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Characteristic Changes in *Korean Native Cattle* Spermatozoa Frozen-Thawed with L-Cysteine and/or Catalase

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ABSTRACT

The objective of this study was to evaluate the characteristics of *Korean Native Cattle* sperm frozen-thawed with L-cysteine and/or catalase. The semen from bulls was collected by the artificial vagina method, and Triladyl containing 20% egg-yolk and/or L-cysteine (L), catalase (C) and L-cysteine + catalase was added to the diluted semen for cryopreservation. The results showed that sperm viability was significantly higher in the L-cysteine + catalase (69.49 ± 3.16%) group than in the control (60.5 ± 3.94%) group ($p<0.05$). Acrosome damage was significantly lower in the L-cysteine (17.12 ± 1.08%) group than in the control (21.46 ± 1.14%), catalase (20.54 ± 0.76%), and L-cysteine + catalase (19.29 ± 0.65%) groups ($p<0.05$). In addition, the level of intact mitochondria in the spermatozoa was significantly higher in the L-cysteine (58.65 ± 1.39%) group than in the control (50.63 ± 2.37%) group ($p<0.05$). The hydrogen peroxide level in the frozen-thawed sperm was significantly lower in the L-cysteine (3.74 ± 1.66%), catalase (4.65 ± 1.87%), and L-cysteine + catalase (8.11 ± 2.15%) groups than in the control (13.22 ± 1.6%) group ($p<0.05$). The glutathione level was significantly higher in the L-cysteine (1.33 ± 0.03%) group than in the control (1.08 ± 0.06 %), catalase (1.05 ± 0.02%) and L-cysteine + catalase (1.11 ± 0.03%) groups ($p<0.05$). In conclusion, L-cysteine and catalase could protect the membrane of *Korean Native Cattle* sperm from damage during sperm cryopreservation. Especially, L-cysteine was more effective for keeping acrosomes and mitochondria intactness during sperm cryopreservation.

(Key words : L-cysteine, catalase, cryopreservation, sperm ability, *Korean Native Cattle*)

INTRODUCTION

Cryopreservation of bull semen has been widely used as an essential tool of the livestock industry, particularly in conjunction with the diffusion of genetic material and the banking of genome resources to preserve precious transgenic lines; however, freezing and thawing processes lead to the generation of reactive oxygen species (ROS) that impair post-thaw motility, viability, intracellular enzyme, fertility, and sperm functions (Zhao and Buhr, 1995; Aitken *et al.*, 1998).

The most common ROS generated by spermatozoa are superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxy lradical (^-OH). H_2O_2 is the most toxic ROS for the spermatozoa of most species because of its ability to penetrate biological membranes (Aitken, 1995). During cryopreservation polyunsaturated fatty acids (PUFA) in the sperm plasma membrane undergo peroxidation, which results in the for mation of ROS (Jones and Mann, 1977). Differences in fatty acid composition and lipid class ratios in spermatozoa among species are important factors to consider in freezing spermatozoa.

The seminal plasma has antioxidant systems composed of catalase (CAT) and reduced glutathione (GSH; Aitken and Baker, 2004; Gadea *et al.*, 2004; Jang *et al.*, 2008); which antioxidants are cytoplasmic, however, this antioxidant ability is restricted due to small cytoplasmic droplet by spermiogenesis small cytoplasmic droplet component that contains antioxidants to scavenge oxidants in sperm. Thus, the antioxidant capacity of sperm may be insufficient to prevent LPO during the freeze-thawing process. Mammalian cells can only utilize cysteine for the enhancement of intracellular glutathione biosynthesis and it has been shown to penetrate the cell membrane easily both *in vitro* and *in vivo* (Mazor *et al.*, 1996). L-cysteine, as a precursor

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of glutathione, has been shown to enhance intracellular glutathione production both *in vivo* and *in vitro* (Jeyendran *et al.*, 1989) and to prevent hydrogen peroxide-mediated loss of sperm motility in bulls (Bilodeau *et al.*, 2001). Also, L-cysteine has been shown to prevent loss of motility, viability, and membrane integrity during sperm liquid storage or in the frozen state (Szczesniak-Fabianczyk *et al.*, 2003). In addition, it has improved porcine oocyte maturation and fertilization *in vitro* (Jeong and Yang, 2001).

Catalase catalyzes the decomposition of H_2O_2 into water and oxygen, thus removing an initiator of chain reactions leading to lipid peroxidation and to the formation of other reactive radicals (Aitken, 1995). Catalase is necessary for decomposition of the toxic product hydrogen peroxide (H_2O_2) produced during the course of anaerobic metabolism of spermatozoa (Salisbury and Lodge, 1963). Thus, addition of catalase enzyme to semen extenders to overcome the increased production of H_2O_2 has received the attention of studies dealing with preservation of semen. Supplementation of the extender with catalase has resulted in the improvement of sperm parameters in several species (Rocaetal., 2005; Michaeletal., 2007); however, there are no reports about the efficacy of enzymatic antioxidant catalase and non-enzymatic antioxidant L-cysteine for cryopreservation of the spermatozoa of *Korean Native Cattle*. Therefore, this study was conducted to investigate the effects of L-cysteine and catalase on the characteristics of the frozen-thawed sperm of Korean native cattle.

MATERIALS AND METHODS

1. Semen Collection

For all experiments herein, Korean native cattle semen was collected using an artificial vagina and a teaser at the Hanwoo (*Korean Native Cattle*) Experiment Station, National Institute of Animal Science, Rural Development Administration, Korea. The collected semen (volume: 5～15 ml; density: 2～10 × 10^8 ml; live ratio >75%) was diluted with TRILADYL® (KRUUS, Cat. No. 340244, Denmark) containing 20% (v/v) egg-yolk. Then, the bull semen was transferred to the laboratory at 4～5℃ within 2 hour for experiments.

2. Sperm Cryopreservation and Thawing

Before the freezing process, Triladyl containing 20% (v/v) egg-yolk and/or L-cysteine (L; 10 mM), catalase (C; 200 IU), and L-cysteine + catalase (L + C; 10 mM + 200 IU) were added to the diluted semen. A second dilution to provide a concentration of 2 × 10^7 sperm per ml was performed with an aliquot of semen diluted with the same freezing extender used in the first dilution. The sperm were loaded into 0.5 ml straws in are frigerator at 4℃. These straws were placed 10 cm above a liquid nitrogen surface where the temperature was approximately −120℃. After cooling for 10 min, all straws were immersed directly into liquid nitrogen (−196℃) for storage. For the experiments, frozen semen straws were thawed for 45 sec in a 37℃ waterbath. The thawed semen samples were immediately transferred into microtubes of 1.5 ml and diluted with Beltsville Thawing Solution (BTS) by centrifugation at 1,500 rpm for 5 min. The sperm pellets were assessed for concentration and resuspended in BTS buffer of 1 ml.

3. Sperm Characteristics Assay

The LIVE/DEAD® sperm Viability Kit (Molecular Probes, Eugene, OR, USA) was used in a fluorescence-based assay to analyze sperm viability. Membrane-permanent SYBR 14 nucleic acid stain labels live sperm with green fluorescent tags, and membrane-impermanent propidium iodide (PI) labels the nucleic acids of membrane-compromised sperm with red fluorescent tags. Diluted semen samples in BTS buffer were prepared in light-tight tubes. A final SYBR-14 concentration of 40 nM was achieved through addition of 2 μl of diluted SYBR-14 dye to each sample of diluted semen. After incubation for 10 min at 37℃, 2 μl of PI was added to the sample of diluted semen to produce a final PI concentration of 2 μM. The diluted semen samples were incubated for 10 min at 37℃ under lightproof conditions. Stained sperm was analyzed by flow cytometry (Fig. 1A).

Sperm acrosome integrity was analyzed by fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) and PI double stain. Post-thaw semen was diluted with phosphate buffered saline (PBS) and the fluorescent probes were added to a 1 ml aliquot of semen with the addition first of 1 μl FITC-PNA. After incubation for 10 min at 37℃, 2 μl of PI was added to the sample of diluted semen to produce a final PI concentration of 2 μM. The diluted semen samples were incubated for 10 min at 37℃ under lightproof conditions. Thereafter, the stained semen was centrifuged at 1,500 rpm for 5 min. The sperm pellets were resuspended in 1 ml of PBS. Stained sperm were analyzed by flow cytometry (Fig. 1B).

To evaluate sperm mitochondrial activation, Rhodamine 123 (Sigma, Cat. No. R8004, USA) and PI double stain were used. Semen samples diluted in PBS were prepared in light-tight tubes. A final Rhodamine 123 concentration of 0.53 mM was achieved through addition of 2 μl of diluted Rhodamine 123 dye to each sample of diluted semen. After incubation for 10 min at 37℃, 2 μl of PI was added to the sample of diluted semen to produce a final PI concentration of 2 μM. The diluted semen samples were incubated for 10 min at 37 ℃ under light-proof conditions. Thereafter, the stained semen was centrifuged at 1,500 rpm for 5 min. The sperm pellets were resuspended in 1 ml of PBS. Stained sperm was analyzed by flow cytometry (Fig. 1C).

The sperm hydrogen peroxide (H_2O_2) level was analyzed by carboxy-2',7'-Dichlorofluorescin diacetate (carboxy-DCFDA) and PI double stain. Post-thaw semen was diluted with PBS containing bovine serum albumin (BSA) and the fluorescent probes were added to a 1 ml aliquot of semen with the addition first of 1 μl carboxy-DCFDA. After incubation for 40 min at 37℃, 2 μl of PI was added to the sample of diluted semen to produce a final PI concentration of 2 μM. The diluted semen samples were incubated for 10 min at 37℃ under lightproof conditions. Thereafter, the stained semen was centrifuged at 1,500 rpm for 5min. The sperm pellets were resuspended in 1 ml of PBS. Stained sperm were analyzed by flow cytometry (BD FACS Calibur; BD, USA). The sperm hydrogen peroxide (H_2O_2) level was evaluated as stained (positive) or non-stained (negative) by carboxy-DCFDA (Fig. 2).

To evaluate the sperm glutathione (GSH) level, an analysis was carried out using Cell Tracker™ Red CMTPX (Invitrogen, Cat. No. C34552, USA) single stain. Semen samples diluted in PBS were prepared in light-tight tubes. A final Cell Tracker™ Red CMTPX concentration of 1 μM was achieved through addition of 1 μl of diluted Cell Tracker™ Red CMTPX dye to each sample of diluted semen. After incubation for 1 hour at 37℃, the stained semen was centrifuged at 1,500 rpm for 5 min. The sperm pellets were resuspended in 1 ml of PBS. Stained sperm were analyzed by flow cytometry. The sperm GSH level was evaluated as stained (positive) or non-stained (negative) by Cell Tracker™ Red CMTPX (Fig.3).

4. Flow Cytometry Analysis

Flow cytometry analysis was performed by using a FACS caliber flow cytometer (BD FACS Calibur; BD, USA). Forward scatter (FSC), side scatter (SSC), green fluorescence (FL1), and red fluorescence (FL2) were measured and collected for each

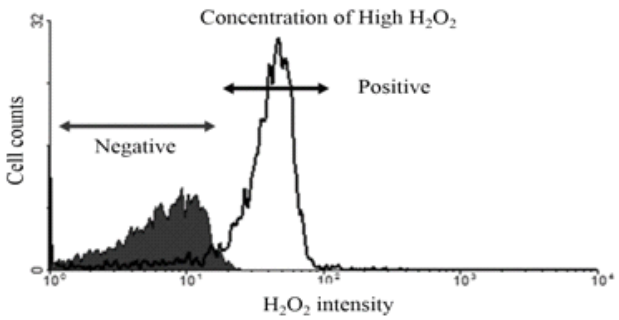

Fig. 2. Histogram from flow cytometry for spermatozoa stained with carboxy-DCFDA. Negative : non-stained; Positive: stained

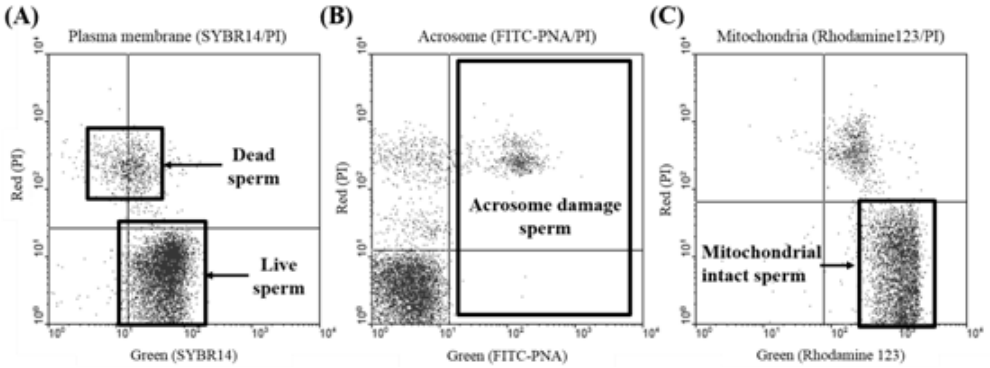

Fig. 1. Methods of flow cytometry analysis for assessing bull sperm physiology. Viability was analysis using the plasma membrane integrity, up black box was means dead sperm (PI positive sperm) and low black box means live sperm (SYBR14 positive sperm) (A), acrosome damage was analysis FITC-PNA and PI doubling staining methods, black box means acrosome damage sperm (FITC-PNA positive sperm) (B), Mitochondrial integrity was measured using the Rhodamine123 and PI double staining, black box show mitochondrial intact sperm (C)

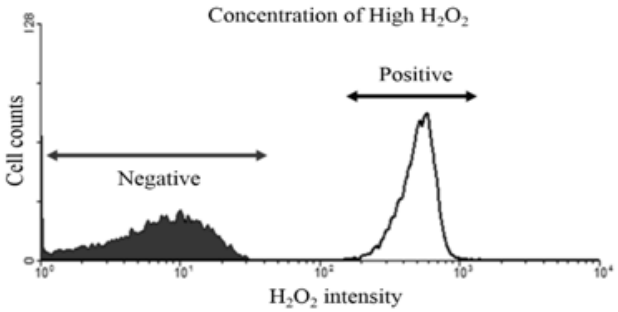

Fig. 3. Histogram from flow cytometry for spermatozoa stained with Cell Tracker™ Red. Negative : non-stained; Positive : stained.

sample. FSC indicated the size of spermatozoa whereas SSC indicated the granularity. FSC and SSC parameters were gated, and only events with scatter characteristics similar to sperm cells were analyzed for fluorescence (Nagy *et al.*, 2003) among the total of 10,000 sperm cells.

5. Statistical Analysis

Statistical analysis was performed with analysis of variance (ANOVA) using SAS (version 9.1, SAS Institute Inc., Cary, NC, USA). Data are presented as mean ± standard error of the mean (S.E.M.). Differences in the mean values of sperm damage after treatment with various treatment conditions were processed using Duncan's multiple range tests. Differences were considered significant when $p<0.05$.

RESULTS

The characteristics of *Korean Native Cattle* sperm frozen-thawed with catalase (10 mM) and/or L-cysteine (200 IU) are shown in Table 1. Sperm viability was significantly higher in the L-cysteine + catalase (69.49 ± 3.16%) group than in the control (60.5 ± 3.94%) group ($p<0.05$). When acrosome integrity was evaluated by acrosome membrane damage in frozen-thawed sperm, the proportion of damaged acrosomes was significantly ($p<0.05$) lower in the L-cysteine (17.12 ± 1.08%) group than in the control (21.46 ± 1.14%), catalase (20.54 ± 0.76%) and L-cysteine + catalase (19.29 ± 0.65%) groups. In addition, mitochondrial membrane integrity was significantly ($p<0.05$) higher in the L-cysteine (58.65 ± 1.39%) group than in the control (50.63 ± 2.37%) group.

The sperm hydrogen peroxide (H_2O_2) level was significantly ($p<0.05$) lower in the L-cysteine (3.74 ± 1.66%), catalase (4.65 ± 1.87%) and L-cysteine + catalase (8.11 ± 2.15%) groups than in the control (13.22 ± 1.6%) group (Fig. 4). Fig. 5 shows the flow cytometry analysis of the difference in hydrogen peroxide (H_2O_2) intensity of the frozen-thawed bull sperm. On the other hand, the GSH level was significantly ($p<0.05$) higher in the group with L-cysteine (1.33 ± 0.03%) than in the control (1.08 ± 0.06%), catalase (1.05 ± 0.02%) and L-cysteine + catalase (1.11 ± 0.0%) groups as shown in Fig. 6. The flow cytometry analysis of the differences in GSH intensity of the frozen-thawed bull sperm is shown in Fig. 7.

DISCUSSION

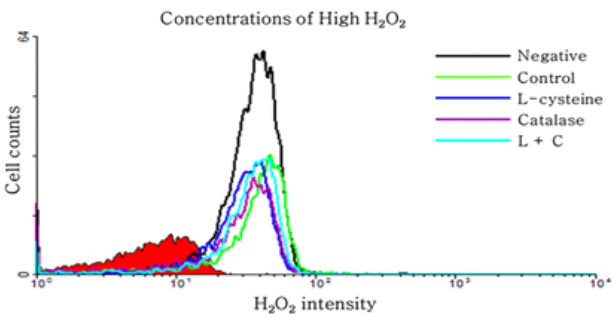

Fig. 4. H_2O_2 levels in *Korean Native Cattle* sperm frozen-thawed with L-cysteine and/or catalase.

Table 1. Characteristics of spermatozoa cryopreserved with catalase and/or L-cysteine in *Korean Native Cattle*

Treatments	Analysis of spermatozoa frozen-thawed (%)		
	Viability	Acrosome damage	Mitochondrial integrity
Control	60.50 ± 3.94^{b}	21.46 ± 1.14^{a}	50.63 ± 2.37^{b}
L-Cysteine [L]	63.78 ± 2.36^{ab}	17.12 ± 1.08^{b}	58.65 ± 1.39^{a}
Catalase [C]	68.00 ± 2.98^{ab}	20.54 ± 0.76^{a}	54.12 ± 1.65^{ab}
L + C	69.49 ± 3.16^{a}	19.29 ± 0.65^{a}	55.70 ± 2.99^{ab}

^{a,b} Figures with different superscripts indicate significant differences from each other treatment group ($p<0.05$).

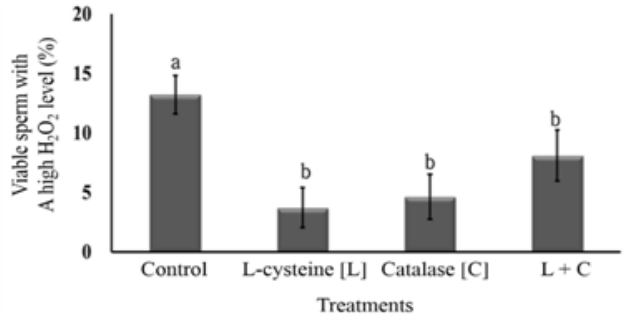

Fig. 5. Hydrogen peroxide (H_2O_2) intensity of *Korean Native Cattle* sperm frozen-thawed with L-cysteine and/or catalase. [a,b] Bars with different super scripts indicate significant differences from each other treatment group (p<0.05).

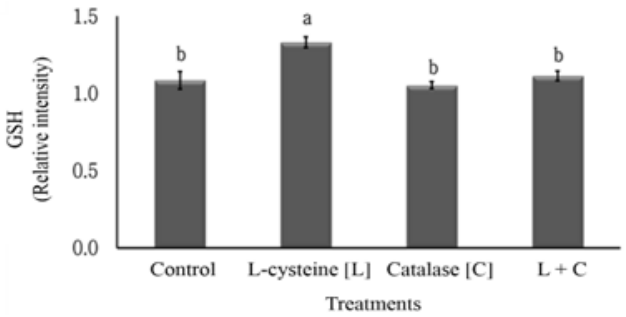

Fig. 6. Glutathione intensity of *Korean Native Cattle* sperm frozen-thawed with L-cysteine and/or catalase. [a,b] Bars with different super scripts indicate significant differences from each other treatment group (p<0.05).

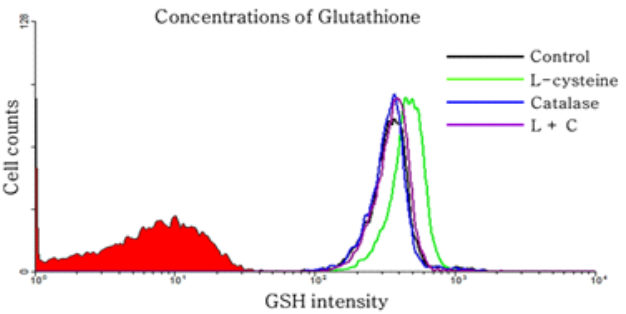

Fig. 7. Flow cytometric analysis of glutathione intensity of frozen-thawed *Korean Native Cattle* sperm.

In this study, an evaluation was made of the effectiveness of L-cysteine and/or catalase on viability, acrosome integrity, mitochondrial integrity, H_2O_2 level, and glutathione level in frozen-thawed spermatozoa of Korean native cattle. It is well known that sperm cryopreservation is important as a valuable tool for creating effective semen banking. It is reported that spermatozoa motility, viability and acrosome integrity are greatly affected by the freezing-thawing procedure (Donnelly *et al.*, 2001; Lee *et al.*, 2011).

Spermatozoa contains high concentrations of PUFA, which reactivity is high to LPO, which causes to a subsequent loss of sperm motility, plasma, acrosome and mitochondrial membrane integrity, and fertilizing ability of the sperm (Chen *et al.*, 1993; Aitken and Fisher, 1994; Lee *et al.*, 2011).

The protective antioxidant system are broken out preferentially in cytoplasmic of sperm, but sperm cells was removed most of their cytoplasmic droplet during the spermiogenesis. As a result, spermatozoa was had less cytoplasmic droplet component containing antioxidants which sperm was easily damaging to ROS and LPO; therefore, sperm are susceptible to LPO during cryopreservation and thawing (Alvarez and Storey, 1989; Storey, 1997).

L-cysteine has an effect to eliminate to ROS in the intracellular protective mechanism, which inhibits capacitation and protects membrane damage in mammal sperm (Johnson *et al.*, 2000). In the spermatozoa of many species, L-cysteine also plays a major role as a protection of plasma damage. Under physiological conditions, protection of mammalian spermatozoa against oxidative stress is provided by an enzymatic antioxidant system, mainly represented by superoxide dismutase (SOD) and CAT present in spermatozoa and the seminal plasma of different species including bulls (Hermelo *et al.*, 1987; de Almeida *et al.*, 1989; Thiangtum *et al.*, 2009).

Spermatozoa are susceptible to oxidative damage under oxidative stress. This damage can be prevented by supplementation of the semen extender with antioxidants (Sikka, 2004). Our results showed that antioxidant supplementation after thawing prevented a decrease of sperm viability and an increase of membrane damage under oxidant stress, and the combination of L-cysteine and catalase significantly improved viability of bull spermatozoa after cryopreservation.

A variety of scavengers are present in spermatozoa and seminal plasma including L-cysteine, GSH, GSH-PX, and catalase (Alvarez and Storey, 1989; Jeulin *et al.*, 1989), which remove alkoxyl and peroxyl radicals and thus protect sperm viability and functions against oxidative damage (Baker *et al.*, 1996; Aitken and Baker, 2004). In the present study, it is clearly shown that L-cysteine produces better results for acrosomal integrity, mitochondrial integrity, and glutathione level compared with the other groups. The addition of L-cysteine

exhibited significant cryoprotective activity on certain sperm parameters such as post-thaw motility and acrosomal and overall morphological integrity, which is similar to the results from studies performed with boar (Szczesniak-Fabianczyk *et al.*, 2003; Funahashi and Sano, 2005), ram (Agarwal *et al.*, 2004), and goat (Aboagla and Terada, 2003) semen.

Based on these results, we can hypothesize that L-cysteine displayed a protective influence on the functional integrity of acrosomes and mitochondria, thereby improving post-thawed sperm viability. The L-cysteine play a role to positive effects on many sperm ability such as membrane stabilizers, antioxidants and several factors. For example, L-cysteine can reactivate ROS and catalyze the detoxification of H_2O_2 and other superoxides (Meister, 1992). Improvement of frozen bull sperm might be explained in this research by L-cysteine. Because GSH level is decreased during the cryopreservation process, addition of L-cysteine which play a precursor of intracellular glutathione in freezing solution is beneficial for frozen-thawed bull semen (Bilodeau *et al.*, 2001). In addition, L-cysteine reduce the lipid peroxidation, which produces free radicals, and thus decrease sperm damage from ROS (Watson 2000; Chatterjee *et al.*, 2001). Besides being active as a glutathione precursor, L-cysteine can also exert a protective effect in the extracellular milieu, primarily due to its nucleophilic and antioxidant properties (De Flora *et al.*, 2001). As catalase activates the decomposition of H_2O_2 into water and oxygen, the generation of radicals by Fe^{2+} is less efficient in the presence of this enzyme. The blocking of this pathway reduces the negative effects of H_2O_2 and other radicals, which are known to decrease sperm motility (O'Flaherty *et al.*, 1997; Bilodeau *et al.*, 2001). In conclusion, L-cysteine and catalase are effective in protecting against membrane damage of the sperm of Korean native cattle during cryopreservation, and especially L-cysteine was more effective for acrosome - and mitochondrial integrity in cryopreservation of the sperm of *Korean Native Cattle*.

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