

Short Communication

Comparison of Cryotop and ReproCarrier products for cryopreservation of bovine blastocysts through survival rate and blastocysts quality

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ABSTRACT Cryopreservation is used for blastocyst preservation of most mammalian embryos and is an important technique for breeding. We aimed to compare the efficiency of the cryopreservation method using the standard Cryotop device and the ReproCarrier device, a domestic product manufactured in Korea. The efficacy of the two devices was analyzed based on the survival rate, intracellular levels of reactive oxygen species (ROS), and apoptosis of the vitrified bovine blastocysts. The survival rates of the vitrified-warmed blastocysts were similar between the ReproCarrier group (58.4 ± 17.7%) and Cryotop group (59.9 ± 14.1%). Intracellular ROS levels and apoptotic index were determined by DCFDA staining and TUNEL assay. Changes in intracellular ROS levels, number of total nuclei, and cellular apoptosis of vitrified blastocysts after cryopreservation were not significantly different between the two groups. These results indicate that the ReproCarrier device method is as effective as the standard Cryotop method for vitrification of bovine blastocysts *in vitro*.

Keywords: bovine blastocyst, cryopreservation, Cryotop, ReproCarrier, vitrification

INTRODUCTION

Cryopreservation of oocytes and embryos in assisted reproductive technology for application in domestic animals and humans is very important to preserve the maternal and paternal genetic complements. Cryopreservation allows widespread use of valuable animal embryos to improve the chances of pregnancy. However, intracellular ice formation in cryopreservation methods for oocytes and embryos can lead to fatal damage. To overcome this issue of intracellular ice formation, slow freezing and

vitrification methods are most widely used (Mucci et al., 2006).

The balance of redox reactions in cells is very crucial for maintaining the metabolic environment and gene expression during embryonic development. However, high levels of reactive oxygen species (ROS) were seen in pre-implantation embryos of cattle (Min et al., 2014). ROS, such as hydrogen peroxide, cause oxidative stress, which is known to cause DNA damage and apoptosis (Takahashi, 2012). Therefore, regulating oxidative stress and ROS production during the vitrification-warming process is criti-

cal for blastocyst survival as well as its quality after the process.

A previous study demonstrated the process of apoptosis of bovine blastocysts (Paschoal et al., 2017). Apoptosis is an important indicator for evaluating the quality of blastocysts. TUNEL analysis, the most used method for identifying apoptotic cells, can confirm apoptosis through the labeling of a wide range of oligonucleosomal DNA fragments produced by endogenous DNase activity during the apoptosis process. Increased percentage of apoptotic cells is also an important indicator of the quality in embryo cryopreservation (Park et al., 2006).

Currently, most of the cryopreservation-related products for slow freezing and vitrification procedures are manufactured in foreign countries (Dos Santos-Neto et al., 2017). Cryotop (Kitazato Corporation, Japan) is a representative device developed for vitrification and cryopreservation of embryos. Cryotop is associated with high survival rate and is widely used for sensitive oocytes and blastocyst stage. However, domestic production of cryopreservation-related products is still insufficient. Hence, this study aimed to compare the efficacy of the ReproCarrier (Biomedical Manufacturing technology Center, Korea), a domestic product, and the Cryotop for vitrification of bovine *in vitro*-produced (IVP) blastocysts by analysis of survival rate and blastocyst quality after thawing.

MATERIALS AND METHODS

Chemicals

Unless noted otherwise, all chemicals used in this study were purchased from Sigma-Aldrich Korea (Sigma-Aldrich Korea, Yongin, Korea).

In vitro production of bovine embryos

In vitro maturation (IVM) of bovine oocytes was performed as described by Min et al. (2014) with slight modification. Briefly, bovine ovaries were acquired from a local slaughterhouse and transported to the laboratory in 0.9% saline containing 75 µg/mL penicillin G sodium salt at 30–35°C. Cumulus-oocyte complexes (COCs) were aspirated from follicles (3–6 mm in diameter) using a disposable 10-mL syringe with an 18-gauge needle. Aspirated COCs with at least three layers of compact cumulus cells and homogeneous cytoplasm were collected using mouth

pipetting, then washed thrice in Tyrode's lactate-*N*-2-hydroxyethylpiperazine-ethanesulfonic acid (TL-HEPES). Approximately 10–15 oocytes were matured in 50 µL of IVM medium in a 60-mm dish (Nunc, Roskilde, Denmark) under paraffin oil (Junsei, Tokyo, Japan) for 20–22 h at 38.5°C under an atmosphere of 5% CO₂ in air. 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, 10 IU/mL human chorionic gonadotropin, 25 µM β-mercaptoethanol, 10 ng/mL epidermal growth factor, 25 µg/mL gentamycin, 10% FBS (Gibco-BRL) and 1 µg/mL estradiol-β. After IVM, 15 oocytes were fertilized with frozen-thawed sperm at a concentration of 2 × 10⁶ cells/mL in 50 µL of fertilization medium. The fertilization medium consisted of Fert-TALP medium supplemented with 0.2 mM Na-pyruvate, 25 µg/mL gentamycin, and 0.6% BSA. In addition, 10 µg/mL heparin, 80 µM penicillamine, 4 µM hypo-taurine, and 2 µM epinephrine were also added to the fertilization drops. 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 with 0.4 mM Na-pyruvate, 1 mM glutamine, 0.3 mg/mL glutathione, 25 µg/mL gentamycin, and 10% FBS for four days at 38.5°C in 5% CO₂ in air.

Vitrification and warming procedure

As one experimental method, cryopreservation was carried out by vitrification with a Cryotop (Kitazato Supply Co, Fujinomiya, Japan, Fig. 1A) using a slightly modified version of the procedure described by Moulavi (2019). Briefly, one or two blastocysts were transferred into equilibration solution (ES) consisting of 7.5% dimethyl sulfoxide (DMSO) and 7.5% ethylene glycol (EG) in PBS supplemented with 20% FBS at room temperature for 5 min. Next, blastocysts were transferred into vitrification solution (VS) consisting of 15% DMSO, 15% EG, and 0.5 M sucrose dissolved in PBS containing 20% FBS. After 40–45 s, the blastocysts were loaded into a Cryotop and sank into liquid nitrogen. The process from exposure to VS to sinking into liquid nitrogen was completed within 1 min at room temperature. Vitrified blastocysts were warmed

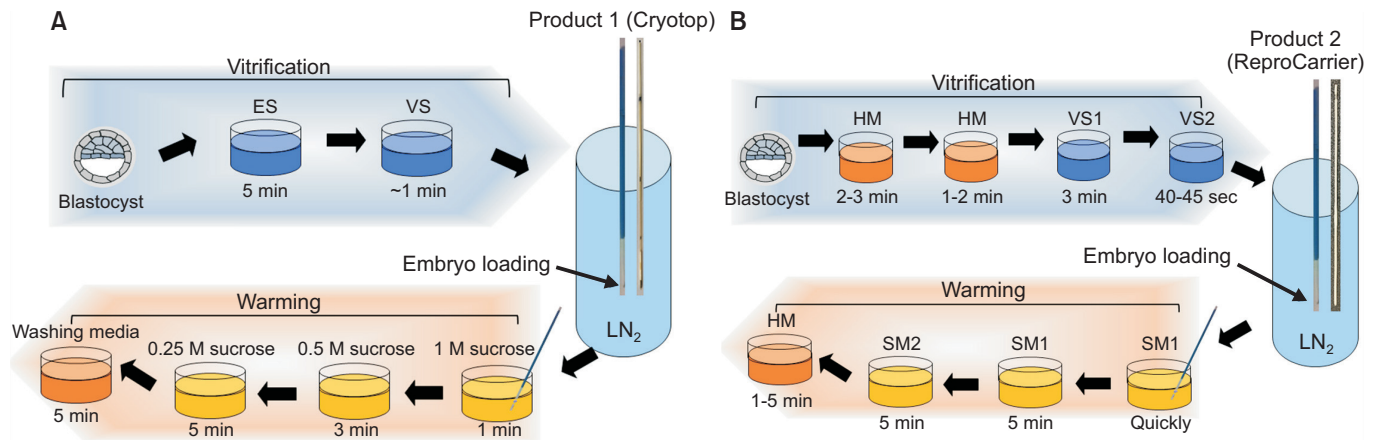


Fig. 1. Experimental diagram of the vitrification methods of bovine blastocysts using Cryotop and ReproCarrier products. (A) Description of vitrification protocol using Cryotop. (B) Graphical description of vitrification experimental methods using ReproCarrier.

by immersing the Cryotop directly into warming solution (1.0 M sucrose in PBS with 20% FBS) for 1 min, after which they were transferred to dilution solution (0.5 M sucrose in PBS with 20% FBS) for 3 min, and then to dilution solution (0.25 M sucrose in PBS with 20% FBS) for 5 min at room temperature. Subsequently, blastocysts were incubated for 5 min in washing solution (PBS with 20% FBS) at room temperature. As another experimental method, the vitrification process was carried out using the ReproCarrier (Fig. 1B). Briefly, one or two blastocysts were transferred into handling medium (HM; Modu Science, Korea) at room temperature for 2-3 min, followed by a second transfer to HM at room temperature for 1-2 min. Next, blastocysts were transferred into vitrification solution 1 (VS1) at room temperature for 3 min, followed by transfer into vitrification solution 2 (VS2) at room temperature for 40-45 s. Thereafter, the blastocysts were loaded into a ReproCarrier and sunk into liquid nitrogen. The process from exposure to VS2 to sinking into liquid nitrogen was completed within 1 min. Vitrified blastocysts were warmed by immersing the ReproCarrier directly into sucrose medium 1 (SM1) solution, followed by immediate transfer to the next SM1 solution at room temperature for 5 min. Next, blastocysts were transferred into sucrose medium 2 (SM2) solution for 5 min. Finally, blastocysts were transferred into HM. Survival of vitrified-warmed blastocysts was determined according to re-expansion rates after 24 h of recovery in culture medium.

Measurement of ROS levels

The level of ROS in each embryo was measured using

the difluorodihydrofluorescein diacetate method (H2DCFDA; Molecular Probes, Eugene, OR, USA) described previously (Choi et al., 2008). Vitrified-warmed blastocysts in IVC medium were washed thrice with 0.1% polyvinylalcohol (PVA) in PBS. Blastocysts were transferred into IVC medium containing 5 μ M H₂DCFDA for 30 min at 38.5°C. The intensity of H2DCFDA was measured with an iRiS Digital Cell Imaging System (Logos Biosystems, Gyeonggi-do, South Korea). The measured fluorescence images were analyzed by Image J software Version 1.38 (National Institutes of Health, Bethesda, MD, USA).

Assessment of apoptosis in blastocysts

Apoptotic cells in vitrified-warmed blastocysts were detected using an *In Situ* Cell Death Detection kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. On day 7, blastocysts were washed with 0.1% PVA in PBS and then fixed in 3.7% formaldehyde in PBS for 1 h at room temperature. Next, blastocysts were permeabilized using 0.5% Triton X-100 for 30 min at room temperature. The fixed blastocysts were incubated in TUNEL reaction medium for 1 h at 38.5°C, then washed and mounted on slide glass with DAPI solution. Stained blastocysts were examined under an iRiS Digital Cell Imaging System (Logos Biosystems, Gyeonggi-do, South Korea).

Statistical analysis

All percentage data obtained in the present study are presented as the mean \pm 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

Comparison of the instructions and features of the Cryotop and ReproCarrier products

Cryotop is composed of “Diamond tip”, “Wider sheet”, “Conjunction reshaped”, “Widened body”, and “Identification marks” (Fig. 2A). The length and weight of Cryotop were measured to be 13 cm and 0.62 g, respectively. Blastocysts mounted wider sheet as flat part (Fig. 2C). The lid of the Cryotop was closed in the liquid nitrogen. The ReproCarrier consists of “Embryo loading well”, “Gas passage way”, and “Identification logo” (Fig 2B). The length and weight of ReproCarrier were measured to be 13.2 cm and 1.11 g, respectively. The blastocysts were mounted into the embryo-loading well (Fig. 2D). The lid of the ReproCarrier was closed before placing it in the liquid

nitrogen. Notably, the ReproCarrier has a distinct site as the loading well to place the embryos as compared to the Cryotop.

Comparison of survival rate, intracellular ROS, and apoptosis level in the vitrified-warmed bovine blastocysts using Cryotop and ReproCarrier

To investigate the efficacy of the Cryotop and ReproCarrier products in survival of the vitrified-warmed bovine blastocysts after their recovery, we evaluated the morphology of normal blastocysts using a microscope (Leica, Solms, Germany) (Fig. 3A). Survival rates of the vitrified-warmed blastocysts were not significantly different between the Cryotop ($59.9 \pm 14.1\%$) group and ReproCarrier ($58.4 \pm 17.7\%$) group (Fig. 3B). Based on this result, both methods were verified to have similar effects on blastocyst survival after cryopreservation.

To confirm the damage caused to the bovine blastocyst by cryopreservation, we measured intracellular ROS levels by H2DCFDA staining of vitrified-warmed bovine blasto-

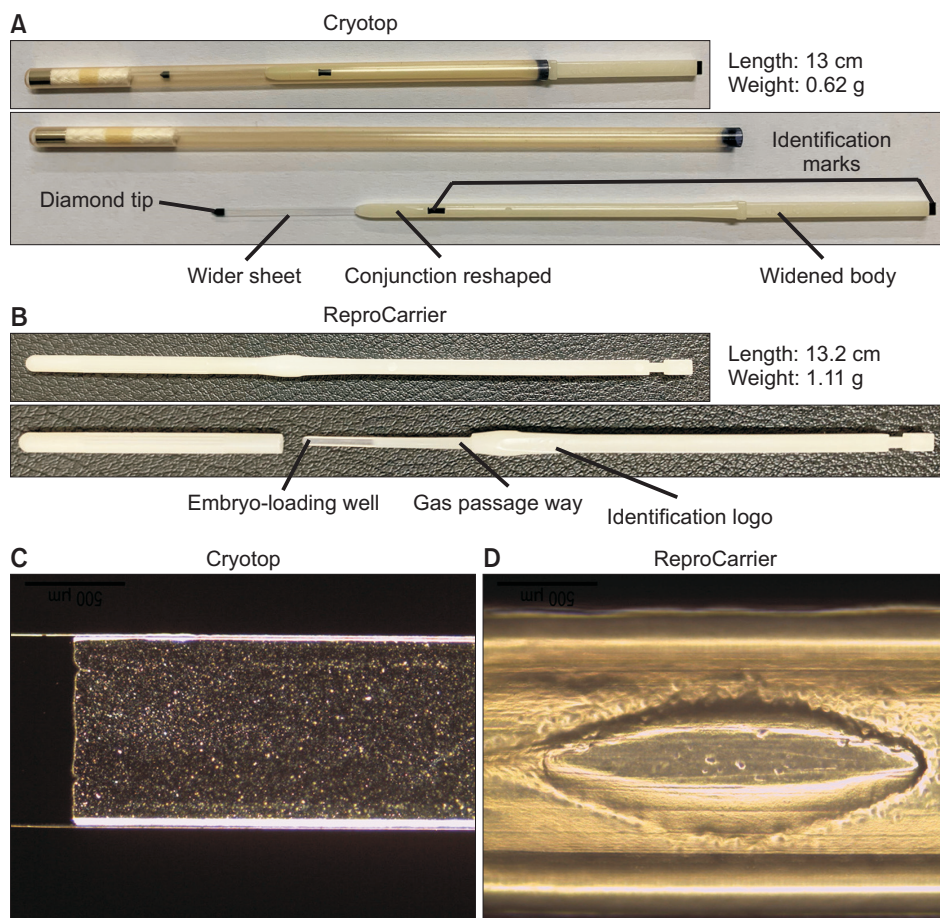


Fig. 2. Comparison of standard features for vitrification products. (A) Length, weight, and standard features of Cryotop. (B) Length, weight, and standard features of ReproCarrier. (C) Bovine blastocysts were mounted flat site on Cryotop. (D) Bovine blastocysts were mounted in the embryo-loading well on the ReproCarrier.

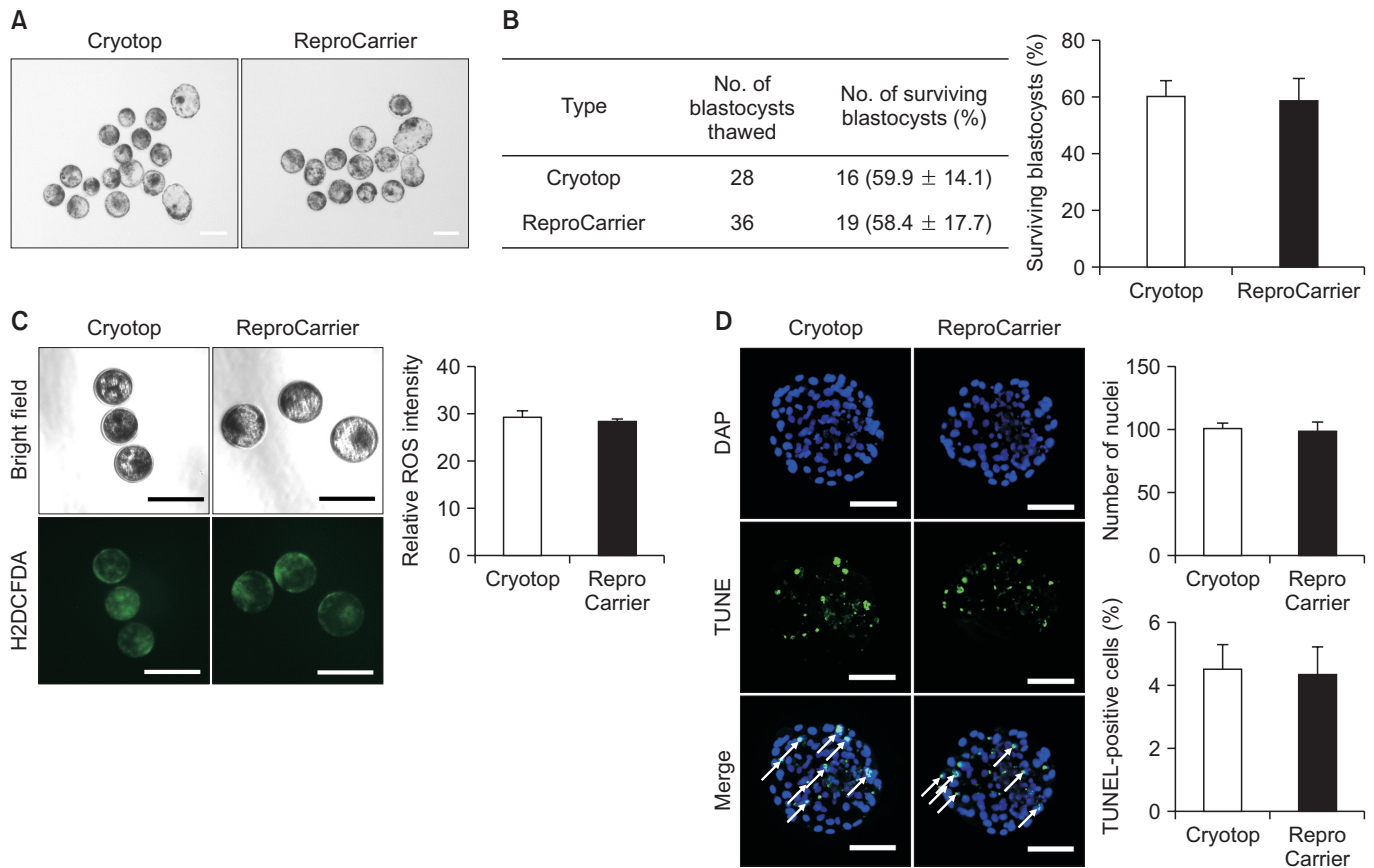


Fig. 3. Comparison of survival rate, intracellular ROS production, and apoptosis level in vitrified-warmed bovine blastocysts obtained using Cryotop and ReproCarrier products. (A) Morphology of vitrified-warmed bovine blastocysts visualized using microscope. (B) The survival rates of vitrified-warmed bovine blastocysts using Cryotop and ReproCarrier. (C) Vitrified-warmed bovine blastocysts using Cryotop and ReproCarrier were evaluated for intracellular ROS (green) by H2DCFDA staining. Scale bar = 100 μ m. Data in the bar graph represent means \pm SD from three independent experiments. (D) Colocalization of DAPI and TUNEL stains at the blastocyst stage. The blastocysts were stained for chromosome (blue) and fragmented DNA (green). The white arrows indicate a merged spot with chromosome and fragmented DNA. Data in the bar graph represent the means \pm SD from three independent experiments. Scale bar = 200 μ m.

cysts (Fig. 3C). The intracellular ROS intensity in blastocysts subjected to Cryotop method showed similar results to that in blastocysts subjected to the ReproCarrier. To investigate fragmented DNA, we performed DAPI and TUNEL staining of vitrified-warmed bovine blastocysts (Fig. 3D). The number of nuclei were similar in the blastocysts of both Cryotop (105.3 \pm 12.3) and ReproCarrier (100.5 \pm 17.5) groups. In addition, the number of TUNEL-positive cells using Cryotop (4.5 \pm 2.5%) and ReproCarrier (4.8 \pm 2.9%) were not significantly different. Thus, cryopreservation-induced damages such as ROS production and fragmented DNA were not significantly different between the Cryotop and ReproCarrier groups.

DISCUSSION

In the present study, we confirmed the similar survival rates of vitrified-warmed bovine blastocysts prepared using the Cryotop and ReproCarrier products. In addition, blastocysts obtained from the ReproCarrier method showed a similar level of cellular apoptosis due to cryo-damage as observed in the Cryotop group. Thus, our results demonstrated the similar efficacy of the ReproCarrier device as a domestic product in comparison to the Cryotop device for vitrification of bovine blastocyst.

Vitrification is a commonly used simple cryopreservation method yielding high survival rates. Cryotop method is the most representative of the vitrification cryopreservation method. Currently, domestic products related to

cryopreservation, such as ReproCarrier are rare. ReproCarrier is a cryopreservation product developed in Korea (Fig. 2B and Fig. 2D). Therefore, we confirmed whether ReproCarrier is suitable for cryopreservation by comparing the survival rate of blastocysts as well as the cryo-damage in vitrified-warmed blastocysts obtained through ReproCarrier or Cryotop products. No difference was found in the viability and quality of blastocysts obtained by the two methods, as evident from the evaluation of ROS production and apoptosis (Fig. 3C, 3D). ROS is known to play a variety of roles during the respiratory process of cells. However, ROS imbalance is known to cause DNA damage, endoplasmic reticulum (ER)-stress (He et al., 2018), and apoptosis in mammalian cells (Ansari et al., 2018). In addition, increased ROS during embryo development has been associated with decreased blastocyst formation (Yang et al., 2018). In other words, regulating ROS production is important to improve blastocyst competence (Moulavi et al., 2019) and survival of vitrified blastocysts (Pereira et al., 2019). During cryopreservation, blastocysts can be damaged by various factors such as membrane, DNA, and/or thermal damage (Inaba et al., 2016). A previous study indicated that endogenous ROS-mediated cellular cryo-damage is related to blastocyst formation rate and survival (Lee et al., 2019). In the current study, we observed that the intracellular ROS production was not significantly different in the ReproCarrier and Cryotop methods.

During IVP progression of bovine embryos, apoptosis is an important indicator of the quality of blastocysts, and an increase in the number of apoptotic cells indicates that the *in vitro* culture environment is inadequate (Nánássy et al., 2008). The TUNEL assay in our study revealed that the total cell number and apoptotic cells were not significantly different in the ReproCarrier and Cryotop groups (Fig. 3D).

CONCLUSION

In conclusion, the results of the present study suggest that vitrified warmed bovine blastocyst obtained using the ReproCarrier method is not significantly different from that obtained using the Cryotop in terms of survival rate of blastocyst formation, number of apoptotic cells, and intracellular ROS levels. Therefore, we suggest that ReproCarrier has similar efficiency to the Cryotop for cryopreservation of bovine blastocysts.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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