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Short Communication

# Effects of Mito-TEMPO on the survival of vitrified bovine blastocysts *in vitro*

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**ABSTRACT** Vitrification methods are commonly used for mammalian reproduction through the long-term storage of blastocyst produced *in vitro*. However, the survival and quality of embryos following vitrification are significantly low compared with blastocyst from *in vitro* production (IVP). This study evaluates that the survival of frozen-thawed bovine embryos was relevant to mitochondrial superoxide derived mitochondrial activity. Here we present supplementation of the cryopreservation medium with Mito-TEMPO (0.1 μM) induced a significant ($p < 0.001$; non-treated group: 56.8 ± 8.7%, re-expanded at 24 h vs Mito-TEMPO treated group: 77.5 ± 8.9%, re-expanded at 24 h) improvement in survival rate of cryopreserved-thawed bovine blastocyst. To confirm the quality of vitrified blastocyst after thawing, DNA fragmentation of survived embryos was examined by TUNEL assay. As a result, TUNEL positive cells rates of frozen-thawed embryos were lower in the Mito-TEMPO treated group (4.2 ± 1.4%) than the non-treated group (7.1 ± 3.5%). In addition, we investigated the intracellular ROS and mitochondrial specific superoxide production using DCF-DA and Mito-SOX staining in survived bovine embryos following vitrification depending on Mito-TEMPO treatment. As expected, intracellular ROS levels and superoxide production of vitrified blastocysts after cryopreservation were significantly reduced ($p < 0.05$) according to Mito-TEMPO supplement in freezing medium. Also, mitochondrial activity measured by MitoTracker Orange staining increased in the frozen-thawed embryos with Mito-TEMPO compared with non-treated group. These results indicate that the treatment of Mito-TEMPO during cryopreservation might induce reduction in DNA fragmentation and apoptosis-related ROS production, consequently increasing mitochondrial activation for developmental capacity of frozen-thawed embryos.

**Keywords:** bovine blastocyst, cryopreservation, Mito-TEMPO, superoxide, vitrification medium

## INTRODUCTION

Cryopreservation as a technique for reproduction is used to preserve the genetic complements on domestic animals and humans (Lee et al., 2014; Kim et al., 2020). Especially, cryopreservation technology provides additional opportunity of pregnancy through the vitrified-warmed process in animal embryos. This is applied as a necessary technique for pregnancy using *in vitro* fertilization (IVF) embryos to solve the difficulty of female infertility (Hara et al., 2018). However, cryopreservation method is accompanied by cryoinjury such as the reactive

oxygen species (ROS) production (Len et al., 2019), DNA fragmentation (Inaba et al., 2016), mitochondria dysfunctions (Gualtieri et al., 2021), and physiological damage from small ice crystals (Huebinger et al., 2016). Therefore, development of new cryopreservation additives is necessary to protect from the ROS production, mitochondrial related cryoinjury, and apoptosis during cryopreserved-thawed process.

Vitrified embryos following freezing are known to increase mitochondrial dysfunctions such as loss of mitochondrial membrane potential (MMP) and adenosine triphosphate (ATP) productions compared with fresh embryos in mammals (Nohales-Córcoles et al., 2016). Mitochondria are important intracellular organelle for cell survival in mammalian cells and embryos (Babayev and Seli, 2015; Xia et al., 2019). Mitochondria play a critical role in producing ATP through the electron transport chain, which assists oocyte maturation and embryo development competence in mammalian embryos (May-Panloup et al., 2021). In addition, the ATP synthesis process accompanies ROS which is a direct by-product from mitochondria (van Hameren et al., 2019). In general, mitochondria-derived superoxide is known to carry out the role of redox-sensitive second messenger for generating signal responses in multiple ways (Shadel and Horvath, 2015). Whereas, excessive oxidative stress by external damage such as freezing and thawing process impair cellular functions and lead to apoptosis (Ott et al., 2007; Lee et al., 2016; Len et al., 2019).

Triphenylphosphonium chloride (Mito-TEMPO) is a potent cell-permeable ROS antioxidant, which is known as a mitochondrial-derived superoxide target scavenger (Yang et al., 2019). Also, Mito-TEMPO is a synthetic compound that can play a role as superoxide dismutase (SOD) mimetic function in mammalian cells and embryos (Asadzadeh et al., 2021). Mito-TEMPO has an antioxidant capacity that protects cells against oxidative damages in human pathologies (Dikalova et al., 2010; Choumar et al., 2011). According to previous study, reduction of superoxide-derived oxidative stress by Mito-TEMPO improved oocyte maturation rate and developmental competence in porcine and bovine embryos (Yang et al., 2018; Park et al., 2020; Yousefian et al., 2021). Moreover, freezing medium with Mito-TEMPO supplementation assisted conservation of post-thawed sperm quality and survival competence in human (Lu et al., 2018; Masoudi et al., 2021). However, studies concerning the protective effects of mitochondrial specific superoxide production by Mito-TEMPO on bovine embryos and blastocyst during cryopreservation have not been reported.

Therefore, we hypothesized that Mito-TEMPO could protect the low survival of cryopreserved-thawed bovine blastocysts in response to damages from the ROS or superoxide production. In the present study, we confirmed the effects of Mito-TEMPO supplementation during the cryopreservation process on survival, intracellular ROS levels, superoxide production and mitochondrial activity in vitrified-warmed bovine embryos.

## MATERIALS AND METHODS

### Chemicals

Unless otherwise stated, all chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

### *In vitro* production of bovine embryos

Bovine ovaries were acquired from a local abattoir and transported to the laboratory in 0.9% saline containing 75 μg/mL penicillin G sodium salt at 36-38℃. Cumulus-oocyte complexes (COCs) were aspirated from follicles (3-6 mm in diameter) using a 10 mL disposable syringe with an 18-gauge needle. Approximately 15 COCs were matured in 50 μL of IVM medium in a 60 mm dish under paraffin oil for 22 h at 38.5℃ under 5% $CO_2$. The medium used for oocyte maturation was TCM-199 (Gibco-BRL, Grand Island, NY, USA) supplemented with 0.2 mM Na-pyruvate, 0.6 mM cysteine, 10 IU/mL pregnant mare's serum gonadotropin (PMSG), 10 IU/mL human chorionic gonadotropin (hCG), 10 ng/mL epidermal growth factor (EGF), 25 μg/mL gentamycin, 25 μM β-mercaptoethanol, 10% FBS (Gibco-BRL), and 1 μg/mL estradiol- β. Following IVM, 15 oocytes were fertilized with frozen-thawed sperm at a concentration of $2 \times 10^6$ cells/mL in 50 μL of fertilization medium (Fert-TALP). The fertilization medium consisted of Fert-TALP medium supplemented with 0.2 mM Na-pyruvate, 25 μg/mL gentamycin, and 0.6% BSA. When sperm were added to the fertilization drops, 10 μg/mL heparin, 80 μM penicillamine, 4 μM hypotaurine, and 2 μM epinephrine (PHE) were also added. After 22 h of insemination, cumulus-enclosed oocytes were stripped using gentle pipetting and transferred to CR1-aa medium containing 0.4 mM

Na-pyruvate, 1 mM glutamine, 0.3 mg/mL glutathione, 25 μg/mL gentamycin, and 0.3% BSA for *in vitro* culture (IVC). After culture for two days, the cleaved embryos were further cultured in medium containing 50 μL of CR1-aa 0.4 mM Na-pyruvate, 1 mM glutamine, 0.3 mg/mL glutathione, 25 μg/mL gentamycin, and 10% FBS for five days at 38.5℃ in 5% $CO_2$ in air.

### Vitrification and warming procedure

Cryopreservation was carried out by vitrification with a Cryotop (Kitazato Supply Co, Fujinomiya, Japen) using a slightly modified version of the procedure described by Kim et al., 2020. Briefly, two or three blastocysts were transferred into equilibration solution (ES) consisting of 7.5% ethylene glycol (EG) and 7.5% dimethylsulfoxide (DMSO) in PBS supplemented with 20% FBS at room temperature for 5 min. Next, blastocysts were transferred into vitrification solution (VS) consisting of 15% EG, 15% DMSO, and 0.5M sucrose dissolved in PBS containing 20% FBS. After 40-45 s, the blastocysts were loaded into a Cryotop and plunged into liquid nitrogen. The process from exposure in VS to plunging into liquid nitrogen was completed within 1 min at room temperature. Vitrified blastocysts were warmed by immersing the Cryotop directly into warming solution (1.0 M sucrose dissolved in PBS containing 20% FBS) for 1 min, then were transferred to dilution solution (0.5 M sucrose dissolved in PBS containing 20% FBS) for 3 min, and then to dilution solution (0.25 M sucrose dissolved in PBS containing 20% FBS) for 5 min at room temperature. Subsequently, blastocysts were incubated for 5 min in washing solution (PBS containing 20% FBS). Survival of vitrified-warmed blastocysts was determined according to re-expansion rates after 24 h of recovery in culture medium. Mito-TEMPO (0.1 μM) was added in ES and VS during vitrification periods. In addition, we used fresh bovine embryos and blastocyst (Non-treated group) from *in vitro* culture (IVC) as controls for comparison with Mito-TEMPO-treated group.

### Assessment of apoptosis in bovine blastocysts

Apoptotic cells in vitrified-warmed blastocysts were detected using an *In Situ* Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany). Vitrified-warmed blastocysts were washed with 0.1 PVA in PBS and then fixed in 3.7% formaldehyde in PBS for 2 days at 4℃. Next, blastocysts were permeabilized using 0.5% Triton X-100 for 30 min at room temperature. The fixed blastocysts were incubated in terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick end-labeling (TUNEL) reaction medium for 1 h at 38.5℃ in the dark, washed and mounted on slide glass with mounting solution containing 1.5 μg/mL 4', 6-diamidino-2-phenylindole (DAPI; Vector Laboratories, CA, USA). DAPI-labeled or TUNEL-positive nuclei were subsequently observed under a fluorescence microscope (Olympus, Tokyo, Japan). Nuclei were recorded as positive for TUNEL labeling only when they showed light green fluorescence on the blue fluorescence background of DAPI.

### Measurement of ROS levels

The ROS levels in vitrified-warmed blastocysts were measured using the dichlorodihydrofluorescein diacetate (H2DCFDA; Molecular Probes, Eugene, OR, USA) and Mito-SOX (Molecular Probes) as previously described (Lee et al., 2011). Vitrified-warmed blastocysts in CR1-aa (with 10% FBS) medium were washed three times with 0.1% polyvinyl alcohol (PVA) in phosphate buffer solution (PBS). Blastocysts were transferred into 1X PBS containing 1 μM H2DCFDA and 1 μM Mito-SOX for 30 min at 38.5℃ under 5% $CO_2$ in air. The intensity of H2DCFDA and Mito-SOX were measured with an iRiS Digital Cell Imaging System (Logos Biosystems, Gyeonggido, South Korea). The measured fluorescence images were analyzed by Image J software Version 1.38 (National Institutes of Health, Bethesda, MD, USA).

### MitoTracker Orange staining

Vitrified-warmed blastocysts were incubated in 1X PBS with 4 μM MitoTracker Orange (Invitrogen, CA, USA) for 30 min at 38.5℃. Stained blastocysts were fixed in 3.7% formaldehyde for overnight at 4℃ and washed thrice with 0.1% PVA in PBS. The intensity of MitoTracker images were acquired using LSM 800 confocal microscope (Zeiss, Jena, Germany). The mitochondrial activity was measured by orange fluorescence quantification using the Image J 1.46r software (NIH, USA). All images for analysis were taken using the same intensity and exposure time.

### Statistical analysis

All percentage data obtained in the present study are presented as the mean ± SD. All data were analyzed using student's *t*-tests and plotted using the GraphPad Prism 5.0

software package (San Diego, CA, USA). Histogram values of densitometry were measured by ImageJ software (NIH, USA).

## RESULTS

### Effect of Mito-TEMPO supplementation during vitrification on the survival rates and apoptosis in frozen-thawed bovine embryos

To investigate the effects of 0.1 μM Mito-TEMPO treatment in a freezing medium, we confirmed frozen-thawed bovine blastocyst developmental and re-expanded rates using microscope (Fig. 1). Representative images of the bovine re-expanded blastocyst following vitrified warming are shown in Fig. 1A. The survival rates of frozen-thawed bovine embryos derived from IVF according to Mito-TEMPO treatment are shown in Table 1. As expected, the re-expanded blastocyst rate and survival rate in Mito-TEMPO exposed frozen-thawed blastocysts (56.8 ± 8.7%, n = 59) were significantly higher ($p < 0.001$) than the non-treated group (77.5 ± 8.9%, n = 63, Fig. 1B and Table 1). We stained nuclei and apoptotic cells using DAPI/TUNEL assay to evaluate frozen-thawed bovine blastocyst quality based on apoptotic positive cells rate (Fig. 2A and Table 2). Total number of nuclei in frozen-thawed bovine blastocysts significantly increased ($p < 0.01$) in Mito-TEMPO exposed group (n = 5.4 ± 1.2) compared with non-treated group (n = 6.7 ± 3.3, Fig. 2B), whereas TUNEL-positive cells in the Mito-TEMPO-exposed group (4.2 ± 1.4%) was lower than the non-treated group (7.1 ± 3.5%, $p < 0.05$, Fig. 2C). These results showed that Mito-TEMPO supplementation in freezing medium improves survival rates through reduction of apoptosis of frozen-thawed bovine embryos.

### Mito-TEMPO protects frozen-thawed bovine blastocyst from cryoinjury caused by mitochondrial-derived superoxide

To examine the expression of intracellular ROS and mitochondrial superoxide, we stained the vitrified-warmed bovine blastocysts in two groups using DCF-DA and Mito-SOX staining. There was an obvious decrease in the intracellular ROS level in embryos following vitrification from freezing medium supplied with Mito-TEMPO compared to the non-treated group ($p < 0.05$, Fig. 3A and 3B). A representative image of the mitochondrial superoxide detection by Mito-SOX staining in the vitrified-warmed blastocysts are shown in Fig. 3A. Decrease in mitochon-

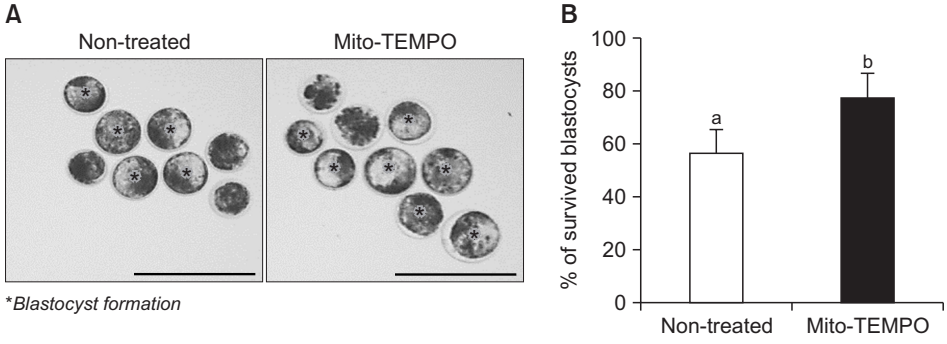


Fig. 1. Effects of Mito-TEMPO on blastocyst survival competence in vitrified-warmed bovine blastocysts. (A) Representative images of survived blastocysts according to the Mito-TEMPO treatment in vitrified-warmed blastocysts. * (asterisks) indicates survived blastocysts after vitrified-warmed process. (B) The percentage of survival rates regard to the Mito-TEMPO exposed in vitrified-warmed bovine blastocysts. Data are expressed as mean ± SD and were analyzed using a *t*-test. Different superscript letters a and b denote significant differences at $p < 0.05$.

Table 1. Survival rates of vitrified-warmed bovine blastocysts with Mito-TEMPO

| Mito-TEMPO (μM) | No. of blastocysts vitrified | No. of blastocysts thawed | % of survived blastocysts (n) |
|---|---|---|---|
| Non-treated | 62 | 59 | 56.8 ± 8.7 (35)[a] |
| 0.1 | 63 | 63 | 77.5 ± 8.9 (49)[b] |

Data are expressed as the mean ± SD, and non-normally distributed data are expressed as the median (interquartile range). Different superscript letters a and b denote significant differences ($p < 0.05$).

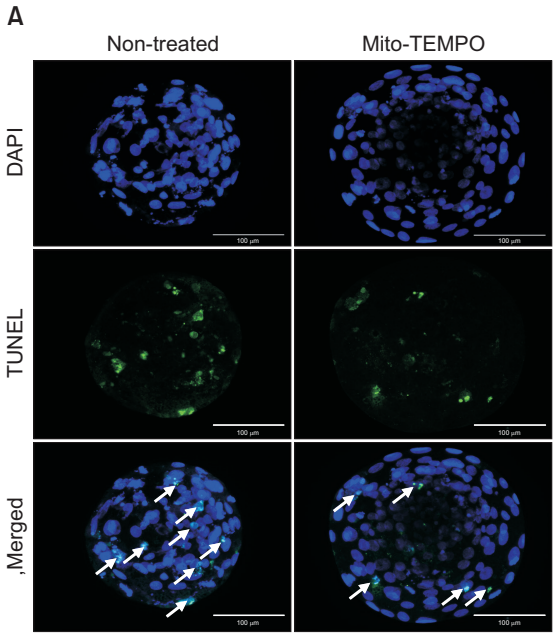


Fig. 2. Changes in cellular apoptosis according to the Mito-TEMPO treatment on vitrified-warmed blastocysts in bovine. (A) Colocalization of DAPI and TUNEL stains at the vitrified-warmed blastocyst stage. The blastocysts were stained for nuclei (blue) and TUNEL-positive cells (green). The white arrows indicate a fragmented DNA of nucleus in blastocysts. (B) The graph indicates the number of nuclei in vitrified-warmed bovine blastocysts. (C) The percentage of TUNEL-positive cells rates depending on the Mito-TEMPO treatment in bovine vitrified-warmed blastocysts are shown. Data are expressed as mean ± SD and were analyzed using a *t*-test. Differences were considered significant at $*p < 0.05$ and $**p < 0.01$.

Table 2. TUNEL-positive cells rates of frozen-thawed bovine blastocysts in Mito-TEMPO treatment

| Mito-TEMPO (μM) | No. of TUNEL-positive cells | % of TUNEL-positive cells |
|---|---|---|
| Non-treated | 6.7 ± 3.3 | 7.1 ± 3.5 |
| 0.1 | 5.4 ± 1.2 | 4.2 ± 1.4* |

Data are expressed as the mean ± SD, and non-normally distributed data are expressed as the median (interquartile range). Differences were considered significant at $*p < 0.05$.

drial superoxide production was clearly observed in Mito-TEMPO exposed embryos following cryopreserved-thawing ($p < 0.05$, Fig. 3C and 3D). Based on these results, we demonstrated that Mito-TEMPO decreased the oxidative stress from intracellular ROS and mitochondrial superoxide in vitrified-warmed bovine embryos during cryopreservation process.

### Mito-TEMPO improves the mitochondrial activity in re-expanded bovine blastocysts after cryopreservation

Mitochondria activity was evaluated by MitoTracker Orange staining in frozen-thawed bovine from Mito-TEMPO treated and non-treated group. The overall intensity of MitoTracker Orange fluorescence expression in blastocysts were significantly increased in the Mito-TEMPO-supplemented group compared with the non-treated group ($p < 0.05$, Fig. 4A and 4B).

## DISCUSSION

In current study, we investigated the effects of Mito-TEMPO through reduction of mitochondrial specific superoxide production using freezing medium supplemented with 0.1 μM Mito-TEMPO for improving survival rate and quality in bovine blastocyst following cryopreserved-thawing. Freezing medium with Mito-TEMPO treatment enhances survival and re-expanding rates of blastocysts through reduction of apoptosis, intracellular ROS, and mitochondrial superoxide in bovine embryos following vitrification. These findings suggest the first evidence related to protective effect of Mito-TEMPO in freezing medium for enhancing survival rate of cryopreserved-thawed bovine blastocyst.

To improve the challenges of current bovine reproduction, many studies have been focusing on increasing cryo-survival rates of *in vitro* produced embryos (IVP) involved in the resumption of development after cryopreservation (Gupta and Lee, 2010; Marsico et al., 2019; Stoecklein et al., 2021). Previous study clearly indicated that thawed blastocyst after cryopreservation shows apoptotic response such as DNA fragmentation compared to *in vitro* blastocysts (Majidi Gharenaz et al., 2016). In present study, we evaluated the ability of re-expansion and survival in vitrified-warmed bovine embryos and correlated them with the apoptotic status derived from ROS and superoxide production (Fig. 1-3). Increased apoptotic

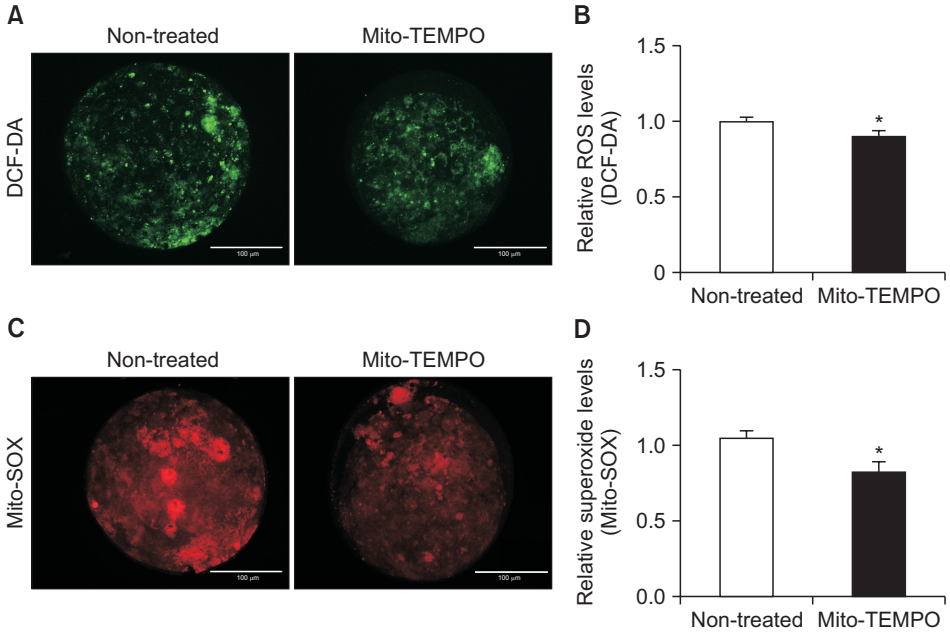


Fig. 3. Comparison of intracellular and mitochondrial ROS expressions in frozen-thawed bovine blastocysts treated Mito-TEMPO. (A) Expression of intracellular ROS (green fluorescence) in vitrified-warmed bovine blastocysts was detected by DCF-DA and analysis using fluorescence microscope. (B) The graph showed relative ROS expression levels on vitrified-warmed blastocyst in bovine. (C) Representative images of mitochondrial superoxide (red fluorescence) in vitrified-warmed bovine blastocysts detected by Mito-SOX and analysis using image system. (D) Relative superoxide expression levels of vitrified-warmed bovine blastocysts treated Mito-TEMPO. Data are expressed as mean ± SD and were analyzed using a *t*-test. Differences were considered significant at $^{*}p < 0.05$.

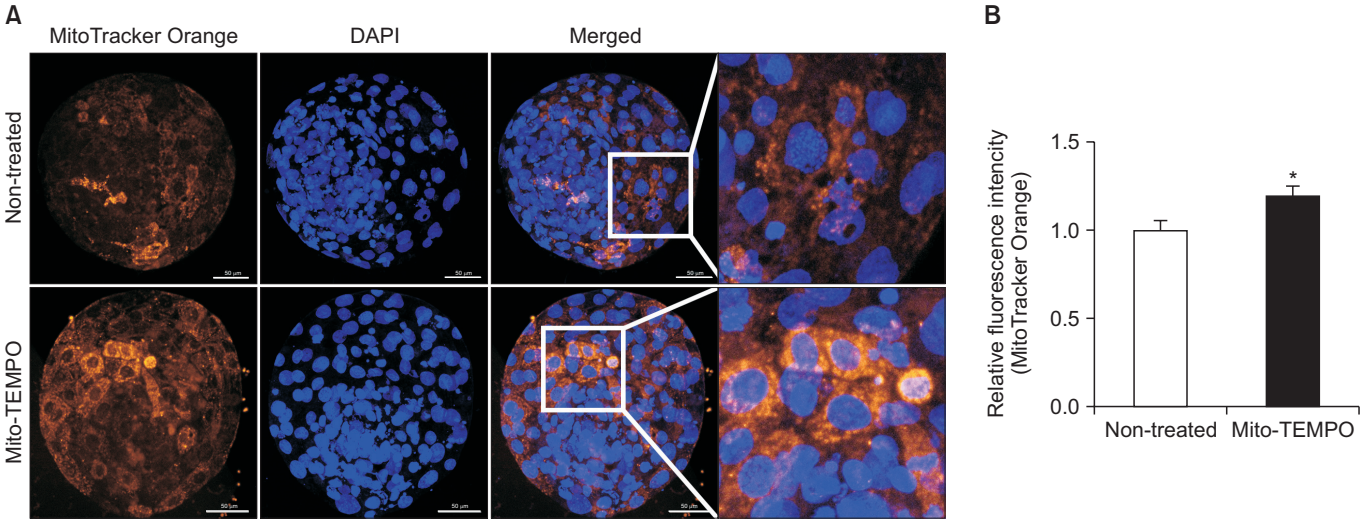


Fig. 4. Confirmation of mitochondrial activity and localization in the frozen-thawed blastocysts with Mito-TEMPO. (A) Representative images of Mitochondria intensity (orange fluorescence) by MitoTracker Orange staining following the Mito-TEMPO treatment in frozen-thawed blastocysts. Mitochondrial expressions and localization were visualized using a fluorescence microscope. (B) The graph indicated relative fluorescence intensity of mitochondria of bovine frozen-thawed blastocyst. Data are expressed as mean ± SD and were analyzed using a *t*-test. Differences were considered significant at $^{*}p < 0.05$.

cells by vitrification-induced abnormal or excessive ROS generation and DNA damage-induced phosphorylated histone H2A (γ-H2AX) accumulation induced disruption of early embryonic developmental competence until expanded blastocysts (Chang et al., 2019). Collectively, low survival rate of vitrified embryos after warming suggests that cryoinjury aggravates embryo quality and developmental capacity through the oxidative stress derived serve ROS accumulation.

Many antioxidants have been effectively employed as a cryoprotective supplement for bovine embryo or frozen-thawed sperm preservation (Al-Mutary, 2021). In previous our study, it is suggested that 0.1 μM Mito-TEMPO exposure can improve early embryonic developmental capacity through mitochondrial superoxide production (Yang et al., 2018). However, effects of Mito-TEMPO supplement

have not yet been assessed for bovine blastocyst during freezing process. In the present study, we confirmed that supplementation with 0.1 μM of Mito-TEMPO reduced intracellular ROS and superoxide from mitochondria in frozen-thawed bovine blastocysts.

The MitoTracker Orange staining is commonly been used to measure mitochondrial activity of embryos (Elahi et al., 2017). It was suggested that the cryopreservation process using bovine embryo or blastocyst could cause irreversible damage to cell organelles, including mitochondria which leads to apoptosis (Gualtieri et al., 2021). Damaged mitochondria have been found to accelerate mitochondrial ROS production, oxidative damage, and disruption of mitochondrial bioenergetics based on low mitochondrial activation (Guo et al., 2013). Our findings demonstrate that viability and mitochondrial activity of frozen-thawed embryos with 0.1 μM Mito-TEMPO were enhanced when compared to non-treated group (Fig. 4). In addition, analysis of mitochondrial activation also showed that Mito-TEMPO supplementation could significantly increase the survival rate of frozen-thawed bovine blastocyst by regulating mitochondrial ROS production. These results suggest that the regulation of Mito-TEMPO between mitochondrial activation and superoxide production in frozen-thawed bovine embryos could enhance viability and survival capacity during cryopreservation.

## CONCLUSION

In conclusion, our findings showed that improvement of survivability in vitrified bovine embryos through reducing mitochondrial superoxide during vitrification process supplemented with Mito-TEMPO. In the present study, it is speculated that cryopreservation could be responsible for ROS and superoxide production which results in apoptosis and reduction of mitochondrial activity. Therefore, our results suggested the potential of Mito-TEMPO as a supplement for improving frozen-thawed blastocysts survival rates in bovine embryos.

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