plastic straws (I.V.M., L'Aigle, France) were heat-softened and pulled manually. The pulled straws were cut at the tapered end with a blade. The inner diameter of the tip was 0.1 mm and the wall thickness was approximately 0.05 mm.

Vitrification solutions

Equilibrium solution (ES) used in vitrification was prepared by dissolving 7.5% dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO, USA) (v/v), and 7.5% ethylene glycol (EG; Sigma), in phosphate-buffered saline (PBS) containing 10% SPS. The vitrification solution (VS) consisted of 15% (v/v) DMSO, 15% (v/v) EG, 0.5 M sucrose, and 10% SPS in PBS. Thawing solutions were 1 M and 0.5 M sucrose (Sigma) and PBS containing 10% SPS.

Vitrification and warming

Embryos were incubated in ES for 10 min before being transferred to VS for 30 s; then, they were loaded into a PNC straw and plunged into liquid nitrogen (LN_2) in a vertical position. The embryos were retained in LN_2 for at least 1 week.

The using PNC straw was warmed by removing it from the LN_2 and immediately placing it in a 37°C bath for 5 s. Using a pulled glass pipette, the embryos retrieved after warming were placed in a 100-µl droplet of warming medium containing 1 M sucrose for 1 min and then in 0.5 M sucrose for 3 min, before being

washed in the washing medium (10% SPS in PBS) two times for 5 min. An embryo was considered morphologically normal in the absence of any signs of abnormal shape, membrane damage, leakage of cellular content and fragmentation, or degeneration of the cytoplasm.

During the experiment, fresh and cryoprotected groups of embryos were cultured separately. The fresh embryos were not exposed to VS or LN_2. The cryoprotected embryos were subjected to all treatments that the vitrified embryos were subjected to, but they were not exposed to LN_2. Ten replicates of the experiment were performed.

Fluorescent staining of embryos to determining mitochondrial $\triangle \Psi$ and viability

The mitochondrial $\triangle \Psi$ of the embryos was measured by 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanide iodide (JC-1; Molecular Probes, Eugene, OR, USA) staining (Crossarizza et al. 1994). Embryos were rinsed with and incubated in a cleavage medium containing 5 μg/ml JC-1 for 15 min at 37°C in the dark. After washing with the cleavage medium, the embryos were examined using an epifluorescence microscope (Axioskop2; Carl Zeiss, Germany).

Viable mitochondria were detected using rhodamine 123 staining. Embryos were incubated with rhodamine 123 solution (Molecular Probes) at a concentration of 10 μg/ml in the cleavage medium (Sage In-Vitro Fertilization Inc.) at 37°C for 15 min in the dark. After washing with the cleavage medium, the embryos were observed at a wavelength of 510 nm by using a confocal imaging system (Model FV300; Olympus, Japan).

The H_2O_2 level in an embryo was measured by making a minor modification to the method of Nasr-Esfahani and Johnson (1991). Two-cell embryos were incubated in cleavage medium supplemented with 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (DCHFDA; Molecular Probes) for 15 min at room temperature. The embryos were then washed in the cleavage medium to remove traces of the dye. Fluorescence emission from the embryos was observed using confocal microscopy (wavelength, 510 nm).

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay

Apoptotic cells in the blastocysts were detected using the In Situ Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany). Fresh and vitrified blastocysts were washed three times with 0.1% polyvinylpyrrolidone in PBS and fixed in 4% (v/v) paraformaldehyde diluted in PBS for 1 h at room temperature. For membrane permeabilization, the fixed embryos were incubated in PBS containing 0.1% (v/v) Triton X-100 for 1 h at 4°C. The fixed embryos were incubated in the TUNEL reaction medium for 1 h at 37°C in the dark. They were then washed and transferred into 2 mg/ml 4′,6-diamidino-2-phenylindole (DAPI). As a positive control, fixed embryos were incubated in the dark in 50 μl of PBS containing 5 μl of RQ1 RNase-free DNase (Promega, Madison, WI, USA) for 30 min at 37°C before conducting the assay. Whole-mount embryos were stained using the TUNEL assay and with DAPI and were then examined under an epifluorescence microscope (Axioskop2; Carl Zeiss). We then determined the number of apoptotic nuclei and the total number of nuclei.

Counting the number of nuclei

The number of nuclei in all blastocysts that developed from fresh, cryoprotected, and vitrified embryos was determined. Briefly, the blastocysts were fixed in 1% formalin solution for about 10 min at room temperature. The blastocysts were then placed on slides with a drop of mounting medium consisting of glycerol and Dulbecco's PBS (DPBS; 3:1) containing 2.5 mg/ml sodium azide and 2 mg/ml Hoechst 33342 (Sigma). Blue and red colors were indicative of ICM and trophectoderm (TE) cells, respectively (data not shown). The number of nuclei in the embryos was counted using an epifluorescence microscope (Axioskop2; Carl Zeiss) at 200 × and 400 × magnifications.

Statistical analysis

All experiments were repeated at least 10 times. All percentage data and data sets obtained in this study were expressed as mean ± standard deviations (SD). The results were analyzed using Student's *t* test and two-way analysis of variance (ANOVA) using the general linear models procedure of the Statistical Analysis System (Cary, NC, USA). A *P* value of <0.05 was considered statistically significant.

Results

Post-vitrification survival and development

When presumptive two-cell mouse embryos were vitrified using the droplet vitrification method, a high number of embryos and morphologically normal embryos were recovered after warming (Figure 1). The survival rates of the vitrified, cryoprotected, and fresh embryos did not differ significantly. The recovered intact embryos were cultured *in vitro* for 72 h until the blastocyst stage. The percentage of two-cell embryos that developed into blastocysts (75.8% ± 14.2%) in the vitrified group was lower than that in the fresh

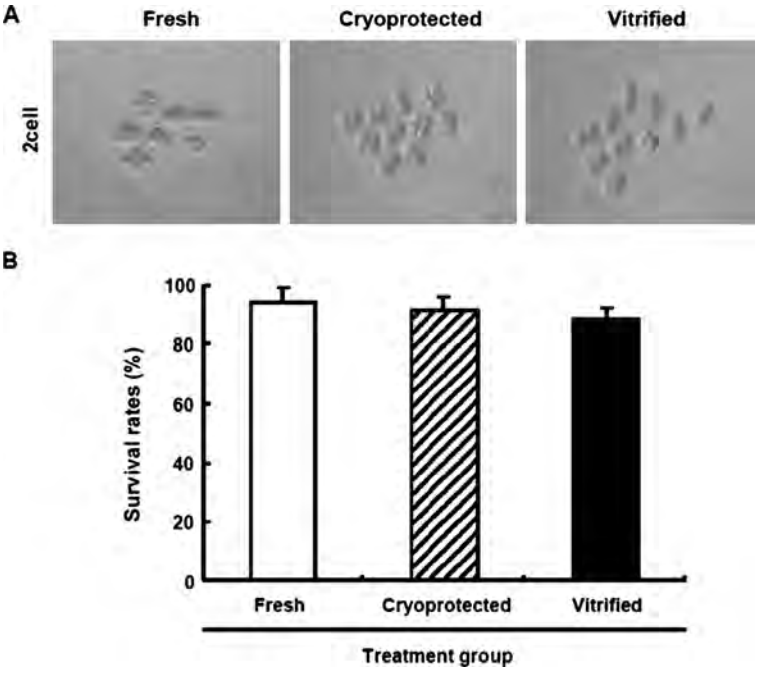

Figure 1. *In vitro* survival rates of 2-cell mouse embryos after vitrification. (A) Images of different 2-cell embryos. (B) Morphological survival was positive if an embryo possessed an intact zona pellucida, plasma membrane, and refractive cytoplasm.

group ($87.8 \pm 11.3\%$), but this observed difference in the percentage of blastocysts was not significant ($P > 0.05$) (Table 1). Thus, no difference in preimplantation development was detected between the fresh and vitrified embryos.

Allocations of ICM versus TE cells and occurrence of apoptosis in blastocysts

In the next experiment, blastocysts were individually stained by performing differential staining method, and then the total cell number was determined. The total cell number of blastocysts derived from vitrified embryos (49.4 ± 2.7) was not different from that of blastocysts derived from fresh embryos (51.3 ± 0.8; Table 1).

Apoptotic cells were identified on the basis of dUTP incorporation at the site of DNA strand breaks, which is a hallmark of apoptosis. The fixed blastocysts were also counterstained with DAPI to determine the DNA pattern in the nucleus. Through these methods, the total cell number and incidence of cell death were determined for both the fresh and vitrified embryos. The total number of cells per embryo and the proportion of cells with fragmented DNA were assessed. The average number of cells per blastocyst was higher in the vitrified blastocysts than in the fresh blastocysts, but this difference was not statistically significant ($P > 0.05$). Low levels of apoptosis were observed in both the fresh and vitrified groups. The incidence of apoptotic cell death in the cryoprotected and vitrified groups was similar to that in the fresh group (Table 2).

Table 1. Blastocyst formation rates of vitrified and warmed 2-cell mouse embryos

Group	Number of 2-cell embryos examined	% of blastocysts	Number of nuclei		
			ICM	TE	Total
Fresh	23	87.8 ± 11.3 (20)	20.9 ± 0.8	30.5 ± 1.5	51.3 ± 0.8
Cryoprotected	35	83.2 ± 7.6 (25)	20.4 ± 0.5	27.8 ± 1.4	48.3 ± 1.1
Vitrified	33	75.8 ± 14.2 (25)	20.3 ± 1.6	29.0 ± 1.1	49.4 ± 2.7

Data are shown as the mean $\pm$ SD. No statistically significant differences were found using two-way ANOVA and Student's *t* test. Two-cell embryos were cultured *in vitro* for 72 h to yield blastocysts. ICM: inner cell mass; TE: trophectoderm.

Table 2. Cell death in blastocysts produced after vitrification of 2-cell mouse embryos

Group	% of blastocytes examined	Number of nuclei	
		DAPI	TUNEL
Fresh	87.8±11.3 (20)	44.5±5.6	3.3±1.9
Cryoprotected	83.2±7.6 (29)	41.7±5.4	5.1±1.5
Vitrified	75.8±14.2 (25)	46.4±5.7	3.1±1.8

Data are shown as the mean±SD. No statistically significant differences were found using two-way ANOVA and Student's *t* test. DAPI: 4′,6-diamidino-2-phenylindole; TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling.

Effect of vitrification on mitochondrial △Ψ and viability in two-cell mouse embryos

The fresh, vitrified, and cryoprotected embryos were stained with JC-1 to detect changes in mitochondrial △Ψ. The red to orange color represents a high mitochondrial △Ψ, whereas green represents a low mitochondrial △Ψ or depolarization. The staining patterns for mitochondrial △Ψ in the fresh, cryoprotected, and vitrified embryos are shown in Figure 2. In all groups, high mitochondrial △Ψ was distributed peripherally along the cell membrane.

The presence of H_2O_2 in individual embryos was assessed by performing fluorescent staining with DCHFDA. Within the cells, DCHFDA is hydrolyzed to form 2′,7′-dichlorodihydrofluorescein (DCHF), which fluoresces on oxidization by H_2O_2 to yield 2′,7′-dichlorofluorescein (DCF). The level of DCF produced is linearly related to the H_2O_2 content of the cells (Nasr-Esfahani and Johnson 1991). A typical fluorescence image of the two-cell mouse embryos is shown in Figure 3. Green fluorescence indicates the presence of H_2O_2. The distribution of H_2O_2 among the vitrified, cryoprotected, and fresh embryos was not different. In the warmed two-cell embryos, viable mitochondria were visualized on rhodamine 123 staining. The staining results showed that mitochondria were evenly distributed throughout the cytoplasm in the fresh, cryoprotected, and vitrified embryos (Figure 4).

Discussion

Vitrification, a relatively simple cryopreservation technique, is now considered a promising technical innovation. Previous reports using different ultra-rapid vitrification methods, including droplet vitrification, have provided encouraging results. The major disadvantages of vitrification were the relatively large volume of the vitrified drop and the delay before the the drop floating on the surface of liquid nitrogen sank; this probably reduced the actual cooling rate (Vajta and Kuwayama 2006).

Most published successful vitrification protocols utilized vitrification solutions containing DMSO and EG in combination at concentrations of up to 15% for each cryoprotectant (Desai et al. 2007). We used EG and DMSO-based vitrification solutions in an attempt to reduce cryodamage. EG has been reported to have low embryonic cytotoxicity (Kuleshova et al. 1999), and diffuses in and out of the embryos very rapidly because of its low molecular weight; hence, embryos may undergo less osmotic stress during vitrification and warming. The polar nature of DMSO and its small and compact structure allows it to penetrate living tissues rapidly without causing significant damage. As expected, both the cryoprotected and vitrified embryos achieved high developmental competence. The rates of blastocyst formation and apoptosis of the vitrified embryos were similar to the rates reported by a previous study that used open pulled straw vitrification method to cryopreserve pronuclear mouse embryos (Zhou et al. 2005).

Arav et al. (2002) have reported that decreasing the vitrification volume minimizes the probability of glass fracture and suggested that fractures appear only when the volume is above 1 μl. We have eliminated these

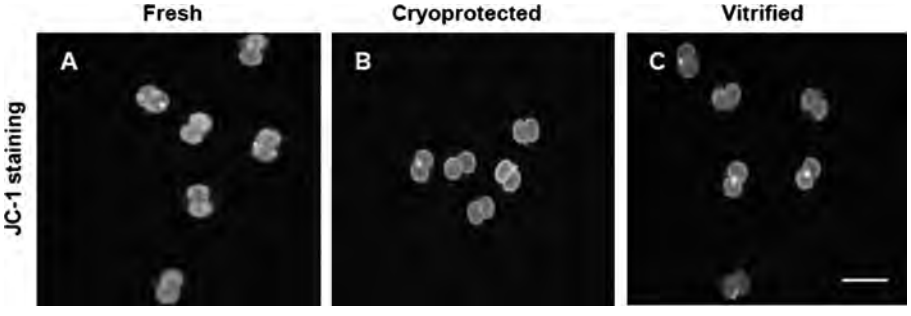

Figure 2. Evaluation of mitochondrial membrane potentials. JC-1 staining was used to assess mitochondrial membrane potential. After vitrification and warming, the 2-cell mouse embryos were stained with JC-1. Representative microscopic images of the fresh (A), cryoprotected (B), and vitrified (C) embryos. The scale bar represents 100 μm.

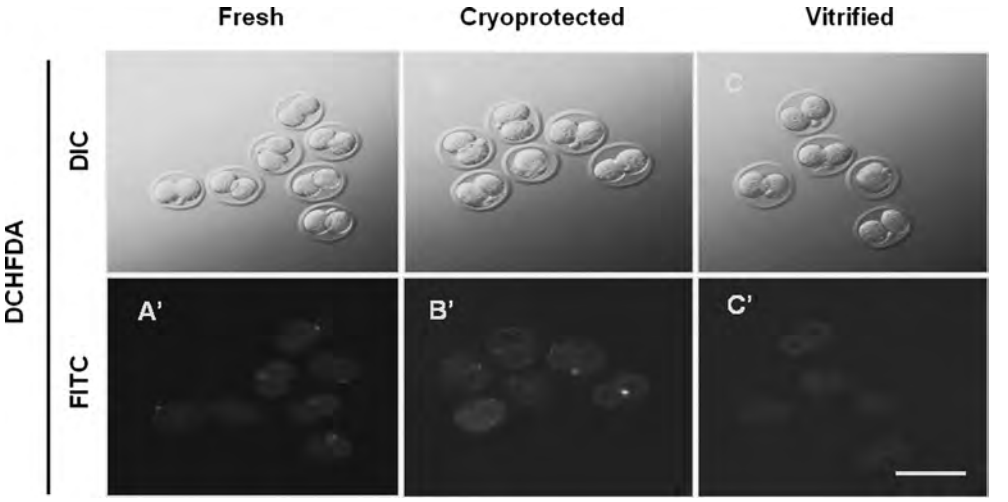

Figure 3. Confocal images of 2-cell mouse embryos. Two-cell mouse embryos were stained with 2′,7′-dichlorodihydrofluorescein diacetate (DCHFDA) to visualize the amount of H_2O_2 within the cells. Confocal images were taken at the same time, magnification $\times 1000$, and parameter settings to compare the fluorescence intensities in the fresh (A and A′), cryoprotected (B and B′), and vitrified (C and C′) groups. The scale bar represents 100 μm.

disadvantages and tried to achieve a higher cooling rate by reducing the volume of the vitrified drop and by immediately immersing it into LN_2. In the present study, the survival rates of the vitrified, cryoprotected, and fresh embryos were not significantly different. The effect of the embryo stage on overcoming cryopreservation procedures is related to cell number (Saha et al. 1996) and kinetic development (Dinnyes et al. 1999). Despite the high survival rate, the blastocyst formation rate of vitrified embryos was still lower than that of the fresh group. This finding may be attributed to the fact that a minor fraction of the embryos is of poorer developmental quality or because some of the embryos are at a developmental stage at which they cannot tolerate cryoinjury.

Total cell number and the ICM/TE ratio are well-established parameters for evaluating blastocyst developmental competence (Thouas et al. 2001). In this study, we differentially stained late blastocysts to analyze implantation potential and observed vitrified and warmed blastocysts. The total cell number, ICM, and TE of the vitrified group were not significantly different from those of the fresh group.

In this study, we documented the effect of the droplet vitrification method on mitochondrial $\triangle\Psi$, viability, and developmental competence of two-cell mouse

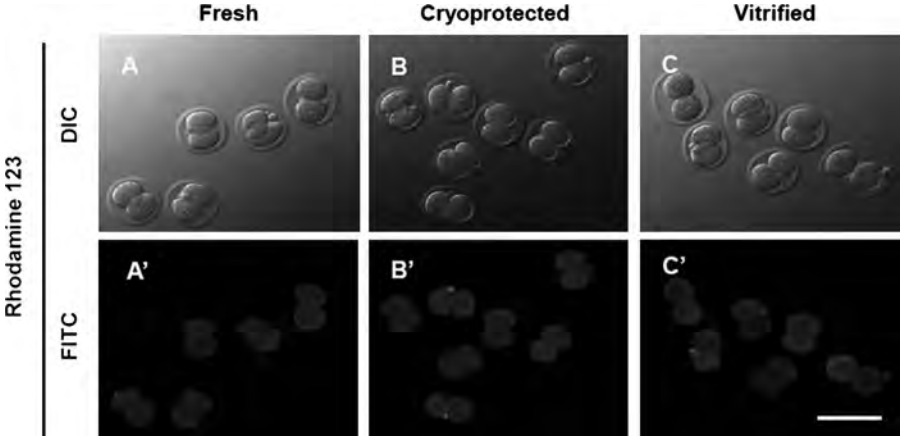

Figure 4. Distribution of viable mitochondria. Viable mitochondria in 2-cell mouse embryos after vitrification and warming were labeled with rhodamine 123. The confocal images of embryos in the fresh (A and A′), cryoprotected (B and B′), and vitrified (C and C′) groups were taken at 1000 × magnification. The scale bar represents 100 μm.

embryos cultured *in vitro*. Similar mitochondrial $\triangle\Psi$ staining patterns were observed in the vitrified and fresh embryos, clearly indicating that our vitrification method did not induce detectable mitochondrial damage in two-cell mouse embryos. In general, all examined blastocysts showed low incidence of apoptosis, possibly because appropriate volumes and a flexible container may have reduced the potential damage caused by mitochondrial redistribution and depolarization.

ROS are able to diffuse through cell membranes and alter most types of cellular molecules such as lipids, proteins, and nucleic acids. This alteration results in multiple consequences, including mitochondrial alterations, embryo cell block, ATP depletion, and apoptosis. ROS-induced apoptosis in embryos has been attributed to changes in mitochondrial functions (Liu et al. 2000). In the present study, no obvious differences in mitochondrial $\triangle\Psi$, H_2O_2 presence, and the distribution of viable mitochondria were observed between vitrified two-cell embryos and fresh embryos. This finding suggests that the use of appropriate vitrification volumes that leads to apparent vitrification during cooling and warming can minimize cryodamage. In conclusion, droplet vitrified two-cell embryos had high post-warming survival and blastocyst formation rates. Interestingly, the mitochondrial $\triangle\Psi$ and viability patterns in the vitrified two-cell embryos were similar to those in the fresh embryos. In addition, the total cell number and apoptosis of the vitrified embryos were not significantly different from those of the fresh embryos. The present findings suggest that the reduction of sample volume may help reduce the cryoprotectant concentration and accelerate the cooling rate, thereby decreasing toxicity and minimizing osmotic shock. Proteomic and metabolomic analysis may provide more information in the relation between embryo quality and the developmental potency of the vitrified embryos.

Acknowledgements

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