Cryopreservation with Trehalose Reduced Sperm Chromatin Damage in Miniature Pig

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ABSTRACT

Miniature pig sperm cryopreservation is continually researched in biotechnology for breed conservation and reproduction. It is important to control the temperature at each stage of cryopreservation and cryoprotectant. It is also necessary to find the optimal cryoprotectant concentration and chemical elements of the extender. Recently, many studies have used various cryoprotectant materials, such as dimethyl sulphoxide (DMSO), ethylene glycol (EG), antifreeze protein (AFP), amides, and glycerol. Glycerol is a commonly used cryoprotectant. However, glycerol has critical cytotoxic properties, including osmotic pressure and it can cause irreversible damage to live cells. Therefore, We focused on membrane fluidity modifications can reduce cell damage from freezing and thawing procedures and evaluated on the positive effects of trehalose to the viability, chromatin integrity, and motility of boar sperm. Miniature pig sperm was separated from semen by washing with modified- Modena B (mMB) extender. After centrifugation, the pellet was diluted with the prepared first extender. This experiment was designed to compare the effects that sperm cryopreservation using two different extenders has on sperm chromatin. The control group used the glycerol only and it was compared with the glycerol and glycerol plus trehalose extender. Sperm viability and motility were evaluated using WST1 assays and computer-assisted semen assays (CASA). Chromatin structure was examined using acridine orange staining. For the motility descriptors, trehalose caused a significant (p<0.01) increase in total motility (57.80 ± 4.60% in glycerol vs. 75.50 ± 6.14% in glycerol + trehalose) and progressive (51.20 ± 5.45% in glycerol vs. 70.74 ± 8.06% in glycerol + trehalose). A significant (p<0.05) increase in VAP (42.70 ± 5.73 μm/s vs. 59.65 ± 9.47 μm/s), VSL (23.06 ± 3.27 μm/s vs. 34.60 ± 6.58 μm/s), VCL (75.36 ± 11.36 μm/s vs. 99.55 ± 12.91 μm/s), STR (54.4 ± 2.19% vs. 58.0 ± 1.63%), and LIN (32.2 ± 2.05% vs. 36.0 ± 2.45%) were also detected, respectively. The sperm DNA fragmentation index was 48.8% to glycerol only and 30.6% to glycerol plus trehalose. Trehalose added group showed higher percentages of sperm motility, stability of chromatin structure than glycerol only. In this study, we suggest that trehalose is effective in reducing freezing damage to miniature pig sperm and can reduce chromatin damage during cryopreservation.

(Key words : trehalose, chromatin damage, sperm cryopreservation, miniature pig)

INTRODUCTION

Cryopreservation is generally a suitable method for long-term storage of various types of cells and tissues (Momose *et al*., 2010). However, cryopreservation causes changes in cell membrane protein structure and enzymatic function, as well as DNA damage (Hwang et el., 2009; Honda *et al*., 2001; Momose *et al*., 2010). Cryopreservation decreases the sperm count, due to cold shock, intracellular ice crystal formation, osmotic stress, and reactive oxygen species (ROS) formation during freezing. (Kumaresan *et al*., 2011; Gutierrez-Perez *et al*., 2011). Chromatin is one of the cell components prone to damage during sperm freezing and thawing (Perez-Cerezales *et al*., 2011). Damaged chromatin may be a result of fatal molecular changes related to protamine conformational changes, induced by increased DNA damage (Gutierrez-Perez *et al*., 2011). Extenders support sperm when the semen is diluted due to physicochemical property stabilization (Irawan *et al*., 2010). Therefore, freezing extenders for boar sperm contain egg yolk and other agents, including buffers, additives and cryoprotectants. Cryoprotectants block the formation of ice crystals during freezing (Irawan *et al*., 2010). The most frequently used cryoprotectant is glycerol. In the specific case of boar sperm, glycerol is used as a carbon source, even in the presence of glucose (Gutierrez-

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Perez *et al*., 2009). However, glycerol can induce changes in the lipid packing structure of the sperm membrane, altering sperm stability and water permeability. These changes can reduce sperm quality (Lopes *et al*., 2009; Draberova *et al*., 2010).

Trehalose is a non-reducing disaccharide that stabilizes and protects cellular membranes and proteins (Gutierrez-Perez *et al*., 2009; Seo *et al*., 2011). It was reported that cells and tissues treated with extracellular trehalose exhibited enhanced survival after cryopreservation (Erdag *et al*., 2002; Kang and Shen, 2011; Chen *et al*., 2011). Research suggests that trehalose positively affects cryopreservation (Gutierrez-Perez *et al*., 2009). The aim of this study was to compare the cryoprotective effect of glycerol and glycerol plus trehalose extenders on the motility, viability, and chromatin structure of miniature pig sperm. We hypothesized that addition of trehalose would allow a decreased glycerol concentration in the extender and result in increased sperm quality. In addition, we investigated the effect of trehalose on sperm chromatin and determined trehalose reduces chromatin damage post frozen-thawed spermatozoa.

MATERIALS AND METHODS

1. Chemical Agents

All chemicals were purchased from Sigma-Aldrich Chemical company (St. Louis, MO, USA), except Equex STM, which was purchased from Nova Chemical Sales (Scituate, MA, USA).

2. Semen Sample Collection

Semen was collected from 8-year-old miniature pig using the gloved hand method at Kangwon National University. After ejaculation, semen was diluted with modified-Modena B (mMB) extender (6 g glucose, 0.45 g EDTA, 1.38 g sodium citrate, 0.2 g sodium bicarbonate, 1 g tris base, 0.5 g citric acid, 0.01 g cysteine, 0.8 g BSA, and 0.06 g kanamycin sulfate; pH 7.0). The semen was grouped into two fractions. One group was used as fresh semen to evaluate sperm concentration, viability, and motility. Progressive motility and viability were checked by CASA (Hamilton Thorne Inc., HTM-HELOS, Beverly, MA, USA) at 100X. The other group was frozen in 0.25-mL straws. Only sperm with a progressive motility of greater than 85% was used.

3. Freezing and Thawing

The semen was divided into two groups to be frozen with glycerol alone and glycerol plus trehalose. Samples were cooled to 15℃ for 1 hr. To remove seminal fluid, each sample was washed 5 times with mMB extender and centrifuged for 10 min at 400 g at 17℃. The supernatant was discarded and the pellet was diluted in lactose-egg yolk solution (LEY extender: 80% v/v lactose, 20% v/v egg yolk, and 100 μg/mL kanamycin sulfate). Pellets were cooled to 4℃ for 1 hr and a dilution solution with the second extender was added, including LEY-glycerol-Orvus-ES-Paste (89.5% v/v LEYGO extender, 9% v/v glycerol, 1.5% v/v Equex STM, and 100 mM; w/v trehalose). Extender and sperm were loaded into 0.25-mL straws and stored in liquid nitrogen. Frozen sperm in straws were thawed for 10 sec at 50℃. Sperm was diluted with Beltsville thawing solution (BTS; 37 mg/mL glucose, 1.25 mg/mL EDTA, 6 mg/mL sodium citrate, 1.25 mg/mL sodium bicarbonate, 0.75 mg/mL potassium chloride, 0.6 mg/mL penicillin, and 1 mg/mL streptomycin).

4. Motility Assay

Sperm motility was checked using CASA (Hamilton Thorne Inc.) and the IVOS software version 10.6, adjusted for boar sperm. Three microliters of freeze-thawed sperm samples, diluted with BTS, were then placed on a slide (20 μm depth, Leja, Nieuw Vennep, The Netherlands) (Pursel and Johnson, 1975). At least 10 predetermined fields were taken for each sperm sample. CASA evaluation yields path velocity (VAP), velocity of straight-line motion (VSL), track speed (VCL), lateral amplitude (ALH), straightness (STR), and linearity (LIN).

5. Viability Assays

The viability of thawed sperm was measured using water-soluble tetrazolium salts (WSTs) according to the manufacturer's instructions. Briefly, the tetrazolium salts are converted to formazan by succinate formazan reductase. The enzyme exists in the mitochondria of viable sperm cells and increased viable cell activity causes an increase in formazan. Formazan generated by active sperm cells was quantified by measuring the absorbance on an ELISA plate reader. The absorbance shows the direct proportion of viable sperm cells. We added sperm cells to a 96-well plate in a final volume of 100 μL/well Whittingham's media. Next, 10 μL preMix WST-1 was added per well and plates were incubated for 180 minutes in a humidified atmosphere (37℃, 5% CO_2). Absorbance of the samples was measured using an ELISA plate reader (Bio-Tek Inc., Winooski, VT, USA) with a wavelength of 420 nm. The absorbance was measured at 0, 30, 60, 120, and 180 minutes.

6. Sperm Chromatin Structure Assay (SCSA)

The chromatin integrity of sperm was assessed by SCSA. SCSA uses acridine orange (AO) and converts from green to red or yellow fluorescence due to DNA denaturation. We prepared Carnoy's fixative (1:3 ratio of glacial acetic acid: absolute methanol) and AO staining solution (0.1% acridine orange, 0.1 M citric acid, 0.3 M sodium phosphate dibasic). Thawed sperm was smeared on a glass slide and dried. Samples were fixed for 2 hrs with Carnoy's fixative. The samples were stained for 5 minutes in the dark and sperm cells were evaluated using a fluorescence microscope. We counted the green sperm and red sperm and determined the intact sperm chromatin as the percentage of green sperm in 500 stained sperm cells.

7. Statistical Analyses

Data were processed using the R statistical program. Data are expressed as the mean ± SD and analyzed using one-way ANOVA to determine the limit of detection of viability and DNA damage for cryopreservation. Differences were considered significant at $p<0.05$.

RESULTS

1. Motility

There were significant differences in motility between glycerol and glycerol plus trehalose. For the motility descriptors, trehalose caused a significant ($p<0.01$) increase in total motility (57.80 ± 4.60% in glycerol vs. 75.50 ± 6.14% in glycerol + trehalose) and progressive (51.20 ± 5.45% in glycerol vs. 70.74 ± 8.06% in glycerol+trehalose) (see Table 1).

A significant ($p<0.05$) increase in VAP (42.70 ± 5.73 μm/s vs. 59.65 ± 9.47 μm/s), VSL (23.06 ± 3.27 μm/s vs. 34.60 ± 6.58 μm/s), VCL (75.36 ± 11.36 μm/s vs. 99.55 ± 12.91 μm/s), STR (54.4 ± 2.19% vs. 58.0 ± 1.63%), and LIN (32.2 ± 2.05% vs. 36.0 ± 2.45%) were also detected, respectively. However, ALH was not significant different (5.10 ± 0.78 μm in glycerol vs. 5.85 ± 0.34 μm in glycerol+trehalose). Therefore, using an extender with trehalose affects sperm motility during cryopreservation.

2. Viability

The viability of thawed sperm from glycerol plus trehalose (0.304) was not significantly different from glycerol (0.2898) (Fig. 1). Therefore, using extender with trehalose does not affect sperm viability during cryopreservation.

3. Chromatin Damage

SCSA showed that using an extender containing trehalose blocks DNA fragmentation. Fig. 2 shows green sperm and red sperms. Green and red mixed sperm have partially damaged DNA. The sperm from glycerol DFI (DNA fragmentation index) was 48.8%, but sperm from glycerol plus trehalose DFI

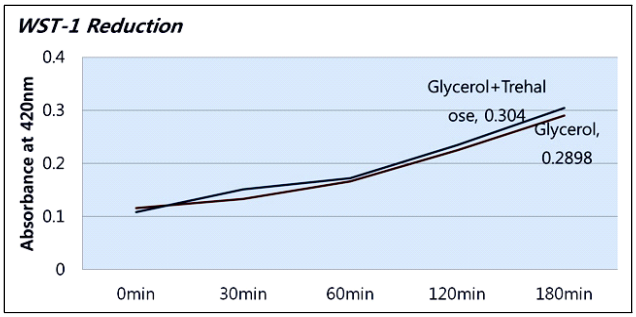

Fig. 1. WST-1 reduction rates of the thawed sperms containing trehalose and glycerol were measured by an ELISA plate reader (Bio-Tek Inc.) with a wavelength of 420 nm. Check the absorbance at 0, 30, 60, 120, 180 minutes of incubation time.

Table1. The parameters are path velocity (VAP), velocity of straight-line motion (VSL), track speed (VCL), lateral amplitude (ALH), straightness (STR), and linearity (LIN)

Group	Motility (%)	Progressive (%)	VAP (μm/s)	VSL (μm/s)	VCL (μm/s)	ALH (μm)	STR (%)	LIN (%)
Glycerol	57.80 ± 4.60	51.20 ± 5.45	42.70 ± 5.73	23.06 ± 3.27	75.36 ± 11.36	5.10 ± 0.78	54.4 ± 2.19	32.2 ± 2.05
Glycerol+trehalose	75.50 ± 6.14	70.74 ± 8.06	59.65 ± 9.47	34.60 ± 6.58	99.55 ± 12.91	5.85 ± 0.34	58.0 ± 1.63	36.0 ± 2.45
P value	0.00163	0.00334	0.0124	0.0105	0.0201	0.12	0.0297	0.0386

Results are expressed as means ± SD of 2 different experiments with a total number of analyzed sperm of 2,549 (glycerol), 2,720 (glycerol+trehalose). Different letters show significant differences.

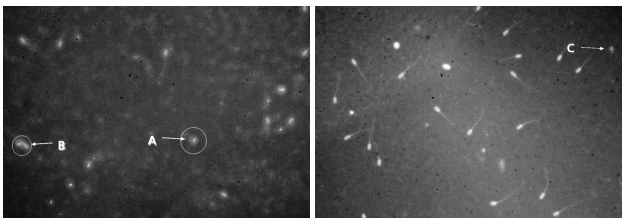

Fig. 2. Thawed sperms stained by acridine orange were examined by fluorescent microscopy (axiovert 200, Carl Zeiss, Germany) using a standard fluorescein isothiocynate (FITC) filter set (excitation wavelength; 450~490 nm; x400). The representative images were fragmented sperm DNA (A), normal sperm (B), and partially damaged sperm (C), respectively.

Table 2. The proportion of intact chromatin and motile sperm cells in glycerol and trehalose. Partially green or red sperm included to the damaged status

Group	Chromatin structure status(%)	
	Glycerol	Glycerol+trehalose
DFI	48.8	30.6

The parameters are DNA fragmentation index (DFI).

was 30.6% (Table 2). The results indicate that using extender with glycerol and trehalose blocks chromatin damage in sperm during cryopreservation.

DISCUSSION

Recently, it has been suggested that trehalose has many cryoprotective effects. In this regard, many species, including boar, ram, goat, rabbit, dog, and mouse, have been studied for acrosome (Gutierrez-Perez *et al*., 2009; Hwang *et al*., 2009), DNA fragmentation (Lee *et al*., 20010), telomere damage (Perez-Cerezales *et al*., 2011), epithelial tissues (Erdag *et al*., 2002; Kang and Shen, 2011; Chen *et al*., 2011), tubulin functions (Draberova *et al*., 2010), stress tolerance (Momose *et al*., 2010), oocyte cryosurvival (Eroglu *et al*., 2002), and many species of sperm quality following cryopreservation (Dalimata and Graham, 1997; Shiva Shankar Reddy *et al*., 2010; Aisen *et al*., 2002). However, there has been no research reported regarding the effect of trehalose on chromatin; therefore, in this study, we focused on chromatin.

To support glycerol as a sperm cryoprotectant, we added trehalose to assist as a cryoprotectant extender. We evaluated the effects of trehalose by measuring viability, motility, and sperm chromatin structure, which indicate the quality of thawed sperm. The data show that using a trehalose extender results in higher values in two post-thawing tests, SCSA and motility assessment. Sperm from glycerol plus trehalose displayed higher motility and progressive than glycerol only. However, ALH was similar suggesting trehalose affects sperm motility during cryopreservation. Using SCSA, we confirmed the effect of trehalose on sperm chromatin during cryopreservation. SCSA found that sperm from glycerol plus trehalose extender has a lower DFI than those from a glycerol only extender. However, the viability of thawed sperm did not show any significant difference between glycerol only and glycerol plus trehalose.

The correlation between sperm motility and the status of sperm chromatin indicates that sperm chromatin is important for sperm motility (Giwercman *et al*., 2003; Corcuera *et al*., 2007). Our experimental results confirm these findings.

Sperm chromatin integrity and motility are related to thawed boar sperm fertility (Donnelly *et al*., 2001; Linhart *et al*., 2005; Love, 2011). Although trehalose does not significantly affect viability, it enhanced the quality of miniature pig sperm chromatin integrity and motility. This suggests that a cryoprotectant including trehalose extender can increase the fertility of miniature pig sperm after cryopreservation.

In conclusion, our results suggest that using an extender with trehalose positively affects the chromatin integrity and motility of miniature pig sperm. Therefore, our results reinforce the hypothesis that trehalose can be used to decrease cold shock during cryopreservation and increase the fertility of freeze-thawed miniature pig sperm. These results will be confirmed using *in vitro* fertilization (IVF) and should strengthen our current results.

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