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The Reduction of Hydrogen Peroxide in Viable Boar Sperm Cryopreserved in the Presence of Catalase

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Abstract : Semen cryopreservation induces the formation of reactive oxygen species (ROS), and the ROS cause sperm damage. We aimed to investigate the effects of the antioxidative enzyme catalase (CAT) on sperm quality and ROS during cryopreservation. Sperm rich fractions collected from five Duroc boars were cryopreserved in freezing extender with (200 or 400 U/mL) or without CAT (control). After thawing, sperm motility, viability, normal morphology, plasma membrane integrity, mitochondrial function and intracellular ROS were evaluated. CAT significantly improved total sperm motility at a concentration of 400 U/mL ($P < 0.05$), but didn't improve progressive sperm motility, viability, morphological defects, plasma membrane integrity and mitochondrial function in frozen-thawed boar sperm. In evaluation of ROS, CAT had no effect on reduction in $\cdot O_2$, but scavenged H_2O_2 in viable frozen-thawed boar sperm at concentrations of 200 and 400 U/mL ($P < 0.05$). In conclusion, CAT was not enough to improve quality of frozen-thawed sperm, but can reduce H_2O_2 generation in viable boar sperm during cryopreservation.

Key words **:** boar sperm, catalase, cryopreservation, ROS.

Introduction

Semen cryopreservation is associated with the increased generation of reactive oxygen species (ROS) in animals (9,22) and higher levels of ROS during cryopreservation cause impairment of sperm function (13). Boar sperm are particularly susceptible to oxidative attack by ROS, because they contain large amounts of polyunsaturated fatty acids (38).

Sperm oxidative damage is the result of an improper balance between ROS generation and scavenging activities. The scavenging potential of the ejaculate is normally maintained by adequate levels of antioxidants present in the seminal plasma (SP) (21,35). However, cryopreserved boar sperm have low antioxidative enzyme defense mechanisms and can be vulnerable to oxidative attack, because SP is generally removed during the cryopreservation process. Accordingly, the generation of ROS during cryopreservation should be minimized in order to improve the quality of cryopreserved sperm in boar.

ROS-induced damage in sperm can be eliminated by the action of enzymatic and non-enzymatic antioxidants (1). Several research studies in animals have reported that antioxi dants minimize the toxic effects of ROS to sperm by direct addition of antioxidative enzymes (i.e., catalase, glutathione peroxidase, superoxide dismutase) to sperm preservation extenders (16,25-27,36). Although there have been several studies on the effect of antioxidative enzymes during sperm cryopreservation, their effect on sperm quality is still contradictory and their action as ROS scavengers needs further investigation.

Hydrogen peroxide (H_2O_2) is considered the most toxic species (2,17) because of its ability to cross membranes freely and inhibit enzyme activities and cellular functions, thus decreasing the antioxidant defenses of the sperm (17). Likewise, H_2O_2 is known as the major ROS responsible for oxidative damage in boar sperm (18). Catalase (CAT) has been reported as a potential H_2O_2 detoxifier (3), but its ability to act as antioxidant in cryopreservation of boar sperm is not well known. Therefore, the aims of this study were to investigate the effects of CAT, as a supplement in sperm freezing extenders on sperm quality and ROS during cryopreservation of boar sperm.

Materials and Methods

Reagents and media

All media components were purchased from Sigma Chemical Co (St. Louis, MO, USA). A stock solution of catalase (CAT; 2×10^5 U/mL) was prepared and preserved at −80°C until used (32). The medium used for sperm extension was Beltsville Thawing Solution (BTS) (30). The media used for sperm cryopreservation were a lactose-egg yolk (LEY) extender (80 mL of 11% lactose solution and 20 mL of egg yolk, pH 6.2) and LEY-glycerol-Orvus-ES-Paste (LEYGO) extender (89.5% [v/v] LEY extender, 9% [v/v] glycerol, and 1.5% [v/v] Equex STM [Nova Chemical Sales, Scituate Inc., MA, USA], pH 6.2).

Experimental design

Each experimental day, stock solutions of CAT were thawed and added to cryopreservation media for a final concentration

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of 200 and 400 U/mL. Semen cooled to 15°C was equally divided into three aliquots and centrifuged. The aliquots were extended each with LEY and LEYGO supplemented with different proportions of CAT: (1) LEY and LEYGO extenders without CAT (control); (2) supplemented with 200 U/mL CAT; (3) supplemented with 400 U/mL CAT. The extended aliquots were then cryopreserved. The effects of CAT on boar sperm cryopreservation were assessed by conventional sperm parameters (sperm motility, viability, and morphology), plasma membrane integrity, mitochondrial membrane integrity and intracellular ROS at 30 min postthawing.

Processing, cryopreservation and thawing of semen

Five fertile Duroc boars were used in this study. One ejaculation from each boar was collected using the gloved-hand method (n = 5). Sperm rich fractions were extended (1:1 [v/v]) in BTS. After collection, the sperm concentration, motility, and morphology were evaluated under light microscopy, and only ejaculates with ≥ 70% motile sperm and ≥ 80% normal morphologies were used. Immediately after evaluation, the diluted sperm-rich fractions were slowly cooled to 15°C for 3 h and cooled semen was transferred to the laboratory within 24 h at 15°C. Semen was processed according to the straw freezing procedure (4) with some modification. Briefly, semen cooled to 15°C was centrifuged at 800 × *g* for 10 min (15°C) and the semen pellet was resuspended with LEY extender to a concentration of 1.5×10^8/mL. After further cooling to 5°C for 90 min, two parts LEY-extended semen were mixed with one part LEYGO extender to a final freezing concentration of 1×10^8/mL. The diluted and cooled semen was then loaded into 0.5-mL French straws (IMV, L'Aigle, France) and placed in liquid nitrogen vapor approximately 3 cm above the level of the liquid nitrogen for 20 min after which the straws were stored directly in the liquid nitrogen. Thawing was achieved by immersing the straws in a water bath at 37°C for 20 sec (12) and thawed sperm suspensions were diluted at 37°C in BTS (1:2 [v/v]). After holding the thawed sperm suspension in a water bath for 30 min, the thawed sperm were evaluated.

Sperm evaluation

Conventional sperm parameters

The percentage of total motile sperm and progressively motile sperm (sperm showing rapid steady forward [RSF] movement) was estimated using microscopic examination (33). The percentage of motile and progressively motile sperm was determined by observing a minimum of 300 sperm, in at least six different fields under a bright field microscope at × 400 magnification. The mean of six successive estimations was recorded as the final motility score. The viability of sperm was assessed by means of the eosin-nigrosin staining method (10). Viability was assessed by counting 200 sperm under a bright field microscope at × 400 magnification. Sperm displaying partial or complete purple staining were considered non-viable; only sperm showing strict exclusion of stain were counted as viable. The morphology of sperm was evaluated using the Diff-Quik kit (International Reagents Corp., Kobe, Japan). Briefly, a drop of semen on a glass slide was drawn out and allowed to air-dry. The slide was then stained with the Diff-Quik kit. At least 200 sperm were evaluated under light microscopy at × 1000 magnification.

Sperm plasma membrane integrity

Sperm plasma membrane integrity was assessed using 6-carboxyfluoresceindiacetate (6-CFDA; Sigma-Aldrich, St. Louis, MO, USA)/propidium iodide (PI; Sigma-Aldrich) fluorescent staining (31). Briefly, 500 μL of semen sample (1×10^6 sperm/mL) were stained with 6-CFDA (5 μL of a 2 μg/mL) and PI (5 μL of a 0.1 mg/mL). Samples were then incubated at 37°C for 15 min and analyzed using flow cytometry. Analyzed sperm were classified as having either an intact plasma-membrane (CFDA+/PI–) or a damaged plasma-membrane (CFDA–/PI+).

Sperm mitochondrial function

Sperm mitochondrial function was assessed using Rhodamine 123 (R123; Molecular Probes Inc., Eugene, OR, USA)/PI (Sigma-Aldrich) fluorescent staining (28). Aliquots of 500 μL of semen sample (1×10^6 sperm/mL) were stained with R123 (3 μL of a 0.1 mg/mL) and PI (5 μL of a 0.1 mg/mL). Samples were then incubated at 37°C for 25 min and analyzed using flow cytometry. Data were expressed as the percentage of viable sperm with intact mitochondrial membrane integrity (R123+/PI–). In addition, the mean fluorescence intensity (MFI) of R123 was measured to evaluate mitochondrial activity in each population (total, viable and dead sperm).

Sperm intracellular $\cdot O_2$ and H_2O_2

Hydroethidine (HE; Molecular Probes Inc.) and 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA; Molecular Probes Inc.) were used to detect $\cdot O_2$ and H_2O_2, respectively, as described by Guthrie and Welch (18). Aliquots of 500 μL of semen (1×10^6 sperm/mL) were mixed either HE or H_2DCFDA to final concentrations of 4 μM and 200 μM, respectively. To simultaneously differentiate living from dead sperm, Yo-Pro-1 (final concentration, 0.05 μM) was added to HE-treated sperm and PI (final concentration, 2 μM) was added to H_2DCFDA-treated sperm. Samples stained with HE and Yo-Pro-1 were incubated at 25°C for 40 min and samples stained with H_2DCFDA and PI were incubated at 25°C for 60 min. After incubation, samples were analyzed using flow cytometry. Data were expressed as the percentage of viable sperm with high $\cdot O_2$ (high ethidium fluorescence) and H_2O_2 (high DCF fluorescence). In addition, the MFI of ethidium and DCF were measured to evaluate intracellular mean $\cdot O_2$ and H_2O_2 per the total (whole) sperm and each sub population.

Flow cytometric analysis

All flow cytometry analyses were performed using a FACScalibur flow cytometer (Becton Dickinson, San José, CA, USA) equipped with a 15 mW air-cooled 488 nm argon-ion laser and Cell Quest Pro software (Becton Dickinson). A total of 10,000

individual sperm-sized events were selected based on forward and side scatter and collected at a flow rate of < 200 events/second. FL1 signals (6-CFDA, R123, Yo-Pro-1 and DCF) were detected between a 500 and 530 nm band pass filter, and FL3 signals (PI and ethidium) were detected through a > 630 nm band pass filter. Evident sperm populations were gated and the quantity of sperm and MFI of each population were determined.

Statistical analysis

Statistical analysis of the data was performed using SPSS software (version 15.0 for Windows; SPSS Inc., Chicago, IL, USA). The Shapiro-Wilk test was utilized to evaluate normality analysis. The non-parametric Friedman test was used for analysis of all data, and the Wilcoxon signed ranks test was used to calculate the difference between samples in cases showing significant differences with the Friedman test. Statistical significance was set at $P < 0.05$ and all data were presented as the mean ± standard error of the mean (SEM).

Results

Treatment with 400 U/mL CAT improved total sperm motility compared with the control ($P < 0.05$), but not compared with the 200 U/mL CAT treatment. Total sperm motility in the 200 U/mL CAT-treated groups was not different from the control (Table 1). There were no significant differences among the three groups (0, 200, and 400 U/mL CAT-treated groups) in progressive motility, viability, morphology, plasma membrane integrity, and mitochondrial function (Tables 1-3).

The percentage of viable sperm with a high intracellular $\cdot O_2$ (V2) was not significantly different among the three groups (4.30 ± 0.56%, 4.62 ± 0.63%, and 4.73 ± 0.75% for 0, 200, and 400 U/mL CAT-treated groups, respectively; Fig 1B). The intracellular $\cdot O_2$ of the CAT-treated groups were not different from that of the control in total (172.76 ± 7.54, 185.20 ± 12.94, and 182.62 ± 11.58 for 0, 200, and 400 U/mL CAT-treated groups, respectively) and viable sperm populations (97.87 ± 5.84, 102.59 ± 7.48, and 103.43 ± 8.26 for 0, 200, and 400 U/mL CAT-treated groups, respectively), but was lower than that of the control in moribund (1009.52 ± 20.90, 869.22 ± 19.58, and 875.21 ± 25.63 for 0, 200, and 400 U/mL CAT-treated groups, respectively) and dead sperm populations (303.16 ± 6.16, 282.54 ± 7.68, and 285.69 ± 7.24 for 0, 200, and 400 U/mL CAT-treated groups, respectively, $P < 0.05$; Fig 1C). There were no significant differences in intracellular $\cdot O_2$ between the CAT-

Table 1. Sperm motility, viability and plasma membrane integrity following catalase (CAT) treatment

CAT treatment	Motility (%)		Viability (%)	Plasma membrane integrity (%)	
	Total	Progressive		Intact	Damaged
0 U/mL	48.00 ± 3.39[a]	42.00 ± 3.39	66.30 ± 2.83	59.61 ± 1.77	39.34 ± 1.42
200 U/mL	50.00 ± 4.18[a,b]	45.00 ± 4.18	68.80 ± 1.50	54.57 ± 2.82	44.48 ± 1.76
400 U/mL	52.00 ± 4.06[b]	47.00 ± 4.06	70.00 ± 1.87	56.79 ± 2.65	42.35 ± 1.61

Within a column, values with different superscripts differ significantly, $P < 0.05$.

Table 2. Sperm morphological defects following catalase treatment

Morphology parameter (%)	0 U/mL CAT	200 U/mL CAT	400 U/mL CAT
Head abnormalities	6.20 ± 1.93	6.60 ± 0.75	6.60 ± 1.69
Acrosome abnormalities	14.00 ± 1.27	11.80 ± 1.83	12.20 ± 1.53
Midpiece abnormalities			
Cytoplasmic residue	10.40 ± 3.37	10.40 ± 2.11	12.80 ± 3.31
Broken, bent, double and thick	2.80 ± 0.74	3.80 ± 1.02	3.00 ± 0.55
Tail abnormalities	0.40 ± 0.40	0.40 ± 0.40	0.60 ± 0.40
Total abnormalities	30.20 ± 4.10	29.20 ± 4.62	29.80 ± 4.53

Table 3. Sperm mitochondrial function following catalase treatment

CAT treatment	Viable sperm with intact mitochondrial membrane (%)	Mitochondrial activity (MFI of R123)		
		Total sperm	Viable sperm	Dead sperm
0 U/mL	62.88 ± 1.75	177.94 ± 7.04	202.70 ± 10.01	148.87 ± 3.49
200 U/mL	58.84 ± 2.28	176.22 ± 6.90	203.34 ± 9.55	149.56 ± 4.25
400 U/mL	60.96 ± 1.05	175.05 ± 4.26	200.31 ± 6.48	147.93 ± 2.58

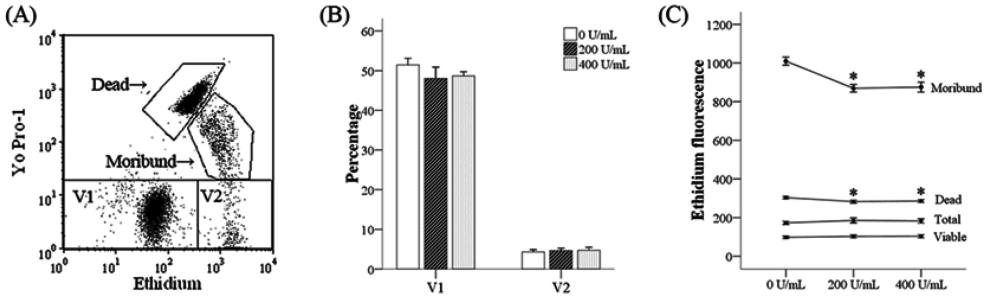

Fig 1. Flow cytometric analysis of sperm labeled with HE/Yo-Pro-1 in 0 U/mL (control), 200 U/mL, and 400 U/mL CAT-treated groups. The labeled sperm was classified into four categories (A). In (A), V1 represents viable sperm with a low intracellular $\cdot O_2$, and V2 represents viable sperm with a high intracellular $\cdot O_2$. The percentage of V1 and V2 (B) and mean fluorescence intensity (MFI) of ethidium in each category (C) were compared among the three experimental groups. Data are presented as the mean ± SEM. Asterisk (*) indicates values that are significantly different from control ($P < 0.05$, n = 5).

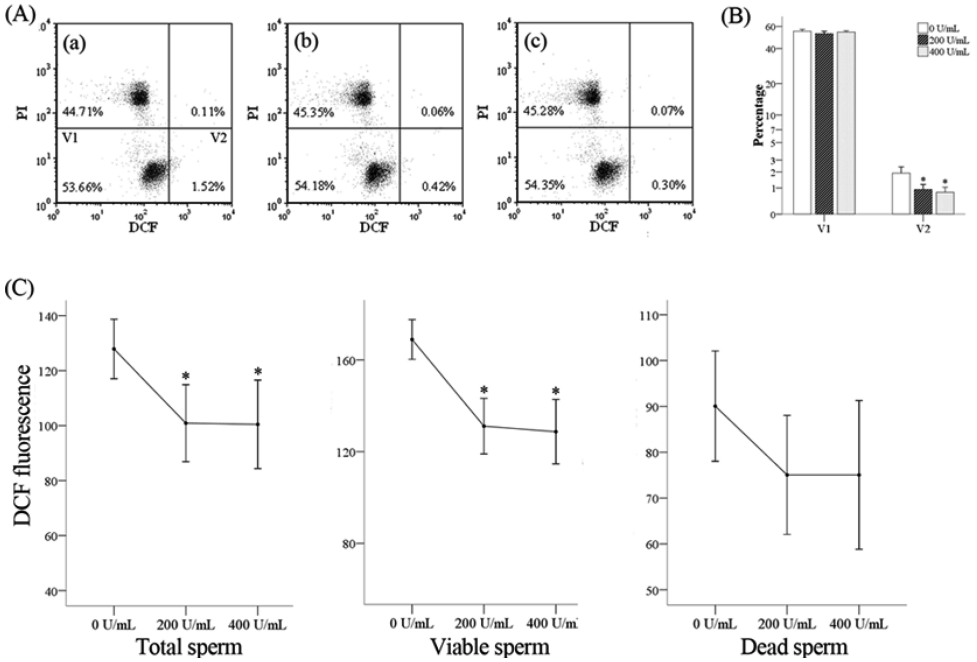

Fig 2. Flow cytometric analysis of sperm labeled with H_2DCFDA/PI in control (a), 200 U/mL (b), and 400 U/mL (c) CAT-treated groups. The labeled sperm were classified into three categories (A). In (A), V1 (lower right quadrant) represents viable sperm with a low intracellular H_2O_2, V2 (lower left quadrant) represents viable sperm with a high intracellular H_2O_2, and the upper quadrants represent dead sperm. The percentage of V1 and V2 (B) and MFI of DCF in each category (C) was compared among the three experimental groups. Data are presented as the mean ± SEM. Asterisk (*) indicates values that are significantly different from control ($P < 0.05$, n = 5).

treated groups in all populations.

The percentage of viable sperm with a high intracellular H_2O_2 (V2) decreased in the CAT-treated groups (0.93 ± 0.26% and 0.80 ± 0.25% for 200 and 400 U/mL CAT-treated groups, respectively) compared with the control (1.91 ± 0.48%, $P < 0.05$; Fig 2B). In addition, the intracellular H_2O_2 of the CAT-treated groups was lower than that of the control in both total (127.76 ± 10.80, 100.90 ± 14.00, and 100.47 ± 16.08 for 0, 200, and 400 U/mL CAT-treated groups, respectively) and viable sperm populations (168.94 ± 8.69, 131.14 ± 12.13, and 128.73 ± 14.08 for 0, 200, and 400 U/mL CAT-treated groups, respectively, $P < 0.05$). In dead sperm population, the intracellular H_2O_2 of the CAT-treated groups was not significantly different from that of the control (90.06 ± 12.04, 75.05 ± 12.99, and 75.05 ± 16.23 for 0, 200, and 400 U/mL CAT-treated groups, respectively; Fig 2C). There were no significant differences in intracellular H_2O_2 between the CAT-treated groups in all subpopulation.

Discussion

Part of the reductions in sperm motility and fertility associated with cryopreservation may be due to oxidative damage from excessive or inappropriate formation of ROS (29,32). Oxi-

dative stress, an imbalance between ROS creation and native antioxidant defense mechanisms, causes decreased sperm motility, membrane fluidity, DNA integrity and mitochondrial function, as well as alteration of sperm metabolism (1), decreased ability to fuse with the oocyte, and compromised pregnancy after in vitro fertilization (20). H_2O_2 is known as the major ROS responsible for oxidative damage in human (3), stallion (7) and boar sperm (18). Therefore, CAT, which transforms H_2O_2 into a harmless product (H_2O) (15), might help prevent oxidative damage induced by cryopreservation and thereby improve sperm qualities.

We found that CAT supplementation of the freezing extender increased total motility, but did not improve other sperm quality parameters (progressive motility, viability, morphological defects, plasma membrane integrity, and mitochondrial function). Our results are in agreement with evidence that 200 U/mL CAT does not improve motility, mitochondrial activity, and acrosome integrity of stallion sperm (8). Likewise, 200 U/mL CAT compromised motility in epididymal cat sperm (37) and addition of 400 U/mL and 800 U/mL CAT was deleterious to the motility of ram sperm (25). At the same time, improvement of frozen-thawed sperm quality by CAT supplementation in other animals (26,32) showed that oxidative stress may play a role in cell damage during cryopreservation. Optimization of CAT concentration may be required to effectively identify the benefits to boar sperm cryopreservation.

To examine the antioxidative ability of CAT on frozen-thawed boar sperm, we used the flow cytometry to measure ROS in each sperm population after cryopreservation following CAT supplementation. Although CAT improved sperm quality only weakly at best, it did diminish H_2O_2 in viable frozen-thawed sperm even at a low concentration of 200 U/mL. CAT supplementation may keep viable frozen-thawed sperm from oxidative damage by reducing the intracellular H_2O_2 of viable sperm after freeze-thawing. The fertilizing ability of viable sperm might be enhanced by CAT supplementation after freeze-thawing in a time-dependent fashion, with CAT preventing impairment of sperm surviving from cryopreservation through antioxidative action. Indeed, the addition of CAT to the extender improves the survival and in vitro fertility of liquid-stored ram sperm (25) and improves total sperm motility, viability, and the ability of frozen-thawed sperm to produce embryos in vitro, with reduced ROS generation using chemiluminescence method (32). In addition, the antioxidant action of CAT in viable frozen-thawed sperm might be of further benefit to sperm in the female reproductive tract, which is characterized by higher levels of oxidative stress (5,39). However, it is still not clear that CAT provides viable boar sperm with sufficient protection from oxidative damage during cryopreservation. Further studies will be necessary to determine if the reduction of H_2O_2 in viable frozen-thawed sperm by CAT is sufficient to improve fertilizing ability after freeze-thawing.

CAT only uses H_2O_2 as a substrate when its concentration is largely above physiological levels, as can happen in oxidative bursts characteristics of stress responses (14,15). The reduction of H_2O_2 in viable frozen-thawed sperm by CAT supplementation provide indirect evidence that cryopreservation may produce enough H_2O_2 to cause cytotoxicity in viable boar sperm. CAT might be a major antioxidant for cryopreserved boar sperm.

CAT shows a high selectivity for its substrate, which is H_2O_2. Unexpectedly, however, CAT reduced the MFI of ethidium in moribund and dead boar sperm. HE is considered to be a more sensitive probe for $\cdot O_2$ than H_2O_2 (34) and HE has been used in many studies for $\cdot O_2$ detection (6,23,24). Others have indicated that under certain conditions HE can serve as a substrate for oxidation by either $\cdot O_2$ or H_2O_2 (18,19). Accordingly, the reduction in MFI of ethidium by CAT in moribund and dead boar sperm might indicate that HE might serve as a substrate for H_2O_2. However, the H_2O_2 in dead sperm did not significantly decrease with CAT supplementation on the basis of the DCF assay. It might be due to removal of H_2O_2 prior to activating the generation of $\cdot O_2$ inside the cells (11) rather than by oxidation of HE by H_2O_2. Further research is required to determine if the reduction in ethidium fluorescence by CAT is due to the H_2O_2-detecting ability of HE or $\cdot O_2$ inactivation following removal of H_2O_2 by CAT.

In conclusion, this study focused on the effect of CAT on ROS in viable boar sperm after cryopreservation. CAT led to a reduction in H_2O_2 in the viable frozen-thawed sperm. Although no obvious effect of CAT on cryopreserved sperm quality was observed, the reduction of H_2O_2 in viable sperm by CAT supplementation might have some positive effects on fertility.

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Catalase 첨가에 따른 돼지 정액 동결 및 융해 후 생존 정자에서 Hydrogen Peroxide의 감소

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요 약 : 정액 동결 과정은 활성산소종의 생성을 유발하며, 생성된 활성산소종은 정자의 손상을 일으키는 것으로 알려져 있다. 따라서 본 연구의 목적은 동결 과정 중 항산화 효소 중 하나인 catalase (CAT)를 첨가함으로써 융해 후 정자의 기능과 활성산소종의 수준에 미치는 효과를 알아보고자 하였다. 5마리 돼지에서 채취한 정액은 0 (대조군), 200, 400 U/mL CAT가 첨가되어 있는 동결 희석액으로 각각 동결하였다. 융해 후, 정자 운동성, 생존성, 정상 형태율, 형질막 온전성, 미토콘드리아 기능, 세포내 ROS를 평가하였다. CAT는 400 U/mL의 농도에서 전체 정자 운동성을 향상시켰지만 ($P < 0.05$), 전진 운동성, 생존성, 기형율, 형질막 온전성, 미토콘드리아 기능의 향상을 나타내지 않았다. 활성산소종의 평가에서, CAT는 융해된 생존 정자의 $\cdot O_2$의 감소에는 효과를 나타내지 않은 반면 H_2O_2를 감소시켰다 ($P < 0.05$). 결론으로 CAT는 동결 및 융해된 정자의 질을 향상시키는 데 큰 효과를 나타내진 않았지만 생존 정자에서 H_2O_2을 제거함로써 생존정자의 산화적 손상을 감소시킬 수 있으리라 판단된다.

주요어 : 돼지 정자, catalase, 동결 보존, 활성산소종