Effects of Trehalose and Sucrose on DNA Integrity of Evaporatively Dried Boar Spermatozoa and Embryo Development After ICSI

Li, Xiao Xia
Department of Animal Science and Biotechnology, College of Agriculture and Life Science, Chungnam National University

Yi, Young Joo
Division of Biotechnology, College of Environmental & Bioresources, Chonbuk National University

Lee, Ji Hey
Department of Animal Science and Biotechnology, College of Agriculture and Life Science, Chungnam National University

Kim, Keun Jung
Department of Animal Science and Biotechnology, College of Agriculture and Life Science, Chungnam National University

他

https://doi.org/10.5109/1526399

出版情報：九州大学大学院農学研究院紀要. 60 (2), pp.385-392, 2015-09-18. Faculty of Agriculture, Kyushu University
バージョン：
権利関係：

J. Fac. Agr., Kyushu Univ., **60** (2), 385–392 (2015)

Effects of Trehalose and Sucrose on DNA Integrity of Evaporatively Dried Boar Spermatozoa and Embryo Development After ICSI

Xiao Xia LI[1], Young Joo YI[2]**, Ji Hey LEE[1], Keun Jung KIM[1], Eun Young KIM[1], Lili ZHUANG[1], Ju Lan CHUN[1], Kyung–Bon LEE[3], Takafumi GOTOH and Min Kyu KIM[1]***

Kuju Agricultural Research Center, Faculty of Agriculture,
Kyushu University, Kuju, Oita, 878–0201, Japan
(*Received May 18, 2015 and accepted May 19, 2015*)

Evaporative–drying, a novel technique for sperm preservation, has many advantages however, the results were inferior to freeze–drying. The objective of this study is to investigate the effects of trehalose or sucrose supplemented into desiccation/preservation solution for sperm dehydration on sperm DNA integrity and the development of porcine embryos after intracytoplasmic injected with evaporatively dried boar spermatozoa. The addition of 0.2 M trehalose or sucrose significantly reduced DNA damage of sperm compared to those of other concentrations. The rate of cleaved embryo and blastocyst formation was significantly increased in oocytes injected with evaporatively dried sperm with 0.2 M trehalose. According to TUNEL assay, total cell number was increased, and the indices of TUNEL, fragmentation and total apoptosis were significantly decreased in blastocysts from ICSI with sperm treated with trehalose. Also, evaporatively dried sperm with trehalsoe were preserved at 4˚C for 1 year, and successfully produced pre–implantation embryos after ICSI that transferred to the recipients became pregnant and maintained pregnancy periods until 3 months. Consequently, the present study found out that trehalose could reduce DNA damage of sperm during dehydration, and oocytes injected with sperm treated with trehalose developed to pre–implantation embryos that might produce normal piglets after embryo transfer (ET).

Key words: Evaporative–drying, spermatozoa, trehalose, ICSI, pig

INTRODUCTION

Generally, mammalian semen are stored by cryopreservation, which is a conventional method for long–term storage, but there are some disadvantages in its application; it is always required liquid nitrogen (LN_2) for storage and transportation, and can be occurred cross contamination through pathogen transmission among stored samples (Tedder. 1995; Bielanski. 2003; Kyuwa *et al*., 2007; Grout *et al*., 2009). Since intracytoplasmic sperm injection (ICSI) has come forth in mice, various approaches have been explored to simplify the sperm preservation, including freeze–drying, evaporative–drying and storage under hyperosmotic conditions (Li *et al*., 2007). In spite of freeze–drying has offered some promising results, its reliance on LN_2 did not alleviate some of the negatives associated with cryopreservation. On the other hands, evaporative–drying has been recently suggested, and that has a significant feature like simple and fast processing, and cost–saving for sample preparation, but the results have not been successful as much as freeze–drying (Li *et al*., 2009). In order to bear the severe weather conditions (cold or dryness), animals and plants accumulate large amount of sugars (glucose, sucrose and trehalose) in their body cells, so that it could give the resistance from freezing or dehydration by glass formation, interacting with membrane components and proteins, and providing energy source (Storey *et al*., 1994; Crowe *et al*., 1998; Oliver *et al*., 2001; Sugimachi *et al*., 2006; Liu *et al*., 2012). Trehalose is a non–reducing disaccharide formed by the linkage of two glucose molecules which is one of the commonly synthesized intracellular protectant (Elbein *et al*., 1974; Crowe *et al*., 2001; Elbein *et al*., 2003). In the present study, we investigated the effects of trehalose or sucrose supplemented into desiccation/preservation solution during sperm dehydration, and examined sperm DNA integrity and embryo development after oocytes injected with evaporative–dried boar spermatozoa.

MATERIALS AND METHODS

Semen Collection and Evaporative Drying of Boar Spermatozoa

Boar semen was collected from proven fertile adult Duroc boars 15–22 months of age. The boars were placed on a routine collection schedule of one collection per week. The sperm–rich fraction of ejaculate was collected into an insulated vacuum bottle. The sperm–rich fractions of ejaculates with greater than 85% motile spermatozoa were used. The semen was centrifuged, and the supernatant was removed. The pellet sperm was washed twice with phosphate–buffered saline (PBS), and washed twice with desiccation/preservation solution Tyrode based desiccation (TrB) buffer (Sitaula *et al*., 2009), consisting

[1] Department of Animal Science and Biotechnology, College of Agriculture and Life Science, Chungnam National University, 220 Gung–dong, Yuseong–gu, Daejeon 305–764, Korea
[2] Division of Biotechnology, College of Environmental & Bioresources, Chonbuk National University, Iksan–si, Jeollabuk–do 570–752, Korea
[3] Department of Biology Education, College of Education, Chonnam National University, Gwangju 500–757, Korea

* Corresponding author: Min Kyu Kim, DVM, Ph.D.(E–mail: kminkyu@cnu.ac.kr)
** Xiao Xia Li and Young Joo Yi have contributed equally to this work.

of 5.69 g/l NaCl, 0.23 g/l KCl, 0.29 g/l $CaCl_2 \cdot 2H_2O$, 0.08 g/l $MgCl_2 \cdot 6H_2O$, 2.09 g/l $NaHCO_3$, 0.04 g/l NaH_2PO_4, 0.9 g/l glucose and 2.3 g/l Hepes by centrifugation at × 700 g for 5 min, and the pellets were diluted with TrB buffer or TrB buffer supplemented with 0.1, 0.2 or 0.5 M three different concentrations of trehalose or sucrose, respectively. Twenty microliter diluted samples were placed on glass slide in clean bench for 30 min. After evaporative–drying, each samples on glass slides were packed with a tinfoil, and stored at 4˚C. All the evaporative–drying experiments were performed at room temperature (RT), and samples on glass slides after drying were shown in Fig. 1. Unless otherwise stated, all chemicals were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO, USA). For rehydration of evaporatively dried spermatozoa, sperm samples rehydrated with the same volume (20 μl) of Dulbecco's phosphate buffered saline containing 0.1% w/v polyvinyl alcohol (DPBS–PVA) for 5 min, and only normal shaped spermatozoa (with an intact head, long and straight tail) were used for ICSI (Fig. 2).

Oocyte Collection and In Vitro Maturation (IVM) of Porcine Oocytes

Ovaries were collected from gilts at a local slaughterhouse and transported to the laboratory within 2 h. Cumulus–oocyte complexes (COCs) were aspirated from follicles of 3 to 7 mm in diameter. The COCs were matured in tissue culture medium (TCM) 199 supplemented with 10% porcine follicular fluid (PFF), 10 ng/ml epidermal growth factor (EGF), 10 IU/ml pregnant mare serum gonadotropin (PMSG) and 10 IU/ml human chorionic gonadotropin (hCG) for 44 h at 38.5˚C in a humidified air of 5% CO_2. COCs were cultured with PMSG and hCG for 22 h, and then they were cultured without these hormones for 22 h.

In Vitro Fertilization (IVF) and Culture (IVC) of Porcine Oocytes

IVF was performed as previously described (Li *et al.*, 2013). Briefly, after oocyte maturation, cumulus cells were removed from the oocytes by repeated pipetting in 0.1% hyaluronidase. The cumulus–free oocytes with first polar body release and uniform cytoplasm were washed three times with the fertilization medium. The fertilization medium was a modified tris–buffered medium (mTBM) consisting of 113 mM NaCl, 3 mM KCl, 7.5 mM $CaCl \cdot 2H_2O$, 5 mM sodium pyruvate, 11 mM glucose, 20 mM Tris, 1 mM caffeine, 0.57 mM L–cysteine and 0.1%

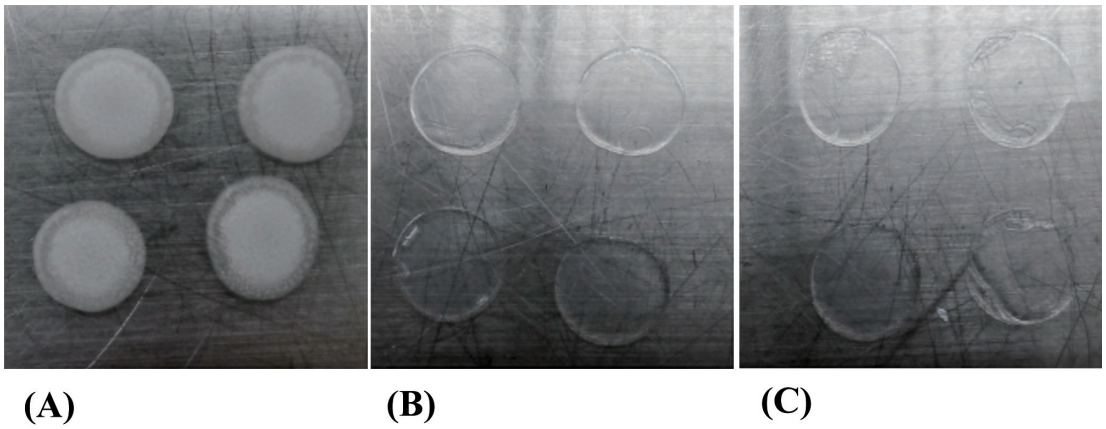

Fig. 1. Evaporatively dried boar spermatozoa after desiccation at RT for 30 min. Evaporatively dried sperm in TrB buffer without trehalose or sucrose (A), in TrB buffer supplemented with 0.2 M trehalose (B) or 0.2 M sucrose (C).

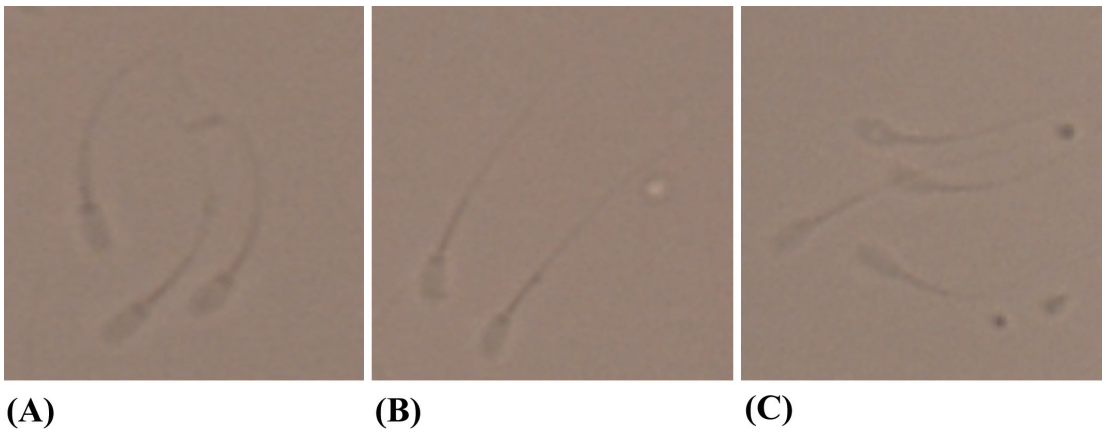

Fig. 2. Evaporatively dried boar sperm after rehydration. Spermatozoa from a control group exhibited abnormal morphology with a bent tail (A). Dehydrated sperm in Trb buffer with 0.2 M trehalose (B) or 0.2 M sucrose (C) were shown mostly normal morphology with a long and straight tail. Magnification= ×400.

w/v bovine serum albumin (BSA). Frozen–thawed (FT) sperm suspensions were washed one time with 1 ml modified TCM–199 medium (mTCM–199) supplemented with 26.2 mM $NaHCO_3$, 3.05 mM glucose, 0.91 mM Na–pyruvate, 2.92 mM, Ca–lactate · $5H_2O$, 75 mg/l kanamycin and 10% v/v fetal bovine serum (FBS), then washed twice with 1 ml mTBM by centrifugation at × 700 g for 3 min. The sperm pellet was resuspended in 1 ml mTBM and the sperm concentration was adjusted to 1×10^6 spermatozoa/ml. The percentage of motile spermatozoa after thawing was 60–80%. Approximately, 15 to 20 oocytes were transferred into 60 μl droplets of mTBM for 30 min, and then co–incubated with 20 μl diluted sperm for 6 h before being transferred to porcine zygote medium–3 (PZM–3) for further culture. The day of IVF was defined as Day 0.

Intracytoplasmic Sperm Injection (ICSI) and Culture (IVC) of Porcine Oocytes

As previously mentioned in our study [15], after oocyte maturation, mechanically denuded oocytes with first polar body release and a uniform cytoplasm were kept in 20 μl droplet of PZM–3 until ICSI was performed under an inverted microscope with micromanipulators. For ICSI, FT sperm suspensions were washed twice with 1 ml prewarmed (38.5˚C) DPBS–PVA by centrifugation at × 700 g for 3 min. The sperm pellet resuspended in 1 ml DPBS–PVA was immediately used for ICSI. One drop of 7 μl DPBS–PVA containing 2 μl sperm suspension and another 7 μl droplet containing 10 to 15 denuded oocytes were placed on the lid of a 50 × 9–mm petri dish and covered with mineral oil. From the edge of the sperm–containing droplet, a motile spermatozoon was aspirated into the injection pipette tail first without an immobilizing treatment such as tail–scoring/cutting and transferred to the drop of oocytes. Metaphase Ⅱ–stage oocyte was held with its polar body at either the 6 or 12 o'clock position using a holding pipette. Injection pipette advanced into the oocyte, and small amount of cytoplasm was aspirated to break the plasma membrane. The aspirated cytoplasm and the spermatozoon were then gently injected into the oocyte. For ICSI with evaporatively dried sperm, sperm were rehydrated for 5 min with prewarmed DPBS–PVA, collected into a 1.5 ml tube and washed twice with 1 ml of DPBS–PVA by centrifugation at × 700 g for 3 min. The sperm pellet resuspended in 1 ml DPBS–PVA and immediately used for ICSI. All the procedures were performed at RT. After ICSI, 10 to 15 zygotes were cultured in a 20 μl drop of PZM–3. The medium drops were covered by mineral oil and incubated at 38.5˚C in a humidified air of 5% CO_2.

TUNEL Assay of Boar Spermatozoa

Terminal deoxynucleotidyl transferase–mediated dUTP nick–end labeling (TUNEL) assay was performed to assess the presence of fragmented DNA accumulation. Boar spermatozoa were labelled with APO–BrdU™ TUNEL assay kit containing Alexa Fluor® 488 anti–BrdU (Life technologies, Seoul, Korea) according to the manufacturer's protocol. After processing, 10,000 cells were evaluated by FAC–scan flow cytometer (BD Biosciences, Seoul, Korea). TUNEL positive cells indicated green fluorescence, and propidium iodide (PI) was added as a counter stain for sperm DNA.

Assessment of Embryonic Development and TUNEL assay in Embryos

The rates of cleavage and blastocyst formation were determined at 48 and 168 h after the onset of IVC, respectively. The embryos were fixed with 4% paraformaldehyde in PBS–PVA for 1 h, and permeated in PBS–PVA with 1.0% Triton X–100 and 0.1% sodium citrate solution for 30 min, after then stained with 4',6–diamidino–2–phenylindole (DAPI) and mounted in mounting medium (Vectashield, Vector Laboratories Inc., CA, USA) at RT in the dark. Embryos were observed under fluorescence microscope (Carl Zeiss, Jena, Germany). TUNEL assay kit was used to assess the presence of apoptotic cells (*In Situ* Cell Death Detection Kit, TMR red, Roche diagnostics, Indianapolis, IN, USA) in blastocysts at day 7. Blastocysts were fixed, permeated, washed with PBS–PVA three times and incubated in TUNEL reaction medium (enzyme solution: label solutions=1:9) for 1 h at 37˚C in the dark. After complete reaction, embryos were stained with DAPI and mounted in mounting medium. The numbers of apoptosis nuclei, fragmentation and total numbers of nuclei were subsequently observed and captured images using scanning laser confocal microscope (LSM5 Live, Carl Zeiss, Jena, Germany) with × 20 objectives connected to an image analysis system (Zeiss LSM5 Live Release ver. 4.2. SP1 Image Browser software, Carl Zeiss). Apoptotic cells were stained with both TUNEL (red signal) and DAPI (blue signal), resulting in a pink color; non–apoptotic cells and fragmented nuclear were stained blue only with DAPI.

Transfer of Oocytes Injected with Evaporatively Drying Spermatozoa

About 150 to 200 putative zygotes produced by ICSI with evaporatively drying sperm with 0.2 M trehalose before 3–5 h of surgery were transferred to both oviducts of recipient gilts. Pregnancy was diagnosed in the recipients by using ultrasonography for detection of the placental cavity after transfer for 1 month.

Statistical Analysis

Statistical analysis was carried out using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). The means of development rate were compared by one way ANOVA. Data are presented as mean values ± standard deviation (SD). $P<0.05$ was considered significant.

RESULTS

Effects of Different Concentrations of Trehalose or Sucrose Supplemented into TrB Buffer on Sperm DNA Integrity

To evaluate the effects of trehalose or sucrose on evaporative–drying of boar spermatozoa, TUNEL assay was performed that can assess single and double stranded breaks on sperm DNA. Sperm positive for DNA

damage indicated green fluorescence as shown in Fig. 3. The sperm plasma–membrane integrity (BrdU/PI) was analyzed by flow cytometry as shown in Fig. 4. Fresh sperm were shown minimal DNA damage with 7.1 and 5.9%, and sperm dried with 0.2 M trehalose resulted in 29.5% TUNEL positive which was significantly lower rate compared to no supplemented (control), 0.1 or 0.5 M trehalose groups (69.7–79.0%, P<0.05; Fig. 4). Also, sperm dried in the presence of 0.2 M sucrose was shown significantly lower percentage of DNA damage than those of control, 0.1 or 0.5 M sucrose (53.3 *vs.* 70.5–73.0%, P< 0.05; Fig. 4).

Intracytoplasmic Injection with Boar Spermatozoa from Evaporative–Drying in the Presence of Varied Concentrations of Trehalose or Sucrose

Boar spermatozoa were dried evaporatively in the presence of different concentrations of trehalose, after then were injected into oocytes (Table 1). The cleavage rate was significantly higher in 0.2 M trehalose group than control (without trehalose), 0.1 or 0.5 M trehalose groups (81.3 *vs.* 76.3–78.6%, P<0.05; Table 1). In blastocyst formation rate, 0.2 M trehalose group was significantly higher than other three groups (16.4 *vs.* 11.0–12.7%, P<0.05; Table 1). Also, ICSI was performed using with evaporatively drying sperm in the presence of various concentrations of sucrose (Table 2). The cleavage and blastocyst rates significantly increased in 0.2 M sucrose group (79.6 and 14.2%) compared to control (without sucrose), 0.1 or 0.5 M sucrose groups (73.4–74.7% in cleavage, and 11.0–12.2% in blastocyst formation, P<0.05; Table 2).

Comparison of the Development and Quality of Embryo on Intracytoplasmic Injection of Sperm Dried in the Presence of 0.2 M Trehalose or Sucrose

As stated above, 0.2 M trehalose or sucrose signifi-

Table 1. Effects of TrB supplemented with different concentrations of trehalose on embryo development in vitro after ICSI

Group[A]	No. of oocyte[B]	No. of cleaved embryo (%, mean ± SD)	No. of blastocyst (%, mean ± SD)
Control	156	119(76.3±1.2^{c})	17(11.0±1.2^{b})
0.1 M	155	119(76.6±2.2^{bc})	18(11.7±1.6^{b})
0.2 M	155	126(81.3±1.9^{a})	25(16.4±2.0^{a})
0.5 M	156	123(78.6±2.3^{b})	20(12.7±2.0^{b})

[A] Evaporative–drying of boar spermatozoa in the absence (control) / presence of trehalose.
[B] Experiments were repeated eight times.
[a–c] Values with different superscripts are significantly different within columns (P<0.05).

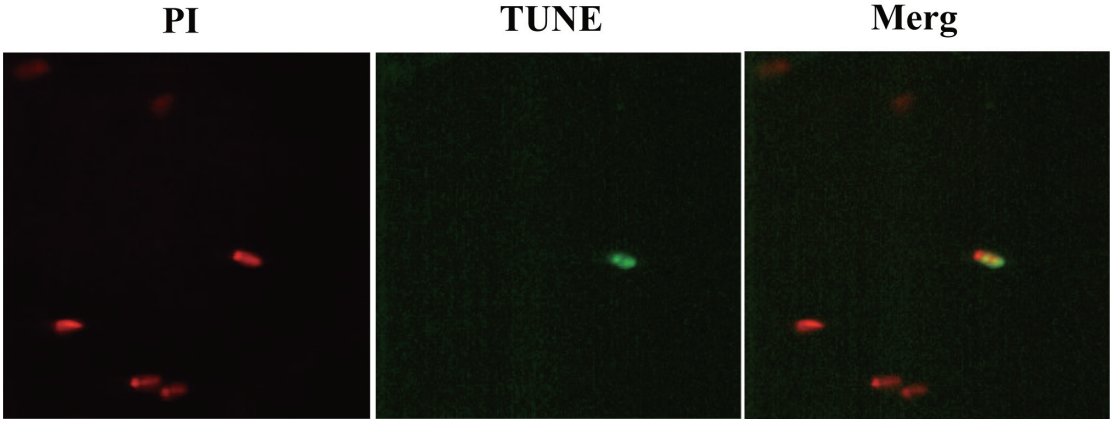

Fig. 3. TUNEL assay of boar spermatozoa. TUNEL positive–sperm indicated green fluorescence, while intact sperm was counterstained with PI (red). Magnification= ×400.

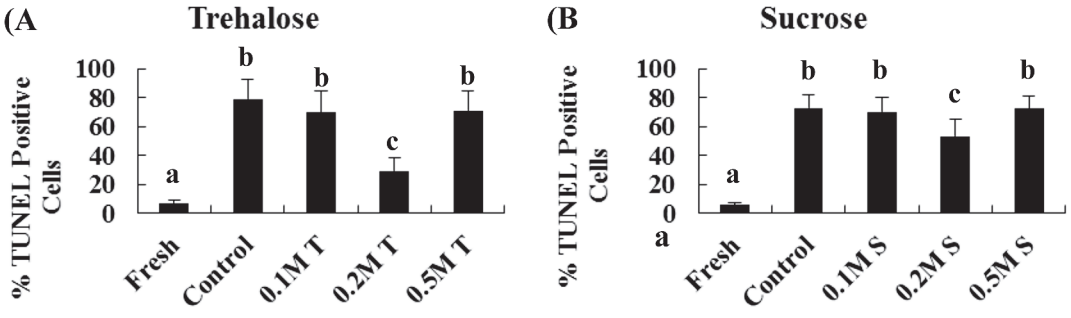

Fig. 4. Effect of different concentrations of trehalose or sucrose on evaporative–drying of boar spermatozoa. Spermatozoa labelled with TUNEL were analyzed by flow cytometry. Data are expressed as mean ± SD of three replicates. Bars with different letters (a–c) differ significantly (p<0.05).

Table 2. Effects of TrB supplemented with different concentrations of sucrose on embryo development in vitro after ICSI

Group[A]	No. of oocyte[B]	No. of cleaved embryo (%, mean ± SD)	No. of blastocyst (%, mean ± SD)
Control	146	109(74.7±1.4[b])	16(11.1±1.2[b])
0.1 M	146	109(74.7±1.5[b])	16(11.0±0.9[b])
0.2 M	147	117(79.6±1.8[a])	21(14.2±1.8[a])
0.5 M	147	108(73.4±1.6[b])	18(12.2±1.8[b])

[A]Evaporative–drying of boar spermatozoa in the absence (control) / presence of trehalose.
[B]Experiments were repeated eight times.
[a,b]Values with different superscripts are significantly different within columns (P<0.05).

Table 3. Effects of TrB supplemented with 0.2 M trehalose or sucrose on embryo development in vitro after ICSI

Group[A]	No. of oocyte[B]	No. of cleaved embryo (%, mean ± SD)	No. of blastocyst (%, mean ± SD)
IVF	256	221(86.3±1.2[a])	81(31.6±1.2[a])
ICSI–FT	254	215(84.6±2.8[a])	49(19.3±2.1[b])
ICSI–control	255	191(75.4±2.4[d])	26(10.4±1.1[e])
ICSI–0.2 M trehalose	253	206(80.8±2.3[b])	40(15.9±1.9[c])
ICSI–0.2 M sucrose	254	197(77.8±1.6[c])	33(13.1±1.3[d])

[A]IVF: *in vitro* fertilization, ICSI–FT: ICSI with frozen–thawed (FT) sperm, ICSI–control: ICSI with evaporatively dried sperm without trehalose or sucrose, ICSI–0.2M trehalose/sucrose: ICSI with evaporatively dried sperm with 0.2 M trehalose or sucrose.
[B]Experiments were repeated eight times.
[a–e]Values with different superscripts are significantly different within columns (P<0.05).

cantly reduced damage of sperm DNA after evaporative–drying (Fig. 4), and moreover, embryo development was increased after ICSI (Table 1 & 2). Therefore, to find out a suitable protectant for evaporative–drying of boar spermatozoa and produce normal embryos *in vitro*, the development and quality of embryo were compared after ICSI with sperm dried with 0.2 M trehalose or sucrose, respectively (Table 3). Significantly higher rates of cleaved embryo (86.3 and 84.6%) and blastocyst (31.6 and 19.3%) were observed in IVF and FT sperm–injected groups than evaporatively dried sperm–injected groups (P<0.05; Table 3). When oocytes were injected with evaporatively dried sperm, the rates of cleavage (80.8 *vs.* 75.4–77.8%) and blastocyst formation (15.9 *vs.* 10.4–13.1%) were significantly higher in 0.2 M trehalose group than control (without trehalose or sucrose) or 0.2 M sucrose groups (P<0.05; Table 3). The images of blastocysts after TUNEL assay were shown in Fig. 5. Total cell number was significantly increased in IVF groups than those of FT sperm–injected or evaporatively dried sperm–injected groups, however, significantly higher cell number

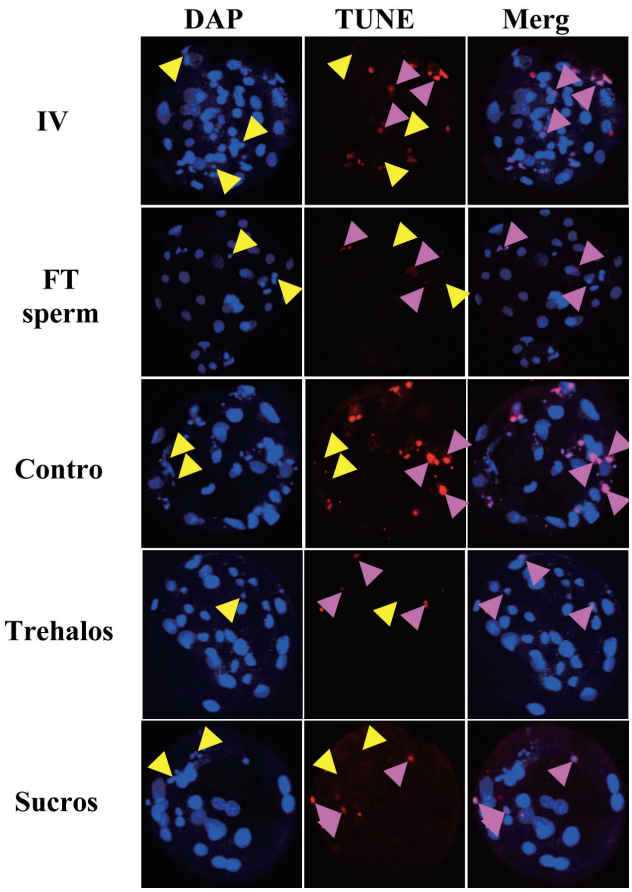

Fig. 5. TUNEL assay of blastocysts derived from IVF, ICSI with frozen–thawed (FT) sperm, and ICSI without (control)/ with trehalose or sucrose. Apoptotic cells indicated red fluorescence (pink arrows), and DNA was stained with DAPI indicated fragmentation (yellow arrows). Magnification= ×200.

was observed in trehalose group among evaporatively dried sperm injected groups (P<0.05; Fig. 6A). Significantly higher indices of TUNEL, fragmentation and total apoptotic cells were shown in IVF or ICSI with evaporatively dried sperm (control) than other groups (P< 0.05; Fig. 6B, C&D). But those indices significantly decreased in trehalose group among evaporatively dried sperm injected groups (P<0.05; Fig. 6B, C&D).

Long–Term Storage of Evaporatively Dried Spermatozoa and Subsequent Embryo Development after ICSI

Boar spermatozoa were dried evaporatively in the presence of 0.2 M trehalose, after then preserved at 4˚C up to 1 year. Embryo development was evaluated after ICSI with sperm stored for different periods (Table 4). The percentages of cleaved embryo and blastocyst formation were significantly decreased in oocyte injected with sperm preserved for 1 year (77.3 and 10.1%) compared to those of sperm stored for 1, 3 and 6 month (80.0–81.9% in cleavage and 14.5–16.2% in blastocyst formation (P<0.05; Table 4). However, boar sperm was successfully stored at 4˚C for 1 year, resulted in producing pre–implantation embryos *in vitro* after ICSI.

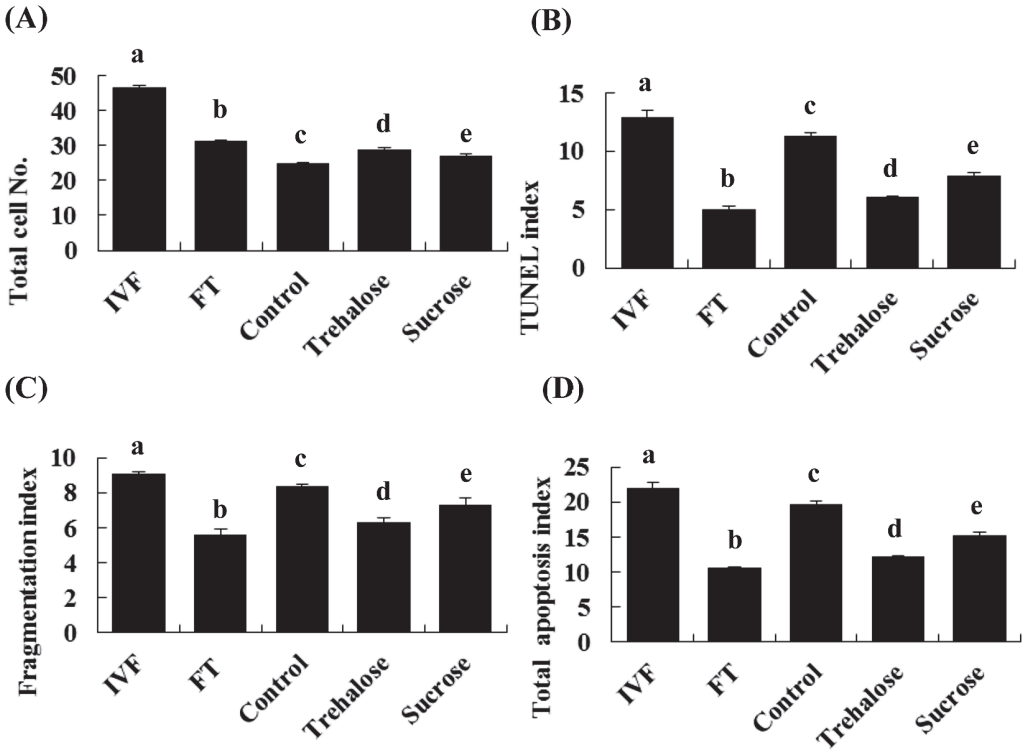

Fig. 6. Effect of trehalose or sucrose in blastocysts derived from IVF, ICSI with frozen–thawed (FT) sperm, and ICSI without (control)/with trehalose or sucrose. The total cell number (A), nuclear apoptosis (B), fragmentation (C) and total apoptosis (D) were examined by TUNAL assay. Data are expressed as mean ± SD of three replicates. Bars with different letters (a–e) differ significantly ($P<0.05$).

Embryo transfer (ET) of Oocytes Injected with Evaporatively Dried Spermatozoa

Oocytes injected with evaporatively dried spermatozoa in the presence of 0.2 M trehalose were surgically transferred into recipient mothers. Higher pregnancy rate and longer pregnancy duration indicated in the recipients received oocytes injected with sperm treated with trehalose than those of control (without trehalose; Table 5).

DISCUSSION

In the present study, we demonstrated that 0.2 M trehalose or sucrose supplemented to TrB desiccation/preservation buffer significantly reduced DNA damage of boar spermatozoa during evaporative–drying, and oocytes injected with sperm dried in the presence of trehalose developed to pre–implantation embryo that transferred to the recipients successfully became pregnant after ET.

Since mammalian sperm have been kept in a state of freezing, there have been attempts to store them in a dry condition. Although spermatozoa have been well preserved by freeze–drying in various animals (Polge *et al.*, 1949; Sherman *et al.*, 1954; Meryman *et al.*, 1960; Uehara *et al.*, 1976; Jeyendran *et al.*, 1981; Wakayama *et al.*, 1998; Kwon *et al.*, 2004; Liu *et al.*, 2004), that technique has required elaborate protocols and high equipment for freezing and drying (Meryman *et al.*, 1960). Later, convectively dried mouse spermatozoa were stored at 4°C, and capable of producing viable fetuses after intracyto-

Table 4. Development of embryo derived from ICSI with evaporatively dried sperm preserved at 4°C for different storage periods

Group[A]	No. of oocyte[B]	No. of cleaved embryo (%, mean ± SD)	No. of blastocyst (%, mean ± SD)
1 month	150	123(81.9±2.2[a])	24(16.2±1.7[a])
3 month	150	122(81.3±1.5[a])	23(15.2±1.7[a])
6 month	151	121(80.0±2.1[a])	22(14.5±1.5[a])
1 year	150	116(77.3±2.1[b])	15(10.1±2.0[b])

[A] Spermatozoa were preserved after evaporative–drying in the presence of 0.2 M trehalose.
[B] Experiments were repeated eight times.
[a,b] Values with different superscripts are significantly different within columns ($P<0.05$).

Table 5. Pregnancy rate after embryo transfer (ET) using oocytes injected with sperm dried in the presence of trehalose

Group[A]	No. of recipient	No. of oocyte transferred	Pregnancy period			
			1 month	2 month	3 month	Total pregnancy rate (%)
Control	10	1698	1	1	1	3(33.3)
0.2 M trehalose	12	2372	0	3	2	5(41.7)

[A] Putative zygotes derived from ICSI with sperm dried in the absence (control) / presence of trehalose were transferred to the recipients.

plasmic injection that proved a feasibility of convective–drying of mammalian sperm (Bhowmick *et al*., 2003). However, there is no report for evaporatively dried sperm from large animal yet.

Trehalose has been used to desiccate some mammalian cells. Many species of animals, insects, fungi, bacteria and yeast generated trehalose as an energy metabolite and a protective agent against desiccation, heat, cold, hypoxia and general cellular stress, even though mammals appeared a lack of the enzymatic pathways to produce it (Ahmadi *et al*., 1999; Guo *et al*., 2000; Chen *et al*., 2001; Gordon *et al*., 2001; Puhlev *et al*., 2001; Wolkers *et al*., 2002; Crowe *et al*., 2003; Elbein *et al*., 2003; Chen *et al*., 2004; Crowe *et al*., 2004; McGinnis *et al*., 2005). Trehalose and sucrose have been always selected as protectants in desiccated preservation studies of mammalian cells because of their protective effects (Womersley *et al*., 1986; Chen *et al*., 2001; Wolkers *et al*., 2001; Acker *et al*., 2002; Eroglu *et al*., 2002; Elliott *et al*., 2006). Sitaula *et al.* reported that trehalose and sucrose increased sperm membrane integrity significantly in bovine spermatozoa, which was consistent with our result. In the present study, the addition of trehalose or sucrose in the desiccation/preservation (TrB) buffer improved sperm integrity after de–/rehydration, probably, those sugars coated sperm that might protect the membrane in a dry condition (Fig. 1&2). Furthermore, desiccation could deteriorate sperm DNA, but in this study, TrB buffer supplemented with 0.2 M trehalose or sucrose significantly reduced DNA damage during evaporative–drying, which was confirmed by TUNEL assay (Fig. 4). McGinnis *et al.* found that in evaporatively dried sperm without trehalose, few number of sperm still survived after a week, which means that molecules in the chromatin are sufficiently packed with high viscosity into a structure, resulted in living several days under harsh dryness. Thus the main role of trehalose is to protect the nuclear matrix, consequently that can prolong storage period. In the present study, we preserved boar spermatozoa at 4˚C after evaporative–drying in the presence of 0.2 M trehalose up to 1 year that produced pre–implantation embryos after ICSI (Table 4), suggesting that trehalose could be useful for the extension of sperm storage.

The blastocysts derived from oocytes after injected with evaporatively dried sperm with 0.2 M trehalose significantly increased total cell number, and decreased the indices of TUNEL, fragmentation and total apoptosis (Fig. 5&6) that might alleviate dehydration stress of sperm, and contribute to embryo development after ICSI (Table 3). Additionally, we performed surgical ET using oocytes derived from ICSI with evaporatively dried sperm, and the recipients were successfully pregnant and maintained pregnancy periods until 3 months (Table 5), suggesting the possibility of producing normal piglets in future.

The present study showed that 0.2 M trehalose or sucrose would protect boar spermatozoa, and reduce deleterious effects during dehydration. Oocytes injected with evaporatively dried sperm with 0.2 M trehalose developed to pre–implantation embryo that transferred to the recipients successfully became pregnant after ET. Our findings suggest that trehalose or sucrose could contribute to sperm preservation and ICSI in human and animal.

ACKNOWLEDGEMENT

This study was supported by a grant from the Next–Generation BioGreen 21 program (No. PJ011359), Rural Development Administration, Republic of Korea.

REFERENCES

Acker, J. P., A. Fowler, B. Lauman, S. Cheley and M. Toner 2002 Survival of desiccated mammalian cells: beneficial effects of isotonic media. *Cell Preserv. Technol*., **1**: 129–140

Ahmadi, A. and S. C. Ng 1999 Developmental capacity of damaged spermatozoa. *Hum Reprod*., **14**: 2279–2285

Bhowmick, S., L. Zhu, L. McGinnis, J. Lawitts, B. D. Nath, M. Toner and J. Biggers 2003 Desiccation tolerance of spermatozoa dried at ambient temperature: production of fetal mice. *Biol. Reprod*., **68**: 1779–1786

Bielanski, A., H. Bergeron, P. C. Lau and J. Devenish 2003 Microbial contamination of embryos and semen during long term banking in liquid nitrogen. *Cryobiology,* **46**: 146–152

Chen, T., J. P. Acker, A. Eroglu, S. Cheley, H. Bayley, A. Fowler and M. Toner 2001 Beneficial effect of intracellular trehalose on the membrane integrity of dried mammalian cells. *Cryobiology,* **43**: 168–181

Chen, Q. and G. G. Haddad 2004 Role of trehalose phosphate synthase and trehalose during hypoxia: from flies to mammals. *J. Exp. Biol*., **207**: 3125–3129

Crowe, J. H., J. F. Carpenter and L. M. Crowe 1998 The role of vitrification in anhydrobiosis. *Annu. Rev. Physiol*., **60**: 73–103

Crowe, J. H., L. M. Crowe, A. E. Oliver, N. Tsvetkova, W. Wolkers and F. Tablin 2001 The trehalose myth revisited: introduction to a symposium on stabilization of cells in the dry state. *Cryobiology,* **43**: 89–105

Crowe, J. H., L. M. Crowe, F. Tablin, W. Wolkers, A. E. Oliver and N. M. Tsvetkova 2004 Stabilization of cells during freeze–drying: the trehalose myth. *In* "Life in the Frozen State", ed. by B. J. Fuller, N. Lane and E. E. Benson, CRC Press, Boca Raton, FL, pp. 581–601

Crowe, J. H., F. Tablin, W. F. Wolkers, K. Gousset, N. M. Tsvetkova and J. Ricker 2003 Stabilization of membranes in human platelets freeze–dried with trehalose. *Chem. Phys. Lipids*, **122**: 41–52

Elbein, A. D. 1974 The metabolism of alpha, alpha–trehalose. *Adv. Carbohydr. Chem. Biochem*., **30**: 227–256

Elbein, A. D., Y. T. Pan, I. Pastuszak and D. Carroll 2003 New insights on trehalose: a multifunctional molecule. *Glycobiology,* **13**: 17R–27R

Elliott, G. D., X. H. Liu, J. L. Cusick, M. Menze, J. Vincent, T. Witt, S. Hand and M. Toner 2006 Trehalose uptake through P2X7 purinergic channels provides dehydration protection. *Cryobiology,* **52**: 114–127

Eroglu, A., M. Toner and T. L. Toth 2002 Beneficial effect of microinjected trehalose on the cryosurvival of human oocytes. *Fertil. Steril*., **77**: 152–158

Gordon, S. L., S. R. Oppenheimer, A. M. Mackay, J. Brunnabend, I. Puhlev and F. Levine 2001 Recovery of human mesenchymal stem cells following dehydration and rehydration. *Cryobiology,* **43**: 182–187

Grout, B. W. and G. J. Morris 2009 Contaminated liquid nitrogen vapour as a risk factor in pathogen transfer. *Theriogenology,* **71**: 1079–1082

Guo, N., I. Puhlev, D. R. Brown, J. Mansbridge and F. Levine 2000 Trehalose expression confers desiccation tolerance on human cells. *Nat Biotechnol,* **18**: 168–171

Jeyendran, R. S., E. F. Graham and M. K. Schmehl 1981 Fertility

of dehydrated bull semen. *Cryobiology*, **18**: 292–300

Kwon, I. K., K. E. Park and K. Niwa 2004 Activation, pronuclear formation and development in vitro of pig oocytes following intracytoplasmic injection of freeze–dried spermatozoa. *Biol. Reprod*., **71**: 1430–1436

Kyuwa, S., T. Nishikawa, T. Kaneko, T. Nakashima, K. Kawano, N. Nakamura, K. Noguchi, T. Urano, T. Itoh and N. Nakagata 2003 Experimental evaluation of cross–contamination between cryotubes containing mouse 2–cell embryos and murine pathogens in liquid nitrogen tanks. *Exp. Anim*., **52**: 67–70

Li, M. W., J. D. Biggers, H. Y. Elmoazzen, M. Toner, L. McGinnis and K. C. Lloyd 2007 Long–term storage of mouse spermatozoa after evaporative drying. *Reproduction,* **133**: 919–929

Li, X. X., D. S. Lee, K. J. Kim, J. H. Lee, E. Y. Kim, J. Y. Park and M. K. Kim 2013 Leptin and nonessential amino acids enhance porcine preimplantation embryo development in vitro by intracytoplasmic sperm injection. *Theriogenology,* **79**: 291–298

Liu, J., G. Y. Lee, J. A. Lawitts, M. Toner and J. D. Biggers 2012 Preservation of mouse sperm by convective drying and storing in 3–O–methyl–D–glucose. *PLoS One,* **7**(1): e29924

Liu, J. L., H. Kusakabe, C. C. Chang, H. Suzuki, D. W. Schmidt, M. Julian, R. Pfeffer, C. L. Bormann, X. C. Tian, R. Yanagimachi and X. Yang 2004 Freeze–dried sperm fertilization leads to full–term development in rabbits. *Biol. Reprod*., **70**: 1776–1781

McGinnis, L. K., L. Zhu, J. A. Lawitts, S. Bhowmick, M. Toner and J. D. Biggers 2005 Mouse sperm desiccated and stored in trehalose medium without freezing. *Biol. Reprod*., **73**: 627–633

Meryman, H. T. 1960 Drying of living mammalian cells. *Ann. N. Y. Acad. Sci*., **85**: 729–734

Meryman, H. T. 1960 Principles of freeze–drying. *Ann. N. Y. Acad. Sci*., **85**: 630–640

Oliver, A. E., O. Leprince, W. F. Wolkers, D. K. Hincha, A. G. Heyer and J. H. Crowe 2001 Non–disaccharide–based mechanisms of protection during drying. *Cryobiology,* **43**: 151–167

Polge, C., A. U. Smith and A. S. Parkes 1949 Revival of spermatozoa after vitrification and dehydration at low temperatures. *Nature,* **164**: 666

Puhlev, I., N. Guo, D. R. Brown and F. Levine 2001 Desiccation tolerance in human cells. *Cryobiology,* **42**: 207–217

Sherman, J. K. 1954 Freezing and freeze–drying of human spermatozoa. *Fertil. Steril*., **5**: 357–371

Sitaula, R., H. Elmoazzen, M. Toner and S. Bhowmick 2009 Desiccation tolerance in bovine sperm: A study of the effect of intracellular sugars and the supplemental roles of an antioxidant and a chelator. *Cryobiology,* **58**: 322–330

Storey, K. B. and T. P. Mommsen 1994 Effects of temperature and freezing on hepatocytes isolated from a freeze–tolerant frog. *Am. J. Physiol*., **266**: R1477–1482

Sugimachi, K., K. L. Roach, D. B. Rhoads, R. G. Tompkins and M. Toner 2006 Nonmetabolizable glucose compounds impart cryotolerance to primary rat hepatocytes. *Tissue Eng*., **12**: 579–588

Tedder, R. S., M. A. Zuckerman, A. H. Goldstone and A. E. Hawkins, A. Fielding, E. M. Briggs, D. Irwin, S. Blair, A. M. Gorman and K. G. Patterson 1995 Hepatitis B transmission from contaminated cryopreservation tank. *Lancet,* **346**: 137–140

Uehara, T. and R. Yanagimachi 1976 Microsurgical injection of spermatozoa into hamster eggs with subsequent transformation of sperm nuclei into male pronuclei. *Biol. Reprod*., **15**: 467–470

Wakayama, T. and R. Yanagimachi 1998 Development of normal mice from oocytes injected with freeze–dried spermatozoa. *Nat. Biotechnol*., **16**: 639–641

Wolkers, W. F., S. A. Looper, A. E. McKiernan, N. M. Tsvetkova, F. Tablin and J. H. Crowe 2002 Membrane and protein properties of freeze–dried mouse platelets. *Mol. Membr. Biol,*. **19**: 201–210

Wolker, W. F., N. J. Walker, F. Tablin and J. H. Crowe 2001 Human platelets loaded with trehalose survive freeze–drying. *Cryobiology,* **42**: 79–87

Womersley, C., P. S. Uster, A. S. Rudolph and J. H. Crowe 1986 Inhibition of dehydration–induced fusion between liposomal membranes by carbohydrates as measured by fluorescence energy transfer. *Cryobiology,* **23**: 245–255