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Protective Effect of Trehalose on Canine Spermatozoa in Cryopreservation

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Cryopreservation of canine spermatozoa allows to preserve male genetic materials and contributes to conserve genetic materials of superior males or extinct animals in breeding management by using the frozen spermatozoa at time needed. However, spermatozoa may undergo multiple steps of possible damage during cryopreservation such as ice crystal formation. The semen extender is composed of buffer, saccharide, cryoprotectant and antibiotics which provide an optimal milieu for spermatozoa. Here, trehalose was treated in the semen extender that was used to freeze freshly collected canine semen, and the effect of trehalose on sperm motility and acrosome integrity were investigated based on different thawing and post thawing incubation conditions. The sperm motility was evaluated by CASA, and the frozen–thawed sperm motility was significantly higher than that in 44 mM glucose treatment group (MOT, ALH and MAD). Thawed sperms in trehalose treatment group maintained their motility at various thawing temperature ($P < 0.05$). During post–thawing incubation periods the frozen–thawed sperms' motility was reduced in time manner (0~8 h). However, the addition of trehalose in semen extender enhanced the stability of sperm motility significantly compared to the 44 mM glucose treatment during post–thawing incubation. In addition, trehalose treatment contribute to maintain intact acrosomes of sperms during thawing and post–thawing incubation effectively. In conclusion, the treatment of trehalose to semen extender reduced the damage of sperm motility and acrosomes which are related to the fertile quality of the frozen–thawed spermatozoa. Therefore trehalose would be a feasible supplementation to protect sperms to maintain their fertile ability during cryopreservation.

Key words: Canine spermatozoa, CASA, Cryopreservation, Semen extender, Trehalose

INTRODUCTION

Artificial insemination of domestic bitches with frozen–thawed semen has been used in clinics as a part of the assisted reproductive techniques and has become an essential technique to preserve the genetic diversity in researches. For example, in canidae, the amount of ejaculated semen is relatively not enough to inseminate female dogs (Farstad, 2009) that ejaculated semen from superior genetic and endangered canidae are preferred to be preserved when it is needed for inseminating female dogs.

Cryopreservation has been applied as a useful tool to preserve sperms. However, cryopreservation is able to induce problems from chilling, freezing, and thawing processes such as cold shock, ice crystal formation, osmotic pressure changes, and oxidative stress (Aitken *et al.*, 1998). When the extracellular ice crystal formation was occurred by the conversion of water to ice during cryopreservation, the plasma membrane of sperms act as a physical boundary to the exterior cellular condition (Farstad, 2009). The formation of ice crystals induce the freeze–dehydration of semen extender that increases the osmotic stress and destabilization of sperm membrane that changes the ultrastructure of phospholipid bilayer. However, under –80°C, highly viscous mixture within and outside the organelles converts into a glassy matrix which is the comparatively well–balanced configuration for long–term preservation (Fuller, 2004). The destabilization of sperm membranes during the cryopreservation is possible to induce cell death with extreme membrane damage during altering liquid to solid phase while freezing (Mazur, 1977).

Semen extender is composed of various ingredients including cryoprotectant, saccharides, amino acids, and antibiotics to protect sperms against the effects of cryopreservation process. Among them, a high concentration of saccharides supports the excretion of water out of the cell that decreases the intracellular ice crystal formation and maintains the osmotic pressure of the semen extenders. In addition, saccharides are used as an energy source for the post–thawed spermatozoa (Garcia and Graham, 1989; Abdelhakeam *et al.*, 1991; Garde *et al.*, 2008). Trehalose is an unreduced disaccharide which is composed by two glucose molecules at α, α–1, 1–glycosidic

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linkage. Trehalose is found in many organisms at high concentration including yeasts and fungal spores which are capable to survive in complete dehydration environments (Watson, 2000; Reddy *et al*., 2010). Trehalose presumably plays a major role in preventing noxious alteration of the cellular membrane during dehydration that is related with the competence of exchanging water through the membrane/solution connection (Chen *et al*., 2006). The addition of trehalose in semen extender is known to enhance the motility and viability of frozen–thawed mammalian sperm cells (Sztein *et al*., 2001; Matsuoka *et al*., 2006). Moreover, trehalose effects on the stabilization of certain cellular proteins and/or lipid in plasma membranes that increases sperm membrane fluidity and provides a synergic effect with glycerol to avoid intracellular ice crystal formation in hypertonic conditions during the cryopreservation process (Liu *et al*., 1998; Uysal and Bucak, 2009). It has been reported that canine spermatozoa are also affected by the cryopreservation process that decreases progressive motility, acrosome integrity, and viability of sperms (Rodriguezmartinez *et al*., 1993).

The objective of this study was to investigate the effect of trehalose on the motility and acrosome integrity of canine spermatozoa when it was supplemented in semen extender during cryopreservation process. Computer–assisted sperm analysis (CASA) and Giemsa staining were used to evaluate sperms depending on various trehalose concentration and incubation temperature conditions.

MATERIALS AND METHODS

Animals

In this study, 8 male beagle dogs (aged 2 years, weight 10 to 15 kg) were used as semen donors. All animal experiments were approved by Institutional Animal Care and Use Committee (IACUC) of Chungnam National University (Approval Number: CNU–00487), and performed according to "The Guide for the Care and Use of Laboratory Animals" published by IACUC of Chungnam National University. Dogs were raised indoors in separated cases with a temperature and ventilation control system. Dogs were fed commercial diet once daily and provided water *ad libitum*.

Semen Collection

Semen was collected once a week by digital manipulation according to the technique described by Kutzler (2005). The first and third fractions of transparent semen (seminal plasma) were removed, and the second sperm–rich fraction of milky colored semen (fresh semen) was yielded from each ejaculate. To be used, the each fresh semen sample has a volume $\geq$ 1.0 mL, concentration $>$ 200 $\times$ 10^6 spermatozoa/mL, and motility $\geq$ 80%.

Semen Extender

The base extender is composed of 198 mM Tris (Tris–hydroxymethyl–aminomethane), 66 mM citric acid, 2.8 mM sodium–benzyl penicillin, 0.8 mM streptomycin sulfate (pH 6.5, 250 mOsm) (Rota *et al*., 1997). The first extender was composed of the base extender with 20% (v/v) egg yolk, and the second extender was composed of the base extender with 20% (v/v) egg yolk and 12% (v/v) glycerol.

Semen dilution and freezing

The fresh semen sample was washed twice with PBS and centrifuged at 750 $\times$ g for 5 min at room temperature (approximately 25°C). After washing, each sperm pellet was re–suspended and diluted with the 1st semen extender. For the control group 44 mM glucose was treated into the 1st semen extender, and for trehalose treatment group 5, 15, 25 or 35 mM trehalose was treated into the 1st semen extender to a concentration of 400 $\times$ 10^6 sperm/mL at room temperature. The 1st extended semen was immediately placed at 4°C for 60 min to be equilibrated. The 1st extended semen was then diluted in a 1:1 ratio with the 2nd semen extender to a final concentration of 200 $\times$ 10^6 sperm/mL. After equilibration, the cooled 2nd extender was loaded into 0.5 mL straws (0.5 mL EcoStraws (REF. 13408/3010), MINITUBE, Germany) placed 4 cm above the surface of liquid nitrogen (–196°C) and frozen horizontally for 10 min. Finally, the frozen semen in straws were plunged into a liquid nitrogen tank and stored until used.

Thawing

Frozen semen in straws was thawed at various temperature conditions (36°C for 60 s, 54°C for 15 s, or 72°C for 8 s) in a water bath. Each thawed semen straw was immediately transferred into a 1 mL tube and incubated in the refrigerator and the water bath at various incubation temperatures (4, 17, 25 or 36°C) for 0, 1, 2, 4 or 8 h.

Evaluation of sperm motile ability by CASA

Thawed semen samples were evaluated by CASA system software of Sperm Analysis Image System® (Medical Supply Co. Ltd., Korea). Thawed semen samples were placed onto a pre–warmed Makler counting chamber (New York Microscope Company Inc., USA) and sperm movement were observed using a 100× objective of phase contrast microscope (EClIPSE E600, Nikon, Japan) and then recorded by a vision camera (HVR–2000C, HyVISION SYSTEM Inc., KOREA) at 37°C. The sperm movement characteristics were measured by CASA; motility (MOT, %), curvilinear velocity (VCL, μm/s), straight–line velocity (VSL, μm/s), average–path velocity (VAP, μm/s), linearity (LIN, %), amplitude of lateral head displacement (ALH, μm/s), straightness (STR, %), beat–cross frequency (BCF, Hz), mean angular displacement (MAD, degree), and wobble (WOB, %). For each evaluation, five replicates were analyzed.

Evaluation of acrosome integrity

Acrosome integrity was evaluated by Giemsa staining solution (GS500, Sigma–Aldrich Chemical Company, USA). A drop of fresh or thawed semen was placed on a glass slide and smeared. The fine smear was air–dried for

10 min. The glass slide was placed into a jar with Giemsa staining solution for 45 min, then rinsed with distilled water, and air–dried. Two hundred sperms were evaluated using a light microscope at 1,000 × magnification according to the manufacturer's methods.

Statistical Analyses

The results were expressed as means ± SD. Means were analyzed using a repeated measurement model analysis of variance (ANOVA), followed by Duncan's post hoc test to determine significant differences among the groups using the SPSS statistics program (version 19.0; IBM®, NY, USA). Differences with values of $P < 0.05$ were considered statistically significant.

RESULTS

The effect of trehalose on sperm motility depending on various concentrations

To optimize trehalose concentration canine fresh semen were frozen in the semen extender trehalose (5, 15, 25, or 35 mM or 44 mM glucose), and the motility of frozen–thawed canine sperms were evaluated using CASA after thawing at 36°C for 60 s (Fig. 1). The sperms from fresh semen displayed significantly high values in overall motile parameters compared to those of frozen–thawed sperms. There were no significant difference found in values of VCL, VSL, VAP, LIN, STR, BCF, and WOB. However, Thawed sperms treated with 25 mM trehalose had significantly higher MOT (84.5%), ALH (1.8 μm), and MAD (15.7 degree) values compared to 44 mM glucose treatment group that was even higher

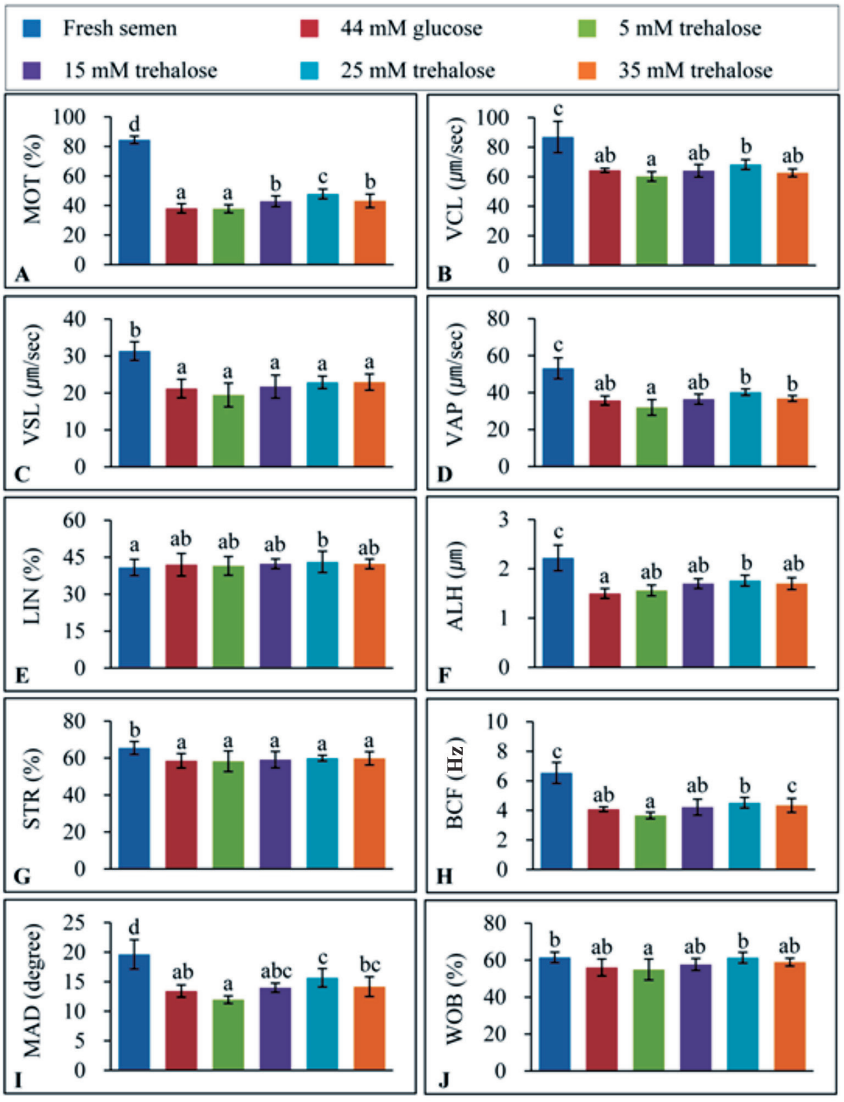

Fig. 1. The effect of trehalose concentration on sperm movement values by CASA. (A) MOT; motility, (B) VCL; curvilinear velocity, (C) VSL; straight–line velocity, (D) VAP; average–path velocity, (E) LIN; linearity, (F) ALH; amplitude of lateral head displacement, (G) STR; straightness, (H) BCF; beat–cross frequency, (I) MAD; mean angular displacement, (J) WOB; wobble. [a–d]: bar with different letters are significantly different, $P < 0.05$. The experiment was repeated five times.

than those in other trehalose treatment groups (Fig. 1. A, F and I). Therefore, 25 mM trehalose was treated to the semen extender in following experiments comparing with 44 mM glucose treatment as a control.

The effect of various thawing temperatures on sperm motility with/without trehalose treatment

The following experiment were carried out to find the effect of trehalose on frozen semen related to thawing temperatures (Fig. 2). When trehalose was treated, thawed sperms at 36°C for 60 s, 54°C for 15 s, or 72°C for 8 s shows significantly higher values of MOT, VCL, VAP, ALH, BCF, and WOB compared to the control. Although there was no significant difference between trehalose and glucose treated groups after thawing at 36°C for 60 s, thawed sperms which were treated with trehalose showed higher VSL and STR at the thawing condition of 54°C for 15 s, or 72°C for 8 s (Fig. 2. C and G). In addition, sperms in the trehalose treatment group showed significantly higher MAD only at the thawing condition of 72°C for 8 s (Fig. 2. I). However, there was no significant motile difference among three different thawing temperatures within each treatment group except WOB.

The effect of various incubation conditions on frozen–thawed sperm motility with/without trehalose treatment

After thawing, it was investigated whether trehalose treatment affects on sperms motility depending on the post–thawing incubation periods. Values of thawed sperm motility were decreased during post–thawing incubation periods in time–manner (Fig. 4). Sperms treated with 25 mM trehalose maintained significantly higher MOT, VCL, VAP, and LIN than those of the control group (44 mM glucose) at all incubation conditions. STR, BCF and WOB were not significantly different

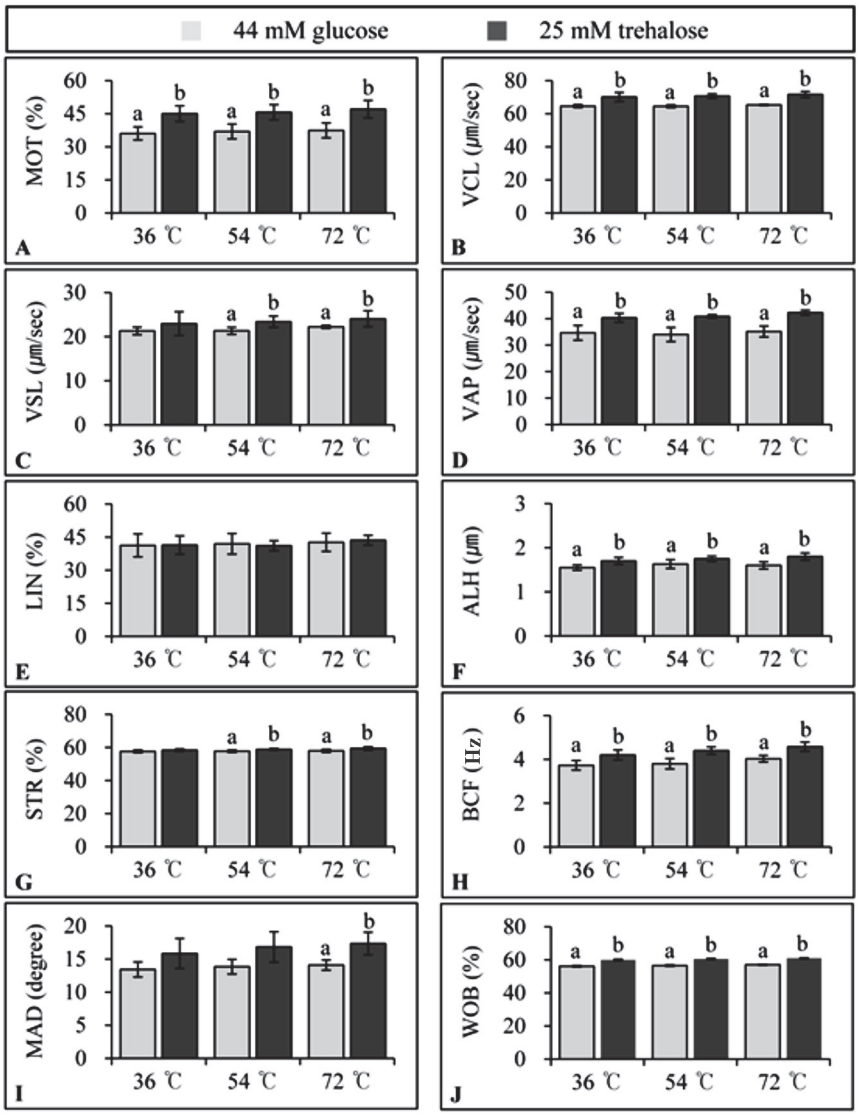

Fig. 2. The effect of thawing condition on sperm movement value by CASA. (A) MOT; motility, (B) VCL; curvilinear velocity, (C) VSL; straight–line velocity, (D) VAP; average–path velocity, (E) LIN; linearity, (F) ALH; amplitude of lateral head displacement, (G) STR; straightness, (H) BCF; beat–cross frequency, (I) MAD; mean angular displacement, (J) WOB; wobble. $^{a–d}$: bar with different letters are significantly different, $P < 0.05$. The experiment was repeated three times.

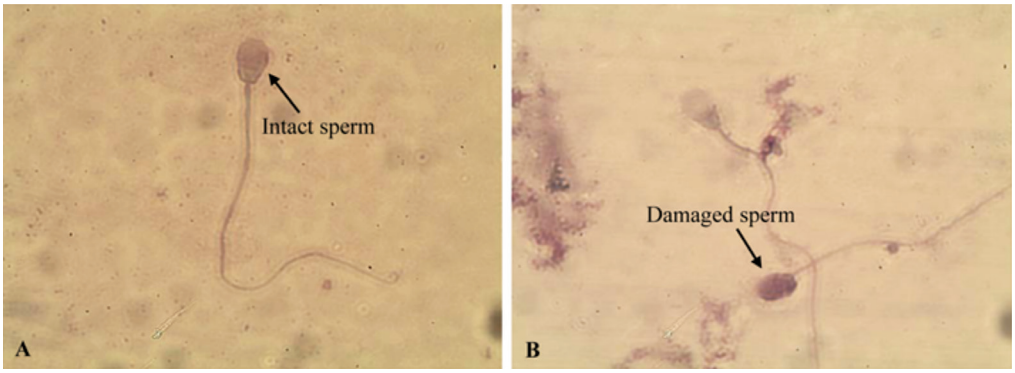

Fig. 3. Acrosome integrity of the frozen–thawed sperm stained by Giemsa. (A) Intact sperm: the sperm head stained pink and the anterior of sperm head (acrosome membrane area) stained light pink, especially. (B) Damaged sperm: the sperm head stained dark purple and no visual boundary line of acrosome membrane.

Table 1. The effect of thawing conditions (temperature and time) on acrosome integrity of frozen–thawed sperm

Groups		Acrosome integrity (%, Mean ± S.D.)
Fresh semen		86.00 ± 3.34 [d]
Glucose 44 mM	36°C/60 s	39.50 ± 3.19 [ab]
	54°C/15 s	38.88 ± 3.35 [a]
	72°C/8 s	38.13 ± 3.42 [a]
Trehalose 25 mM	36°C/60 s	45.75 ± 2.75 [c]
	54°C/15 s	44.88 ± 2.50 [c]
	72°C/8 s	43.88 ± 2.06 [bc]

[a–d] : columns with different letters are significantly different, $P < 0.05$

This experiment was repeated three times.

between trehalose and glucose treatment groups, and not affected by various incubation periods in both treatment groups. In addition, various incubation periods did not induce significant difference of MOT, VCL, VAP and LIN within each treatment groups.

Acrosome integrity of frozen–thawed sperms

Acrosome integrity of thawed sperms was checked by giemsa staining. The intact acrosome is stained pink except the anterior of sperm head (acrosome membrane area) which is stained light pink relatively, but a damaged acrosome is stained dark purple (Fig. 3). In fresh semen, 86% sperms contained intact acrosomes (Table 1). The rate of intact acrosomes of frozen–thawed sperms was significantly higher in the 25 mM trehalose treatment group than those in 44 mM glucose treatment at any thawing condition tested (Table 1). Next, the acrosome integrity of frozen–thawed sperms was investi-

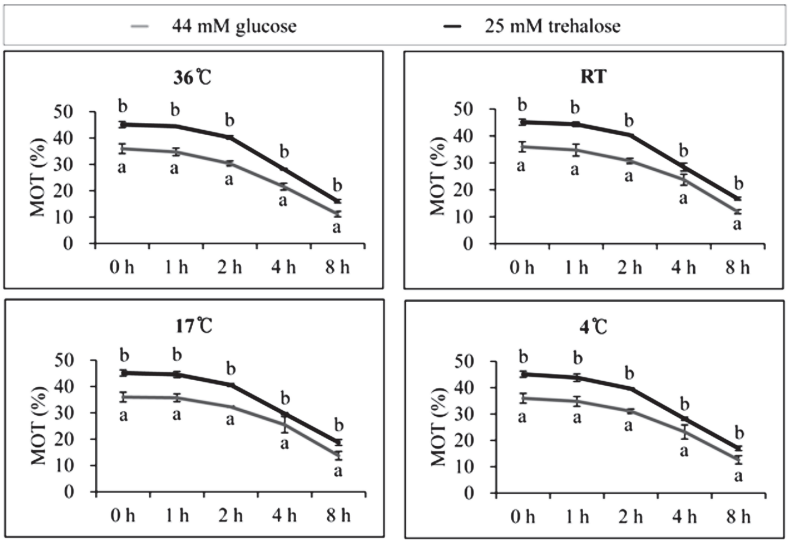

Fig. 4. Variation of sperm motility in frozen-thawed semen which were maintained under different incubation conditions. This study was conducted under various incubation temperatures; 36°C : water bath, RT : room temperature (25°C), 17°C : semen refrigerator and 4°C : normal refrigerator. The motility of sperms was measured by CASA program. [a, b]: bar (according to incubation time within each group) with different letters are significantly different, $P < 0.05$. The experiment was repeated three times.

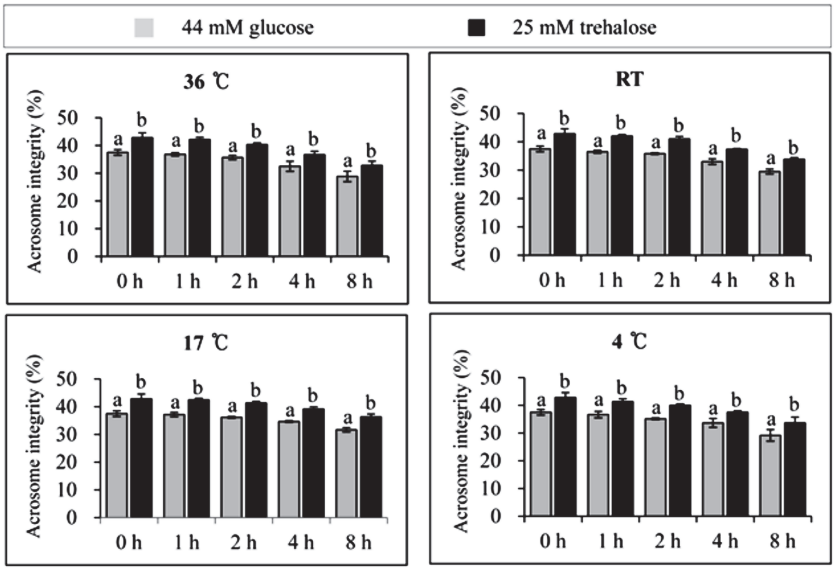

Fig. 5. Acrosome integrity of sperms in frozen-thawed semen which were maintained under different incubation conditions. This study was conducted under various incubation temperatures; 36°C : water bath, RT : room temperature (25°C), 17°C : semen refrigerator and 4°C : normal refrigerator. Acrosome integrity of sperms was averaged by the rate of intact and damaged acrosome in sperms. [a, b]: bar (according to incubation time within each group) with different letters are significantly different, $P < 0.05$. The experiment was repeated three times.

gated at various post–thawing incubation periods (0~8 h) and temperatures (36, RT, 17 and 4°C) after thawing at 36°C for 60 s (Fig. 5). During post–thawing incubation, the rate of intact acrosomes was gradually decreased in time manner. There was significantly higher percentage of sperms with intact acrosomes in 25 mM trehalose treated group compared to those in 44 mM glucose treated group regardless of post–thawing incubation conditions. In addition, within each treatment group, there was no difference of intact acrosome depending on incubation temperatures.

DISCUSSION

The sperm cryopreservation process induces irreparable injury that decreases the motility and fertility of spermatozoa (Watson and Martin, 1975). One of the principal causes diminishing sperms quality is the intracellular ice crystallization formed during the freezing and thawing process (De Leeuw *et al*., 1993). It has been found that water performs a significant function in the conservation of the structural and functional integrity of biological membranes during cryopreservation. The elimination of intracellular waters into the external environment throughout freezing, drying, and dehydration processes can lead to structural and functional modifications in the cell membranes.

A number of previous studies discovered that trehalose can protect the spermatozoa quality from damage by freezing and thawing in cryopreservation. Trehalose has been known to reduce the rapid physical and morphological changes of phospholipid membranes during the alteration of the water state. Trehalose arranges a hydrogen bond between the sugar hydroxyl group and the phospholipid polar head group that increase the membrane fluidity by which the water molecules move through a membrane under freezing and thawing conditions (Lee *et al*., 1989; Iwahashi *et al*., 1995; Giraud *et al*., 2000). In addition, the phospholipid bilayer induces the glass phase of membranes leading to greater endurance of the spermatozoa against freeze–thawing damage (Molinia *et al*., 1994; Patist and Zoerb, 2005). Trehalose has a higher glass transition temperature than that of other disaccharides that limits the transitional action of the bimolecular membrane. With that, trehalose is known to induce the stabilization of lipid membrane (Patist and Zoerb, 2005) by resisting the osmotic pressure and lowering the occurrence of ice crystal formation (Aisen *et al*., 2002).

Fertilization of human and animal oocytes has been shown to be positively correlated with sperm motility. Sperm motile measurement parameters are commonly used to evaluate the fertile potential of sperms (Amann, 1989; Bongso *et al*., 1989). For example, Milligan *et al*. (1980) reported that spermatozoa from fertile and infertile men could be distinguished from each other based on their sperm velocity measurements. It is also reported that sperm speed measurement is correlated with the penetration of zona–free hamster eggs and human oocytes *in vitro* (Fetterolf and Rogers, 1990). CASA has been used to evaluate sperm motility and morphology for predicting sperm's fertile ability in clinics and researches. It was reported by Barrat *et al*. (1993) that the total number of spermatozoa and VAP can be used as predictors of pregnancy. Moreover, a positive correlation between the total number of motile spermatozoa

and VCL has previously been observed (Gerris and Khan, 1987). VCL is the average velocity measured over the actual point–to–point track followed by the cell in micrometers per second (Verstegen *et al*., 2002) and VCL alone may predict fertility (Liu *et al*., 1988).

In this study, the effect of trehalose was investigated by using CASA whether it reduces the damage of sperm fertile ability induced during cryopreservation process. Previously, Yildiz *et al*. (2000) and Yamashiro *et al*. (2007) reported that the addition of trehalose in semen extender improved the sperm quality. By treating trehalose in semen extender, thawed sperm motility resulted in significantly higher compared to those with glucose treatment (MOT and ALH). In addition, sperms frozen in semen extender with trehalose always maintained significantly higher motile ability (MOT, VCL, VAP, ALH, BCF and WOB) when thawing at various temperatures. STR and MAD were also highly maintained by trehalose treatment at the thawing condition of 72°C for 8 s although there was no significant difference of those at the thawing condition of 36°C for 60 s. Moreover thawed sperms were not affected by different thawing temperature when treated with trehalose. Sperms were also highly motile in trehalose treated group compared to those in glucose treated group during post–thawing incubation periods. The frozen–thawed sperms which received a cold shock at 4°C during post thawing incubation were also more motile with trehalose treatment than those treated with glucose. The result supports that the supplementation of trehalose in semen extender would reduce the damage of sperm motility which is induced by cryopreservation at various thawing temperatures and duration.

Acrosome contains enzymes which are responsible to penetrate the zona pellucida of oocytes during fertilization. The reduction of intact acrosome of spermatozoa influences the fertilization capability. Plasma and acrosome membrane are damaged by cryopreservation process including mitochondrial sheath and axoneme (Salamon and Maxwell, 2000). In frozen–thawed spermatozoa, plasma and outer acrosome membrane are very cryo–sensitive with biochemical variations such as loosing lipoproteins and amino acids, decreasing in loosely bound cholesterol proteins, decreasing in acrosomal proteolytic activity, and reducing ATP and ADP synthesis (Salamon and Maxwell, 1995). Therefore, cryopreservation affects the ratio of the cholesterol/phospholipid and substance of the lipid bilayer in the membrane of frozen–thawed spermatozoa (Medeiros *et al*., 2002). Consequently, cryopreservation process exert negative influence on the stability of acrosome membrane and acrosome capacity. Trehalose treatment induced increased rate of intact acrosome. In addition thawed spermatozoa with trehalose treatment were more stable even with different thawing condition. Trehalose also supported the thawed spermatozoa to maintain higher percentage of intact acrosome during post thawing incubation periods. It inferred that trehalose would protect acrosome integrity during thawing and post thawing incubation period in cryopreservation.

Cryopreservation is a useful tool to preserve spermatozoa, and has been performed in clinics and research field for artificial insemination, *in vitro* fertilization, and intracytoplasmic sperm injection. Besides the effectiveness, cryopreservation process negatively affect to the sperm motile ability that decrease their fertile ability. In this study trehalose was treated in semen extender to reduce the damage on spermatozoa which can be occurred during cryopreservation process. Supplementation of trehalose into semen extender improved the sperm motility and also acrosome integrity of frozen–thawed sperms. Therefore trehalose would be an effective supplementation to protect sperms fertile against the possible damage during cryopreservation that will enhance the efficiency of artificial reproductive techniques.

AUTHOR CONTRIBUSIONS

Kang–Sun Park conceived of the presented idea, designed the experiment, carried out the experiment, and wrote the manuscript.

Ju Lan Chun conceived of the presented idea, carried out the experiment, developed the theory, and wrote the manuscript.

Eun Young Kim and Ji Hye Lee contributed to sample preparation and performed the experiment.

Nobuhiko Yamauchi discussed the results and contributed to the final version of the manuscript.

Kyung–Bon Lee conceived the original idea, discussed the results, supervised the project, and contributed to the final version of the manuscript.

Min Kyu Kim conceived the original idea, discussed the results, supervised the project, and contributed to the final version of the manuscript.

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