Original Article

Effect of Catalase and Superoxide Dismutase on Motility, Viability and Acrosomal Integrity of Canine Spermatozoa during Storage at 5^0C

Khongsak Thiangtum[1, 2*] Tatsuya Hori[3] Eiichi Kawakami[3]

Abstract

Preservation of canine spermatozoa at 5^0C is a helpful method for dog breeding management. However, in many species, oxidative damage due to generation of reactive oxygen species (ROS) and long term storage which specifically cause DNA damage, and lipid peroxidation during preservation techniques can cause a decline of sperm motility and fertility capacity. In this study, the effect of antioxidants catalase (CAT) and superoxide dismutase (SOD) on motility, viability and acrosomal integrity of chilled canine semen were investigated. Semen was collected by digital manipulation from 4 dogs (two ejaculates/dog). After removal of seminal plasma, spermatozoa were diluted in egg yolk Tris-fructose citrate solution (EYT-FC) with or without CAT, SOD or the combination of CAT and SOD at dosages of 100, 400 and 1,600 U/ml in each antioxidant. Diluted spermatozoa were kept at 5^0C for 7 days. Sperm motility, viability and acrosomal integrity in the EYT-FC with CAT or/and SOD and without the antioxidants (control) were not significantly different at same point of time. At day 7, the percentage of mean compared between control and treatment groups for sperm motility were 65.6% and 65.0-66.9%, viability were 83.6% and 82.1-84.9% and acrosomal integrity were 87.2% and 85.9-88.5%, respectively. In conclusion, adding CAT and SOD or the combination of CAT and SOD in EYT-FC did not significantly improve the maintenance of motility, viability and acrosomal integrity during dog sperm storage at 5^0C for 7 days.

Keywords: canine spermatozoa, catalase, chilled semen, superoxide dismutase

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บทคัดย่อ

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Khongsak Thiangtum[1, 2*] *Tatsuya Hori*[3] *Eiichi Kawakami*[3]

การเก็บรักษาน้ำเชื้อสุนัขที่อุณหภูมิ 5 องศาเซลเซียส เป็นวิธีการที่เป็นประโยชน์ต่อการจัดการผสมพันธุ์ในสุนัข อย่างไรก็ตาม ผลกระทบจากอนุมูลอิสระที่เกิดขึ้นระหว่างขบวนการเพื่อเก็บรักษา และการเก็บรักษาอสุจิไว้เป็นเวลานาน ซึ่งสร้างความเสียหายต่อดีเอ็นเอ และการเกิดขบวนการลิปิดเปอร์ออกซิเดชั่น สามารถส่งผลให้อัตราการเคลื่อนที่ และประสิทธิภาพในการผสมของตัวอสุจิลดลงในสัตว์หลายชนิด การศึกษานี้จะดูผลของสารต้านอนุมูลอิสระ คาตาเลส และซูเปอร์ออกไซด์ดิสมิวเทส ต่ออัตราการเคลื่อนที่ อัตราการมีชีวิต และคุณภาพของอะโครโซม ของอสุจิสุนัขที่แช่เย็น โดยทำการรีดเก็บน้ำเชื้อจากสุนัข 4 ตัว ตัวละ 2 ครั้ง แยกเอาส่วนน้ำเลี้ยงอสุจิทิ้ง จากนั้นจึงละลายอสุจิด้วยสารละลายน้ำเชื้อชนิดทริสฟรุกโตสซิเตรท (Tris-fructose citrate solution, EYT-FC) ที่ไม่มีส่วนผสมของสารคาตาเลส และซูเปอร์ออกไซด์ดิสมิวเทส (กลุ่มควบคุม) หรือที่มีส่วนผสมของ คาตาเลส ขนาดความเข้มข้น 100, 400 และ 1,600 ยูนิต/มล. หรือที่มีส่วนผสมของซูเปอร์ออกไซด์ดิสมิวเทสขนาดความเข้มข้น 100, 400 และ 1,600 ยูนิต/มล. หรือที่มีส่วนผสมของทั้งคาตาเลสและซูเปอร์ออกไซด์ดิสมิวเทส ขนาดความเข้มข้น 100, 400 และ 1,600 ยูนิต/มล. อสุจิในสารละลายน้ำเชื้อถูกแช่เย็นที่อุณหภูมิ 5 องศาเซลเซียส เป็นเวลา 7 วัน จากการศึกษาไม่พบความแตกต่างอย่างมีนัยสำคัญทางสถิติ ของอัตราการเคลื่อนที่ อัตราการมีชีวิต และอัตราความสมบูรณ์ของอะโครโซมของอสุจิระหว่างกลุ่มควบคุม กับกลุ่มที่มีส่วนผสมของสารคาตาเลส หรือสารซูเปอร์ออกไซด์ดิสมิวเทส หรือคาตาเลสและซูเปอร์ออกไซด์ดิสมิวเทสในแต่ละช่วงเวลาของการศึกษา โดยในวันที่ 7 ของการแช่เย็น พบว่าอสุจิในกลุ่มควบคุมเปรียบเทียบกับกลุ่มที่ละลายในสารละลายน้ำเชื้อที่มีส่วนผสมของคาตาเลส หรือ/และซูเปอร์ออกไซด์ดิสมิวเทส มีค่าเฉลี่ยของอัตราการเคลื่อนที่ เท่ากับร้อยละ 65.6 และ 65.0-66.9 มีค่าเฉลี่ยอัตราการมีชีวิต เท่ากับร้อยละ 83.6 และ 82.1-84.9 มีค่าเฉลี่ยอสุจิที่มีอะโครโซมสมบูรณ์ เท่ากับร้อยละ 87.2 และ 85.9-88.5 จากการศึกษาครั้งนี้สรุปได้ว่า การเติมสารคาตาเลส หรือ/และ ซูเปอร์ออกไซด์ดิสมิวเทส ในสารละสารน้ำเชื้อ EYT-FC ไม่ได้ช่วยรักษาอัตราเคลื่อนที่ อัตราการมีชีวิต และอัตราความสมบูรณ์ของอะโครโซม ของอสุจิสุนัข ที่เก็บรักษาที่อุณหภูมิ 5 องศาเซลเซียส ในระยะเวลา 7 วัน

คำสำคัญ: อสุจิสุนัข คาตาเลส น้ำเชื้อแช่เย็น ซูเปอร์ออกไซด์ดิสมิวเทส

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Introduction

Preservation of dog sperm at 5^0C is a helpful method in dog breeding management since we can conserve the motility and viability up to 4-6 days in extender (Iguer-ouada and Verstegen, 2001). This preserved semen can overcome the limitation of live animal transportation. However, using chilled dog semen is limited by its decrease in motility and fertilizing capacity related to storage time. There are many reasons for the decrease in fertilizing capacity of chilled dog semen. One reason is oxidative damage to spermatozoa during sperm processing and storage. Since sperm membrane is rich in polyunsaturated fatty acids, it can easily undergo lipid peroxidation in the presence of reactive oxygen species (ROS). Peroxidation of sperm membrane phospholipids leading to change in membrane fluidity then results in loss of motility. Finally, the peroxidized sperm cells lack the membrane dynamics of fusogenicity and is deficient in fertilization of oocytes (Lenzi et al., 2002).

Fortunately, mammalian spermatozoa have an enzymatic antioxidant system to protect themselves against oxidative stress. This system consisting of glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase (CAT) physiologically controls the balance between ROS production and neutralization (Lenzi et al., 2002). The amount of each enzyme which is present both in

seminal plasma and spermatozoa is different among species. In human, antioxidants in semen are primarily of post-testicular origin and probably serve to protect ejaculated spermatozoa from oxidative stress such as that which occurs in the female reproductive tract (Zini et al., 2002). In equine, seminal plasma contains a high activity of catalase that is derived primarily from prostatic secretion (Ball et al., 2000). Furthermore, seasonal variation of catalase activity in seminal plasma was reported in ram (Marti et al., 2007), boar (Gilum et al., 2011) and bull (Asadpour et al., 2012). In canine, Cassani et al. (2005) and Neagu et al. (2011) reported the important antioxidant SOD like activity in seminal plasma, spermatic and prostatic fraction of dog semen. This enzyme is an important component of the enzymatic antioxidant system that protects spermatozoa against the deleterious effect of ROS in dog semen. However, preservation procedures for dog semen that require removing of seminal plasma may reduce or impair the antioxidant capability of spermatozoa. It was interesting to examine whether or not adding antioxidants to extender might help to improve the maintenance of motility of chilled dog semen.

Various antioxidants have been tested in attempts to prevent oxidative stress in semen from a variety of species. Previous studies in human (Rossi et al., 2001), ram (Maxwell and Stojanov, 1996) and boar (Fabianczyk et al., 2003; Peña et al., 2003; Funahashi and Sano, 2005) found the positive effect to sperm motility when adding antioxidant to semen extender but not in horses (Aurich et al., 1997; Ball et al., 2001), cat (Thiangtum et al., 2009) and cattle (Verberckmoes et al., 2005) semen preservation. However, varied effects were found in each species depending on type and concentration of antioxidants. For chilled dog semen, vitamin E, 5-(4-dimethylamino-phenyl)-2-phenyl-penta-2, 4-dienoic acid (B16) and butylated hydroxytoluence had beneficial effect on preservation of semen quality (Michael et al., 2009; Sahashi et al., 2011) but not vitamin C (Michael et al., 2008). However, there are no reports indicating SOD and the combination of CAT and SOD including its different concentration for chilled dog semen. Therefore, the aim of present study was to evaluate the effect of adding various dosages of antioxidant CAT, SOD and the combination of CAT and SOD on motility, viability and acrosomal integrity of canine semen during storage at 5^0C.

Materials and Methods

Animals: Four male dogs aged 4-10 (7.5±1.5) years were used in this study. These animals were housed in pens with ample runs under natural lighting. Commercial dry dog food (Master foods Co. Ltd., Tokyo, Japan) was provided twice a day and drinking water was given *ad libitum*. This study was conducted under the guidelines of the Animal Care and Use Committee of the Nippon Veterinary and Animal Science University.

Semen collection and assessment: Semen was collected using digital manipulation. Three fractions were separated by changing the collecting tube during semen collection. The interval of one week was obtained for semen collection from same dog. After collection, separated fractions were measured for volume. Raw semen then was evaluated for sperm concentration, motility, viability, morphology, and acrosome integrity. The concentration of sperm was determined by hemocytometer counts. Sperm motility was determined objectively by light microscope (VBS-FT-2 Nikon, Japan) at x400 magnification using the sperm motility examination plate (NFA-71, Fujihira Industry) and keep warm on 37^0C warm plate (Type-III, Fujihira Industry). Progressive motility was scored in percentage. To determine sperm viability and morphology, a drop of semen and eosin stain was mixed together on a clean microscope slide and then was smeared. The smear was examined using bright field microscope at x400 magnification. Dead spermatozoa which cell membrane allowed entering of stain were pink in colour while the live spermatozoa were transparent. The percentages of dead/alive and normal/abnormal sperm were assessed for viability and morphology, respectively.

For acrosomal evaluation, 20 µl of sperm samples were fixed in 0.5 ml of 4% paraformaldehyde solution at room temperature for 15 min then stored at 4^0C until processed. Fixed sperm then were centrifuged for 8 min at 5,000 rpm and the supernatant was discarded. Pellets were washed twice with 0.5 ml of 0.1M ammonium acetate (pH= 9.0) and the pellet resuspended in approximately 50 µl of the ammonium acetate solution. An aliquot of this suspension was smeared onto microscope slides and allowed to dry at room temperature. After drying, the slides were flooded with Coomassie Blue stain 0.22% [Coomassie® Brillian blue G-250 (MP Biomedical, Inc., Ohio, USA) in 50% methanol, 10% glacial acetic acid and 40% deionized water (Larson and Miller, 1999)] for 90 sec, rinsed with deionized water. From each sample, 200 spermatozoa were assessed individually for acrosomal integrity using bright field microscopy at x1000. The acrosomal integrity was classified in three groups; intact acrosome (homogenious stain at acrosomal region), damaged acrosome (patchy stain or some small vacuole in acrosomal region) and non-intact acrosome (no stain at acrosomal region).

Addition of CAT and SOD into extender and storage at 5^0C: The effect of CAT and SOD on canine spermatozoa during storage at 5^0C was studied in eight ejaculates from four dogs (two ejaculates/dog). After evaluation of raw semen, seminal plasma was removed from sperm rich fraction by centrifuging at 1,500 rpm for 5 min. Supernatant was discarded and sperm pellet was then resuspended in egg yolk Tris-fructose citrate solution (EYT-FC, pH: 6.5, osmotic pressure: 300 mOsm/l) (Tsutsui et al., 2003) to a concentration of $40x10^6$. The composition of EYT-FC is described in Table 1. Volume of 500 µl resuspended semen was aliquoted to ten 1.5 ml microcentrifuge tubes. To study the effect of antioxidants on chilled dog spermatozoa, one tube was added with 500 µl of EYT-FC without antioxidants (control) and each remaining 9 tubes were added with 500 µl of EYT-FC containing various dosages of antioxidants as follows; 200, 800 and 3,200 U/ml of bovine liver CAT (Wako Pure Chemical Industries, Ltd., Japan), 200, 800 and

3,200 U/ml of SOD from bovine erythrocytes (EMD Biosciences, Inc. La Jolla, CA) and 200, 800 and 3,200 U/ml of the combination of CAT and SOD. Thus, concentration of CAT, SOD and the combination of CAT and SOD in EYT-FC extender would be reduced to 100, 400 and 1,600 in each treatment. A total of 10 microcentrifuge tubes containing a final concentration of $20x10^6$ sperm/ml extended semen was then placed in plastic box containing with 25^0C water. The water in the external tubes prevented temperature variation during sperm assessment. This box was then placed in programmable cooling refrigerator (UH-JF, Chino Ltd., Tokyo, Japan). Extended semen was cooled down from 25^0C to 5^0C within 1 hour and then stored at 5^0C for 7 days. Sperm motility and viability were evaluated at 24 hour interval for 7 days. Acrosomal integrity was evaluated at 0 h and day 7 of storage.

Statistical analysis: Statistical analysis of the results was performed using SPSS (version 11.5) software. To compare sperm motility, viability and acrosomal integrity between control and treatment groups over time, data were analyzed using repeated measurement of variance (ANOVA) followed by Duncan's test. Difference of $p< 0.05$ was taken as significant.

Results

Our dogs used in this study gave a good quality of raw semen (Table 2). The average percentage of sperm motility, viability and acrosomal integrity were 88.1±2.8, 93.3±1.4 and 96.8±0.5, respectively. Sperm motility gradually decreased when diluted spermatozoa in EYT-FC were stored at 5^0C for 7 days. At day 7, the average percentage of sperm motility was less than 70 in the control and all treatment groups. However, there was no significant difference between the control and treatment groups when any dosage of CAT, SOD or the combination of CAT and SOD were added at any time (Table 3).

Similar to the sperm motility, the viability of diluted spermatozoa in EYT-FC was not significantly different between the control and treatment groups when stored at 5^0C for 7 days. The sperm viability from the control and every treatment groups were still high (> 80%) after being stored for 7 days (Table 4).

After being stained with Coomassie Blue, sperm acrosome was easily classified. The percentage of acrosomal intact sperm decreased after diluted spermatozoa were stored at 5^0C for 7 days in EYT-FC. However, the average percentage of acrosomal intact sperm at day 7 was still higher than 80% in the control and all treatment groups. There was no significant difference between the control and treatment groups (Table 5).

Table 1 Composition of EYT-FC extender

Ingredient	EYT-FC
Citric acid (g)	1.3
Tris aminomethane (g)	2.4
Fructose (g)	1
Egg-yolk (ml)	20
Distilled water (ml)	80
Penicillin G potassium (IU)	100,000
Streptomycin sulfate (g)	0.1

Table 2 Raw semen quality in dogs (n= 4)

Parameter	Mean±SEM	Range
Total number of sperm ($x 10^6$)	595.2±66.9	367.0-952.0
Sperm motility (%)	88.1±2.8	70.0-95.0
Viable sperm (%)	93.3±1.4	85.5-97.5
Normal morphology sperm (%)	96.8±0.4	95.2-98.0
Sperm with intact acrosome (%)	96.8±0.5	95.0-98.5

Semen samples were collected 2 times.

Table 3 Percentage of sperm motility (mean±SEM) in EYT-FC extender with the addition of various concentrations of CAT or SOD or the combination of CAT and SOD

Antioxidant	Sperm motility (%)							
	0 day	1 day	2 days	3 days	4 days	5 days	6 days	7 days
Control	86.3±2.1	81.9±2.1	78.8±1.3	78.8±0.8	79.4±1.1	76.3±2.1	73.1 ± 1.3	65.6 ± 2.2
CAT (100 U/ml)	85.0±2.1	82.5±1.6	78.1±1.6	78.8±0.8	76.9±2.1	76.3±1.8	73.1 ± 1.9	65.0 ± 2.1
CAT (400 U/ml)	83.8±2.8	81.9±1.6	78.1±1.3	79.4±0.6	78.1±1.6	75.6±2.0	72.5 ± 1.9	65.6 ± 2.6
CAT (1,600 U/ml)	85.6±2.2	81.9±1.6	79.4±1.8	78.8±0.8	78.1±1.3	73.8±1.6	73.1 ± 1.3	65.0 ± 2.7
SOD (100 U/ml)	85.6±2.0	82.5±1.6	78.1±1.3	78.1±0.9	75.6±1.1	74.4±1.5	71.9 ± 1.6	66.3 ± 2.3
SOD (400 U/ml)	85.6±2.4	81.9±1.6	77.5±1.6	79.4±0.6	76.9±1.6	72.5±1.6	71.3 ± 1.8	66.3 ± 2.8
SOD (1,600 U/ml)	85.6±2.0	81.9±1.6	79.4±1.5	78.8±0.8	78.1±1.6	73.8±2.1	72.5 ± 1.9	65.6 ± 2.2
CAT+SOD (100 U/ml)	83.8±2.6	82.5±1.6	78.8±1.6	77.5±1.6	75.6±1.5	73.1±1.6	70.6 ± 1.4	67.5 ± 1.6
CAT+SOD (400 U/ml)	85.0±1.8	82.5±1.6	78.1±1.3	79.4±1.1	76.9±1.9	73.1±1.9	71.3 ± 1.8	66.9 ± 1.6
CAT+SOD (1,600 U/ml)	84.4±1.8	80.6±1.5	80.0±1.3	78.1±0.9	75.6±2.0	73.1±1.3	70.6 ± 2.0	65.6 ± 1.8

CAT: Catalase, SOD: Superoxide dismutase

Table 4 Percentage of sperm viability (mean±SEM) in EYT-FC extender with the addition of various concentrations of CAT or SOD or the combination of CAT and SOD

Antioxidant	Sperm viability (%)							
	0 day	1 day	2 days	3 days	4 days	5 days	6 days	7 days
Control	91.8±2.1	89.9±1.2	89.9±0.8	89.0±1.4	88.6±1.5	87.4±1.6	84.2±2.2	83.6±2.4
CAT (100 U/ml)	92.1±2.0	88.0±1.7	87.6±1.4	86.6±2.2	88.2±2.0	86.4±2.6	84.4±1.8	82.1±2.2
CAT (400 U/ml)	89.9±2.1	87.9±1.1	88.4±1.6	89.9±1.3	89.7±1.9	85.1±1.8	84.6±1.7	83.3±2.1
CAT (1,600 U/ml)	91.1±2.3	90.1±1.5	88.0±1.1	88.4±1.8	88.6±1.3	85.5±1.6	85.6±1.3	83.4±1.8
SOD (100 U/ml)	90.9±1.9	88.5±1.2	88.8±1.0	88.6±0.7	88.4±1.7	87.4±1.9	82.8±2.6	84.9±1.5
SOD (400 U/ml)	91.6±2.0	89.4±1.3	88.5±1.0	89.5±0.8	89.4±1.2	86.4±2.0	82.4±2.2	83.4±2.5
SOD (1,600 U/ml)	90.4±1.9	88.8±1.3	88.1±1.3	89.4±1.3	89.3±1.5	87.1±1.3	82.6±2.5	82.3±2.2
CAT+SOD (100 U/ml)	91.6±2.2	87.6±1.6	87.0±1.0	90.1±0.9	88.8±1.8	87.2±1.5	83.5±1.7	83.9±2.0
CAT+SOD (400 U/ml)	91.3±2.1	87.3±1.7	86.4±1.2	88.8±1.4	86.6±1.8	86.3±1.8	84.6±1.7	82.9±2.5
CAT+SOD (1,600 U/ml)	91.3±1.8	88.4±1.2	87.4±1.3	88.5±1.4	88.2±1.7	88.1±1.1	86.3±1.2	82.9±2.4

CAT: catalase, SOD: superoxide dismutase

Table 5 Percentage of sperm with intact acrosome (mean±SEM) in EYT-FC extender with the addition of various concentrations of CAT or SOD or the combination of CAT and SOD

Antioxidant	Intact acrosome (%)	
	0 day	7 days
Control	96.9±0.5	87.2±3.6
CAT (100 U/ml)	96.3±0.7	86.9±3.3
CAT (400 U/ml)	96.4±0.3	86.3±3.0
CAT (1,600 U/ml)	95.9±0.9	88.5±2.9
SOD (100 U/ml)	97.1±0.5	86.7±3.0
SOD (400 U/ml)	96.6±0.6	86.3±3.1
SOD (1,600 U/ml)	96.0±1.2	84.9±3.0
CAT+SOD (100 U/ml)	96.3±0.7	86.4±3.1
CAT+SOD (400 U/ml)	96.1±0.7	85.9±3.0
CAT+SOD (1,600 U/ml)	96.9±0.4	86.2±3.4

CAT: catalase, SOD: superoxide dismutase

Discussion

Preservation of dog semen at 5^0C has become a routine procedure for dog breeding since it can conserve the motility and viability of dog spermatozoa up to 4-6 days, preserve and several extenders can be used. Moreover, the cost is lower when compared with frozen semen. However, the major disadvantage of this method is the decrease in motility and fertility capability when long term storage is performed. To maintain sperm motility and fertilizing capability of chilled semen, many previous studies examined a composition of semen extenders such as source of sperm energy (Ponglowhapan et al., 2004; Verstegen et al., 2005) and cold-shock protective agents (Beccaglia et al., 2009). In this study, EYT-FC with and without antioxidants were used for the preservation of dog semen. Although sperm motility gradually decreased when diluted semen was stored at 5^0C, it still maintained above 75% at day 4 of storage. This result supported previous studies on preservation of chilled dog spermatozoa in extenders (Iguer-ouada and Verstegen, 2001). An important finding in this study showed that EYT-FC extender containing 100, 400 and 1,600 U/ml of CAT or SOD or the combination did not help to maintain sperm motility during storage at 5^0C. Moreover, neither sperm viability nor acrosomal integrity was different from the control group when various concentration of antioxidants CAT and SOD was added to EYT-FC extender. Interestingly, the viability of spermatozoa stored at 5^0C for 7 days was still high (>80%) while the sperm motility decreased more (~ 65%). These finding may support a previous report which non-motile sperm could still recover motility and remain fertile when adequate medium was added (Verstegen et al., 2005). Sugar containing in extender, however, play an important energy source influence on chilled canine sperm motility (Ponglowhapan et al., 2004; Verstegen et al., 2005). Nevertheless, many factors may influence the deterioration of chilled semen quality especially the lipid peroxidation due to oxidative stress during sperm storage in cooling condition.

Many antioxidants have been tested in attempts to prevent oxidative stress in semen from variety of species. SOD and CAT are antioxidant enzymes which are found in a variety of aerobic cells (Fridovich, 1978). SOD can prevent the hazardous effect of reactive oxygen species by catalyzing the dismutation of $O_2^{\bullet -}$ to H_2O_2 when CAT will catalyze H_2O_2 to H_2O and O_2 (Hugo, 1974). Adding antioxidant CAT and SOD to extender resulted in positive effect on chilled ram semen. Moreover, a combination of CAT and SOD had positive effect on the survival of ram spermatozoa during storage at 5^0C (Maxwell and Stojanov, 1996). However, it did not improve the motility when diluted ram semen was incubated at 38^0C (Upreti et al., 1997). In contrast, addition of 5 U/ml of CAT in extender could reduce the sperm motility loss when frozen-thawed bovine spermatozoa were incubated at 38^0C (Bilodeau et al., 2002). In equine, the addition of CAT did not affect the maintenance of sperm motility during storage at 5^0C (Aurich et al., 1997; Ball et al., 2001). The present study did not find any positive effects when adding CAT, SOD or the combination of CAT and SOD to

EYT-FC on chilled canine semen. Similarly, the addition of CAT to Tris-Lecithin extender did not improve chilled dog semen (Beccaglia et al., 2009). In contrast, moderate benefit was reported when catalase at 100 U/ml was added to Tris-glucose egg yolk (Michael et al., 2009). Furthermore, this study was conducted in normospermic dog while some studies reported positive effect of adding SOD and CAT to medium when asthenozoospermic dog sperm were incubated at 38^0C (Kawakami et al., 2007). These results, however, indicated the species variation and the influence of environment including temperature and extenders to the effect of CAT and SOD on spermatozoa.

Although the exact causes are unclear why adding CAT and SOD to extender did not affect the chilled dog sperm quality in this study, possible reasons may be, firsty, endogeneous antioxidant activities was enough for neutralizing reactive oxygen species during chilled semen process and storage at 5^0C. According to this hypothesis, SOD and CAT activity was found in seminal plasma, spermatic and prostatic fraction of canine semen (Cassani et al., 2005; Kawakami et al., 2007). Secondly, the lipid peroxidation of spermatozoal membrane may not increase during canine semen storage at 5^0C like the finding in horse chilled semen (Kankofer et al., 2005). Adding CAT or SOD did not affect chilled semen quality. Alternatively, all possible reasons described above may concurrently occur in chilled dog semen. However, further studies are needed to confirm these hypotheses.

In addition, the concentration of antioxidants added to extender should be considered since high dosage of antioxidants may be harmful to spermatozoa due to the change in physiological condition of semen extender. In ram, survival spermatozoa will increase when the dosage of SOD and CAT added to extender increases. However, CAT at dosage higher than 200 U/ml was toxic to ram spermatozoa (Maxwell and Stojanov, 1996). In contrast, the present study did not finding any different effects on canine spermatozoa when CAT, SOD or the combination of CAT and SOD at dosages of 100, 400 and 1,600 U/ml were added to EYT-FC extender. High dosage of catalase or superoxide dismutase at 1,600 U/ml used in this study had no deleterious effect on chilled canine semen.

In conclusion, this study indicated that adding CAT, SOD or the combination of CAT and SOD to EYT-FC extender did not have positive effect on the maintainance of motility, viability and acrosomal integrity of canine spermatozoa during storage at 5^oC. Further investigation should be conducted in chilled semen from dog which high percentage of sperm abnormalities are produced. Moreover, more studies in canine sperm antioxidant system are requested.

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