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Efficient Cryopreservation of Porcine Blastocysts produced by *In Vitro* Fertilization

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ABSTRACT

Cryopreservation has been applied successfully in many mammalian species. Nevertheless, pig embryos, because of their greater susceptibility to cryoinjuries, have shown a reduced developmental competence. The aim of this study was to evaluate the survival status of vitrified-warmed porcine embryos. Forced blastocoele collapse (FBC) and non-FBC blastocysts are vitrified and concomitantly cultured in culture media which were supplemented with/without fetal bovine serum (FBS). Porcine vitrified-warmed embryos were examined in four different methods: group A, non-FBC without FBS; group B, non-FBC with FBS; group C, FBC without FBS; group D, FBC with FBS. After culture, differences in survival rates of blastocysts derived from vitrified-warmed porcine embryos were found in group A~D (39.5 (A) vs 52.5 (B) and 54.8 (C) vs 66.7% (D), respectively, $p<0.05$). Reactive oxygen species (ROS) level of survived blastocysts was lower in group D than that of another groups ($p<0.05$). Moreover, total cell number of survived blastocysts was higher in group D than that of other groups ($p<0.05$). Otherwise, group D showed significantly lower number of apoptotic cells than other groups (2.0±1.5 vs 3.2±2.1, 2.8±1.9, and 2.7±1.6, respectively, $p<0.05$). Taken together, these results showed that FBS/FBC improves the developmental competence of vitrified porcine embryos by modulating intracellular levels of ROS and the apoptotic index during the vitrification/warming procedure. Therefore, we suggest that FBS and FBC are effective treatment techniques during the vitrification/warming procedures of porcine blastocysts.

(Key words : vitrification, forced collapse blastocoele, apoptosis, blastocyst, porcine)

INTRODUCTION

An efficient cryopreservation for porcine embryos is necessary to optimize cryopreservation systems for stable survival rates and for their widespread use in the embryo transfer industry. However, *in vitro*-derived porcine embryos have low developmental competence and poor quality compared with *in vitro*-derived other mammalian embryos, which may have been caused by suboptimal cryopreservation systems (Cuello *et al.*, 2007). Many studies have been published with the purpose of improving survival rates of porcine embryos via improvement of cryopreservation conditions (Du *et al.*, 2007; Fujino *et al.*, 2008; Cuello *et al.*, 2010). Recently, although successful birth of piglets from vitrified and warmed porcine blastocysts derived from IVF has been reported (Mito *et al.*, 2015), the problems of lower embryonic survival after cryopreservation are still remains.

Theoretically, forced blastocoele collapse (FBC) of blastocysts cavity is based on contraction-expansion cycles of the trophectoderm of the blastocyst (Min *et al.*, 2013; Min *et al.*, 2014). The mechanisms of blastocoel collapse and recovery of trophectoderm rupture are not yet fully understood. Regardless of the causes of blastocyst collapse, FBC blastocysts have a potential to gradually recover their spheroidal shape and the cytoplasm may become transformed in spherical structure due to lack of water (Mukaida *et al.*, 2006). Several studies have shown that there was an increase in the survival rate of blastocysts in response to FBC of blastocysts cavity using a glass micro-needle (Vanderzwalmen *et al.*, 2002), a 29-gauge needle (Son *et al.*, 2003) and a hand-drawn Pasteur pipette (Hiraoka *et al.*, 2004). Furthermore, there is formation of ice crystals due to the presence of water in blastocoele cavity, which can

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harm blastocysts quality in cryopreservation (Li *et al.*, 2012).

The exact mechanisms in fetal bovine serum (FBS) responsible for the biological effect on the development of embryos are not known. On the other hand, several studies have indicated that culture medium supplemented with FBS can cause morphological and physiological differences in embryos compared to those produced *in vivo* or in serum-free media (Mucci *et al.*, 2006). However, FBS is an extremely complex containing a variety of energy substrates, amino acids, vitamins and growth factors that may support survival and developmental competence of mammalian embryos in culture (Men *et al.*, 2005).

The objective of this study was to evaluate the developmental competence and qualities of frozen-thawed porcine blastocysts derived from FBS supplement in porcine embryo culture medium and vitrification by using forced collapse of the blastocoele.

MATERIALS AND METHODS

1. Chemicals

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

2. *In Vitro* Production of Porcine Embryos

Porcine ovaries were obtained from at a local abattoir and transported to the laboratory in 0.9% saline supplement with 75 μg/ml potassium penicillin G at approximately 30～35℃. Immature cumulus-oocyte complexes (COCs) were aspirated from follicles between 3 and 6 mm in diameter using an 18-gauge needle into a disposable 10 ml syringe (Funahashi *et al.*, 1994). Undamaged COCs with the same quality cytoplasm and surrounded by cumulus cells were selected by mouth pipetting and then washed three times in TL-HEPES medium. Overall, approximately 50～60 COCs were matured in 500 μl of IVM medium in a 4-well dish (Nunc, Roskilde, Denmark) at 38.5℃ and under 5% CO_2 in air. BSA free North Carolina State University (NCSU) 23 medium supplemented with 10% follicular fluid, 0.57 mM cysteine, 10 ng/ml β-mercaptoethanol, 10 ng/ml epidermal growth factor (EGF), 10 IU/ml pregnant mare serum gonadotropin (PMSG) and 10 IU/ml human chorionic gonadotropin (hCG) was used for oocyte maturation (Petters and Wells, 1993). After culturing for 22 h, COCs were washed three times and then further cultured in PMSG and hCG-free maturation medium for 22 h. After completion of IVM, IVF of porcine oocytes was performed as described by Abeydeera and Day (1997). The IVF medium, modified Tris-buffered medium (mTBM), consisted of 113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2$, 5 mM sodium pyruvate, 11 mM glucose, 20 mM Tris, 2.5 mM caffeine sodium benzoate and 1 mg/ml BSA. Fresh semen was kindly supplied once a week by an artificial insemination company (Dar by Porcine AI Center, Anseong, Korea) and kept at 17℃ for 5 days. Semen was then washed three times by centrifugation with Dulbecco's phosphate buffered saline (DBPS, Gibco BRL, Grand Island, NY, USA) supplemented with 1 mg/ml BSA (Fraction V), 100 mg/ml penicillin G, and 75 mg/ml streptomycin sulfate. At the end of washing, the spermatozoa were resuspended in mTBM at pH 7.8. Oocytes were washed three times in mTBM with 2.5 mM caffeine sodium benzoate and 1 mg/ml BSA (fatty acid free), after which they were placed into 48 μl of mTBM under paraffin oil. Next, 2 μl of diluted spermatozoa were added to a 48 μl drop of medium containing 15～20 oocytes to give a final concentration of 1.5×10^5 sperms/ml. Finally, the oocytes were co-incubated with spermatozoa for 6 h at 38.5℃ and under 5% CO_2 in air. For all experiments, the embryos were cultured in 50 μl drops of PZM-3 medium with 3 mg/ml BSA at 38.5℃ and under 5% CO_2 in air. After 48 h of culture, cleaved embryos were further cultured in a 50 μl drop of PZM-3 medium supplemented with 3 mg/ml BSA at 38.5℃ and under 5% CO_2 in air for 4 days. Blastocyst formation was evaluated under a stereomicroscope at 6 days after insemination.

3. FBC of Blastocysts Cavity

Before beginning the cryopreservation, FBC of porcine *in vitro*-derived blastocysts was performed using pulled Pasteur pipet. After holding the *in vitro*-derived blastocyst with the flat side of 29-gauge needle and placing the inner cell mass (ICM) at the 12 or 6 o'clock position, a pulled Pasteur pipet was pushed through the trophectoderm cell into the blastocoel cavity until it shrunk. Contraction of the *in vitro*-derived blastocysts was observed between 30 sec to 1 min. After complete shrinkage of the blastocoel, *in vitro*-derived blastocysts frozen-thawed and stored in liquid nitrogen (LN_2) tank.

4. Vitrification Procedure

Cryopreservation was carried out by vitrification with a cryotop (Kitazato Supply Co, Fujinomiya, Japan) as described by Kuwayama *et al.* (2007) with slight modifications. Briefly,

porcine *in vitro*-derived 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, one or two blastocysts were transferred into vitrification solution (VS) consisting of 15% EG, 15% DMSO and 0.5 M sucrose dissolved in PBS containing 20% FBS. After 20～30s, the blastocysts were loaded into a cryotop and plunged into LN_2. The process from exposure in VS to plunging into LN_2 was completed within 1 min. Vitrified blastocysts were then warmed by immersing the cryotop directly into warming solution (1.0 M sucrose dissolved in PBS containing 20% FBS) for 1 min, after which they 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. Subsequently, blastocysts were incubated for 5 min in the washing solution (PBS containing 20% FBS). Forced blastocoele collapse (FBC) and non-FBC blastocysts are vitrified and concomitantly cultured in culture media which were supplemented with/without FBS. Therefore, we divided four experimental groups (group A, non-FBC without FBS; group B, non-FBC with FBS; group C, FBC without FBS; group D, FBC with FBS). Survived and hatched rates of vitrified-warmed blastocysts were determined according to re-expansion rates after 24 h of recovery in culture medium.

5. TUNEL Assay

Apoptotic cells in blastocysts were detected using an *in situ* Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany). Vitrification-derived blastocysts were washed three times with 0.1% PVP in PBS and then fixed in 4% (v/v) paraformaldehyde diluted in PBS for 1 h at room temperature. For membrane permeabilization, fixed embryos were incubated in PBS containing 0.1% (v/v) Triton X-100 for 1 h at 4℃. Fixed embryos were preincubated in TUNEL reaction medium for 1 h at 38.5℃ in the dark, washed and mounted on slides with mounting solution containing 1.5 μg/ml 4', 6-diamidino-2-phenylindole (DAPI; Vector Laboratories). 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 fluorescent background of DAPI. The person counting cells and apoptotic nuclei was unaware of the experimental group.

6. Measurement of ROS Levels

The level of H_2O_2 in each embryo was measured using the difluorodihydrofluorescein diacetate method (H_2DCFDA; Invitrogen, Molecular Probes, Willow, USA) described previously (Choi *et al.*, 2008). H_2DCFDA produced an intermediate difluorodihydrofluorescein (DCF) after reaction with ROS, DCF upon oxidation produced difluorofluorescein which can be monitored in fluorescence microscope (Crow, 1997). At day 6, IVP blastocysts were recovered and used for the experiment. After three washes in IVC medium, blastocysts were transferred into IVC medium containing 5 μM H_2DCFDA for 20 min at 38.5℃. A stock solution of H_2DCFDA dissolved in dimethylsulfoxide (DMSO) was then diluted in IVC medium, after which the permeabilized blastocysts in H_2DCFDA were washed three times with 0.1% PVA-dPBS and placed into a 50 μl drop covered with mineral oil. The fluorescent emissions from the embryos were recorded as TIFF files using a cooled CCD camera attached to a fluorescent microscope (IX 51, Olympus, Tokyo, Japan) with filters at 488 nm forexcitation and 520 nm for emission. The recorded fluorescent images were processed (subtract background) and analyzed (measure integrated density) using Image J software version 1.38 (National Institutes of Health, Bethesda, MD). A total of 20 blastocysts were examined in each treatment group.

7. Statistical Analysis

All experiments were repeated more than three times. Survival rate of vitrified-thawed porcine IVF blastocysts was analyzed by the Chi-square test. Other all percentage data are presented as the means±SD. The Student's *t*-test was used for comparison between two groups, and Duncan's multiple range tests was used for multiple comparisons. A probability value of $p<0.05$ was considered significant.

RESULTS

1. FBC/FBS Enhances the Rates of Survival in Cryopreserved Expanded Porcine Blastocysts

As shown in Table 1, the rates of survival of blastocysts were significantly higher in group D than in group A, B and C of vitrification full expanded blastocysts at $p<0.05$ level. Consistent with that, the rates of survival blastocysts were also significantly higher in group D than in non-FBC group A, B and C of vitrification total expanded blastocysts at $p<0.05$

Table 1. Survival rates of vitrified-thawed porcine IVF blastocysts after 24 h culture in the treatment or non-treatment of FBS and FBC

Group		No. of embryos examined	No. (%) of blastocysts		
			Full expanded	Partial expanded	Total survived
Non-FBC	w/o FBS	58	3 (5.2)[a]	20 (34.5)	23 (39.7)[a]
	w/w FBS	60	9 (15.0)[b]	25 (41.7)	34 (56.7)[b]
FBC	w/o FBS	62	9 (15.0)[b]	26 (43.3)	35 (56.5)[b]
	w/w FBS	62	16 (25.8)[c]	28 (45.2)	44 (70.9)[c]

[a~c] Values with different superscripts in the same column denote a significant difference relative to other groups ($p<0.05$).

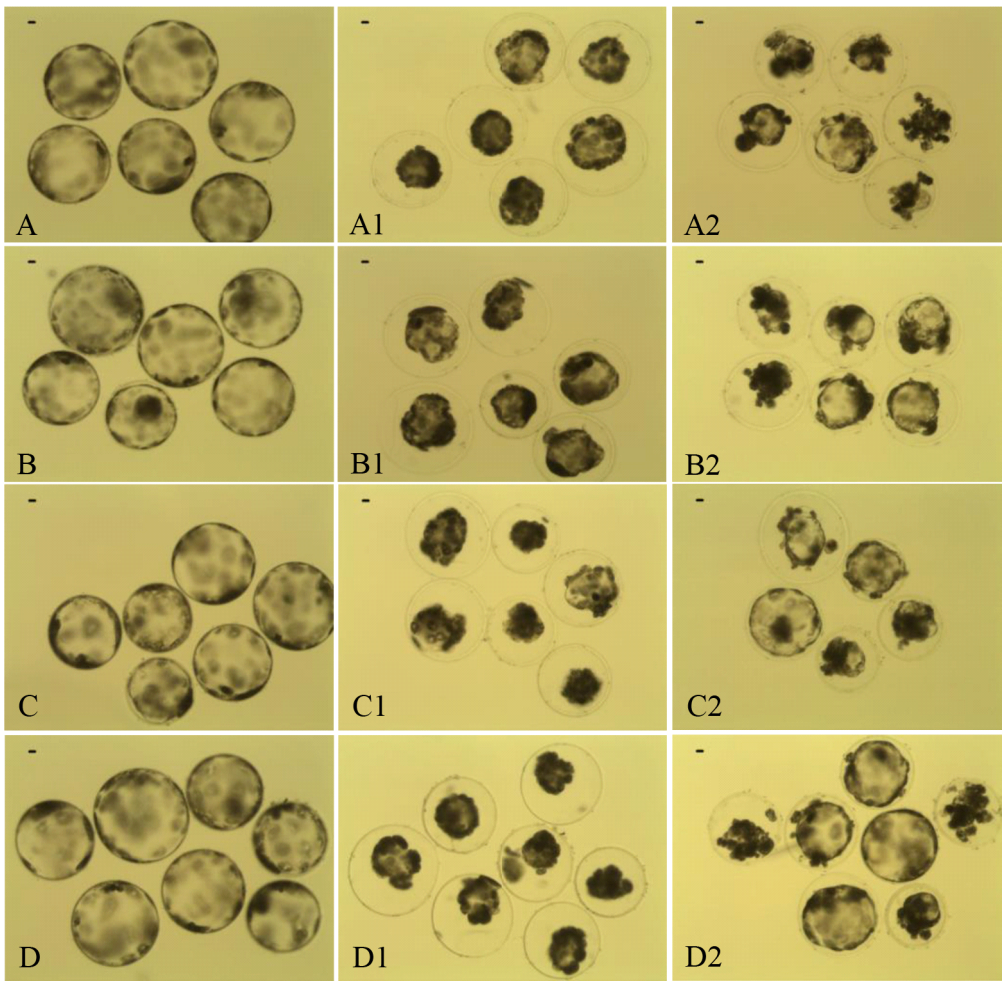


Fig. 1. Morphological patterns in non-FBC and FBC treated frozen-thawed porcine blastocysts. Fresh blastocysts derived from IVF (A～D) and frozen-thawed porcine blastocysts at 0 h culture (A1～D1). Non-FBC frozen-thawed porcine blastocysts cultured with PZM-BSA for 24 h (A2) and culture with PZM-BSA+10% FBS (B2). Morphological patterns in FBC treated frozen-thawed porcine blastocysts after 24 h cultured with PZM-BSA (C2) and cultured with PZM-BSA+10% FBS (D2). Scale bars=50 μm.

level. Consequently, the full and total expanded rates of frozen-thawed blastocysts were increased by using FBC/FBS of blastocysts cavity during cryopreservation.

2. Total Cell Numbers and Apoptosis in Blastocysts derived from Group A~D of Cryopreserved Porcine Expanded Blastocysts

In group D of frozen-thawed porcine expanded blastocysts,

total cell number was significantly higher than that of group A, B and C (46.6±3.8 vs. 40.2±3.4, 43.2±3.4 and 44.6±3.9, respectively; $p<0.05$) as shown in Table 2 and Fig. 2. The index of TUNEL-positive cells of appear green in apoptotic cells decreased in frozen-thawed expanded blastocysts derived from group D compared with the group A, B and C (2.0±1.5 vs. 3.2±2.1, 2.8±1.9 and 2.7±1.6, respectively; $p<0.05$) as shown in Table 2 and Fig. 2.

3. Effects of FBC/FBS Treatment on Expression Levels of ROS in Cryopreserved Porcine Expanded Blastocysts

We also investigated the intracellular levels of ROS in cryopreserved porcine expanded blastocysts treated with or without FBC/FBS. In group D of frozen-thawed porcine blastocysts expression of ROS was significantly lower than that of group A, B and C ($p<0.05$) as shown in Fig. 3A. The index of ROS decreased in frozen-thawed porcine blastocysts derived from group D compared with the group A, B and C ($p<0.05$) as shown in Fig. 3B.

DISCUSSION

This study showed the survival potential of frozen-thawed porcine blastocysts. The cryopreservation of *in vitro* porcine embryos has the potential for application in numerous biotechnologies. However, frozen-thawed porcine embryos are considered to be more sensitive to chilling damage than *in vivo* embryos because of low developmental competence embryos during maturation. In the present study, we developed an efficient cryopreservation method for porcine preimplantation embryos using the FBC and FBS system. We found that frozen-thawed FBC/FBS blastocysts were associated with improved embryo developmental competence and qualities such as increased total cell numbers and reduced numbers of apoptotic nuclei.

The expanded blastocysts have a fluid-filled blastocoele, which is known to cause damage to embryos via intra-blastocoelic ice formation during cryopreservation. A possible explanation for low developmental rates of cryopreserved embryos is that inappropriate dehydration and permeation of cryoprotectant might cause ice crystal formation in the cooling and warming steps during cryopreservation (Mukaida *et al.*, 2006). On the other hand, the beneficial effects of reducing the blastocoele fluid before cryopreservation prevents ice crystal formation

Table 2. Effect of different culture systems on total cells and apoptosis of vitrified-warmed porcine IVF blastocysts

Group		No. of embryos examined	Apoptosis (nuclei)	
			DAPI	TUNEL
Non-FBC	w/o FBS	20	40.2±3.4[a]	3.2±2.1[a]
	w/w FBS	20	43.2±2.4[a]	2.8±1.9[a]
FBC	w/o FBS	20	44.6±3.9[a]	2.7±1.6[a]
	w/w FBS	20	46.6±3.8[b]	2.0±1.5[b]

[a,b] Values with different superscripts in the same column denote a significant difference relative to other groups ($p<0.05$).

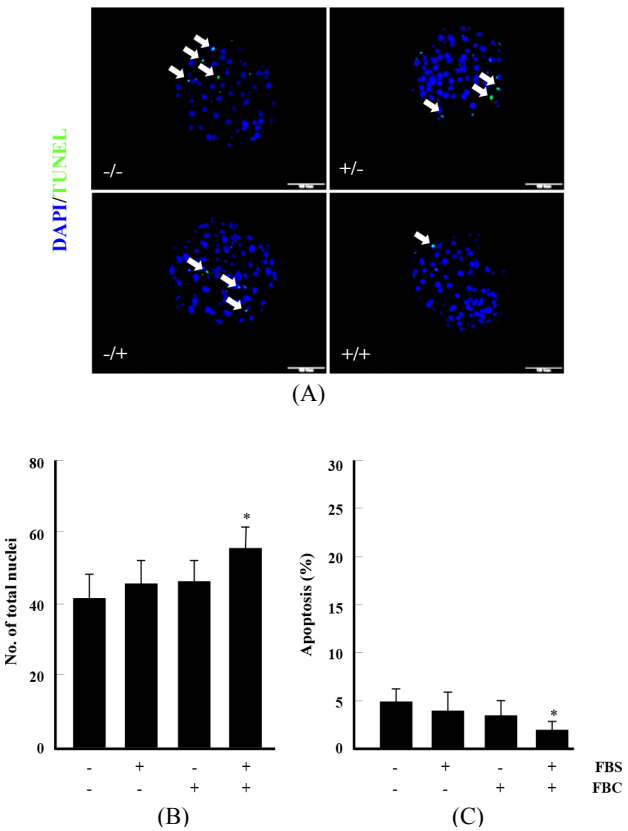
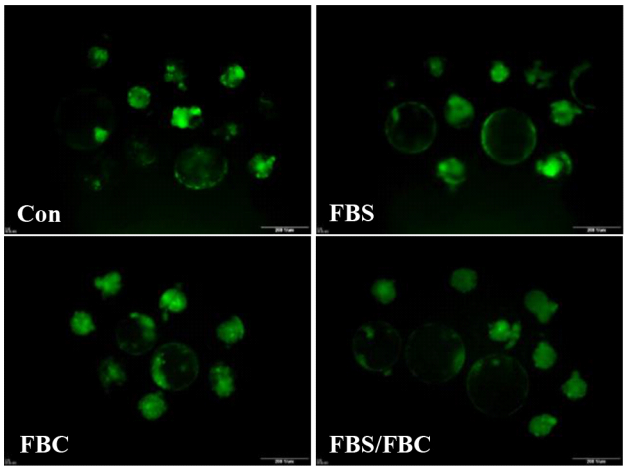
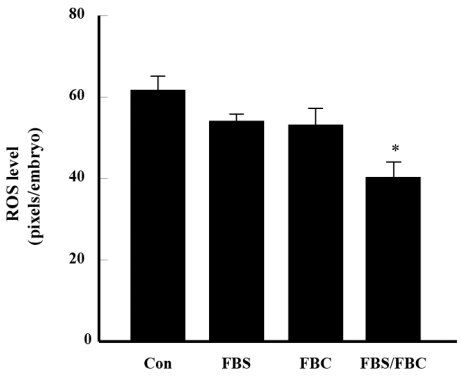


Fig. 2. Comparison of apoptotic characteristics in porcine frozen-thawed blastocysts derived from FBS and FBC treatment groups. The chromatin content is stained by DAPI (blue), fragmented DNA is labeled by the TUNEL reaction, and colocalization with DAPI appears sky-blue (A). Total cells number (B) and number of apoptotic nuclei (C) in porcine blastocyst stage embryos derived from frozen-thawed porcine blastocysts. Data are the mean±SD. Statistically significant differences are indicated by asterisks ($p<0.05$). Scale bars =100 μm.



(A)



(B)

Fig. 3. Comparison of antioxidant characteristics in porcine frozen-thawed blastocysts derived from FBS and FBC treatment groups. Fluorescence microscopy imaging of intracellular ROS expression (A) and level of ROS (B) in blastocysts. Data are the mean±SD. Statistically significant differences are indicated by asterisks (p<0.05). Scale bars=100 μm.

injuries caused during the normal cryopreservation (Vanderzwalmen *et al.*, 2002; Lee *et al.*, 2006). To overcome these problems during cryopreservation, several studies have demonstrated that the rates of survival were improved after FBC of the blastocyst cavity before cryopreservation (Son *et al.*, 2003; Hiraoka *et al.*, 2004). However, the effects of forced blastocoele collapse followed by vitrification on survival of *in vitro* porcine blastocysts have not been reported. Significant differences between the forced blastocoele collapse group C, D and w/o forced blastocoele collapse group A, B in developmental ability and embryonic quality were observed in porcine frozen-thawed blastocysts (p<0.05).

The influence of FBS on early embryo developmental competence depends on its origin, batches, and even the concentration used. On the other hand, there are reports of adverse effects of FBS supplementation on an embryo resistance to vitrification procedure cryodamage (Pereira *et al.*, 2005; Gomez *et al.*, 2008). Embryos produced with FBS supplementation have abnormally accumulated intracellular lipids compared with those produced *in vivo* or in serum-free systems. Embryos produced in serum-free systems have less accumulation of intracellular lipids and better survival when they were subjected to cryopreservation. However, porcine blastocysts produced with FBS supplementation did have an increased ability to regain their original morphology after freezing/warming. We demonstrated that frozen-thawed porcine expanded blastocysts in the FBS group showed higher full expanded rates than those of non-FBS group (Table 1). The blastocysts produced in the presence of FBS may help increase the metabolism of intracellular lipids. Consequently, blastocysts derived from this system had increased ability to survive cryopreservation.

TUNEL assay enables the detection of apoptosis in mammalian embryos based on the morphological observation of common features of apoptosis, including condensation or fragmentation of nuclei, or determination of the presence of specific DNA degradation products (Makarevich and Markkula 2002; Neuber *et al.*, 2002). Sudano *et al.* (2011) showed that cryopreservation leads to physical, chemical, and thermal damage, which can cause apoptosis in blastocysts. Consistent with these previous reports, our results showed that the incidence of apoptosis rates in the group D was lower than in the group A, B and C (p<0.05). Furthermore, the total cell numbers of blastocysts were confirmed, using the DAPI and TUNEL because the blastocysts with a number of these cells are important parameters for embryo development (Hao *et al.*, 2004). Therefore, we observed that the total cell numbers in group D were higher than that of group A, B and C (p<0.05). Taken together, these results suggest that the significant differences between the group D and group A, B and C in embryonic quality were observed in *in vitro* porcine frozen-thawed blastocysts derived from FBS and FBS (p<0.05).

ROS are primarily generated in the mitochondria as by-products of normal cell metabolism, and the principal source of ROS is hydrogen peroxide. Damaged mitochondria can increase ROS production. Low levels of ROS in embryos are necessary for normal cellular functions, whereas higher levels of ROS damage in embryos (Tareq *et al.*, 2012; Khalil *et al.*, 2013).

Furthermore, the present study showed that ROS levels were significantly higher in the group D than the group A, B and C (Fig. 3). Taken together, treatment with FBC/FBS also had an apparent effect on reduction of ROS level in porcine blastocysts during vitrification procedure. These findings might be the reduction of culture condition damages mitochondria, leading to the production of excessive ROS.

In conclusion, the present study is the first report of successful cryopreservation of porcine blastocysts using a FBC and FBS procedure. Moreover, the survival and quality of blastocyst indicated that the FBC and FBS procedure led to satisfactory improvement of porcine *in vitro*-derived blastocysts.

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