JAST (Journal of Animal Science and Technology) TITLE PAGE

Upload this completed form to website with submission

ARTICLE INFORMATION	Fill in information in each box below
Article Type	Research article
Article Title (within 20 words without abbreviations)	MIT-001 mitigates vitrification-induced mitochondrial stress and regulates F-actin reorganization, enhancing the survival and development of vitrified-warmed bovine blastocysts
Running Title (within 10 words)	MIT-001 enhances survival of vitrified bovine blastocysts
Author	Dae-Wook Kim[1,2,#], Hyo-Jin Park[1,2,#], Seul-Gi Yang[2,3], Jae-Ho Lee[4,5] and Deog-Bon Koo[1,2,3,*] [#]These authors contributed equally to this work.
Affiliation	[1]Department of Biotechnology, College of Engineering [2]Center for Infertility [3]Department of Companion Animal Industry, College of Natural and Life Sciences, Daegu University, 201 Daegudae-ro Jillyang, Gyeongsan, Gyeongbuk 38453, Republic of Korea. [4]CHA Fertility Center Seoul Station, Seoul 04637, Republic of Korea. [5]Department of Biomedical Sciences, CHA University, Pocheon 11160, Republic of Korea.
ORCID (for more information, please visit https://orcid.org)	Dae-Wook Kim (https://orcid.org/0009-0002-5616-930X) Hyo-Jin Park (https://orcid.org/0000-0003-3287-2914) Seul-Gi Yang (https://orcid.org/0000-0003-3247-2443) Jae-Ho Lee (https://orcid.org/0000-0001-8735-444X) Deog-Bon Koo (https://orcid.org/0000-0001-7825-9598)
Competing interests	No potential conflict of interest relevant to this article was reported.
Funding sources State funding sources (grants, funding sources, equipment, and supplies). Include name and number of grant if available.	This research was supported by the National Research Foundation of Korea (NRF), funded by the Korean government (MSIT) (NRF-2021R1C1C2009469 and NRF-2022R1A2C1002800), and the Basic Science Research Program through the NRF, funded by the Ministry of Education (RS-2023-00246139), Republic of Korea.
Acknowledgements	Not applicable.
Availability of data and material	Upon reasonable request, the datasets of this study can be available from the corresponding author.
Authors' contributions Please specify the authors' role using this form.	Conceptualization: Park HJ. Data curation: Kim DW, Park HJ. Formal analysis: Kim DW. Methodology: Kim DW, Yang SG, Park HJ, Lee JH. Validation: Kim DW, Yang SG, Park HJ. Investigation: Kim DW. Writing - original draft: Kim DW, Park HJ, Koo DB. Writing - review & editing: Kim DW, Park HJ, Koo DB.
Ethics approval and consent to participate	This article does not require IRB/IACUC approval because there are no live animal participants.

CORRESPONDING AUTHOR CONTACT INFORMATION

For the corresponding author (responsible for correspondence, proofreading, and reprints)	Fill in information in each box below
First name, middle initial, last name	Deog-Bon Koo
Email address – this is where your proofs will be sent	dbkoo@daegu.ac.kr
Secondary Email address	
Address	Jillyang, Gyeongsan, Gyeongbuk 38453, Republic of Korea
Cell phone number	+82-10-4632-5528
Office phone number	+82-53-850-6557
Fax number	+82-53-850-6559

ABSTRACT

In this study, we investigated whether MIT-001, a small-molecule reactive oxygen species (ROS) scavenger, improves the re-expansion and viability of bovine blastocysts in response to vitrification-induced mitochondrial dysfunction and stress. Therefore, this study aimed to analyze the protective effects of MIT-001 on the mitochondrial function of bovine blastocysts following vitrification warming. In this experiment, MIT-001 (0.1 μM) was allocated to three culture conditions based on treatment period: (I) warming only (WARM); (II) vitrification only (VITR); and (III) both vitrification and warming (VITR-WARM), compared with the control (Non-treated). Survival analysis of cryopreserved bovine blastocysts revealed that MIT-001 supplementation during the warming period (WARM group) significantly improved ($p < 0.01$; Non-treated: 57.3 ± 2.3% vs WARM: 74.2 ± 7.3%) post-warm survival rates. It is noteworthy that surviving blastocysts in the WARM groups demonstrated significantly ($p < 0.05$) lower TUNEL positive cells (%) and a higher ratio of expanded blastocyst development compared to the other groups. Intracellular ROS, as well as mitochondrial and nuclear superoxide levels, were significantly reduced ($p < 0.001$) in the MIT-001-treated WARM group, accompanied by enhanced mitochondrial activation (MitoTracker Orange staining). Simultaneously, mitochondrial membrane potential (MMP), assessed using JC-1 staining, was elevated, whereas a reduction in mitochondrial fission marker dynamin-related protein 1 (DRP1) expression was observed in surviving blastocysts from the MIT-001-supplemented WARM group ($p < 0.01$). In addition, MIT-001 improved cytoskeletal stability by decreasing the aggregation thickness of filamentous actin (F-actin, $p < 0.001$; Non-treated: 10.73 μm vs. WARM: 6.03 μm) in bovine blastocyst of the WARM group. Finally, the enhanced developmental potential of vitrified-warmed blastocysts was linked to increased phospho-p38 mitogen-activated protein kinase (MAPK) expression exclusively in the WARM group compared to the other groups. Consequently, MIT-001 mitigates cryopreservation-induced cellular stress by improving mitochondrial function and regulates F-actin stabilization to enhance the viability and developmental potential of vitrified-warmed bovine blastocysts. These findings highlight the potential of MIT-001 to support cellular recovery and developmental capacity during cryopreservation, suggesting that it may play an effective protective role in bovine blastocyst cryopreservation.

Keywords: MIT-001, Vitrification, Reactive oxygen species, Mitochondrial function, F-actin, Bovine blastocyst

INTRODUCTION

MIT-001, also known as NecroX-7, is a novel mitochondria-targeted reactive oxygen species (ROS) scavenger developed by MitoImmune Therapeutics Inc. (Seoul, South Korea), and has been shown to protect mammalian cells from oxidative damage [1]. MIT-001 inhibits ROS generation and calcium accumulation in mitochondria, thereby suppressing the release of molecules associated with cell damage and apoptosis [2, 3]. Until recently, the activation of ROS or mitochondrial protection by MIT-001 in vitrified-warmed bovine blastocysts had not been investigated.

Embryo cryopreservation offers flexibility in gamete management, which is crucial for livestock production and infertility treatment. It is especially advantageous for preserving and transporting livestock genetic resources, thereby improving reproductive potential, facilitating genetic enhancement, and contributing to biodiversity conservation [4]. However, cryopreserved embryos often exhibit reduced viability and quality compared with fresh embryos because of intracellular ice crystal formation during vitrification [5]. Additionally, the use of cryoprotectants prior to vitrification, along with optimal culture conditions (e.g., temperature), significantly affects blastocyst viability [6]. Post-warming re-expansion is a critical indicator of the recovery and survival of cryopreserved embryos [7].

During early embryonic development, mitochondrial function and quantity are tightly regulated by a quality control system that plays a crucial role in maintaining mitochondrial homeostasis. Mitochondria play a vital role in ROS balance during oxidative phosphorylation, and their dysfunction results in the harmful accumulation of superoxide anions within cells. Interestingly, vitrification and warming have been linked to elevated ROS levels and mitochondrial impairment in various species, including pigs, cattle, and humans. Mitochondrial dysfunction can have profound implications for embryo viability, as mitochondria are critical not only for ATP production but also for regulating apoptosis and controlling ROS generation [8]. Currently, there is no study on the protective effects of ROS removal by MIT-001 in vitrified-warmed bovine blastocysts, and recent research suggests that mitochondrial dysfunction and low membrane potential after vitrification/warming are hallmark features of cryoinjury [8, 9]. Therefore, we investigated the effect of MIT-001, a potential antioxidant agent targeting mitochondria, on the survival rate of cryopreserved bovine blastocysts *in vitro.*

Additionally, mitochondrial fission is necessary for mitochondrial quality control, leading to blastocyst developmental competence in porcine embryos during *in vitro* culture (IVC) progression [10]. In mammals, it is triggered by dynamin-related protein 1 (DRP1), which forms ring-like structures around the constriction points of dividing mitochondria [11]. However, in contrast to its role in cell survival, DRP1-induced mitochondrial fragmentation contributes to the release of proapoptotic factors during apoptosis [12]. Indeed, excessive mitochondrial

fission is linked to apoptosis, and modulation of DRP1 is a promising strategy for improving early embryonic development.

p38 mitogen-activated protein kinases (MAPK) are activated in response to various cellular stressors, including oxidative stress, cytokines, and DNA damage, often culminating in apoptosis and altered cell differentiation [13]. Cortical F-actin stabilization plays a critical role in blastocyst development and re-expansion after vitrification and warming. Studies on murine and porcine preimplantation embryos have indicated that p38 MAPK activation stabilizes F-actin and mitochondrial function, thereby improving embryo development and survival [14, 15].

Given the effects of ROS production and mitochondrial dysfunction on cryoinjury, we evaluated MIT-001's protective effects of against vitrification and warming. Specifically, we assessed the blastocyst viability, cytoskeletal integrity, mitochondrial function, re-expansion potential, and p38 MAPK activation. The goal was to determine whether MIT-001 could mitigate ROS-induced damage, preserve cytoskeletal structure, and improve overall blastocyst quality, offering new insights into enhancing cryopreservation outcomes in assisted reproductive technology and livestock genetic resource conservation.

MATERIALS AND METHODS

Chemicals and animals

Unless otherwise stated, all chemicals utilized in this study were obtained from Sigma-Aldrich (St. Louis, MO, USA). Bovine ovaries were obtained from a local slaughterhouse (Gimhae, Gyeongnam, Korea). No experiments were performed on live animals.

Experimental design of the survival criteria for blastocysts

Bovine embryos were produced using the methods described by Jeong et al. [16]. for embryo development and vitrification-warming. Blastocysts generated through the IVC process were classified into four stages based on their average diameter (Supplementary Fig. 1, Early: 150 μm, Mid: 161 μm, Late: 175 μm and Expanded: 222 μm), ranging from the earliest to the fully expanded stage. Survival was determined based on re-expansion rates after 24 h of post-warming culture. The survival of blastocysts after cryopreservation and post-warming was evaluated according to these criteria, including the average diameter of each group.

Experimental design of MIT-001 treated groups

Bovine blastocysts (n = 180) were assigned to three groups based on the MIT-001 (1 μM) treatment period: (I) warming only (WARM), (II) vitrification only (VITR), and (III) both vitrification and warming (VITR-WARM), compared with the Non-treated control group. After MIT-001 treatment, surviving bovine blastocysts were used for experimental analysis (Supplementary Fig. 2).

In vitro production (IVP)

Bovine ovaries were collected from a local slaughterhouse and transported to the lab in 0.9% saline solution, supplemented with 75 μg/mL potassium penicillin G sodium salt, maintained at 36–38 °C. Cumulus-oocyte complexes (COCs) were aspirated from follicles measuring 3–6 mm in diameter using a 10 mL disposable syringe fitted with an 18-gauge needle. Approximately, 15 COCs were matured in 50 μL of *in vitro* maturation (IVM) medium in a 60 mm dish under paraffin oil for 22 h at 38.5 °C in an atmosphere containing 5% CO_2. The medium for oocyte maturation consisted of Medium 199 (Gibco-BRL, Grand Island, NY, USA) supplemented with 0.2 mM sodium pyruvate, 0.6 mM cysteine, 10 IU/mL pregnant mare serum gonadotropin (PMSG), 10 IU/mL human chorionic gonadotropin (hCG), 10 ng/mL EGF, 25 μg/mL gentamicin, 25 μM β-mercaptoethanol, 10% fetal bovine serum (FBS; Gibco-BRL), and 1

μg/mL estradiol-β. After IVM, 15 oocytes were fertilized using vitrified-warmed sperm at a concentration of 2×10^6 cells/mL in 50 μL of fertilization medium (Fert-TALP). The fertilization medium contained 0.2 mM sodium pyruvate, 25 μg/mL gentamicin, and 0.6% bovine serum albumin (BSA). Additionally, 10 μg/mL heparin, 80 μM penicillamine, 4 μM hypotaurine, and 2 μM epinephrine (PHE) were added during sperm addition. After 22 h of insemination, cumulus-enclosed oocytes were stripped by vortexing and then transferred into Charles Rosekrans amino acid (CR1-aa) medium containing 0.4 mM sodium pyruvate, 1 mM glutamine, 0.3 mg/mL glutathione, 25 μg/mL gentamicin, and 0.3% BSA for IVC. After two days of culture, cleaved embryos were further cultured in 50 μL of CR1-aa medium supplemented with 0.4 mM sodium pyruvate, 1 mM glutamine, 0.3 mg/mL glutathione, 25 μg/mL gentamicin, and 10% FBS for five days at 38.5 °C in 5% CO_2.

Vitrification and warming procedure

Vitrification was performed using Cryotop (Kitazato Supply Co., Fujinomiya, Japan), following a modified method of Jeong et al., 2021 [16]. Briefly, blastocysts were transferred to an equilibrated solution (ES) containing 7.5% ethylene glycol (EG) and 7.5% dimethyl sulfoxide (DMSO) in phosphate-buffered saline PBS (Gibco-BRL) with 20% FBS for 5 min at room temperature. Blastocysts were transferred to a vitrification solution (VS) containing 15% ethylene glycol (EG), 15% dimethyl sulfoxide (DMSO), and 0.5 M sucrose in PBS with 20% FBS. After 25 s, the samples were placed on the cryotop and immediately submerged in liquid nitrogen. The entire process, from VS exposure to immersion in liquid nitrogen, was maintained for 1 min. The vitrified blastocysts were warmed by immersing the Cryotop in a warming solution (1.0 M sucrose in PBS with 20% FBS) for 1 min, followed by immersion in a diluted solution (0.5 M sucrose in PBS with 20% FBS) for 3 min. They were then treated with a further diluted solution (0.25 M sucrose in PBS with 20% FBS) for 5 min at room temperature, followed by incubation in a washing medium (PBS containing 20% FBS) for 5 min. The survival of vitrified-warmed blastocysts was determined according to the rate of re-expansion after 24 h of recovery in the culture medium. The blastocysts were divided into four groups (Non-treated, WARM, VITR, VITR-WARM) depending on the presence of MIT-001 (1 μM) during the vitrification and/or warming steps.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

Apoptotic cells in vitrified-warmed blastocysts were detected using a TUNEL assay with an *in situ* Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany). Blastocysts were washed with 0.1% PVA in PBS

and subsequently fixed in 3.7% formaldehyde in PBS for 2 h at 38.5 °C. Blastocysts were permeabilized with 0.5% Triton X-100 for 30 minutes at 38.5 °C. The fixed blastocysts were incubated in TUNEL reaction medium for 1 h at 38.5 °C in the dark, followed by washing and mounting on glass slides with 1.5 μg/mL 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA). Images were captured using an iRiS™ digital cell imaging system (Logos Biosystems Inc., Anyang, Korea). Nuclei were considered TUNEL-positive only if they exhibited light-green fluorescence against a blue DAPI background

Measurement of ROS levels

ROS levels in the vitrified-warmed blastocysts were measured using dihydroethidium (DHE; Invitrogen, CA, USA, D11347), dichlorodihydrofluorescein diacetate (H_2DCF-DA), and Mito-SOX (Invitrogen, M36008). Vitrified-warmed blastocysts in CR1-aa medium (with 10% FBS) were washed three times with 0.1% PVA in PBS. Blastocysts were transferred into 1X PBS containing 10 μM DHE, 5 μM H_2DCF-DA and 1 μM Mito-SOX for 30 min at 38.5 °C. The intensities of H_2DCF-DA and MitoSOX were measured using an LSM 800 confocal microscope (Zeiss, Jena, Germany). Fluorescence images were analyzed using ImageJ 1.46r software (NIH, MD, USA).

MitoTracker Orange staining

Vitrified-warmed blastocysts were incubated in 1X PBS with 4 μM MitoTracker Orange (Invitrogen, M7514) for 30 minutes at 38.5 °C. The incubated blastocysts were washed with 0.1% PVA in PBS. Stained blastocysts were fixed in 3.7% formaldehyde for 2 h at 38.5 °C and washed three times with 0.1% PVA in PBS. The MitoTracker image intensity was captured using an LSM 800 confocal microscope (Zeiss). Mitochondrial activity was assessed by quantifying orange fluorescence using the ImageJ 1.46r software (NIH). All images were obtained at consistent intensities and exposure times.

Immunofluorescence (IF) staining

Vitrified-warmed blastocysts were washed three times with 0.1% PVA in PBS and fixed in 3.7% formaldehyde in PBS. Fixed blastocysts were then washed three times in 0.1% PVA in PBS and permeabilized with 0.5% Triton X-100 for 30 min at 38.5 °C. After permeabilization, blastocysts were blocked with a blocking solution (0.1% PVA in PBS containing 1% BSA, 0.1% Tween-20, and 0.1% Triton X-100). The blocked blastocysts were incubated with the following primary antibodies: 1:200 dilution of anti-phospho-p38 MAPK (Thr180/Tyr182) (Cell Signaling, MA, USA)

and 1:100 dilution of anti-DRP1 (Santa Cruz, CA, USA) overnight at 4 °C. The blastocysts were then incubated with goat anti-rabbit IgG (Santa Cruz Biotechnology) diluted 1:200 in blocking solution for 2 h at room temperature. Image data were quantified using ImageJ 1.46r software (NIH). All images were obtained at the same intensity and exposure time.

Analysis of mitochondrial membrane potential (ΔΨm)

Embryos were washed three times with 0.1% PVA in PBS and incubated IVC medium with JC-1 (100:1) (Cayman Chemical, MI, USA) for 30 min at 38.5 °C. Washed embryos were fixed in 3.7% formaldehyde in PBS for 2 h at 38.5 °C. The aggregated form (J-aggregate) of mitochondria emitted red fluorescence, while the monomers (J-monomer) emitted green fluorescence.

Filamentous actin (F-actin) staining

Vitrified-warmed blastocysts were washed with 0.1% PVA in PBS and then fixed in 3.7% formaldehyde in PBS for 2 h at 38.5 °C. Blastocysts were permeabilized with 0.5% Triton X-100 for 30 min at room temperature, and after washing were incubated with 5 μg/ml phalloidin-FITC for 1 h at 38.5 °C. After washing, embryos were counterstained with 1.5 μg/ml of 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame) for 1 min and mounted on glass slides. Images were captured using a Zeiss LSM 800 confocal microscope, and the fluorescence intensity of F-actin in the embryos was quantified using the ImageJ 1.46r software (NIH).

Statistical analysis

All experiments were conducted in triplicates. All data obtained in this study are presented as the means ± standard deviation (SD). Percentage data were analyzed using the Student's *t*-test and one-way ANOVA, followed by Tukey's multiple comparison test. Image data (TUNEL assay, DHE, DCF-DA, Mito-SOX, Mito-Tracker, JC-1 staining, DRP1, phospho-P38 MAPK, and F-actin) were analyzed using GraphPad Prism 5.0 software (San Diego, CA, USA). Densitometry histogram values were measured using ImageJ 1.46r software (NIH). Differences were considered significant at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

RESULTS

MIT-001 treatment during warming restores the quality of post vitrified-warmed bovine blastocysts

To investigate the effect of MIT-001 on cryopreservation survival, blastocysts were vitrified 7 days after IVC. After vitrification-warming, the blastocysts were classified according to their developmental stages and diameters. Blastocysts with a minimum diameter of 150 μm after post-warming were selected as the criterion for survival analysis (Supplementary Fig. 1 and 2). During the 24 h warming period after cryopreservation, the percentage of expanded blastocysts (Ex: 41.4 ± 21.5%, n = 24) following MIT-001 supplementation was similar to the results observed in IVC bovine embryos (IVC, Ex; 48.2 ± 19.2 %, n = 88) prior to cryopreservation (Fig. 1 and Supplementary Fig. 1). The results revealed an increase in the proportion of expanded blastocysts (Ex) in the WARM group compared to the other groups (Fig. 1A; Non-treated: 29.0 ± 14.4%. vs. WARM: 41.4 ± 21.5% vs. VITR: 28.5 ± 14.3% vs. VITR-WARM: 29.0 ± 13.0%), while the proportion of early blastocysts (E) was lower (Fig. 1A; Non-treated: 25.4 ± 5.7% vs. WARM: 8.0 ± 8.5% vs. VITR: 29.5 ± 15.5% vs. VITR-WARM: 23.7 ± 15.3%). Furthermore, the expanded blastocyst diameter was significantly improved (Non-treated: 233.9 ± 8.4 μm vs. WARM: 264.3 ± 22.9 μm vs. VITR: 242.7 ± 15.6 μm vs. VITR-WARM: 238.0 ± 4.3 μm) in the WARM group (Figs. 1B and 1C). However, the analysis of blastocyst diameter within each stage demonstrated that there was no significant difference in early blastocysts (Fig. 1D).

To evaluate the effects of MIT-001 on the cryopreservation of bovine blastocysts, we compared the survival rates of blastocysts exposed to various MIT-001 treatments during vitrification and warming (Table 1; Fig. 2A). The results showed a significantly higher survival rate in the WARM group ($p < 0.01$, Non-treated: 57.3 ± 2.3% vs. WARM: 74.2 ± 7.3% vs. VITR: 56.2 ± 6.4% vs. VITR-WARM: 54.3 ± 5.6%), which received MIT-001 only during the warming process. To further assess the quality of vitrified-warmed blastocysts, we conducted DAPI/TUNEL staining to measure the total number of nuclei and DNA damage-induced apoptosis (Table 2; Figs. 2B–2D). We found that the WARM group exhibited a significantly higher number of nuclei ($p < 0.01$) and a lower percentage of TUNEL-positive cells, compared to the other groups ($p < 0.01$, Non-treated: 4.0 ± 1.0% vs. WARM: 2.2 ± 0.7% vs. VITR: 3.6 ± 1.4% vs. VITR-WARM: 4.2 ± 1.4%). These results suggest that MIT-001 supplementation during the warming process effectively reduced apoptosis in vitrified-warmed bovine blastocysts, thereby enhancing their overall viability.

Antioxidant effect of MIT-001 on re-expanded and survival blastocysts after vitrification-warming

To assess intracellular ROS and superoxide in the nucleus and mitochondria, which are representative of cryoinjury, fluorescence expression was analyzed in surviving blastocysts from the MIT-001-treated groups during vitrification and warming (Fig. 3). The results revealed that the WARM group exhibited significantly lower ($p < 0.001$) intracellular ROS levels, as determined by DCF-DA staining (Figs. 4A and 4B). Nuclear superoxide levels, as assessed by DHE staining, were significantly lower in the WARM group compared with the non-treated ($p < 0.01$) and VITR

groups (Figs. 3C and 3D). The patterns of mitochondria-specific superoxide production were similar (Figs. 3E and 3F). These findings suggest that the addition of MIT-001 during the warming process effectively mitigated oxidative stress induced by intracellular ROS, and excessive superoxide generation in the nuclei and mitochondria of vitrified-warmed bovine blastocysts.

MIT-001 enhances mitochondrial function and p-P38 MAPK-derived F-actin structures in vitrified-warmed bovine blastocysts

To investigate the effects of MIT-001 treatment on mitochondrial function in vitrified-warmed bovine blastocysts, we performed immunofluorescent staining for MitoTracker Orange, JC-1, and DRP1 (Fig. 4). The activation of mitochondria using MitoTracker Orange and MMP by JC-1 staining showed significantly higher fluorescence intensity in the WARM group ($p < 0.01$) compared with the Non-treated group (Figs. 4A–4D). IF staining with DRP1 as a mitochondrial fission marker was significantly reduced in the WARM group ($p < 0.001$) compared to the other groups (Figs. 4E – 4F). To evaluate the crucial F-actin aggregation and structure during the re-expansion process of vitrified-warmed bovine blastocysts induced by MIT-001, phalloidin-FITC staining, and IF staining of p-phospho (p)-p38 MAPK, an upregulator of F-actin, were performed (Fig. 5). The fluorescence expression of the (p)-p38 MAPK protein increased only in the WARM group ($p < 0.01$). In addition, analysis of the average length of total F-actin thickness and aggregated F-actin thickness (> 6 μm) was significantly reduced ($p < 0.001$) in filament thickness in the WARM group (Figs. 5C - 5E; Non-treated: 10.73 μm vs. WARM: 6.03 μm vs. VITR: 8.23 μm vs. VITR-WARM: 10.33 μm). Interestingly, bovine blastocysts cultured with MIT-001 (WARM group) during the warming process after vitrification showed a significant increase ($p < 0.01$) in the co-localization of F-actin and (p)-p38 MAPK compared to the other groups (Fig. 5F and 5G). These results suggest that the addition of MIT-001 during the warming process mitigates cytoskeletal alterations and mitochondrial dysfunction in vitrified-warmed bovine blastocysts.

DISCUSSION

MIT-001 is a novel compound that functions as a mitochondrial-targeted ROS scavenger and has been reported to restore mitochondrial function in various cell types. Here, we demonstrated that MIT-001 treatment during the warming of bovine blastocysts improved mitochondrial function and blastocyst viability by reducing intracellular ROS and mitochondrial-derived superoxide. MIT-001 treatment during the warming period reduced DNA damage, restored cytoskeletal structure, and lowered ROS levels in vitrified-warmed bovine blastocytes. Additionally, MIT-001 decreased DRP1 expression, improved mitochondrial activity, and significantly increased membrane potential and p38 MAPK phosphorylation. The protective effects of MIT-001 on bovine blastocysts were particularly pronounced during re-expansion at specific developmental stages post-vitrification, suggesting that warm-stage-specific treatment with MIT-001 enhanced blastocyst viability (Fig. 6).

Long-term storage of embryos through freezing and vitrification using assisted reproductive technologies is possible; however, research is still needed to improve the quality and survival of blastocysts after warming [5, 6]. Various studies have indicated that vitrification significantly disrupts the structures of the cytoskeleton [17], mitochondria [8, 18], and cell membranes [19, 20], leading to cytological changes in embryos and blastocysts. Recently, it has been established that cellular damage, mitochondrial function, and ROS regulation are crucial factors influencing the re-expansion and survival of cryopreserved bovine blastocysts. A previous study demonstrated that vitrified blastocysts exhibit increased DNA fragmentation and apoptosis compared to *in vitro*-produced blastocysts [21]. Similarly, reduced survival and re-expansion rates in vitrified porcine blastocysts are closely associated with apoptosis, as evidenced by increased mitochondrial dysfunction and homeostasis [22]. Furthermore, embryos that survive vitrification exhibit defects in cytoskeletal structural stabilization and mitochondrial cytoprotective mechanisms, resulting in increased ROS generation and oxidative stress [23]. Based on this study, the low survival rate of vitrified embryos suggests that cryoinjury impairs embryo quality and developmental competence through oxidative stress, contributing to ROS accumulation. Our study showed that MIT-001 decreased ROS levels and superoxide production after vitrification-warming, but only during the warming period.

Cryopreservation, a key technology in assisted reproduction, induces rapid physicochemical changes in germ cells particularly osmotic stress, pH imbalance, and temperature fluctuations during the freezing and thawing processes [24]. These changes contribute to mitochondrial dysfunction by increasing mPTP opening, ROS accumulation, and intracellular calcium levels, while decreasing mitochondrial membrane potential and ATP production [8]. Such oxidative stress impairs fertilization and embryonic development and is especially known to cause cell damage in

bovine embryos, thereby reducing the survival rate of frozen-thawed blastocysts [25]. MIT-001, a mitochondrial-targeted ROS scavenger, appears to be most effective when administered during the thawing phase, where oxidative stress is acutely elevated. While direct evidence specific to cryopreserved embryos is limited, this phase likely provides a critical window for antioxidant intervention, as metabolic activity and ROS generation surge rapidly upon warming. This timing coincides with a rapid surge in oxidative stress, and our results demonstrate that MIT-001 effectively mitigated intracellular stress and improved the survival and quality of bovine blastocysts by counteracting these changes. In contrast, during the freezing phase, metabolic activity in embryos is largely suppressed, resulting in reduced ROS production and mitochondrial activity [26]. Therefore, the scavenging function of MIT-001 under these conditions appears to be limited, which may explain the lack of significant improvement compared to the control group. Moreover, excessive antioxidant exposure in the absence of sufficient ROS may interfere with physiological ROS signaling and cellular homeostasis. Several studies have shown that an excessive reduction of ROS can impair intracellular signaling, mitochondrial functions, and paradoxically decrease cell viability [27-30]. These findings demonstrate that the addition of MIT-001 during the warming process significantly enhanced the survival rate by reducing oxidative stress, leading to improved quality of vitrified-warmed blastocysts (Tables 1 and 2, Fig. 1 and 3). Therefore, our study demonstrates that administration of MIT-001 during the warming phase significantly reduced ROS levels, improved mitochondrial function, and stabilized cytoskeletal structure. These results suggest that the warming phase represents a key intervention window where MIT-001 can effectively alleviate oxidative stress, while untimely or excessive treatment in the freezing or freeze-thaw phases may fail to support cell survival or even disrupt essential redox signaling pathways. Collectively, these findings emphasize that the efficacy of antioxidant treatment depends critically on both timing and dosage.

Various antioxidants, such as melatonin [31] and resveratrol [18], have been utilized as cryoprotectants for the cryopreservation of mammalian blastocysts or spermatozoa to improve early embryo developmental competence and viability [26]. Moreover, supplementation with antioxidants during IVC of bovine blastocysts may contribute to successful blastocyst production [32]. A previous study demonstrated that MIT-001 exerted anti-inflammatory and antioxidant effects on the mitochondria of mouse ovaries by inhibiting mitochondrial peroxide generation [33]. However, few studies have investigated the effects of MIT-001, a mitochondrial-derived peroxide scavenger, on the developmental competence and cryosurvival of bovine blastocysts. Therefore, this study aimed to standardize the survival of blastocysts at the developmental stages prior to vitrification by categorizing them based on their diameter and evaluating the cryoprotective effects of MIT-001 when added to the warming medium. By assessing the potential

to mitigate cryo-induced damage and enhance post-warming blastocyst viability using MIT-001, our findings may provide insights into the optimization of embryo cryopreservation strategies for improved reproductive outcomes.

Mitochondrial function is not only associated with ATP supply and ROS regulation during early embryo development [26], but is also known to play a crucial role in cytoskeletal stabilization [15, 34], mitochondrial activation, and dynamic responses [24] during the survival of vitrified-warmed blastocysts. DRP1, a key mitochondrial fission factor, is distributed around mitochondria and designated for division [11]. Excessive mitochondrial fragmentation caused by DRP1 hyperactivation has been reported to induce apoptosis during early embryo development, adversely affecting developmental efficiency [11, 12]. Yu et al., [1] demonstrated that MIT-001 restores vitrified-warmed bovine blastocysts by enhancing mitochondrial function and cytoskeletal organization [1]. Similarly, we demonstrated that MIT-001 promotes mitochondrial function and maintains MMP in bovine vitrification-warmed blastocysts. Previous studies have indicated that appropriate phosphorylation of p38 MAPK contributes to the stability of F-actin and is associated with the developmental competence of preimplantation embryos, thereby enhancing embryo viability [15]. F-actin cytoskeleton has been reported to be involved in the activation of p38 MAPK [13, 14]. In a previous study, we found that F-actin reinforcement improved blastocyst development during early embryonic development in pigs [15]. Additionally, previous results indicate that p38 MAPK activity is required for successful preimplantation development, as its inhibition leads to the reversible arrest of preimplantation development [13-15]. Our results suggest that the increase in p38 MAPK phosphorylation and reduction in F-actin polymerization in the MIT-001 group indicate that MIT-001 enhances cryo-survival by stabilizing F-actin (Fig. 5). These results indicate that MIT-001 enhanced the viability of vitrified bovine blastocysts by promoting the phosphorylation of p38 MAPK, thereby stabilizing F-actin, mitigating mitochondrial superoxide production, and safeguarding mitochondrial integrity (Fig. 6).

CONCLUSION

This study demonstrates that cryoinjury-induced oxidative stress and cytoskeletal disruption significantly compromise the survival and developmental competence of vitrified bovine blastocysts. Notably, supplementation with MIT-001 exclusively during the warming stage significantly improved blastocyst viability by reducing apoptosis and enhancing mitochondrial and cytoskeletal integrity, whereas treatment during vitrification alone or throughout both stages showed no such benefit. These results indicate that the timing of antioxidant intervention is critical, with

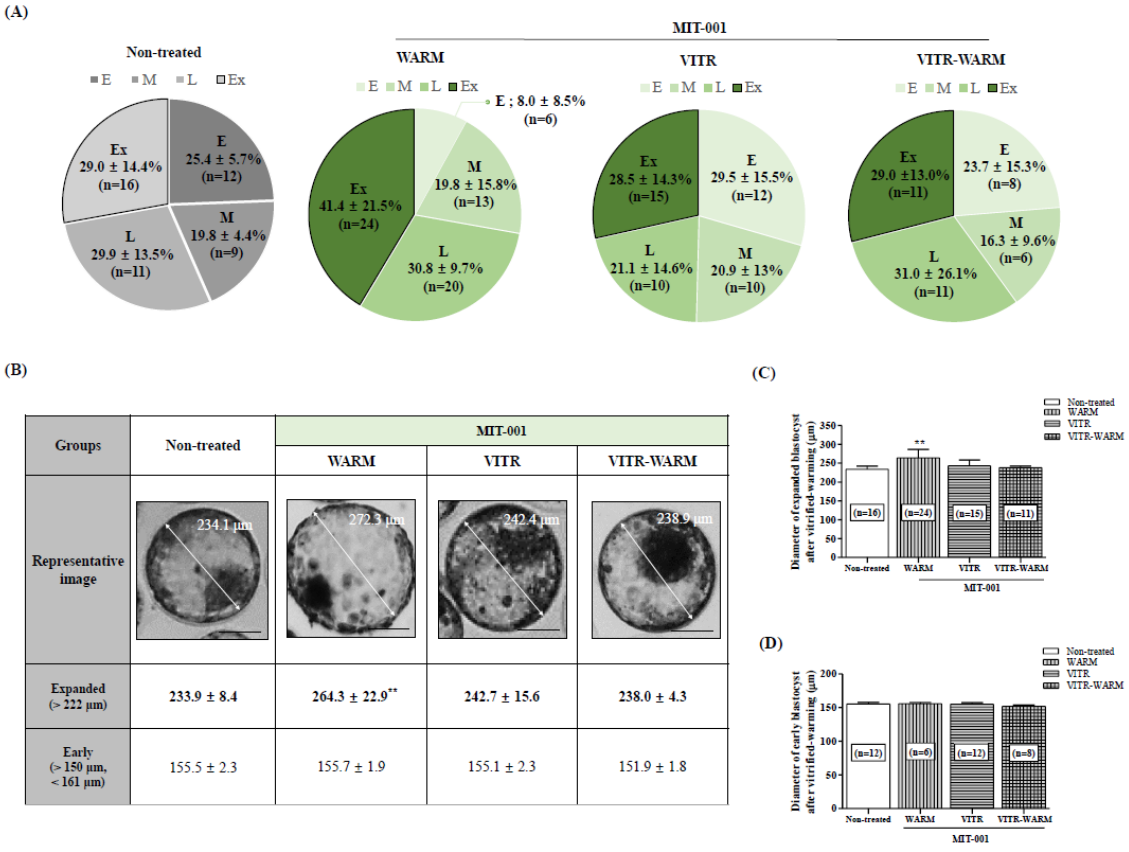
the warming stage representing a key window of vulnerability to oxidative stress. Our findings suggest that MIT-001 is a promising warming-specific antioxidant strategy for improving cryopreservation outcomes.

REFERENCES

1. Yu WD, Kim YJ, Cho MJ, Kim GJ, Kim SH, Kim MJ, et al. MIT-001 restores human placenta-derived mesenchymal stem cells by enhancing mitochondrial quiescence and cytoskeletal organization. International Journal of Molecular Sciences. 2021;22(10):5062 https://doi.org/10.3390/ijms22105062
2. Im K-I, Kim N, Lim J-Y, Nam Y-S, Lee E-S, Kim E-J, et al. The free radical scavenger NecroX-7 attenuates acute graft-versus-host disease via reciprocal regulation of Th1/regulatory T cells and inhibition of HMGB1 release. Journal of Immunology. 2015;194(11):5223-32
3. Grootaert MO, Schrijvers DM, Van Spaendonk H, Breynaert A, Hermans N, Van Hoof VO, et al. NecroX-7 reduces necrotic core formation in atherosclerotic plaques of Apoe knockout mice. Atherosclerosis. 2016;252:166-74
4. Wang N, Li CY, Zhu HB, Hao HS, Wang HY, Yan CL, et al. Effect of vitrification on the mRNA transcriptome of bovine oocytes. Reproduction in domestic animals. 2017;52(4):531-41 https://doi.org/10.1111/rda.12942
5. Huebinger J, Han H-M, Hofnagel O, Vetter IR, Bastiaens PI, Grabenbauer M. Direct measurement of water states in cryopreserved cells reveals tolerance toward ice crystallization. Biophysical journal. 2016;110(4):840-9 https://doi.org/10.1016/j.bpj.2015.09.029
6. Donnay I, Auquier P, Kaidi S, Carolan C, Lonergan P, Mermillod P, et al. Vitrification of in vitro produced bovine blastocysts: methodological studies and developmental capacity. Animal Reproduction Science. 1998;52(2):93-104 https://doi.org/10.1016/S0378-4320(98)00098-0
7. Inaba Y, Miyashita S, Somfai T, Geshi M, Matoba S, Dochi O, et al. Cryopreservation method affects DNA fragmentation in trophectoderm and the speed of re-expansion in bovine blastocysts. Cryobiology. 2016;72(2):86-92 https://doi.org/10.1016/j.cryobiol.2016.03.006
8. Gualtieri R, Kalthur G, Barbato V, Di Nardo M, Adiga SK, Talevi R. Mitochondrial dysfunction and oxidative stress caused by cryopreservation in reproductive cells. Antioxidants. 2021;10(3):337 https://doi.org/10.3390/antiox10030337
9. Somfai T, Nakai M, Tanihara F, Noguchi J, Kaneko H, Kashiwazaki N, et al. Comparison of ethylene glycol and propylene glycol for the vitrification of immature porcine oocytes. Journal of Reproduction and Development. 2013;59(4):378-84 https://doi.org/10.1262/jrd.2013-015
10. Yeon J-Y, Min S-H, Park H-J, Kim J-W, Lee Y-H, Park S-Y, et al. Mdivi-1, mitochondrial fission inhibitor, impairs developmental competence and mitochondrial function of embryos and cells in pigs. Journal of Reproduction and Development. 2015;61(2):81-9 https://doi.org/10.1262/jrd.2014-070
11. Smirnova E, Griparic L, Shurland D-L, Van Der Bliek AM. Dynamin-related protein Drp1 is required for mitochondrial division in mammalian cells. Molecular biology of the cell. 2001;12(8):2245-56
12. Frank S, Gaume B, Bergmann-Leitner ES, Leitner WW, Robert EG, Catez F, et al. The role of dynamin-related protein 1, a mediator of mitochondrial fission, in apoptosis. Developmental cell. 2001;1(4):515-25
13. Zarubin T, Han J. Activation and signaling of the p38 MAP kinase pathway. Cell research. 2005;15(1):11-8 https://doi.org/10.1038/sj.cr.7290257
14. Paliga AJ, Natale DR, Watson AJ. p38 mitogen-activated protein kinase (MAPK) first regulates filamentous actin at the 8–16-cell stage during preimplantation development. Biology of the Cell. 2005;97(8):629-40 https://doi.org/10.1042/BC20040146
15. Joe S-Y, Yang S-G, Lee J-H, Park H-J, Koo D-B. Stabilization of F-actin cytoskeleton by paclitaxel improves the blastocyst developmental competence through P38 MAPK activity in porcine embryos. Biomedicines. 2022;10(8):1867 https://doi.org/10.3390/biomedicines10081867
16. Jeong J-H, Yang S-G, Park H-J, Koo D-B. Effects of Mito-TEMPO on the survival of vitrified bovine blastocysts in vitro. Journal of Animal Reproduction and Biotechnology. 2021;36(4):299-306 https://doi.org/10.12750/JARB.36.4.299
17. Bogliolo L, Murrone O, Piccinini M, Ariu F, Ledda S, Tilocca S, et al. Evaluation of the impact of vitrification on the actin cytoskeleton of in vitro matured ovine oocytes by means of Raman microspectroscopy. Journal of assisted reproduction and genetics. 2015;32:185-93 https://doi.org/10.1007/s10815-014-0389-7
18. Hara T, Kin A, Aoki S, Nakamura S, Shirasuna K, Kuwayama T, et al. Resveratrol enhances the clearance of mitochondrial damage by vitrification and improves the development of vitrified-warmed bovine embryos. PLoS One. 2018;13(10):e0204571 https://doi.org/10.1371/journal.pone.0204571
19. Nedambale T, Dinnyes A, Groen W, Dobrinsky J, Tian X, Yang X. Comparison on in vitro fertilized bovine embryos cultured in KSOM or SOF and cryopreserved by slow freezing or vitrification. Theriogenology. 2004;62(3-4):437-49 https://doi.org/10.1016/j.theriogenology.2003.10.020
20. Coutinho A, Mendes C, Caetano HVA, Nascimento A, Oliveira V, Hernadez-Blazquez F, et al. Morphological changes in mouse embryos cryopreserved by different techniques. Microscopy Research and Technique. 2007;70(4):296-301 https://doi.org/10.1002/jemt.20404

21. Castillo-Martín M, Yeste M, Pericuesta E, Morató R, Gutiérrez-Adán A, Bonet S. Effects of vitrification on the expression of pluripotency, apoptotic and stress genes in in vitro-produced porcine blastocysts. Reproduction, Fertility and Development. 2015;27(7):1072-81 http://dx.doi.org/10.1071/RD13405
22. Chen Y-N, Dai J-J, Wu C-F, Zhang S-S, Sun L-W, Zhang D-F. Apoptosis and developmental capacity of vitrified parthenogenetic pig blastocysts. Animal reproduction science. 2018;198:137-44 https://doi.org/10.1016/j.anireprosci.2018.09.012
23. Estudillo E, Jiménez A, Bustamante-Nieves PE, Palacios-Reyes C, Velasco I, López-Ornelas A. Cryopreservation of gametes and embryos and their molecular changes. International Journal of Molecular Sciences. 2021;22(19):10864 https://doi.org/10.3390/ijms221910864
24. Whaley D, Damyar K, Witek RP, Mendoza A, Alexander M, Lakey JRT. Cryopreservation: an overview of principles and cell-specific considerations. Cell Transplantation. 2021;30:963689721999617. https://doi.org/10.1177/0963689721999617
25. López-Damián EP, Jiménez-Medina JA, Alarcón MA, Lammoglia MA, Hernández A, Galina CS, Fiordelisio T. Cryopreservation induces higher oxidative stress levels in Bos indicus embryos compared with Bos taurus. Theriogenology. 2020;143:74-81. https://doi.org/10.1016/j.theriogenology.2019.12.001
26. Len JS, Koh WSD, Tan S-X. The roles of reactive oxygen species and antioxidants in cryopreservation. Bioscience reports. 2019;39(8):BSR20191601 https://doi.org/10.1042/BSR20191601
27. Schieber M, Chandel NS. ROS function in redox signaling and oxidative stress. Current Biology. 2014;24(10):R453–R462. https://doi.org/10.1016/j.cub.2014.03.034SCIRP+4
28. He L, He T, Farrar S, Ji L, Liu T, Ma X. Antioxidants maintain cellular redox homeostasis by elimination of reactive oxygen species. Cellular Physiology and Biochemistry. 2017;44(2):532–553. https://doi.org/10.1159/000485089
29. Szechyńska-Hebda M, Zarrin Ghalami R, Kamran M, Van Breusegem F, Karpiński S. To be or not to be? Are reactive oxygen species, antioxidants, and stress signalling universal determinants of life or death? Cells. 2022;11(24):4105. https://doi.org/10.3390/cells11244105
30. Rodrigues AQ, Picolo VL, Goulart JT, Silva IMG, Ribeiro RB, Aguiar BA, et al. Metabolic activity in cryopreserved and grafted ovarian tissue using high-resolution respirometry. Scientific Reports. 2021;11:21517. https://doi.org/10.1038/s41598-021-01082-z
31. Yang S-G, Joe S-Y, Bae J-W, Heo G-D, Park H-J, Koo D-B. Melatonin protects against Mdivi-1-induced abnormal spindle assembly and mitochondrial superoxide production during porcine oocyte maturation. Frontiers in cell and developmental biology. 2021;9:693969 https://doi.org/10.3389/fcell.2021.693969
32. Nagao Y, Harada Y, Yamaguchi M, Igarashi A, Ooshima Y, Kato Y. Antioxidant treatment during preservation of bovine ovaries increased the development potential of embryos. Zygote. 2010;18(4):315-21 http://doi.org/10.1017/S0967199409990384
33. Kang M-H, Kim YJ, Cho MJ, Jang J, Koo YD, Kim SH, et al. Mitigating age-related ovarian dysfunction with the anti-inflammatory agent MIT-001. International Journal of Molecular Sciences. 2023;24(20):15158 https://doi.org/10.3390/ijms242015158
34. Jeong J-H, Park H-J, Yang S-G, Koo D-B. The antioxidant capacity of Mito-TEMPO improves the preimplantation development and viability of vitrified-warmed blastocysts through the stabilization of F-actin morphological aspects in bovine embryos. Journal of Animal Reproduction and Biotechnology. 2022;37(4):231-8 https://doi.org/10.12750/JARB.37.4.231

FIGURE LEGENDS



Groups	Non-treated	MIT-001		
		WARM	VITR	VITR-WARM
Representative image				
Expanded (> 222 μm)	233.9 ± 8.4	264.3 ± 22.9**	242.7 ± 15.6	238.0 ± 4.3
Early (> 150 μm, < 161 μm)	155.5 ± 2.3	155.7 ± 1.9	155.1 ± 2.3	151.9 ± 1.8

Figure 1. Assessment of expansion degree and diameter according to MIT-001 treatment in vitrified-warmed bovine blastocysts. (A) Proportions of survived blastocysts at each developmental stage 24 h after warming. (B - D) Representative images of expanded blastocysts and graphs showing the diameter of early and expanded blastocysts in each group. Data are presented mean ± SD and analyzed using one-way ANOVA. Differences were considered significant at $^{**}p < 0.01$; p-values were analyzed using Tukey's multiple-comparison test. Scale bar = 100 μm.

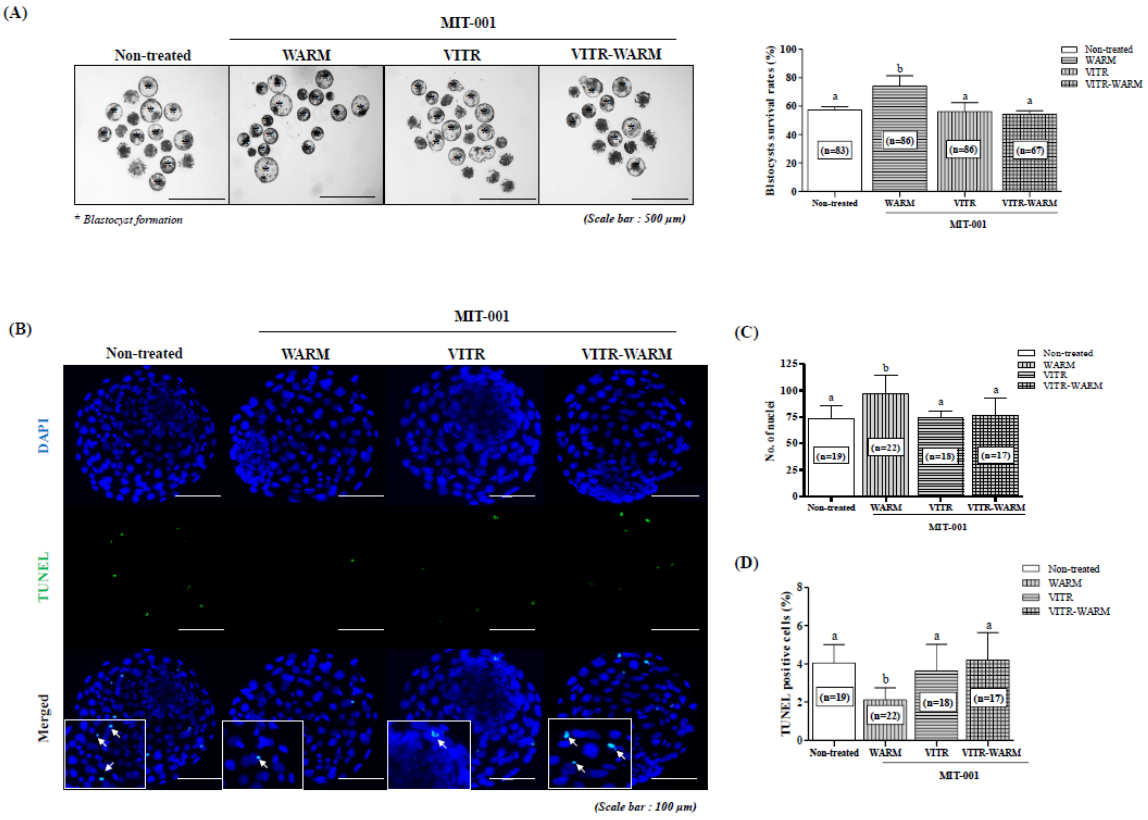


Figure 2. Survival and apoptosis analysis of vitrified-warmed bovine blastocysts following MIT-001 treatment. (A) Representative images of survival blastocysts subjected to vitrified-warmed process after treatment with 1 μM MIT-001 during vitrification and/or warming steps. Asterisks indicate blastocyst formation. Differences in superscript letters a and b indicate significant differences at $p < 0.05$. (B) DAPI (blue) and TUNEL (green, white arrows) staining of vitrified-warmed blastocysts. (C and D) Quantification of total nuclei and the percentage of TUNEL-positive cells in vitrified-warmed blastocysts. Data are presented mean ± SD and analyzed using one-way ANOVA. Different superscript letters within the same column indicate significant differences among treatment groups ($p < 0.05$), and *p*-values were analyzed using Tukey's multiple comparison test.

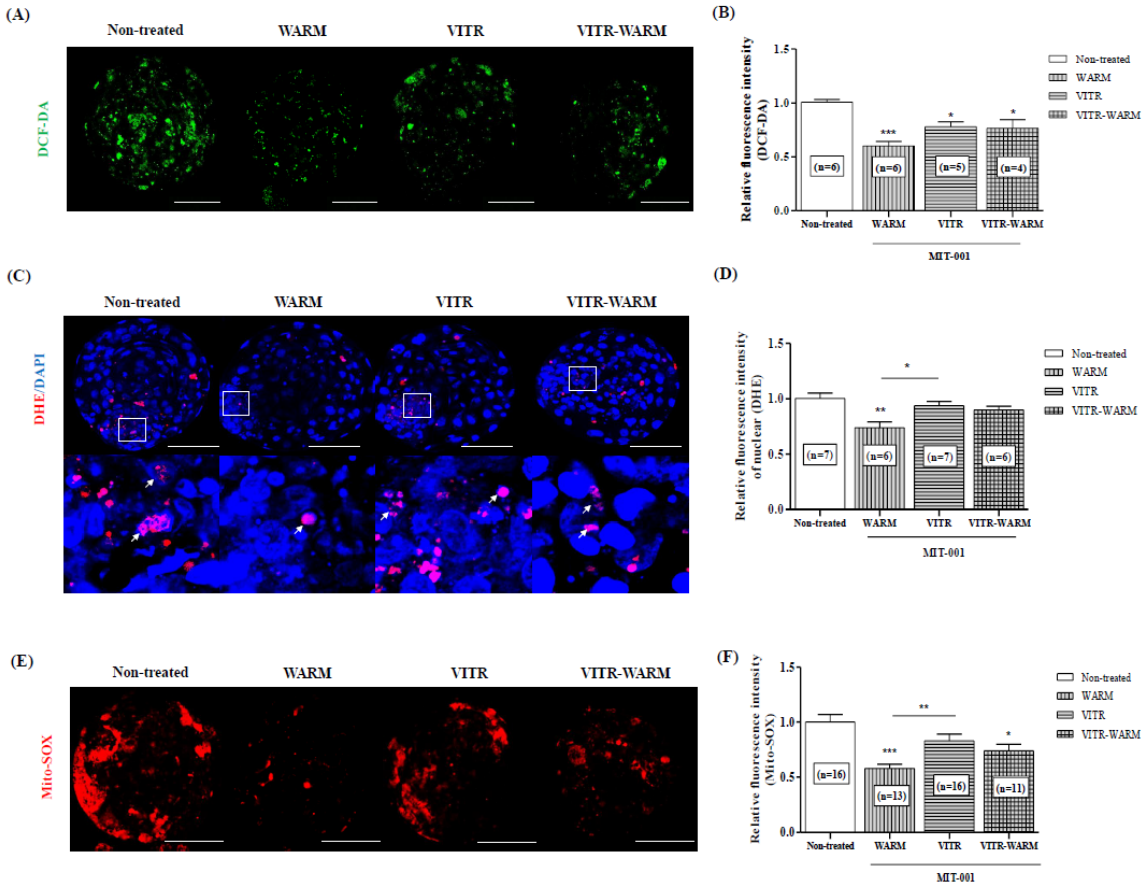
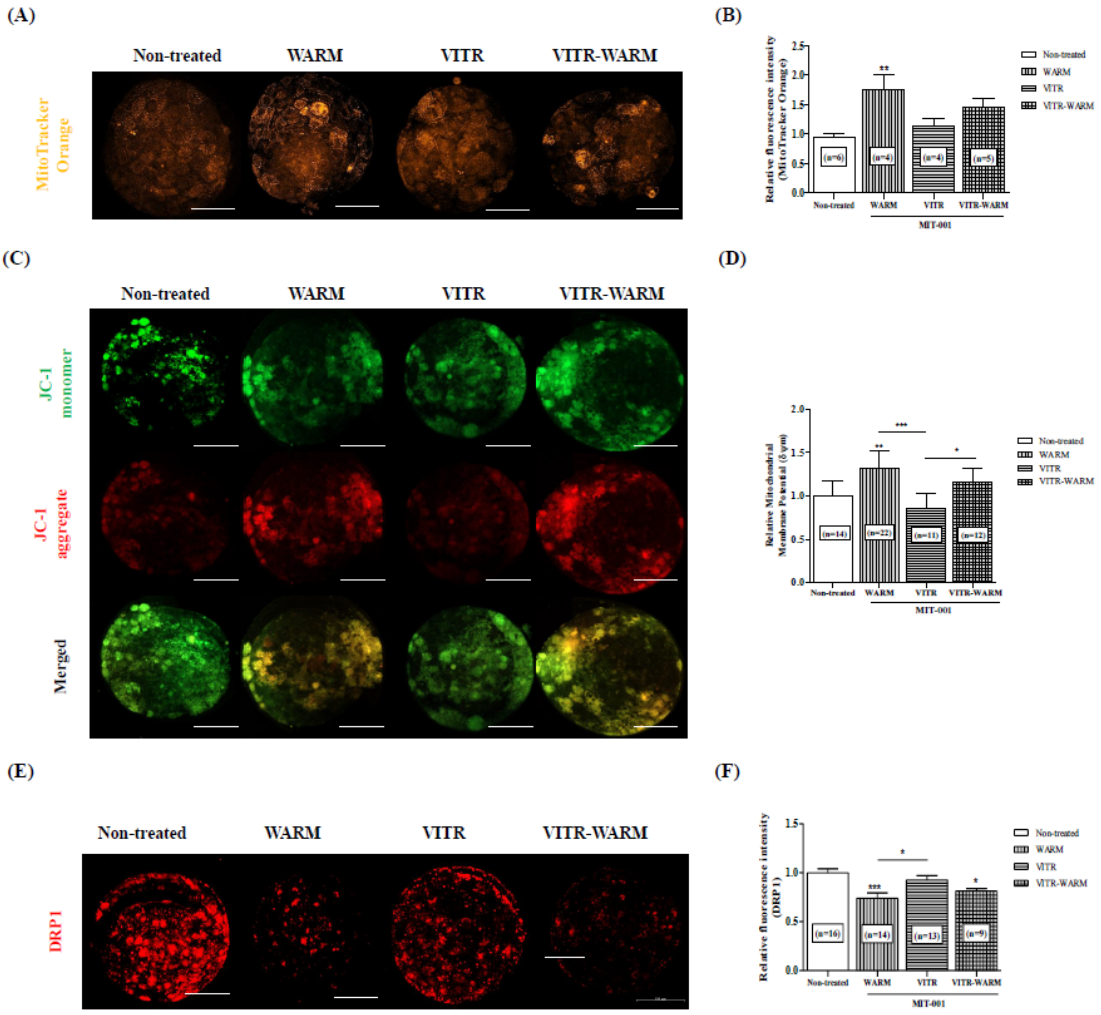


Figure 3. ROS levels in vitrified-warmed bovine blastocysts treated with MIT-001. (A) Intracellular ROS (green) was detected by DCF-DA staining. (B) Quantification of intracellular ROS in vitrified-warmed blastocysts. (C) Fluorescence intensity of nuclear superoxide (red), with white arrows indicating regions of colocalization detected using DHE and DAPI staining. (D) Quantification of the fluorescence intensity showing nuclear superoxide levels. (E) Mitochondrial superoxide (red) was detected by MitoSOX staining. (F) Quantification of mitochondrial superoxide levels after MIT-001 treatment. Data are presented as mean ± SD and analyzed using one-way ANOVA. Differences were considered significant at $^{**}p < 0.01$ and $^{***}p < 0.001$, and p-values were analyzed using Tukey's multiple comparison test. Scale bar = 100 μm.



460

461 **Figure 4. Mitochondrial function analysis in vitrified-warmed bovine blastocysts treated with MIT-001.** (A and
462 B) Mitochondrial intensity (orange) was visualized using MitoTracker Orange staining. (C and D) Representative
463 images and quantification of mitochondrial membrane potential (ΔΨm) using JC-1 staining. (E and F) DRP1
464 expression (red) was detected by immunofluorescence staining. Data are presented as mean ± SD and analyzed using
465 one-way ANOVA. Differences were considered significant at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$; p-values were
466 analyzed using Tukey's multiple comparison test. Scale bar = 100 μm.

467

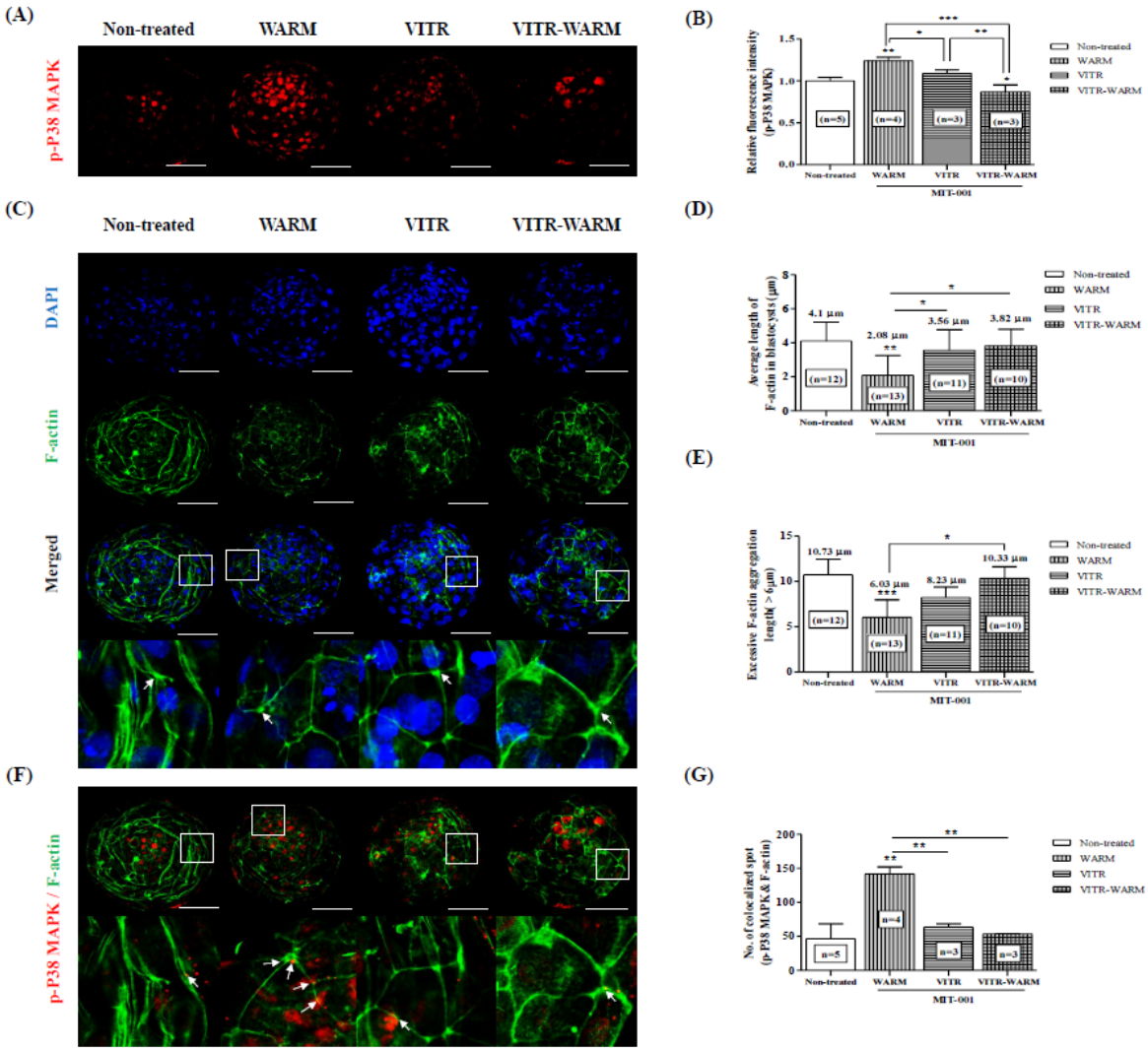


Figure 5. Effects of MIT-001 on F-actin aggregation and p38 MAPK activation in vitrified-warmed bovine blastocysts. (A and B) Expression of phospho (p)-p38 MAPK (red) was detected by immunofluorescence staining. (C) Representative images of F-actin expression (green and white arrows) were visualized by immunofluorescence staining. (D and E) Quantification of total and aggregated F-actin thickness (>6 μm). (F) Images of (p)-p38 MAPK (red) and F-actin (green), with white arrows indicating regions of colocalization. (G) Quantification of colocalization spots, showing the number of colocalized regions between (p)-p38 MAPK and F-actin The data are displayed in bar graphs as the mean ± SD and were analyzed using one-way ANOVA. Statistical significance was set at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$, with p-values evaluated using Tukey's multiple comparison test. Scale bar = 100 μm.

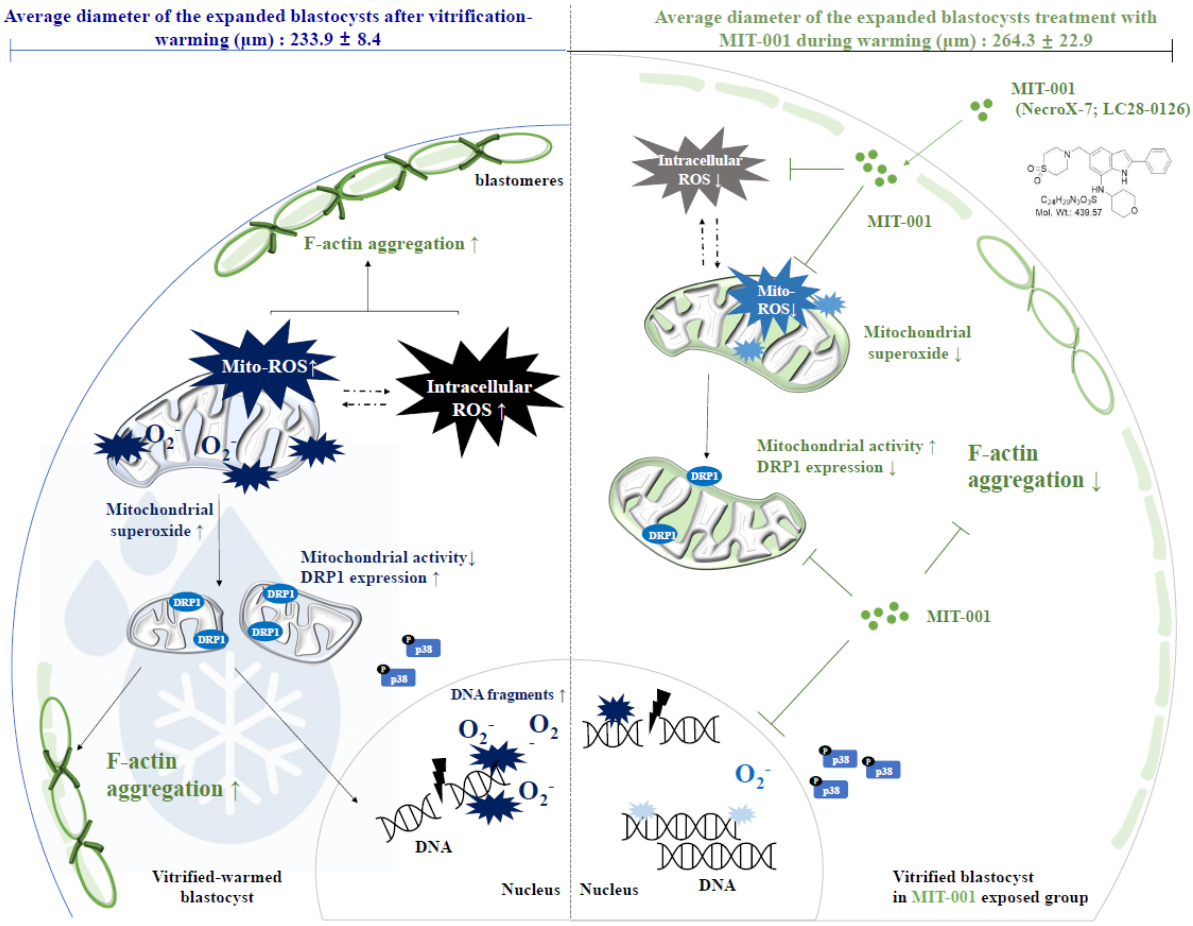


Figure 6. Schematic representation of the effects of MIT-001 in vitrified-warmed bovine blastocysts. Vitrification warming increases intracellular ROS, mitochondrial superoxide, and F-actin polymerization, leading to decreased blastocyst survival and cell diameter. This cellular stress activates p38 MAPK, which is primarily localized in the cytoplasm and promotes its translocation to the nucleus, where it regulates the expression of various genes associated with apoptosis. Treatment with MIT-001 significantly reduced ROS production, improved mitochondrial function, and inhibits p38 activation, thereby decreasing apoptosis and enhancing the survival and quality of the vitrified-warmed blastocysts. Furthermore, MIT-001 suppressed F-actin polymerization, maintained cytoskeletal integrity, and promoted a cellular environment conducive to embryonic development.

Table 1. Survival rates in vitrified-warmed bovine blastocysts treated with MIT-001

Groups	MIT-001 (1 μM) treatment		No. of blastocysts vitrified	No. of blastocysts warmed	No. of survived blastocysts (%)
	Vitrification	Warming			
Non-treated	-	-	**83**	**83**	**48 (57.3 ± 2.3)[a]**
WARM	-	+	**86**	**86**	**65 (74.2 ± 7.3)[b]**
VITR	+	-	**86**	**86**	**47 (56.2 ± 6.4)[a]**
VITR-WARM	+	+	**67**	**67**	**36 (54.3 ± 5.6)[a]**

Data are expressed as mean ± SD of three independent experiments. Different superscript letters denote significant differences ($p < 0.05$).

Table 2. TUNEL-positive cells rates in vitrified-warmed bovine blastocysts treated with MIT-001

Groups	MIT-001 (1 μM) treatment		No. of TUNEL positive cells	% of TUNEL positive cells
	Vitrification	Warming		
Non-treated	-	-	**3.4 ± 2.3**	**4.0 ± 1.0[a]**
WARM	-	+	**2.7 ± 1.3**	**2.2 ± 0.7[b]**
VITR	+	-	**2.8 ± 2.1**	**3.6 ± 1.4[a]**
VITR-WARM	+	+	**3.0 ± 2.0**	**4.2 ± 1.4[a]**

Data are expressed as mean ± SD of three independent experiments. Different superscript letters within the same column indicate significant differences among treatment groups ($p < 0.05$). TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.