Molecules and Cells

Maintained MPF Level after Oocyte Vitrification Improves Embryonic Development after IVF, but not after Somatic Cell Nuclear Transfer

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Levels of maturation-promoting factor (MPF) in oocytes decline after vitrification, and this decline has been suggested as one of the main causes of low developmental competence resulting from cryoinjury. Here, we evaluated MPF activity in vitrified mouse eggs following treatment with caffeine, a known stimulator of MPF activity, and/or the proteasome inhibitor MG132. Collected MII oocytes were vitrified and divided into four groups: untreated, 10 mM caffeine (CA), 10 μM MG132 (MG), and 10 mM caffeine +10 μM MG132 (CA+MG). After warming, the MPF activity of oocytes and their blastocyst formation and implantation rates in the CA, MG, and CA+MG groups were much higher than those in the untreated group. However, the cell numbers in blastocysts did not differ among groups. Analysis of the effectiveness of caffeine and MG132 for improving somatic cell nuclear transfer (SCNT) technology using cryopreserved eggs showed that supplementation did not improve the blastocyst formation rate of cloned mouse eggs. These results suggest that maintaining MPF activity after cryopreservation may have a positive effect on further embryonic development, but is unable to fully overcome cryoinjury. Thus, intrinsic factors governing the developmental potential that diminish during oocyte cryopreservation should be explored.

Keywords: caffeine, maturation-promoting factor, MG132, mouse oocytes, vitrification

INTRODUCTION

Cryopreservation of mammalian oocytes has been successfully applied for long-term storage of surplus eggs in mammalian assisted reproductive technology (ART), including in humans. During application of a slow cooling process, which is traditionally used to enhance the efficiency of cryopreservation, it is important to reduce ice crystal formation through dehydration of intracellular water in oocytes by short exposure to high concentrations of cryoprotectant agents (CPA) (Bernard and Fuller, 1996). Vitrification, an alternative cryopreservation tool, has recently been shown to prevent ice formation and involves the use of a high concentration of CPAs and extremely high cooling rate. This method has replaced the slow freezing protocol and shows high survival rates and favorable clinical outcomes. However, this technology also may have several limitations, such as the risk of contamination by external pathogens because of direct contact with liquid nitrogen and increased cell toxicity due to

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high CPA concentrations. To overcome these weaknesses, researchers have developed numerous cryo-devices and modified CPA systems.

Another drawback of oocyte vitrification is diminished developmental potential because of the decreased quality of embryos after warming. Indeed, for cryopreservation, oocytes should be exposed to multiple CPAs and allowed extra incubation and recovery time. Maturation-promoting factor (MPF), a complex formed by the interaction of cyclin B1 with cyclin-dependent kinase, plays an essential role in cell cycle regulation, maintaining the meiotic arrest of oocytes and cell division after fertilization (Dupre et al., 2002; Labbe et al., 1989; Pines, 1999; Uchida et al., 1996). Moreover, mitogen-activated protein kinase (MAPK), which is responsible for initiating germinal vesicle breakdown, is essential for maintaining the meiotic arrest of oocytes at the MII stage (Tripathi et al., 2010; Van Blerkom, 1989). Although it has been reported that MPF activity is decreased in MII ovine oocytes after vitrification, which may compromise oocyte development after *in vitro* fertilization (IVF) (Ariu et al., 2014; Succu et al., 2007), the activity of MAPK after vitrification has not been throughly investigated.

Caffeine is widely used to increase MPF and MAPK activities, and it has been reported that treatment of ovine and porcine oocytes with caffeine increases MPF activity (Lee and Campbell, 2006; Kikuchi et al., 2000; Kim et al., 2013; Kwon et al., 2008). In oocytes, caffeine induces the dephosphorylation of MPF by inhibiting the transcription factor Myt1 and activity of the G2 checkpoint kinase Wee1, converting inactive MPF into its active form. Caffeine is also well known to maintain MAPK activity (Kikuchi et al., 1999; 2000; Lee et al., 2006; Prather and Racowsky, 1992), and has been reported to prevent premature aging of oocytes (Zhang et al., 2011). In this latter context, exposure of monkey or human fresh oocytes to caffeine was shown to improve embryonic development following somatic cell nuclear transfer (SCNT) (Mitalipov et al., 2007; Tachibana et al., 2013). In addition, Ono et al. (2011) showed that the proteasome inhibitor MG132 maintains MPF activity, reporting that the addition of MG132 to aged mouse oocytes improves oocyte competence. Thus, the objective of the present study was to analyze the effects of supplementing CPA solutions with caffeine and/or MG132 to maintain MPF activity on the embryonic development of vitrified/warmed MII oocyte-derived mouse embryos after IVF and SCNT.

MATERIALS AND METHODS

Approvals of animal experiment

The protocols for the use of animals in these studies were approved by the Institutional Animal Care and Use Committee (IACUC) of CHA University (Project No. IACUC-160054) and all experiments were carried out in accordance with the approved protocols.

Animals

Six-week-old female and 12-week-old male B6D2F1 mice (Orient-Bio Inc., Korea) were kept under controlled temperature and light conditions (12 h light and 12 h dark) with free access to food and water.

Collection of mature oocytes

Female mice were superovulated by intraperitoneal injection of 5 IU pregnant mare serum gonadotropin (PMSG; Sigma-Aldrich, USA), followed after 48-50 h by injection of 5 IU human chorionic gonadotropin (hCG; Sigma-Aldrich). Cumulus-enclosed oocytes were retrieved and collected from oviducts 13-15 h after administering hCG, and cumulus cells were removed from eggs after treatment with 0.1% hyaluronidase (Sigma-Aldrich). Denuded eggs were maintained in human tubal fluid (HTF; Quinn, 1995) media containing 10% Knock-out Serum Replacement (KSR) (Gibco, USA) under mineral oil (Sage *In vitro* Fertilization, Inc., USA) at 37℃ in a humidified 5% CO_2 incubator.

Vitrification procedure and experimental groups

The vitrification procedure was based on the method described by Cha et al. (2011). Quinn's Advantage Medium (with HEPES; Sage) supplemented with 20% (v/v) KSR was used as the base medium for vitrification and warming solution. The combination of ethylene glycol (EG; Sigma-Aldrich) and dimethylsulfoxide (DMSO; Sigma-Aldrich), included in the vitrification solution, were used as CPAs. MII oocytes were pre-equilibrated with 1.3 M EG and 1.1 M DMSO for 2.5 min, and then equilibrated with 2.7 M EG, 2.1 M DMSO and 0.5 M sucrose (Sigma-Aldrich) for 20 seconds. All processes were performed at 37℃. Ten oocytes were loaded onto the electron microscopic (EM) cooper grids (PELCO, USA) and plunged into slush nitrogen (SN_2), produced in the LN_2 chamber of Vit-master (IMT, Ness Ziona, Israel) by applying negative pressure. Vitrified oocytes were kept in the LN_2 tank until application of a 4-step warming process in which oocyte-loaded EM grids were sequentially transferred to warming solution containing 0.5, 0.25, 0.125 and 0 M sucrose, respectively, 2.5 min interval at 37℃.

The optimal concentration of caffeine for use in the vitrification solution was determined by monitoring the survival and embryonic development of vitrified oocytes after cryopreservation. MII oocytes were collected and vitrified under four experimental conditions: untreated (control), 10 mM caffeine-supplemented (CA, Sigma-Aldrich), 10 μM MG132-supplemented (MG, Calbiochem), and 10 mM caffeine +10 μM MG132-supplemented (CA+MG). Fresh oocytes were included for comparison. Warmed oocytes were further washed with fresh medium and incubated in each experimental medium for an additional 2 h prior to insemination or enucleation.

Assay of MPF and MAPK activity after vitrification and warming

MPF assays were performed according to the method described by Lee et al. (2012), with some modifications based on instructions of the MESACUP Cdc2/Cdk1 Kinase Assay Kit (MBL, Japan). Thirty fresh, vitrified/warmed oocytes from the four groups (Control, CA, MG, and CA+MG) were prepared for each sample. Oocytes were washed several times in 0.1% polyvinylpyrrolidone (PVP) in Dulbecco's phosphate-buffered saline (dPBS), then placed into 5 μl of cell lysis buff-

er (PRO-PREP, iNtRON Biotechnology, Korea) and stored at -80℃ until analyzed. Samples were mixed with 45 μl of kinase assay buffer A containing 25 mM HEPES (pH 7.5, 10 mM $MgCl_2$), 10% biotinylated MV peptide (SLTSSSPGGATC), and 0.1 mM ATP. The mixture was incubated for 30 min at 30℃, and the reaction was stopped by addition of 200 μl PBS containing 20% phosphoric acid. After centrifugation, the reaction mixture was transferred to microwell containing immobilized anti-phosphoMV peptide monoclonal antibodies and incubated for 60 min at 25℃. A horseradish peroxidase (HRP) substrate was then added to the microwell and the color intensity of the resulting solution was measured at 492 nm.

Phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2) in fresh and vitrified/warmed oocytes from the four groups was also detected using an anti-phospho-ERK1/2 antibody. Thirty oocytes were rinsed in 0.1% PVP-DPBS, dissolved in 15 μl of 2x sample buffer, and frozen at -20℃. Proteins in samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gels, and transferred to a polyvinylidene difluoride (PVDF) membrane (BIO-RAD, USA). The membrane was blocked with 5% bovine serum albumen (BSA) in TBST (0.1% Tween-20, 10 mM Tris pH 7.4) for 1 h at room temperature. For detection of phosphorylated ERK1/2, the membrane was incubated with anti-ERK1 (pT202/pY204) and anti-ERK2 (pT185/pY187) antibodies (Abcam, England), diluted 1:1000 in Can Get Signal solution 1 (TOYOBO, Japan), at 4℃. The membrane was then incubated with HRP-conjugated anti-mouse secondary antibody (BIO-RAD), diluted 1:3000 in Can Get Signal solution 2, for 1 h at room temperature. Peroxidase activity was visualized by enhanced chemiluminescence (ECL) using Western Blotting Luminol Reagent (Santa Cruz Biotechnology Inc., USA) and developed on Amersham Hyperfilm ECL (GE Healthcare, UK).

Stripped membranes were reprobed with an anti-ERK1+ERK2 antibody (1:1000; Abcam) at 4℃ overnight. Visualized bands were quantified by densitometry using NIH-Image J software.

IVF and embryonic development

Sperm was collected from the cauda epididymis of 12-week-old male mice. Mature MII eggs in HTF containing 10% KSR were inseminated with capacitated spermatozoa ($1\text{-}2 \times 10^6$/ml) by incubation for 6 h. Fertilized eggs were transferred to KSOM Medium (Millipore) under mineral oil and incubated at 37℃ in a humidified 5% CO_2 atmosphere. The cleavage rate was recorded on day 2, and the number of embryos that developed to the blastocyst stage was assessed on day 5. Experiments were repeated at least three times.

Counting cell numbers in blastocysts

The number of cells in each blastocyst in all groups was counted by first staining with anti-Oct3/4 antibody (Santa Cruz) to identify inner cell mass (ICM) cells and counterstained with 4',6-diamidino-2-phenylindole (DAPI; Dako, USA) to label nuclei. Five days after IVF, the zona pellucida (ZP) was removed using an acid Tyrode solution (pH 2.5; Sigma-Aldrich). ZP-free blastocysts were fixed in 3.7% paraformaldehyde in 0.02% Triton X-100 in DPBS containing 0.1% BSA (DPBS-BSA) for 30 min at 4℃ and permeabilized with 0.1% Triton X-100. Blastocysts were transferred to 5% normal goat serum for 2-4 h at 4℃ and then incubated overnight at 4℃ with anti-Oct3/4 antibody (Santa Cruz). Blastocysts were then incubated with secondary antibody and co-stained with Alexa Fluor 594 Phalloidin (Molecular Probes, USA) and DAPI. Blastocysts were mounted on a glass-bottomed dish in PBS and imaged using a Zeiss Axiovert 200M fluorescence microscope with Apotome and a 40x oil-immersion objective lens (Carl Zeiss, Germany). Z-stack images (15-20) of individual blastocysts were obtained and analyzed using Axiovision software 4.6 (Carl Zeiss). Oct3/4- and DAPI-positive cells were counted as ICMs; DAPI-only positive cells were counted as the total cell number. The number of trophectoderms (TEs) was calculated as total cell number minus ICM number.

Chromatin configuration after oocyte vitrification

Oocytes were fixed in 3.7% paraformaldehyde in 100mM PIPES, 0.01% Triton X-100, 0.1% BSA for 30 min at 4℃ and permeabilized in 0.1% Triton X-100. Oocytes were blocked in 5% normal goat serum for 1 h, and then incubated first with anti-α-tubulin primary antibody (1:100; Sigma-Aldrich) or anti-β-tubulin (1:100; BioLegend, USA) at 4℃ overnight and then with Alexa Fluor 555-conjugated goat anti-mouse IgG or rabbit-IgG (Molecular Probes). After counterstaining with DAPI (Dako), oocytes were mounted with Vectashield mounting medium (Vector Laboratories, USA), and fluorescence images were obtained with a Zeiss Axiovert 200M microscope equipped with Apotome and a 100x oil-immersion objective lens (Carl Zeiss).

Embryo transfer and monitoring implantation

Embryos cultured to the 2-cell stage under each of the four treatment conditions were transferred into the uteri of 0.5-day pseudo-pregnant female ICR mice (6-8 weeks old) that had been mated with vasectomized male ICR mice. In order to analyze the embryonic effect on post-implantation development, implantation rates were checked at 7.5 days post coitus (dpc). For visualization of implantation site, 1% Chicago blue dye (0.1 ml) was injected intravenously through the tail vein. After 5 min, mice were euthanized and the intact uterus was excised into normal saline, adhering fat was dissected away, and the tissue was photographed.

SCNT and embryonic development

Vitrified/warmed MII oocytes were incubated at 37℃ for 1-2 h, and then were enucleated in M2 medium containing 5 μg/ml cytochalasin B (CB). Freshly collected cumulus cells, used as nuclear donors, were injected into enucleated oocytes using a Piezo-driven micromanipulator (Prime Tech, LTD, Japan). After nuclear transfer, the reconstructed eggs were artificially activated for 6 h by incubating in M16 medium containing 10 mM $SrCl_2$, 2 mM EGTA, and 5 μg/ml CB (Millipore). Two-cell block was overcome by injecting reconstructed eggs with ~10 pl of lysine (K)-specific demethylase 4A (Kdm4a) mRNA solution (2 μg/μl) using a Piezo-driven micromanipulator (Chung et al., 2015). Reconstructed eggs

were cultured in KSOM, and their embryonic development to the blastocyst stage was examined 120 h after activation.

Statistical analysis

Unless otherwise specified, data are expressed as means ± SEM. Most data were analyzed for statistical significance using a one-way analysis of variance (ANOVA; Duncan-test) followed by a *t*-test, if necessary. Implantation data were analyzed for statistical significance using the Chi-square test. *P* values < 0.05 were considered to be statistically significant.

RESULTS

Effects of caffeine and/or MG132 on MPF and MAPK activity of oocytes after vitrification

After warming, MPF and MAPK activities were evaluated by enzyme-linked immunosorbent assay and Western blotting. Thirty oocytes were analyzed in each group. MPF and MAPK activities in vitrified/warmed oocytes from each of the four groups (untreated, CA, MG, and CA+MG) were compared with those of fresh oocytes. MPF activities in CA, MG, and CA+MG group oocytes were very similar to that in non-vitrified fresh oocytes, and was much higher than that in control (untreated) group oocytes (Figs. 1A and 1B). However, there was no significant difference in MAPK activity among groups (Figs. 1C and 1D).

Effects of caffeine and/or MG132 on oocyte vitrification and development

To determine the optimal concentrations of caffeine and MG132 for improving vitrification of mouse oocytes, we supplemented vitrification and warming solutions with different concentrations of these agents, subjected warmed oocytes to IVF, and cultured the blastocyst to blastocysts. Oocytes treated with 10 mM caffeine during vitrification/warming showed a higher blastocyst formation rate (84.0 ± 1.4%) than untreated oocytes (74.0 ± 2.5%), although there were no differences in survival, fertilization, or cleavage (Table 1). Oocytes treated with 10 μM MG132 during vitrification/warming also exhibited an improved

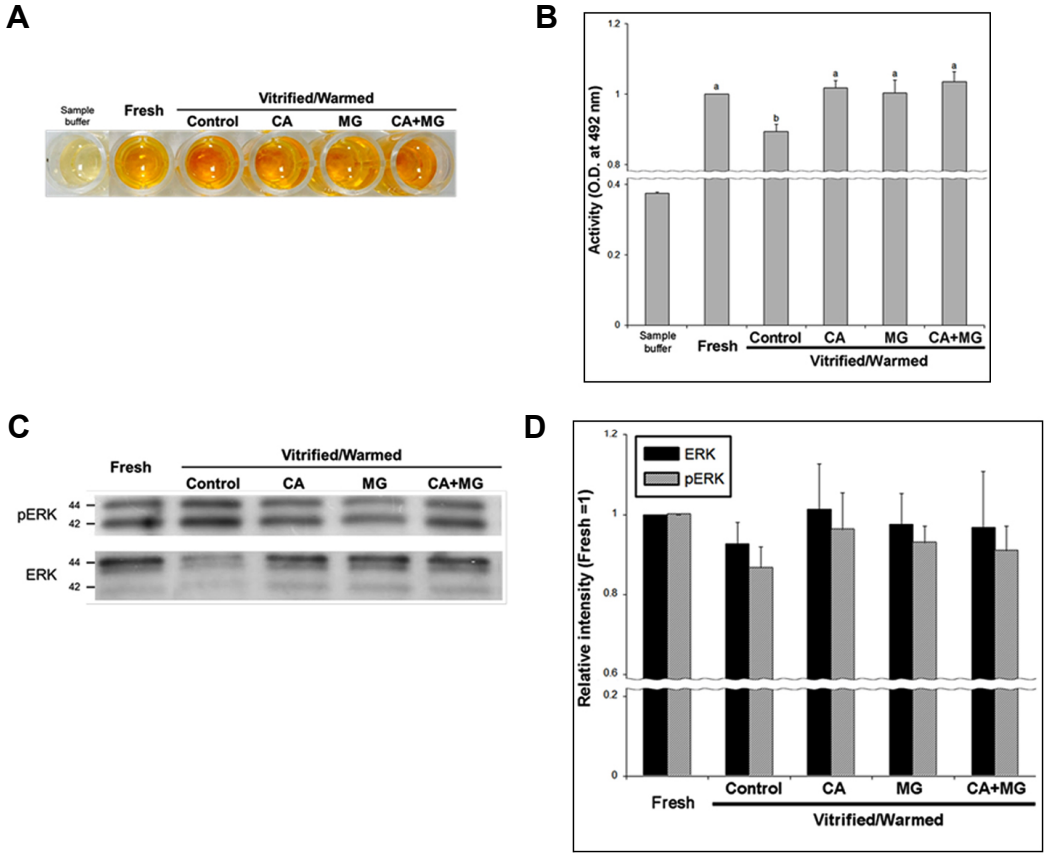

Fig. 1. Effects of caffeine/MG132 on MPF and MAPK activity in vitrified/warmed mouse oocytes. Vitrified/warmed oocytes were pre-incubated in CPA solution without supplements (untreated control) or containing 10 mM caffeine (CA), 10 μM MG132 (MG) and 10 mM caffeine + 10 μM MG132 (CA+MG) for 2 h, and then collected in 5 μl of sample buffer before vitrification/warming to assess kinase activity. (A) Results of enzyme-linked immunosorbent assays for Cdc2 kinase. (B) Changes in MPF activity in fresh oocytes and oocytes in cryopreserved untreated control, CA, MG and CA+MG groups. (C-D) ERK and pERK expression were not significantly different among groups. Western blots showed similar changes in expression. Thirty oocytes were analyzed for each group (n = 5 replicates/group). Data are shown as the means ± SEM.

blastocyst formation rate (90.6 ± 5.5%) compared with oocytes in the untreated group (72.4 ± 3.7%, Table 2).

To establish an optimal medium for vitrification and warming, collected MII oocytes were vitrified/warmed and divided into four groups: untreated (control), 10 mM caffeine (CA), 10 μM MG132 (MG), and 10 mM caffeine +10 μM MG132 (CA+MG). The blastocyst formation rate was increased in the CA, MG, and CA+MG groups compared to that in the untreated control group (Table 3). Interestingly, however, the quality of blastocysts was very similar in all groups. In addition, the numbers of ICM and TE cells, as well as total cell numbers, in blastocysts were not significantly different among groups (Figs. 2A and 2B). However, because the blastocyst formation rate was highest in the CA+MG group, we used this group for subsequent functional studies.

Effects of vitrification in the presence of caffeine/MG132 on chromatin configuration in mouse oocytes

Changes in chromatin configuration were determined after vitrification and supplementation with 10 mM caffeine +10 μM MG132 (CA+MG group) by fixing and analyzing warmed oocytes. As shown in Fig. 3, spindle structures (metaphase chromosomes and actin filaments) of oocytes from fresh and CA+MG groups were intact and well-

Table 1. Embryonic development of vitrified/warmed oocytes in solutions supplemented with different concentrations of caffeine

Treatment		Concentration (mM)	No. of oocytes	Survival (%/oocytes)	Fertilization (%/survived)	Cleavage (%/fertilized)	Blastocyst (%/cleaved)
Fresh		0	75	75 (100 ± 0)^{a}	69 (93.0 ± 3.2)^{a}	66 (96.1 ± 1.6)^{a}	59 (90.0 ± 4.1)^{a}
Vitrified/	Caffeine	0	74	71 (96.3 ± 1.5)^{a}	57 (80.3 ± 1.9)^{b,c}	51 (89.3 ± 3.1)^{a}	38 (74.0 ± 2.5)^{b}
warmed		1	75	71 (95.1 ± 1.2)^{a}	58 (81.8 ± 5.3)^{b}	55 (95.5 ± 1.8)^{a}	34 (62.6 ± 3.1)^{c}
		10	78	75 (99.3 ± 1.5)^{a}	64 (86.0 ± 2.7)^{a,b}	61 (95.8 ± 1.7)^{a}	51 (84.0 ± 1.4)^{a}
		20	73	58 (78.8 ± 2.9)^{b}	41 (71.0 ± 3.5)^{c}	39 (93.5 ± 4.2)^{a}	20 (51.4 ± 2.3)^{d}

Data are shown as mean (%) ± SEM of four replications.
Blastocysts were observed at 120 h after *in vitro* fertilization.
^{a,b,c}Within the same column, value with different superscripts are significantly different ($p < 0.05$)

Table 2. Embryonic development of vitrified/warmed oocytes in solutions supplemented with different concentrations of MG132

Treatment		Concentration (μM)	No. of oocytes	Survival (%/oocytes)	Fertilization (%/survived)	Cleavage (%/fertilized)	Blastocyst (%/cleaved)
Fresh		0	70	70 (100 ± 0)^{a}	65 (92.7 ± 3.7)^{a}	65 (100 ± 0)^{a}	58 (90.2 ± 2.5)^{a}
Vitrified/	MG132	0	74	72 (96.2 ± 2.3)^{a,b}	70 (95.8 ± 2.6)^{a}	64 (90.8 ± 5.5)^{a}	45 (72.4 ± 3.7)^{a,b}
warmed		1	75	71 (96.2 ± 2.3)^{a,b}	63 (88.0 ± 7.3)^{a}	61 (96.7 ± 3.3)^{a}	33 (60.0 ± 7.6)^{b}
		10	75	74 (99.4 ± 0.6)^{a}	69 (93.1 ± 3.7)^{a}	67 (97.6 ± 1.8)^{a}	61 (90.6 ± 5.5)^{a}
		25	74	67 (91.8 ± 3.8)^{b}	62 (90.0 ± 5.5)^{a}	58 (93.3 ± 5.9)^{a}	40 (78.6 ± 10.6)^{a,b}

Data are shown as mean (%) ± SEM of six replications.
Blastocysts were observed at 120 h after *in vitro* fertilization.
^{a,b}Within the same column, values with different superscripts are significantly different ($p < 0.05$)

Table 3. Embryonic development of vitrified/warmed oocytes in solutions supplemented with caffeine and/or MG132

Treatment		No. of oocytes	Survival (%/oocytes)	Fertilization (%/survived)	Cleavage (%/fertilized)	Blastocyst (%/cleaved)
Fresh		156	156 (100 ± 0)^{a}	139 (88.8 ± 2.0)^{a}	134 (97.3 ± 2.7)^{a}	121 (89.7 ± 0.7)^{a,b}
Vitrified/	Control	150	140 (93.4 ± 4.6)^{a}	116 (86.6 ± 4.5)^{a}	104 (88.2 ± 6.5)^{b}	73 (70.2 ± 2.1)^{c}
warmed	CA	155	152 (98.7 ± 0.7)^{a}	134 (89.1 ± 3.1)^{a}	127 (94.6 ± 0.2)^{a}	101 (82.8 ± 4.2)^{b}
	MG	160	156 (97.8 ± 1.3)^{a}	138 (89.7 ± 1.4)^{a}	132 (95.3 ± 0.8)^{a}	112 (87.1 ± 2.6)^{a,b}
	CA+MG	170	163 (96.5 ± 1.2)^{a}	139 (89.9 ± 3.5)^{a}	129 (92.2 ± 1.4)^{a,b}	117 (91.9 ± 1.3)^{a}

Data are shown as mean (%) ± SEM of six replications.
Blastocysts were observed at 120 h after *in vitro* fertilization.
^{a,b}Within the same column, values with different superscripts are significantly different ($p < 0.05$)
Control: untreated; CA: 10 mM caffeine; MG: 10 μM MG132; CA+MG: 10 mM Caffeine +10 μM MG132

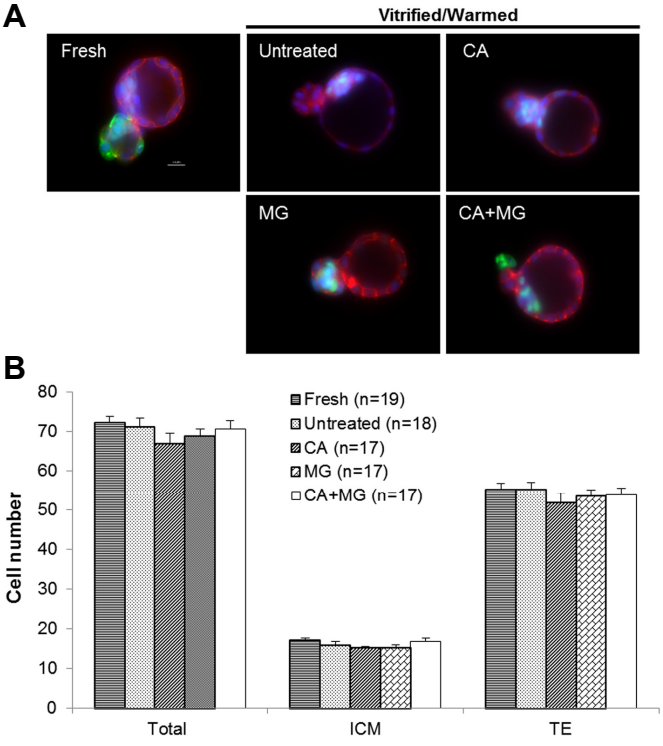

Fig. 2. Comparison of number of total cells, ICM cells, and TE cells. (A) Total cells, ICM cells, and TE cells were counted in fresh oocytes and cryopreserved oocytes from untreated, 10 mM caffeine (CA), 10 μM MG132 (MG), and 10 mM, caffeine +10 μM MG132 (CA+MG) groups. Number of total cells, ICM cells, and TE cells in blastocysts derived from vitrified/warmed oocytes. Blastocysts were observed 120 h after IVF. ICM cells were stained with anti-Oct3/4(green), and total cells were stained nucleus (DAPI, blue) and actin-phalloidin (red). (B) Means of total cell numbers, inner cell mass (ICM), and trophectoderm (TE) of blastocysts from fresh oocytes and cryopreserved oocytes. Differences were not statistically significant. Data are shown as the means ± SEM.

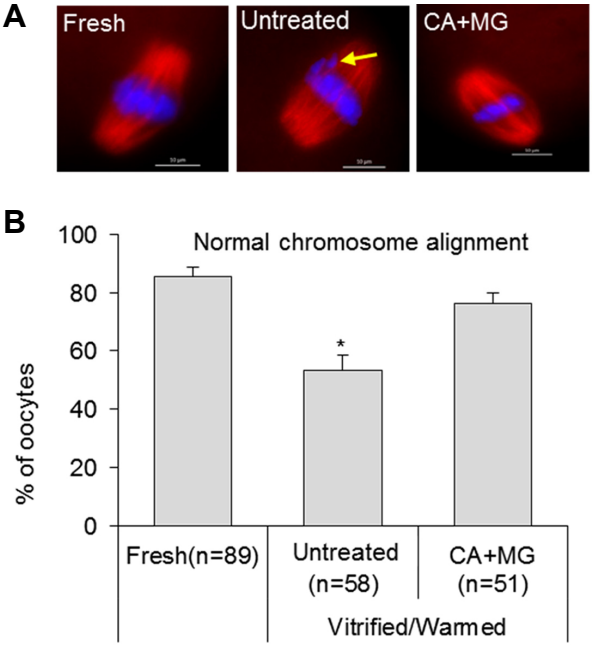

Fig. 3. Chromosome alignment configuration in fresh oocytes and cryopreserved oocytes from untreated and 10 mM caffeine +10 μM MG132 (CA+MG) groups. (A) Comparison of chromosome alignment configurations. Anti-tubulin immunostaining (red) indicates spindles and DAPI staining (blue) indicates chromosomes. Yellow arrow indicates an abnormal chromosome alignment in the untreated group. Scale bar = 10 μm. (B) Percentage of oocytes derived from vitrified/warmed oocytes (*$P < 0.05$: Fresh and CA+MG *vs.* untreated). Data are shown as the means ± SEM.

maintained compared with those from the control (untreated) group. In fact, as shown in Figs. 3A and 3B, the percentage of oocytes with a normal chromatin configuration was higher in the fresh (85.7 ± 3.0%) and CA+MG (76.3 ± 3.7%) groups than in the untreated group (53.4 ± 5.1%, $P < 0.05$).

Implantation rate of embryos derived from oocytes vitrified in the presence of caffeine/MG132

To confirm the safety of caffeine and MG132 for use on cryopreserved eggs, we examined the CA and CA+MG groups in functional studies. To analyze the effects of these treatment on post-implantation development, we transferred 2-cell embryos from each experimental group into the right uterus of a surrogate mother. To reduce individual variation, we also transferred 2-cell embryos from the untreated control group into the left uterus of the same surrogate mother. The implantation rate of the CA group (56.5%) at 7.5 day post conception (dpc) was significantly higher than that of the control group (43.6%, $P < 0.05$; Figs. 4A and 4B). The implantation rate of the CA+MG group (56.5%) was also significantly higher than that of the control group (43.6%, $P < 0.05$; Figs. 4C and 4D).

Development of SCNT-derived embryos using oocytes vitrified in the presence of caffeine/MG132

To assess the effectiveness of caffeine and/or MG132 in improving cryopreserved eggs for use in SCNT technology, we again tested the two experimental groups, CA and CA+MG. Consistent with our previous results, injection of Kdm4a mRNA overcame the 2-cell block in embryos cloned

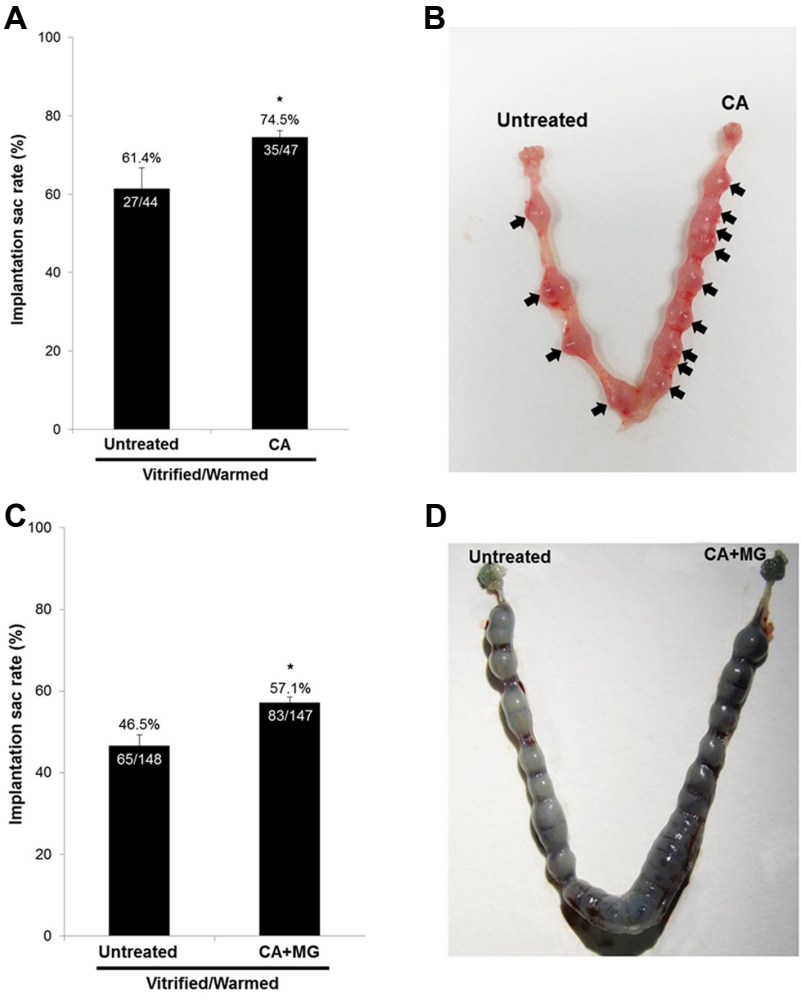

Fig. 4. Comparison of implantation rates in untreated, 10 mM caffeine (CA) and 10 mM caffeine + 10 μM MG132 (CA+MG) treatment groups. (A, B) Implantation sac rate of *in vitro*-fertilized 2-cell embryos derived from vitrified/warmed oocytes (untreated group: left uterus; CA group: right uterus). (C, D) Implantation sac rate of *in vitro*-fertilized 2-cell embryos derived from vitrified/warmed oocytes (untreated group: left uterus; CA + MG treatment group: right uterus). (∗$P < 0.05$)

Table 4. Development rate of somatic cell nuclear transfer (SCNT)-derived mouse embryos obtained from vitrified/warmed oocytes

Injection		Kdm4a (μM)	No. SCNT oocytes	No. with PN formation[+]	No. 2-cell embryos[#]	No. 2-cell block embryos (%)[#]	No. Blastocysts (%)[ζ]
Vitrified/	Fresh	2	99	96	92	3 (3.3 ± 1.8)[a]	61 (66.3 ± 4.3)[a]
warmed	Control	2	92	85	81	3 (3.7 ± 2.1)[a]	44 (54.3 ± 6.1)[a]
	CA+MG	2	95	90	78	2 (2.6 ± 1.4)[a]	34 (43.6 ± 7.7)[a]
Vitrified/	Fresh	2	60	59	58	2 (3.4 ± 0.3)[a]	40 (69.0 ± 5.5)[a]
warmed	Control	2	65	59	54	2 (3.7 ± 0.1)[a]	27 (50.0 ± 1.9)[a]
	CA	2	65	59	55	2 (3.6 ± 0.2)[a]	26 (47.3 ± 0.7)[a]

[a] Within the same column, values with same superscript letters are not significantly different ($P > 0.05$; $n = 5$)
Kdm4a : Injection of Kdm4a mRNA
[+] Based on the number of reconstructed oocytes
[#] Based on the number of pronuclear embryos
[ζ] Based on the number of 2-cell embryos
Control: untreated; CA: 10 mM Caffeine; MG: 10 μM MG132; CA+MG: 10 mM Caffeine+10 μM MG132

from cryopreserved eggs. However, supplementation with caffeine, with or without MG132, did not improve the rate of blastocyst formation in cloned mouse eggs (Table 4). Moreover, the quality of blastocysts did not differ between the experimental groups and untreated control group (data not shown).

DISCUSSION

For the cryopreservation of mammalian oocytes and embryos, vitrification has come to replace the slow freezing protocol because of its improved survival rates and clinical outcomes. However, cytotoxic damage induced during vitrifying/warming by high concentrations of CPAs can impact outcomes. In addition, various intrinsic factors, such as changes in gene expression or protein phosphorylation should be considered because of their potential to reduce metabolic damage induced during cryopreservation. In the present study, we found that MPF activity in mouse MII oocytes was decreased, while MAPK activity was unchanged, during vitrifying/warming. Moreover, maintaining MPF activity in vitrified/warmed MII oocytes by supplementation of CPA solution with caffeine and/or MG132 improved embryonic development *in vitro* and *in vivo*.

Specifically, we found that 10 mM caffeine was an optimal concentration for maintaining the developmental capacity of vitrified oocytes (Table 1) and confirmed that MPF activity was sustained under these conditions (Figs. 1A and 1B), as previously reported (Ariu et al., 2014; Lee et al., 2012). Supplementation with 10 μM MG132 also maintained MPF activity and resulted in a high rate of blastocyst formation from vitrified oocytes. Similar results were obtained in cattle, where treatment with MG132 during the end of oocyte maturation was shown to increase cleavage rates and the percentage of oocytes that became blastocysts without affecting blastocyst cell number (You et al., 2012). Interestingly, the highest rate of embryonic development was observed in the caffeine and MG132 co-supplemented group, suggesting that co-treatment with caffeine and MG132 had a synergistic effect on maintaining of MPF activity. In addition, co-treatment with caffeine and MG132 during vitrification may support the maintenance of spindle structures in MII oocytes after warming (Fig. 3), suggesting that MPF and ERK are involved in maintaining the stability of the chromosome configuration in cryopreserved MII oocytes (Iwamoto et al., 2016; Tong et al., 2003). In contrast, it has been reported that, although MG132 exposure maintains MPF activity during oocyte maturation in monkey, subsequent development is adversely affected (Mitalipov et al., 2007). An additional study from the latter research group showed that maintenance of MPF levels during oocyte manipulation by treatment with only caffeine, which is less toxic, resulted in high cleavage and blastocyst formation rates in monkey and human SCNT embryos (Mitalipov et al., 2007; Tachibana et al., 2013). In fact, the effects of MG132 on embryonic development remain controversial, although it should be noted that the results achieved using our mouse system represent an improvement on previous studies. Therefore, additional studies are needed to confirm the effect of MG132, as few studies have examined oocyte cryopreservation.

In the present study, we found that maintaining MPF activity by supplementation with caffeine and/or MG132 during cryopreservation increased the rate of blastocyst formation, but without changing total cell numbers or morphology (Fig. 2). In addition, the rate of blastocyst formation was not changed in SCNT embryos. In our previous study, we reported that increasing histone demethylation by injecting Kdm4a mRNA overcame reprogramming barriers of the SCNT procedure and substantially reduced the rate of 2-cell block (Chung et al., 2015). Consistent with this, we found that injection of Kdm4a mRNA dramatically decreased the rate of 2-cell block in our SCNT embryos using cryopreserved eggs, but failed to completely overcome the block of embryonic development to blastocysts (Table 4). As described above, high MPF activity may secure the spindle structures after cryopreservation, as previously reported (Kwon et al., 2010). These results suggest that elevated MPF activity may play a role in the development of embryos derived from cryopreserved oocytes, but additional cryo-injury to oocytes remains to be overcome. Therefore, decreases in intrinsic factors that contribute to developmental potential during oocyte cryopreservation may occur, which should be examined in future studies. In addition, for SCNT, we removed the spindle structure of vitrified/warmed MII oocytes for enucleation and replaced it with somatic cells. This difference compared with the IVF procedure may explain why maintenance of MPF during oocyte cryopreservation has different effects on the development of embryos derived from IVF and SCNT.

In conclusion, our results confirmed that the maintenance of MPF activity by supplementation of CPA solution with caffeine and/or MG132 during vitrification/warming have beneficial effects on the developmental competence of mouse oocytes after IVF. However, further studies are needed to avoid cryo-damage that leads to decreases in the developmental potential of cryopreserved oocytes.

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