

Effect of superoxide dismutase, catalase, and glutathione peroxidase supplementation in the extender on chilled semen of fertile and hypofertile dogs

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This study investigated the correlation between oxidative stress status and key canine sperm parameters and the effect of addition of a superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) combination in egg yolk tris-citrate glucose (EYT-G) extender on semen during 10 days of storage at 4°C. Ten Boxer dogs were divided into two groups, fertile (F) and hypofertile (H), depending on pregnancy and live birth rate status in the previous year. Semen evaluation was performed on the day of collection (D0) and after 5 (D5) and 10 (D10) days of cooled storage. Sperm motility, kinetic parameters, and DNA integrity were assessed. A correlation between oxidative status and key semen parameters in both F and H groups was observed. Total and progressive motilities were significantly higher in the treated (SOD, CAT, and GPx addition) versus control groups at D10 in both F and H groups, and at D5 in the H group. DNA integrity was significantly higher in both treated groups (H and F) at D5 and D10. In conclusion, the addition of SOD, CAT, and GPx in the extender allows preservation of semen quality for up to 10 days of storage at 4°C in both fertile and hypofertile dogs.

Keywords: antioxidants, canine sperm, fertility, oxidative stress, semen preservation

Introduction

Artificial insemination (AI) is practiced frequently in domestic dog breeding for genetic improvement of breeds, to satisfy the demands of private owners, and for the preservation of endangered canids. AI has been accepted worldwide, supporting *ex situ* conservation programs for endangered canine species [21]. Canine AI can be done with fresh, chilled, or frozen semen. Currently, the use of cooled semen is increasing in canines as it can be stored for relatively long periods resolving problems associated with AI timing and shipping semen over long distances. Shipped semen can be utilized for trans-vaginal and trans-cervical insemination, and it requires inexpensive equipment and provides improved pregnancy rates [33]. AI techniques in breeding programs involving chilled canine semen require reliable long-term storage conditions to preserve sperm quality. In order to preserve spermatozoa during storage, their metabolic activities

need to be reduced by using an appropriate medium and storage temperature. Hori *et al.* [18] demonstrated that semen qualities can be maintained for up to 48 h when canine semen samples are extended with egg yolk tris-citrate fructose (EYT-F) or glucose (EYT-G) and stored at a temperature in the range of 4°C to 12°C. Several extenders to preserve fertilizing capacity of canine semen have been successfully tested, but further studies are indispensable to improve the quality of cooled stored semen [9,22].

There is little available information about the molecular mechanisms leading to death of spermatozoa during refrigeration, and apoptosis has recently been shown to be involved [24,39]. Also, it has been reported that cryopreservation results in death by apoptosis of equine, bovine, and human spermatozoa [28,31,32,36].

Sperm survival during refrigeration depends on oxygen consumption and metabolism, which increase the amount of reactive oxygen species (ROS) [3]. Although low ROS amounts

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are needed by spermatozoa to acquire fertilizing capabilities, excessive ROS production can damage sperm motility, sperm-oocyte fusion, and impair fertilization [5]. Due to the high sensitivity of spermatozoa to oxidative stress, ROS concentration is controlled by endogenous antioxidants, thereby avoiding ROS overload and preserving semen quality [16,29,37]. These antioxidant systems consist of enzymatic antioxidants, such as SOD, CAT, and GPx, and non-enzymatic antioxidants (vitamin A, C, E, and uric acid). A study on dogs confirmed the presence of endogenous antioxidants in the seminal plasma of pre-spermatogenic, spermatogenic, and post-spermatogenic fractions, with SOD representing the major enzymatic antioxidant in all dog ejaculate fractions, whereas GPx activity was present in the sperm-rich and post-spermatogenic fractions, and CAT activity was deficient [38]. Another study [23], however, demonstrated CAT activity in dog ejaculates and showed that the addition of SOD and CAT in the dilution extender of canine semen improved sperm quality.

The aim of this study was to investigate the effect of addition of SOD, CAT, and GPx in EYT-G extender on the survival of spermatozoa of dogs with different oxidative status. Sperm motility, kinetic parameters, and DNA integrity were evaluated during 10 days of storage at 4°C.

Materials and Methods

Animals

In order to select 10 dogs to place into each of two study groups, fertile (F) and hypofertile (H), a pilot study was conducted on 20 male Boxer dogs. A complete history, physical examination, and semen evaluation were performed on each dog. The age range of the selected dogs was 2 to 7 years (mean \pm SE, 4.5 \pm 1.2 years). The pregnancy and live birth rates of the preceding year after natural breeding and the fertility-related semen parameters of undiluted fresh semen such as concentration, motility, and morphology of spermatozoa were used to distinguish hypofertile and fertile dogs. Among the 20 dogs, the 5 with higher pregnancy and live birth rates (at least 1 live birth with at least 4 live and viable puppies) and with fertility-related parameters above average were included in the F group. The 5 dogs with no record of a live birth in the preceding year and with fertility-related parameters below average were included in the H group.

The dogs were housed in a domestic situation in Naples, Italy. Commercial dog food was given once daily, and water was given thrice daily (morning, afternoon, and evening). The owners of the dogs included in the study provided written consent, and the study was conducted in conformity with the animal study guidelines of the Naples Veterinary Medicine and Animal Production Department (D.lgs.26-04/03/2014).

Experimental design

In the first step of our study, we evaluated the quality of the dogs' ejaculates and their systemic oxidative state. To this aim, one ejaculate and one blood samples were harvested from each dog, for a total of 10 semen samples and 10 blood samples (from 5 fertile and 5 hypofertile dogs).

In the second step, the effect of enzymatic antioxidant supplementation on the cooling resistance of semen collected from fertile and hypofertile dogs was evaluated. To this aim, ejaculate from each dog in the F and H groups was divided into 2 aliquots (hypofertile and fertile controls [HC and FC]; hypofertile and fertile experimental [HE and FE]) and prepared as described below for storage at 4°C with or without antioxidant supplementation in the extender. Diluted and refrigerated samples were stored for 10 days. For each aliquot, semen quality (motility and kinetic parameters) and DNA fragmentation were evaluated at day 0, day 5, and day 10 of storage.

Oxidative stress status evaluation

Blood samples were collected via cephalic vein, and sera collected after centrifugation were frozen, stored at -20°C , and analyzed within one month from collection.

Pro-oxidant and antioxidant status of dogs was evaluated on serum by using the d-ROMs test and the OXY-adsorbent test. All test kits were purchased from Diacron International (Italy) [10].

d-ROMs test: Pro-oxidative status was evaluated by applying the d-ROMs test, a photometric test that allows the determination of reactive oxygen metabolite (ROM) concentration, in particular, hydroperoxide concentration. The d-ROMs test measures the oxidant ability of a serum sample (10 μL) toward a particular substance (modified aromatic amine) that is used as an indicator (chromogen, N,N-diethylparaphenyldiamin). The intensity of the developed color, photometrically quantified at 505 nm, is directly proportional to the concentration of the ROMs, according to the Lambert-Beer's law and is expressed as Carratelli units (1 CARR U = 0.08 mg hydrogen peroxide/dL).

OXY-adsorbent test: Antioxidant capacity (OXY) was measured as the ability of plasma to counteract the massive oxidation induced by a solution of hypochlorous acid (HClO), a powerful and physiological oxidant able to mimic situations that occur *in vivo*. Unreactive HClO radicals further react with the chromogen solution of N,N-diethylparaphenyldiamin and form a colored complex, which is measured at 505 nm. The results were expressed as $\mu\text{mol HClO/L}$.

OSi evaluation: The degree of oxidative stress was expressed by the oxidative stress index (OSi) [2], which is calculated as a ratio between pro-oxidants and antioxidants by using the following formula:

$$\text{OSi} = \text{ROMs/OXY} \times 100.$$

Collection of semen and first evaluation of undiluted fresh semen

Dog semen was obtained manually by masturbation at room temperature (25°C) [3,18] after the males had been accustomed to their housing and to the individual collecting the semen.

The semen was collected into sterile plastic test tubes (Falcon tube; Minitübe, Germany) and stored in a water bath at 37°C for 1 h. The pH and volume of each ejaculate were measured. Semen samples were divided into three fractions, and the second fraction, called the sperm-rich fraction, was used in this study. This fraction was initially evaluated macroscopically and then examined in detail under a microscope. Sperm concentration was determined by using a hemocytometer (Burker chamber) and sperm motility was examined on glass slides on a heated stage at 37°C under a Nikon TE 2000 inverted microscope (Nikon, Italy) connected to a Basler Vision Technology A312 FC camera (Basler, Germany) under phase-contrast at 400× [18]. Sperm viability and morphology were assessed by applying an eosin viability stain (Europath, Italy). Abnormal morphology was determined by examining at least 100 spermatozoa at 400× magnification under phase-contrast microscopy. Total and progressive motilities were evaluated by an expert observer using a phase-contrast microscope with a heated stage at 37°C at 100× magnification on ten randomly selected fields for each sample.

Semen processing for cool storage

Storage methods were performed as previously reported [40]. Samples were centrifuged at 650 × g for 5 min to remove seminal plasma and, then, divided into two aliquots. The sperm concentration of each aliquot was adjusted to 100 × 10⁶ sperm/mL in EYT-G extenders. The EYT-G extender was composed by tris(hydroxymethyl)-aminomethane (2.422 g), citrate acid monohydrate (1.360 g), glucose (1 g), gentamycin (100 mg), egg yolk (20 mL), and distilled water (80 mL), and, if needed, pH was adjusted to 7.0 with hydrogen chloride. Each experimental aliquot was supplemented with 15 IU/mL of GPx, 15 IU/mL of CAT, and 15 IU/mL of SOD. The total amount of antioxidants added to EYT-G was based on previous studies in human and horses [1,13]. The experimental (FE and HE) and control (FC and HC) aliquots were placed in a syringe without air and stored in the fridge at 4°C for 10 days. Semen quality tests were performed at the time of semen collection (D0), and at 5 (D5) and 10 (D10) days of post-cooling storage at 4°C. To prevent a rapid temperature drop, samples were stored in a beaker containing 500 mL of water at room temperature. In addition, to reduce the effects of ambient temperature, the experiments were conducted in a room with a controlled temperature of 20°C.

Semen quality analysis

Semen was examined before and after cooling at different

times, and total and progressive motilities (%), kinetic parameters, and DNA integrity (%) were evaluated as described below.

Moreover, semen analysis was performed by using the Sperm Class Analyzer CASA system (SCA; Microptic, Spain). Briefly, a 10-μL aliquot of semen sample was placed in a Makler chamber on a heated stage at 37°C, after pre-incubation at 37°C for 10 min. At least 200 spermatozoa were counted to evaluate the percentages of motile (MOT) and progressively motile (PMOT) spermatozoa, as well as rapid (RAP), medium (MED), slow (SLOW), and static (STATIC) spermatozoa, and sperm kinetic parameters, *i.e.*, curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), linearity (LIN = VSL/VCL), and straightness (STR = VSL/VAP). The SCA settings were adjusted according to the manufacturer's instructions. All manual and SCA measurements were performed by trained and licensed medical technologists. The SCA results were used when the difference between manual and SCA values was less than 20%. In cases where the difference was greater than 20%, the manual results were reported [17].

DNA fragmentation evaluation

As shown in Fig. 1, DNA fragmentation was evaluated by using the APO-BrdU TUNEL Assay Kit (Molecular Probes, A35125; Invitrogen, USA) as described by Boni *et al.* [8]. Sperm cells were prepared and fixed by using paraformaldehyde as required by the APO-BrdU terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate (dUTP)-nick end-labeling (APO-BrdU TUNEL) assay.

Samples were centrifuged at 1,100 × g for 10 min and the sperm pellets were re-suspended to a final concentration of 1 ×

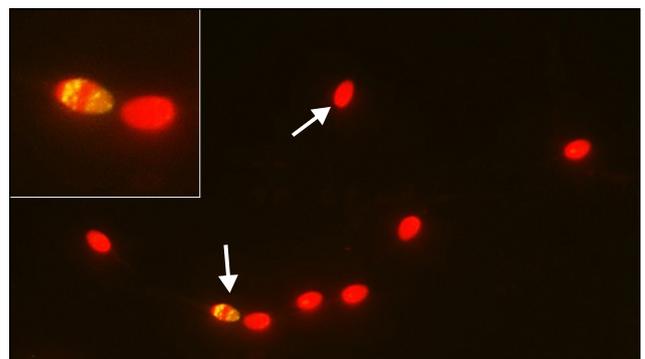


Fig. 1. Representative image of canine spermatozoa stained with an APO-BrdU TUNEL Assay Kit with anti-brdU Alexa Fluor 488 and using fluorescence microscopy at 400× magnification. Propidium iodide stains the nucleus of all sperm cells red and anti-brdU Alexa Fluor 488 stains spermatozoa with fragmented DNA in yellow. TUNEL-negative (red) and TUNEL-positive (yellow).

10^6 cells in 0.5 mL of phosphate-buffered saline (PBS). Then, to each sperm suspension was added 5 mL of 1% (w/v) paraformaldehyde in PBS, after which, it was placed on ice for 15 min, centrifuged twice for 5 min at $600 \times g$. The obtained pellet was re-suspended in 0.5 mL of PBS and permeabilized through the addition of 5 mL of ice-cold ethanol 70% (v/v). Fixed samples were stored at -20°C and stained within 1 week.

TUNEL-labeled spermatozoa were analyzed under a fluorescence microscope (Nikon Eclipse 90i; Nikon) equipped with a mercury lamp (100 W). Final detection of BrdU incorporation at DNA break sites was achieved through an Alexa Fluor 488 dye-labeled anti-BrdU antibody (Invitrogen, Italy). The Alexa Fluor 488 dye has excitation and emission maxima of 495/519 nm. Propidium iodide was excited at 488 nm and detected via a 560 long-pass filter, according to previously published methods [15]. For each sample, 10 slides were prepared and independently analyzed by two expert cytologists counting at least 100 spermatozoa for each slide at $400\times$ final magnification.

Statistical analysis

Statistical analysis was carried out by using non-parametric statistic tests in statistical analysis software (IBM SPSS Statistics ver. 22.0; IBM, USA). Correlations between semen parameters of fresh diluted semen and OSi were determined by using Spearman's test. Interpretation of obtained Spearman's correlation coefficients was based on: $0 \leq r < 0.6$: low correlation; $0.6 \leq r \leq 1$: high correlation.

The effect of storage time on semen parameters was tested by applying the Friedman ANOVA test in both control and experimental groups. Further analysis was carried out to determine whether the quality parameters of semen in the

treated groups (FE and HE) were significantly different from those of the control group (FC and HC, respectively) by using the Wilcoxon signed rank test. Significance was set at $p \leq 0.05$ and $p \leq 0.01$.

Results

Fresh undiluted semen

Fertility-related semen parameters such as volume and sperm concentration, viability, total and progressive motilities, and morphology of spermatozoa in fresh undiluted semen of each of the ten dogs are reported in Table 1.

Oxidative stress status

The pro-oxidant, antioxidant, and oxidative stress status of both groups are presented in Table 2, as d-ROMs, OXY adsorbent, and OSi results, respectively. There were statistically significant differences between groups with respect

Table 2. Results of the oxidative stress evaluation of fertile and hypofertile groups

	Fertile group	Hypofertile group
d-ROMs (CARR U)*	55.8 ± 19.8^a	99.7 ± 4.8^b
OXY-adsorbent ($\mu\text{mol HClO/mL}$)	138.7 ± 38.9^a	139.7 ± 22.6^a
OSi (d-ROMs/OXY)	39.4 ± 6.7^a	66.7 ± 3.8^b

Data are presented as mean \pm SD. d-ROMs, pro-oxidative status; CARR U, Carratelli units; OXY-adsorbent, antioxidant capacity; HClO, hypochlorous acid; OSi, oxidative stress index. ^{a,b}Statistical differences ($p < 0.05$) between groups. *1 CARR U = 0.08 mg hydrogen peroxide/dL.

Table 1. Individual parameters of undiluted fresh semen from 5 fertile and 5 hypofertile dogs

Dogs	Volume (mL)	pH	Concentration (sperm $\times 10^6/\text{mL}$)	Viability (%)	Total motility (%)	Progressive motility (%)	Morphological alteration (%)
Fertile							
1	6	6.5	357	90	90	80	5
2	10	6.6	280	90	85	70	8
3	5	6.4	435	95	95	80	10
4	8	6.6	312	95	90	80	4
5	10	6.5	324	85	80	70	5
Mean \pm SD	7.8 ± 2.3	6.5 ± 0.1	341.6 ± 59.0	91.0 ± 4.2	88.0 ± 5.8	76.0 ± 5.5	6.4 ± 2.5
Hypofertile							
1	3	6.3	180	45	40	20	20
2	2	6.2	254	40	35	30	25
3	4	6.3	280	45	35	25	20
4	3	6.4	280	40	30	15	25
5	2	6.3	196	45	40	20	25
Mean \pm SD	2.8 ± 0.8	6.3 ± 0.1	238.0 ± 47.2	91.0 ± 2.7	36.0 ± 4.2	22.0 ± 5.7	23.0 ± 2.7

to d-ROMs and OSi.

Correlation between quality parameters of fresh semen and OSi: The correlations between each seminal parameter and OSi were evaluated separately in the F and H groups. In the H group, there was a significant positive correlation between OSi and SLOW ($r=1.000$), as well as OSi and STATIC ($r=1.000$), and a significant negative correlation between OSi and MOT ($r=-1.000$) and OSi and RAP ($r=-1.000$). In the F group, there was a significant positive correlation between OSi and SLOW ($r=0.800$) and a significant negative correlation between OSi and PMOT ($r=-0.811$). No significant correlation was detected between OSi and DNA fragmentation in either the F or

H groups.

Semen quality analysis in fertile and hypofertile groups: Fresh semen quality parameters of the F and H group dogs are presented in Fig. 2. The comparison between F and H groups showed significant differences ($p < 0.05$) for the following parameters: MOT, PMOT, RAP, MED, SLOW, STATIC, OSi, and DNA fragmentation. OSi was higher in the H group than in the F group. The data also showed a higher percentage of DNA fragmentation in spermatozoa of the H group than those of the F group ($p < 0.05$).

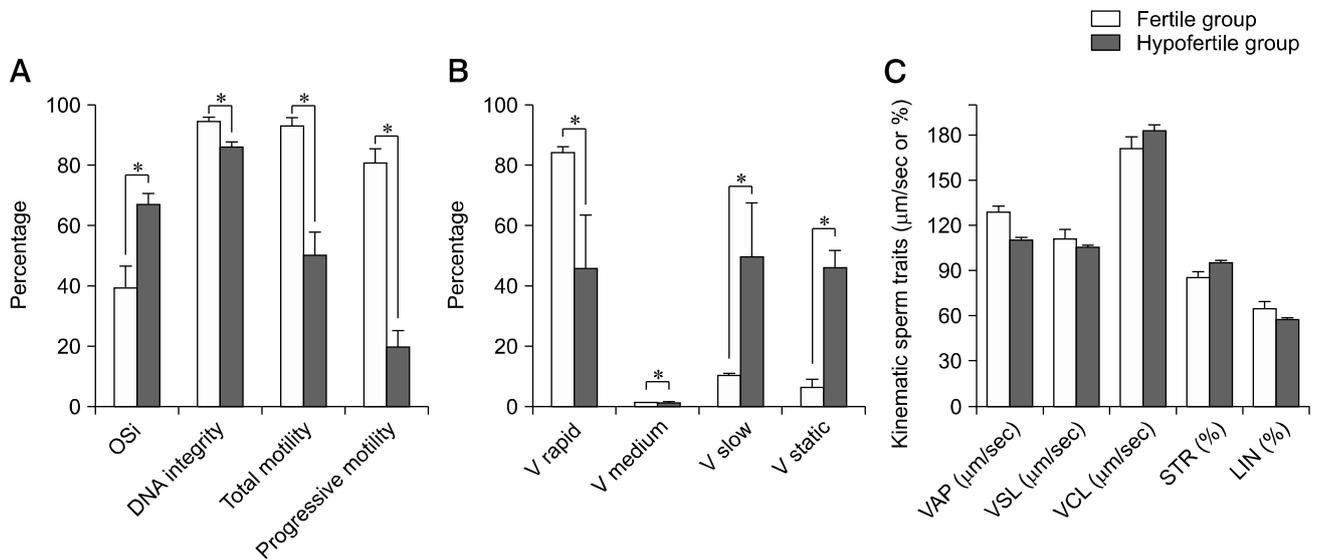


Fig. 2. Results of analysis of fresh diluted semen parameters in fertile and hypofertile groups: percentages of oxidative stress index (OSi), DNA integrity, total and progressive motilities (A); velocity parameters (B), and motion variables (C). VAP, average path velocity; VSL, straight line velocity; VCL, curvilinear velocity; STR, straightness; LIN, linearity. Significant differences between groups are denoted by * $p < 0.05$.

Table 3. Sperm velocity parameter results: measured by Sperm Class Analyzer CASA system within 5 and 10 days of cooled storage in control and experimental groups of both hypofertile and fertile dogs

Groups	RAP		MED		SLOW		STATIC	
	Day 5	Day 10	Day 5	Day 10	Day 5	Day 10	Day 5	Day 10
Hypofertile control	3.4 ± 0.5 ^a	0 ± 0 ^b	2.6 ± 0.5 ^a	2.4 ± 0.5 ^b	4.8 ± 0.8 ^a	2.2 ± 0.4 ^b	88.8 ± 1.1 ^a	95.4 ± 0.5 ^b
Hypofertile experiment	10.6 ± 1.3 ^{a,*}	6.6 ± 1.3 ^{b,*}	2.6 ± 0.5 ^a	2.2 ± 0.4 ^b	5.2 ± 1.1 ^a	5.2 ± 1.6 ^{b,*}	81.6 ± 1.6 ^{a,*}	86 ± 1.9 ^{b,*}
Fertile control	30.1 ± 2.2 ^a	28.0 ± 1.9 ^b	6.4 ± 1.1 ^a	6.9 ± 2.0 ^b	13.6 ± 2.6 ^a	14.9 ± 1.9 ^b	50.3 ± 3.0 ^a	50.0 ± 2.2 ^b
Fertile experiment	11.4 ± 1.1 ^{a,*}	16.7 ± 1.5 ^{b,*}	4.9 ± 1.6 ^a	6.0 ± 2.0 ^b	10.7 ± 2.0 ^a	9.7 ± 2.4 ^{a,*}	73.4 ± 1.9 ^{a,*}	67.0 ± 2.9 ^{b,*}

Data are presented as mean ± SD. ^{a,b}Statistical differences ($p < 0.05$) between days of each sperm motion characteristic parameter. RAP, rapid; MED, medium. *Statistical differences ($p < 0.05$) between control and experiment group.

Cooled semen storage

As expected, total and progressive motilities and DNA integrity were significantly ($p < 0.01$) reduced during cooled storage in each group, both in the control and experimental groups of fertile and hypofertile dogs. Significant changes associated with the storage time were found in sperm velocity parameters (RAP, MED, SLOW, and STATIC) and in sperm motion characteristics (VCL, VAP, and VSL), as shown in Tables 3 and 4, respectively. Straightness (STR) and linearity (LIN) were unaffected by time in fertile dogs in both the treated and control groups. However, straightness (STR) and linearity (LIN) of spermatozoa from hypofertile dogs showed a decrease with time; that decrease was greater in the HC group than in the HE group, especially at D5 of cooled storage. Furthermore, total and progressive motilities were significantly higher in the treated (FE and HE) groups than in the control (FC and HC) groups at D10, as well as at D5 in hypofertile dogs (HE group; panels A and B in Fig. 3). DNA fragmentation was significantly lower in treated (FE and HE) groups than in control (FC and HC) groups at D5 and D10 of cooled storage ($p < 0.05$; panel C in Fig. 3).

Discussion

A fine balance between ROS production and an antioxidant system's ability to scavenge ROS is critical for normal cellular functions [12]. On this basis, a previous study in canines suggested the presence of correlations among individual oxidative status, endogenous antioxidant concentration in semen, and quality of semen that could influence the ability of sperm to withstand cryoinjury [30].

The influence of systemic and seminal oxidative status on semen quality parameters has been reported in humans [14] but not in dogs. As expected, in this study, differences in systemic oxidative status were evident between hypofertile and fertile dogs. Furthermore, for the first time, our results correlated systemic oxidative stress, measured by OSi, with semen quality in a canine species.

Although pregnancy rates remain the ultimate endpoint to evaluate the fertilizing capacity of canine semen, sperm motility and DNA integrity are commonly used to assess the quality of fresh and cooled semen. Sperm DNA damage is higher in infertile men than in fertile men, and such damage has been correlated with poor reproductive outcomes. Currently, in human assisted reproduction, sperm DNA damage, expressed as a DNA fragmentation index, can distinguish fertile and infertile men in clinical practice [14]. Our results show a similar difference in DNA integrity between hypofertile and fertile dogs.

Although the percentage of DNA fragmented spermatozoa in fresh semen was not correlated to OSi in either hypofertile and fertile dogs, we did obtain indirect evidence of a correlation between DNA integrity and oxidative stress during cooled

Table 4. Sperm motion characteristic parameters: measured by using the Sperm Class Analyzer CASA system at 5 and 10 days of cooled storage in control and experimental groups of hypofertile and fertile dogs

Groups	VCL ($\mu\text{m}/\text{sec}$)		VAP ($\mu\text{m}/\text{sec}$)		VSL ($\mu\text{m}/\text{sec}$)		STR (%)		LIN (%)	
	Day 5	Day 10	Day 5	Day 10	Day 5	Day 10	Day 5	Day 10	Day 5	Day 10
Hypofertile control	155.8 \pm 3.4 ^a	109.3 \pm 1.9 ^b	87.9 \pm 2.7 ^a	93.3 \pm 1.7 ^b	73.0 \pm 2.2 ^a	70.4 \pm 0.2 ^b	83.0 \pm 1.9 ^a	75.4 \pm 1.6 ^b	46.8 \pm 1.3 ^a	64.4 \pm 0.9 ^b
Hypofertile experiment	141.5 \pm 2.3 ^{a,*}	105.7 \pm 2.5 ^{b,*}	84.8 \pm 2.5 ^{a,*}	95.7 \pm 1.7 ^{b,*}	80.0 \pm 2.6 ^{a,*}	73.0 \pm 0.4 ^{b,*}	94.2 \pm 1.4 ^a	76.3 \pm 1.0 ^b	59.6 \pm 4.8 ^{a,*}	69.1 \pm 1.9 ^{b,*}
Fertile control	129.8 \pm 5.2 ^a	120.3 \pm 5.0 ^b	101.1 \pm 5.5 ^a	94.2 \pm 3.4 ^b	85.0 \pm 3.2 ^a	81.8 \pm 1.0 ^b	83.8 \pm 3.1 ^a	86.8 \pm 3.2 ^a	65.4 \pm 2.9 ^a	68.1 \pm 2.4 ^a
Fertile experiment	114.3 \pm 3.6 ^{a,*}	108.1 \pm 1.6 ^{b,*}	92.9 \pm 2.4 ^{a,*}	94.0 \pm 2.2 ^b	76.5 \pm 2.1 ^{a,*}	78.1 \pm 2.9 ^{b,*}	82.3 \pm 2.5 ^{a,*}	83.0 \pm 1.8 ^a	66.9 \pm 2.8 ^a	72.1 \pm 2.4 ^{a,*}

Data are presented as mean \pm SD. ^{a,b}Statistical difference ($p < 0.05$) between days of storage of each sperm motion characteristic parameter. VCL, curvilinear velocity; VAP, average path velocity; VSL, straight line velocity; STR, straightness (VSL/VCL). *Statistical difference ($p < 0.05$) between control and experiment groups.

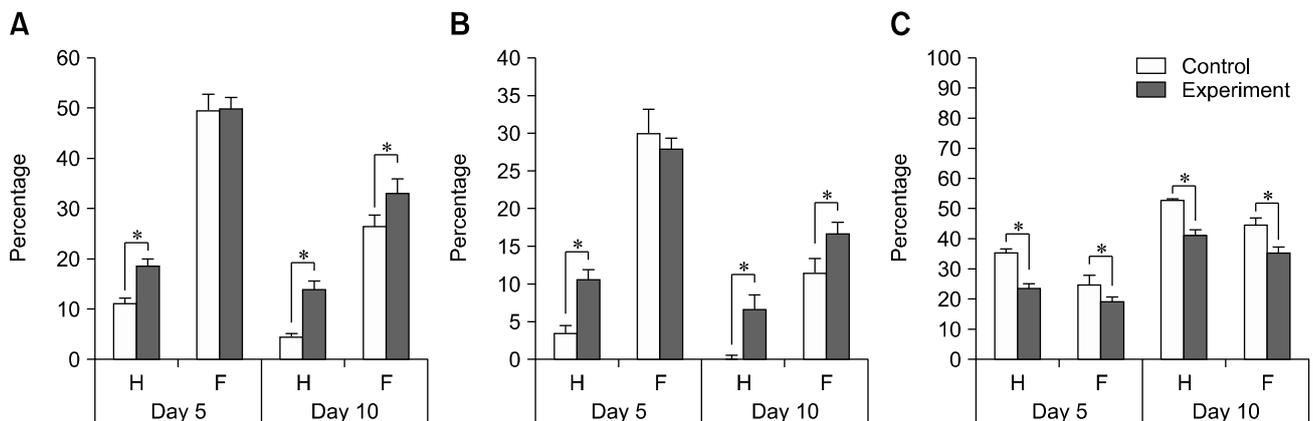


Fig. 3. Results of assessment of percentages of total (A) and progressive (B) motilities and DNA fragmentation (C) of diluted semen after 5 and 10 days of cooled storage in control and experimental groups of hypofertile (H) and fertile (F) dogs. Significant differences ($*p < 0.05$) between control and experimental groups.

storage, as the addition of antioxidants reduced the amount of DNA fragmentation. In contrast to our results, a previous study in canines suggested that the addition of vitamins C and E did not prevent DNA damage [26].

In the present study, the TUNEL assay was chosen to directly estimate DNA breakage, as it has been previously demonstrated to be a valid test in canines [20]. Herein, the addition of a combination of endogenous antioxidants to canine semen of subjects with different oxidative status was tested for the first time to improve semen preservation during cool storage. Several studies have reported that the addition of antioxidants to semen can improve the quality of preserved sperm [6,7,19]. Although in human and horses the positive effects of single endogenous antioxidant supplementation have been tested [1,13], currently, there is little information available on the synergistic effects of different antioxidant combinations on the quality of canine semen stored under chilled conditions. Beneficial effects of a combination of antioxidants, including CAT, SOD, and GPx, on the quality of cryopreserved semen in rainbow trout (*Oncorhynchus mykiss*) have been reported [25]. Endogenous antioxidants differ among species [27], and SOD, CAT, and GPx activities have been demonstrated in canine semen [38]. In addition, a previous study [11] showed that cold storage compromised the SOD activity in the dog sperm cells; moreover, GPx appeared to work efficiently in conjunction with SOD to scavenge the ROS in chilled dog semen. Thus, it has been suggested that the addition of a GPx and SOD combination to a dog semen can protect sperm viability and DNA integrity [11]. Possibly, the synergistic effect of these antioxidants can preserve semen during chilled storage by lowering the concentration of ROS.

Herein, sperm motility and DNA integrity after 5 and 10 days of cooled storage were preserved by the addition of low concentrations of SOD, CAT, and GPx to the extender. Several

studies have assessed the motility of chilled canine semen within 5 days of collection [18,34,35]. Apoptosis is a major cause of sperm damage during cryopreservation [3]. Although the causes of such DNA damage have not been fully elucidated, several lines of evidence suggest that oxidative stress has a key role in the underlying etiology. Spermatozoa are particularly vulnerable to oxidative stress since they generate ROS and are rich in targets for oxidative attack. Furthermore, as spermatozoa are transcriptionally inactive and have little cytoplasm, they are deficient in both antioxidants and DNA-repair systems [4]. Compared to previous studies, the addition of low concentrations of SOD, CAT, and GPx to the dilution medium had allowed cooled storage of semen for 10 days with preserved sperm motility and DNA integrity. The prolonged preservation of cooled semen allows longer distance semen shipments and more precise timing of AI, thereby, further increasing fertility rates. Moreover, antioxidant addition might also further benefit spermatozoa within the female reproductive tract. It would be desirable to validate the results of such *in vitro* study through the assessment of the fertilizing ability of chilled semen supplemented with these antioxidants *in vivo*. To this end, it would be necessary to objectivize various parameters among female factors, male factors (*e.g.*, individual and F and H groups), ovulation monitoring, and insemination techniques.

Taken together, the data obtained in this study demonstrate for the first time a correlation between oxidative stress and semen quality parameters in a canine species. Moreover, we have shown that supplementation of extenders with CAT, SOD, and GPx can preserve sperm motility and DNA integrity thereby improving cooled storage, especially in dogs with poor semen quality.

Acknowledgments

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Conflict of Interest

The authors declare no conflicts of interest.

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