Research Report

Effects of nonenzymatic and enzymatic antioxidants in cryopreserved canine semen extended with docosahexaenoic acid

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Abstract

Polyunsaturated fatty acids (PUFAs), such as docosahexaenoic acid (DHA), could be an alternative to improve cryopreserved canine sperm by increasing membrane fluidity. However, PUFAs also increase sperm susceptibility to lipid peroxidation. Supplementation of the extender with DHA and antioxidants could be an alternative. We evaluated supplementation of antioxidants (vitamin E and catalase) of cryopreserved canine semen extended with DHA. In Experiment 1, ejaculates from 8 dogs were divided into 4 aliquots, with increasing concentrations of DHA in the extender (0, 1, 5, and 10 µM) and cryopreserved. Sperm motility increased in 5 µM DHA and it was used for Experiment 2; ejaculates from 8 dogs were divided into 4 groups: control (5 µM DHA), vitamin E + DHA, catalase + DHA, and vitamin E + catalase + DHA. Samples were evaluated for susceptibility to lipid peroxidation, sperm kinetics, mitochondrial function, DNA integrity, and plasma and acrosomal membrane integrity. Vitamin E + DHA group had beneficial effects on the characteristics of sperm kinetics whereas the catalase + DHA group was harmful to sperm mitochondria. Vitamin E + catalase + DHA group decreased oxidative stress and damage to the plasma membrane and had low mitochondrial membrane activity. Addition of DHA + vitamin E in canine semen extender improved sperm quality after cryopreservation.

Keywords: Lipid peroxidation, oxidative stress, dog, sperm kinetic, polyunsaturated fatty acid

Introduction

Growing interest in increasing reproductive efficiency and societal value of dogs has led to a greater search for reproductive biotechnologies. Thus, male gamete preservation is a valuable tool since semen cryopreservation enables and facilitates its transport, storage for prolonged periods, and cost reduction.[1]

During spermatogenesis, sperm plasma membrane and other specific structures are prepared to react adequately to female genital tract and oocyte.[2] Sperm membrane is composed of a bilayer of lipids, mainly polyunsaturated fatty acids (PUFAs), especially docosahexaenoic acid (DHA) that are of great importance to sperm, as they provide necessary fluidity characteristics for events associated with sperm permeability and fertilization,[3] such as motility and acrosomal reaction.[4] Plasma membrane fluidity depends on many factors, such as temperature, cholesterol content, and the degree of saturation of the carbon bonds.[2] Saturated fatty acids guarantee greater rigidity whereas unsaturated ones provide greater fluidity to the membrane due to double bonds,[5] an essential characteristic for reproductive events. In addition, lipid composition of sperm membrane in several species influences resistance to cold shock during refrigeration and cryopreservation.[6] Therefore, polyunsaturated fatty acids are also directly involved in protecting the membrane during cryopreservation.[7,8] An alternative would be to supplement the samples with PUFAs during cryopreservation, protecting sperm against cryoinjury.[9] However, the high percentage of PUFAs makes sperm susceptible to oxidizing agents, leading to lipid peroxidation (due to their large amount of unsaturation, they are more easily oxidized).[5] Additionally, for canine semen preservation, it is necessary to remove seminal plasma, and with that important antioxidants are removed.[10] When reactive oxygen species (ROS) act on the unsaturated fatty acids' double bonds, a lipid peroxidation chain reaction begins and, if not interrupted, leads to loss of membrane permeability and, consequently, sperm function.[11] Based on these assertions, sperm

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Citation: Clinical Theriogenology 2025, 17, 12690, http://dx.doi.org/10.58292/CT.v17.12690

treatment with PUFAs during cryopreservation could provide ambiguous results, but if PUFAs are incorporated into the sperm membrane, this would make it even more susceptible to ROS attack.

Thus, a plausible alternative would be the association between PUFAs and antioxidants during sperm cryopreservation, aiming to prevent the enhancement of oxidative events.[12] Enzymatic and nonenzymatic antioxidants have specificity for certain oxidizing agents; therefore, prior to antioxidant treatment, it is essential to detect which ROS the sperm is most susceptible to, in order to perform a specific antioxidant treatment to eliminate this ROS.[13] Also, hydrogen peroxide and hydroxyl radical were the most harmful ROS for canine sperm.[13] Therefore, for an efficient antioxidant treatment, an association between enzymatic and nonenzymatic antioxidants would quell the aforementioned ROS.[13] Catalase and vitamin E are examples of antioxidants that are extremely effective in neutralizing hydrogen peroxide and hydroxyl radical, respectively.[14–16]

As the largest lipid-soluble antioxidant of sperm, vitamin E (α-tocopherol) acts on the plasma membrane;[17] it protects the double bond of unsaturated fatty acids against chain oxidation, donating electrons to a hydroxyl radical.[18] It is the most efficient antioxidant to break this chain, reacting with the peroxyl radical, thereby preventing the reaction of this radical with another unsaturated fatty acid.[19] It can also act as a membrane stabilizing agent, forming complexes with its phospholipids.[20] Because of its lipophilic and antioxidant properties, it has a vital role in protecting biological membranes from peroxidative damage.[19] Catalase is part of an enzyme system that degrades hydrogen peroxide into oxygen and water.[21] Hydrogen peroxide is an extremely toxic ROS due to its ability to cross the membrane freely and to inhibit the enzymatic activities of cellular functions, thus decreasing the antioxidant defenses of sperm.[22,23]

Objective of this study was to determine the ideal concentration of DHA, test it in combination in the extender containing 2 antioxidants (vitamin E and catalase) in cryopreserved canine semen, and to verify a possible synergistic effect of these substances in sperm oxidative homeostasis and functionality.

Material and methods

Study was approved by the Bioethics Committee of the School of Veterinary Medicine and Animal Science, University of São Paulo (Protocol number 7693020215). Reagents were purchased from Sigma–Aldrich (St. Louis, MO).

Animals

Eight dogs (7 Belgian Malinois and 1 Labrador Retriever) of proven fertility with a body weight of 28-32 kg and 2-6 years were used. Dogs belonged to Canine Reproduction and Distribution Center of the 2nd Army Police Battalion (Osasco, Sao Paulo - Brazil). Dogs were periodically collected for the assisted reproductive program. Dogs were fed Royal Canin Max Adult® twice a day.

Experiment 1: Effect of supplementation with polyunsaturated fatty acid (docosahexaenoic acid [DHA]) on canine semen extender

Eight ejaculates were collected and diluted in a cryopreservation medium to arrive at a final concentration of 100 x 10^6 sperm per ml. Each ejaculate was divided into 4 aliquots and allocated to control group (0 µM DHA) and 1 µM DHA, 5 µM DHA, and 10 µM DHA groups. After dilution, samples underwent one-step sperm cryopreservation using an automated machine.

Experiment 2: Effect of vitamin E and catalase supplementation in cryopreserved canine semen in extender containing DHA

Eight ejaculates were collected and diluted in a cryopreservation medium for dogs to arrive at a final concentration of 100 x 10^6 sperm per ml. Each ejaculate was divided into 4 aliquots and allocated to control group (without antioxidant) and antioxidant groups (0.6 mM vitamin E, 300 U/ml catalase, and 0.6 mM vitamin E plus 300 U catalase/ml^1). After dilution, samples underwent one-step sperm cryopreservation using an automated machine.

Semen collection, cryopreservation, and thawing

Sperm-rich fraction was collected using the digital manipulation method. Each ejaculate was assessed for motility, vigor, and concentration patterns under light microscopy (Nikon, Eclipse E200, Tokyo, Japan) and for volume via a graduated tube. Sperm concentration (10^6 sperm/ml) was determined in the Neubauer chamber. Samples contaminated with urine or blood or with motility below 70% were excluded.

Seminal plasma was removed by centrifuging semen at 500 x g for 5 minutes (Centribio®). Pellets were divided into 4 aliquots and suspended at a final concentration of 100 x 10^6 sperm/ml with 263 mM tris-yolk-fructose-citric acid (TRIS-hydroxymethyl-aminomethane [Trizma-base; T-6791 Sigma]), 84.7 mM citric acid monohydrate (T-1909), 63 mM D-fructose (F-2543), egg yolk 20%, gentamicin 10 mg/ml (G-1264), and glycerol 5% and used for each treatment mentioned in the respective experiments.

After dilution, seminal aliquots were packed in 0.5 ml straws (IMV Technologies, France) and allocated in an automated cryopreservation machine TK3000® Compacta (TK Tecnologia em Congelação LTDA; Uberaba, MG, Brazil). Subsequently, the samples were refrigerated to 5°C (0.25°C/minute); straws were then positioned 3 cm above liquid nitrogen (–20°C) and immersed in liquid nitrogen. Samples were stored in liquid nitrogen cylinders and packed in racks. One week after cryopreservation and storage, samples were thawed at 37°C for 30 seconds and subjected to sperm evaluation.

Sperm evaluation

Sperm were evaluated for kinetics, plasma and acrosomal membrane integrity, mitochondrial function (activity and mitochondrial membrane potential), DNA integrity, and oxidative status.

Computerized analysis of sperm kinetic patterns

Kinetic patterns were assessed using Computerized Sperm Analysis (CASA; Ivos 12.3, Hamilton-Thorne®, Beverly, MA) with equipment settings for dogs (Table in Appendix). For the analysis, 6 µl of the sample was pipetted in a glass slide with a coverslip heated to 37°C and ~ 1,000 sperm were evaluated. Following variables were considered: motility (%), progressive motility (%), VAP (velocity average pathway, µm/second), VSL (velocity straight line, µm/second), VCL (curvilinear velocity, µm/second), ALH (amplitude of lateral head displacement, µm), BCF (beat cross-frequency, Hz), STR (straightness %) and LIN (linearity %). Sperm speed was classified into 4 groups: fast (%), medium (%), slow (%) and static (%).[24]

Functional sperm tests

Flow cytometry (Guava EasyCyteTM Mini System, Guava® Technologies, Hayward, CA) was used to test sperm function with the concentration fixed at 25 x 10^6 sperm cells/ml after dilution with TRIS egg yolk. Gate details are provided (Figure in Appendix). A total of 10,000 events per sample were analyzed, and data corresponding to yellow (PM1 photodetector: 583 nm), red (PM2 photodetector: 680 nm), and green fluorescent signals (PM3 photodetector: 525 nm) were recorded after a logarithmic amplification. Data were analyzed by FlowJo® v10.2 software (Flow Cytometry Analysis Software, Tree Star Inc, Ashland, OR).[25]

Plasma and acrosomal membranes were evaluated with propidium iodide (PI) and FITC probes conjugated to *Pisum sativum* agglutinin (FITC-PSA), respectively. This association of fluorophores allocates the sperm population into 4 groups: intact membrane and intact acrosome (IMIA), intact membrane and damaged acrosome (IMDA), damaged membrane and intact acrosome (DMIA) and damaged membrane and damaged acrosome (DMDA). Samples were stained with 0.5 mg/ml PI in NaCl 0.9% and 100 mg/ml FITC-PSA (FITC-PSA L-0770) in a 10% sodium azide solution in DPBS. Samples were analyzed by flow cytometry after 10 minutes, excited at 488 nm and detected at 630-650 nm (PI) and 515-530 nm (FITC). Results were expressed as percentage.

Mitochondrial membrane potential was assessed by flow cytometry. Evaluation was made using JC-1 probe (5,5′,6,6′-tetrachloro-1,1′,3,3′ 201-tetraethyl-benzimidazolylcarbocyanine chloride; Invitrogen, Eugene, OR). For the assay, 0.5 µl of the fluorescent probe JC-1 (76.5 mM) was added to the samples and incubated at 37°C for 5 minutes before analysis on the flow cytometer, where there was excitation at 488 nm and detection at 583 nm. Samples were classified into percentages of sperm with high (JC-1 high), intermediate (JC-1 intermediate), and low (JC-1 low) mitochondrial membrane potential.

Mitochondrial activity of 100 sperm were determined using the DAB technique (3′3 diaminobenzidine) in phase contrast microscopy at 1,000 x magnification[26] and classified into 4 classes, according to the percentage of stained midpiece: completely stained, indicating high mitochondrial activity (DAB I); more than 50% colored intermediate piece, indicating average activity (DAB II); more than 50% unstained intermediate piece, indicating low activity (DAB III); and intermediate piece completely unstained, indicating absence of mitochondrial activity (DAB IV). Results were expressed as a percentage (%) and these classes were used to provide an index of mitochondrial activity.

Chromatin stability was tested by sperm chromatin structure assay (SCSA),[27] modified[28] for dogs. Samples were incubated with 50 µl of TNE buffer (0.01 M Tris-HCl, 0.15 M NaCl, 1 mM EDTA and distilled water, pH 7.4) and 100 µl acid detergent (0.08 M HCl, 0.15 M NaCl, Triton X-100 0.1% in distilled water, pH 1.2). After 30 seconds, acridine orange (6 µg/ml stock solution) was added, and each sample was analyzed on flow cytometry after 5 minutes of incubation at 37°C, excited at 488 nm and detected at 630-650 nm (yellow) and 515-530 nm (green). Debris were excluded using a green fluorescence/direct dispersion filter; then the percentage of sperm with DNA alteration (AO+) were selected in an αT histogram. To determine the AO positive (AO+) population, a positive control was incubated with hydrochloric acid (1.2 M in acid detergent, pH 0.1) for 5 minutes to induce DNA fragmentation in almost 100% of sperm.[25]

Evaluation of sperm resistance to oxidative stress

Susceptibility to lipid peroxidation was assessed by the thiobarbituric acid [TBA] reactive substance (TBARS) test that was adapted.[29] Samples were incubated with iron sulfate (4 mM) and vitamin C (20 mM) at 37°C for 90 minutes. After these inductions, 10% trichloroacetic acid (600 ml) was added. Samples were centrifuged at 20,800 x g for 15 minutes at 5°C to isolate proteins and debris. Subsequently, 800 µl of the supernatant was removed and transferred to cryotubes. Then, 1% TBA (800 µl) was added to the tubes, which were incubated at 95°C in a water bath for 15 minutes. In this reaction, malondialdehyde (MDA; the primary product of lipid peroxidation) and TBA produce a pink-colored complex. This staining was quantified on the spectrophotometer (Ultrospec 3300 Pro® Amersham Biosciences, Woburn, MA) at a wavelength of 532 nm. Results were expressed in nanograms of TBARS/10^6 sperm.

To detect ROS, a penetrating fluorescent probe was used, which, when oxidized by intracellular free radicals, binds to DNA and emits a higher intensity of green fluorescence (CellROX® green, Molecular Probes, Eugene, OR). Samples were stained with CellROX® green (final concentration of 5 µM) for 30 minutes at 37°C, and PI was added (final concentration, 6 µM) in the last 10 minutes. Samples were analyzed by flow cytometry, excited at 488 nm, and detected at 630- 650 nm (PI) and 515-530 nm (CellROX® green).

Sperm data were analyzed for negative oxidative stress and intact plasma membrane (NOIM), negative oxidative stress and damaged plasma membrane (NODM), positive oxidative stress and intact plasma membrane (POIM), and positive oxidative stress and damaged plasma membrane (PODM), and expressed as a percentage.

Data analysis

Data were analyzed by the SAS System for Windows (SAS, Institute Inc., Cary, NC, 2000). Treatment effects were tested for normality of residues (Gaussian distribution) and homogeneity of variances using the Guided Data Analysis application. Means were compared using the ANOVA method applying the Least Significant Difference test for multiple comparisons. Results were presented as means with their respective standard errors of the mean; significance was set at $p < 0.05$.

Results

Experiment 1

Groups treated with 5 or 10 µM DHA had higher percentage of sperm with progressive motility (Figure 1A) and rapid speed (Figure 1B) compared to 1 µM group but were similar to control group. The group treated with 10 µM DHA had lower percentage of sperm with slow movement (Figure 1C) and was not different from control. There was a lower percentage of static sperm in 5 µM DHA (Figure 1D). Other sperm kinetic (Table 1) and function (Table 2) variables were not different. There was no difference among groups in lipid peroxidation rates from TBARS and oxidative stress from CellROX® (Table 3).

Thus, based on the results, 5 µM DHA was selected for Experiment 2 to test the effect of antioxidants vitamin E and catalase in samples of cryopreserved canine semen in an extender containing DHA.

Experiment 2

The group treated with vitamin E had higher velocity average pathway (VAP - Figure 2A), velocity straight line (VSL - Figure 2B), total sperm motility (Figure 2C), progressive sperm motility (Figure 2D), and rapid sperm movement speed (Figure 2E) compared to control group. Additionally, the group treated with vitamin E did not differ from other treatments for these variables, except for progressive sperm motility, where the percentage of sperm in the group treated with catalase was significantly lower (Figure 2D). Other kinetic variables were not different among groups (Table 4).

There was no difference in mitochondrial function between control and vitamin E group in any variable (Figures 3A-3D). However, catalase and vitamin E + catalase groups had lower percentage of high mitochondrial activity (DABI) compared to control and vitamin E groups (Figure 3A) and a higher percentage of low mitochondrial activity (DABIII) compared to control group (Figure 3C). Catalase group also had a higher percentage of mitochondrial inactivity (DABIV) compared to control group (Figure 3D). Control group had higher mitochondrial activity index than vitamin E + catalase and catalase group, vitamin E had higher index than catalase group (Figure 3E). No other sperm functional test had a difference among groups (Table 5).

The vitamin E + catalase group had higher percentage of sperm with negative oxidative stress and intact plasma membrane (NOIM) compared to control group (Figure 4A).

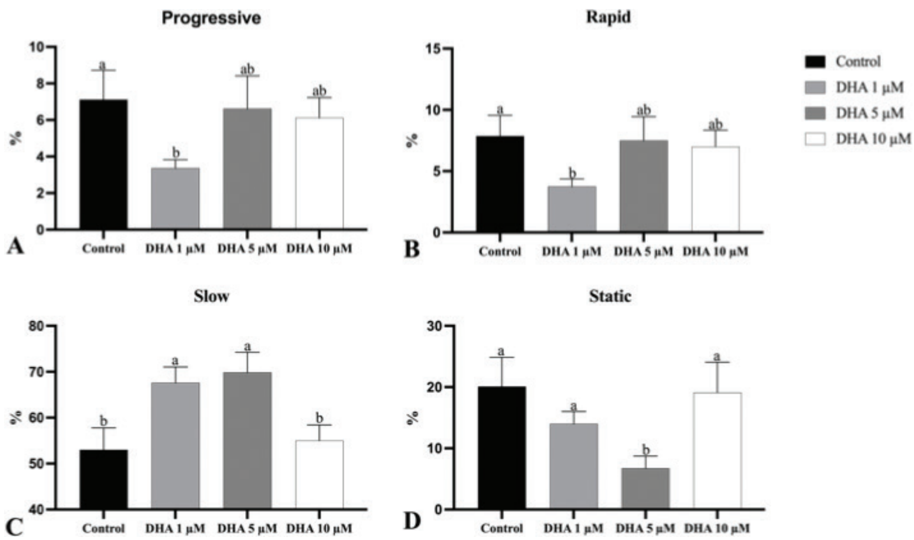

Figure 1. Effect of various concentrations of docosahexaenoic acid (DHA) on sperm kinetics variables
[a,b]Within an end point, columns without a common superscript differed ($p \leq 0.05$)

Table 1. Effect of various concentrations of docosahexaenoic acid (DHA) on sperm kinetics variables

Variable	Control	DHA 1 µM	DHA 5 µM	DHA 10 µM
VAP (µm/second)	80.07 ± 2.66	76.74 ± 1,96	80.16 ± 3.03	78.25 ± 2.38
VSL (µm/second)	69.04 ± 2.42	65.56 ± 2.00	69.35 ± 3.00	66.79 ± 2.37
VCL (µm/second)	127.52 ± 5.35	124.82 ± 5.27	128.11 ± 4.59	126.30 ± 4.33
ALH (µm)	7.66 ± 0.32	7.06 ± 0.34	7.25 ± 0.29	7.55 ± 0.30
BCF (Hz)	26.59 ± 1.27	25.92 ± 1.04	26.96 ± 0.90	27.06 ± 0.38
STR (%)	85.25 ± 0.92	84.50 ± 1.59	85.50 ± 1.12	84.50 ± 1.07
LIN (%)	55.50 ± 1.61	54.25 ± 2.45	55.75 ± 1.73	54.75 ± 1.85
Motility (%)	27.12 ± 4.00	18.50 ± 2.54	23.25 ± 3.13	26.00 ± 3.51
Medium (%)	19.12 ± 2.65	14.62 ± 1.93	15.87 ± 1.77	18.75 ± 2.42

Citation: Clinical Theriogenology 2025, 17, 12690, http://dx.doi.org/10.58292/CT.v17.12690

Table 2. Effect of various concentrations of docosahexaenoic acid (DHA) on functional tests of mitochondrial activity, mitochondrial membrane potential and integrity of sperm plasma, and acrosomal membranes

Variable (%)	Control	DHA 1 μM	DHA 5 μM	DHA 10 μM
DABI	90.00 ± 1.58	90.00 ± 1.70	88.12 ± 1.53	89.62 ± 1.21
DABII	6.37 ± 1.35	5.00 ± 0.68	6.37 ± 0.68	6.25 ± 0.56
DABIII	2.75 ± 0.67	3.25 ± 1.23	4.12 ± 0.72	3.12 ± 0.91
DABIV	0.87 ± 0.29	1.75 ± 0.56	1.37 ± 0.50	1.00 ± 0.27
JC1High	5.82 ± 3.56	4.30 ± 1.53	7.56 ± 3.45	7.26 ± 2.99
JC1Low	73.14 ± 3.85	78.93 ± 3.02	72.22 ± 6.37	71.62 ± 4.16
JC1Intermediate	21.02 ± 3.70	16.76 ± 2.82	20.26 ± 3.98	21.13 ± 3.22
SCSA	6.94 ± 1.01	5.10 ± 1.18	5.80 ± 1.67	13.34 ± 7.27
IMDA	2.48 ± 0.94	2.07 ± 0.63	2.16 ± 0.71	2.52 ± 0.95
DMDA	64.20 ± 4.93	65.12 ± 3.50	64.15 ± 3.80	58.17 ± 11.11
DMIA	21.25 ± 3.40	22.20 ± 2.90	22.45 ± 3.89	24.53 ± 5.13
IMAM	12.04 ± 3.58	10.60 ± 1.05	11.26 ± 2.51	14.80 ± 5.48

Table 3. Effect of various concentrations of docosahexaenoic acid (DHA) in response to oxidative stress features

Variable	Control	DHA 1 μM	DHA 5 μM	DHA 10 μM
POIM (%)	3.96 ± 1.22	4.00 ± 0.72	6.69 ± 2.08	3.51 ± 0.53
PODM (%)	25.80 ± 8.72	33.95 ± 4.90	31.13 ± 5.63	31.22 ± 8.04
NODM (%)	66.32 ± 8.45	58.67 ± 4.92	58.08 ± 6.22	62.13 ± 8.10
NOIM (%)	3.92 ± 0.82	3.38 ± 0.49	4.06 ± 0.66	3.14 ± 0.51
TBARS (ng/10^6sperm)	234.31 ± 26.73	254.35 ± 32.27	246.56 ± 22.64	205.50 ± 16,81

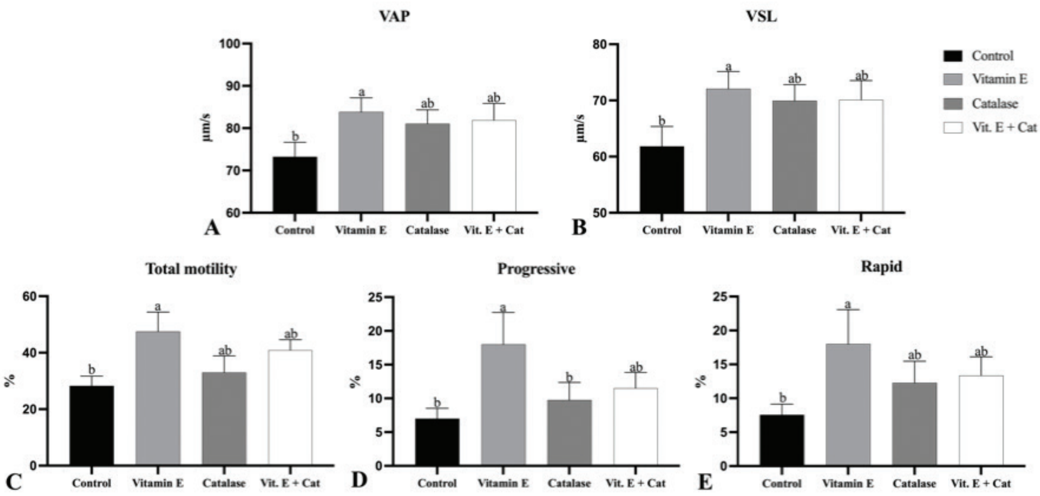

Figure 2. Effect of vitamin E and catalase and their combination on sperm kinetics variables in an extender containing DHA [a,b]Within an end point, columns without a common superscript differed ($p \leq 0.05$)

Positive oxidative stress and damaged plasma membrane (PODM - Figure 4B), positive oxidative stress and intact plasma membrane (POIM - Figure 4C), negative oxidative stress and damaged membrane (NODM - Figure 4D) and lipid peroxidation (TBARS - Figure 4E) were not different among groups.

Discussion

Synergistic effects of PUFAs and enzymatic and nonenzymatic antioxidants added to the canine semen extender were evaluated to prevent exacerbated lipid peroxidation caused by the addition of these fatty acids.[30] In Experiment 1, 5 μM of DHA

Table 4. Effect of vitamin E and catalase and their combination on sperm kinetics variables of in an extender containing DHA

Variable	Control	Vitamin E	Catalase	Vit E + Cat
VCL (μm/s)	118.87 ± 4.84	126.51 ± 3.38	124.76 ± 3.27	124.64 ± 6.02
ALH (μm/s)	7.6 ± 0.26	7.14 ± 0.23	7.34 ± 0.27	7.32 ± 0.4
BCF (Hz)	27.6 ± 0.79	26.8 ± 0.81	25.56 ± 0.77	26.07 ± 1.16
STR (%)	83.29 ± 1.36	84.71 ± 0.78	85.5 ± 0.87	84.62 ± 0.5
LIN (%)	54.14 ± 2.21	57.57 ± 1.39	57.75 ± 1.54	57.5 ± 1.55
Medium (%)	20.71 ± 2.39	22.43 ± 2.57	22.25 ± 3.02	22.75 ± 3.37
Slow (%)	63.29 ± 3.75	54.14 ± 6.16	50.62 ± 5.6	51.75 ± 3.38
Static (%)	8.14 ± 1.22	9.0 ± 1.98	16.0 ± 4.3	12.37 ± 5.45

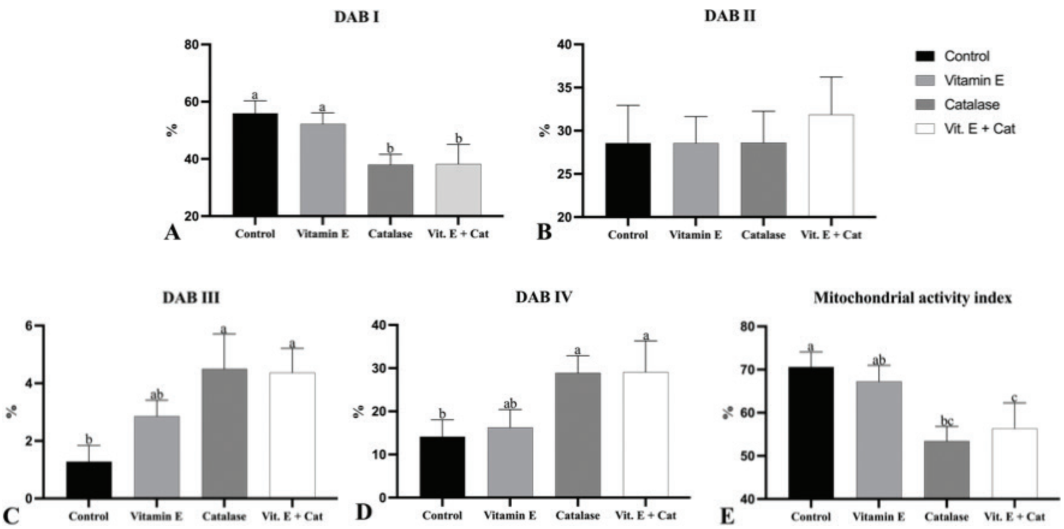

Figure 3. Effect of vitamin E and catalase and their combination on sperm mitochondrial function in an extender containing DHA
[a-c]Within an end point, columns without a common superscript differed ($p \leq 0.05$)

Table 5. Effect of vitamin E and catalase and their combination to functional tests of sperm mitochondrial membrane potential and plasma and acrosomal membrane integrity in an extender containing DHA

Variable (%)	Control	Vitamine E	Catalase	Vit E + Cat
JC1High	36.80 ± 8.99	38.66 ± 8.49	32.43 ± 5.44	28.09 ± 3.96
JC1Low	35.74 ± 2.82	38.26 ± 3.61	44.94 ± 3.91	42.26 ± 2.32
JC1Intermidiate	27.46 ± 7.54	32.03 ± 7.22	22.66 ± 3.88	29.66 ± 3.98
SCSA	1.36 ± 0.25	1.14 ± 0.32	1.56 ± 0.37	2.01 ± 0.81
DMDA	33.67 ± 2.56	30.78 ± 2.48	35.74 ± 3.26	37.12 ± 6.63
IMDA	2.86 ± 0.32	2.71 ± 0.12	3.44 ± 0.42	3.16 ± 0.39
DMIA	17.81 ± 2.06	18.94 ± 3.33	18.54 ± 2.61	20.25 ± 2.92
IMIA	45.68 ± 3.28	50.40 ± 3.09	42.26 ± 4.40	44.21 ± 3.62

had the lowest percentage of static sperm, with same progressive and rapid motility as control group. Motility parameters analyzed by CASA have high potential in clinical applications since there were significant relationships between aspects of sperm movement and cervical mucus penetration, fertilization in vitro and in vivo.[31] Also, sperm with fast and linear movements had greater resistance to cryopreservation and are more likely to overcome female reproductive tract barriers to enable fertilization.[32]

Epididymal bull sperm response to DHA addition was good[9,33] and DHA has a role in sperm plasma membrane stabilization in many domestic species.[34] Human semen incubated with various concentrations of DHA (7.2, 14.4, 28.8, 57.6, or 115.2 μM)

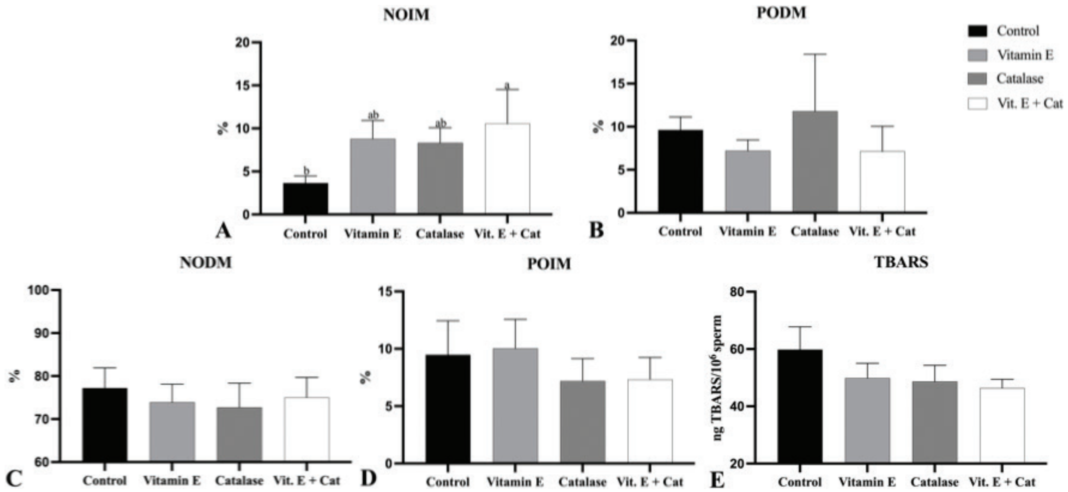

Figure 4. Effect of vitamin E and catalase and their combination to sperm oxidative stress features

had no significant impact on sperm viability, with the exception of the highest concentration tested (115.2 µM) that was cytotoxic.[35] As in the present study, DHA supplement to semen extender also increased progressive motility of boar sperm.[36]

In Experiment 2, sperm kinetics were significantly different; the vitamin E group had superior results compared to control group. Vitamin E helps to maintain the structural integrity of sperm architecture and prevents mitochondrial dysfunction by inhibiting oxidative stress, preventing reproductive dysfunctions;[37] it is an effective antioxidant and has a very important role in protecting the integrity of sperm membrane structure and the structural integrity of mitochondria and tails.[37]

Oral supplementation of vitamin E improved semen quality in dogs with low seminal plasma superoxide dismutase.[38] Vitamin E addition to extender in cattle semen at a concentration of 1 mg/ml provided protection to sperm plasma membrane from bulls with good quality semen, preserved metabolic activity and cell viability.[39] Similarly, in boars, a protective effect on post thaw sperm motility and viability was observed when vitamin E was added to the diluter (concentrations of 200 µM1^{-1}) to the extender.[40] In dogs, higher values for total motility, rapid steady forward and viability were obtained when vitamin E was added at a concentration of 0.3 mM.[1]

Treatment groups had lower levels of oxidative stress compared to control group, but catalase was most harmful to canine sperm. A reductive stress effect on mitochondria caused by excess of antioxidants may increase ROS and induce mitochondrial malfunction,[41] and it may be the cause for lower levels of progressive motility and mitochondrial activity index in catalase group and higher percentage of high mitochondrial activity and low percentage of low mitochondrial activity in vitamin E + catalase group. Both oxidative stress and reductive stress conditions have detrimental effects on male fertility, resulting in alteration of semen parameters such as concentration, motility, or normal sperm morphology.[42] In a recent study, the same authors demonstrated that the establishment of a reductive environment due to high antioxidant concentration has a deleterious impact on sperm motility, vitality, and mitochondrial activity.[42] Addition of 20 IU catalase/ml to bull sperm extender improved sperm quality; higher sperm motility, lower sperm DNA fragmentation and ROS production, a higher mitochondrial membrane potential, and higher percentage of sperm with intact acrosome and plasma membranes,[43] suggesting that the concentration used in this study was too high. In fact, lower concentrations of antioxidants improved human sperm parameters and chromatin quality when used at a concentration of 100 IU/ml.[44]

Enzymatic and nonenzymatic antioxidants are efficient and have improved post thaw semen quality. However, for many antioxidants, their function or mechanism of action at the cellular level are not clearly known.[45] For this reason, the antioxidant treatment of semen is still a field that needs to be studied. We identified the beneficial effects of vitamin E and the deleterious effects of high concentrations of catalase. In conclusion, the addition of 5 µM DHA and 0.6 mM vitamin E to canine semen extender improved semen quality after cryopreservation.

Conflict of interest

None to declare.

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Appendix

Table. Settings utilized to assess dog sperm kinetics (CASA; Hamilton-Thorne®, Ivos 12.3, USA)

Settings	Image capture
Frames per second (Hz)	60
Number of frames	30
	Cell detection
Minimum contrast	75
Minimum cell size (pix)	6
	Progressive cells
Path velocity (VAP - µm/s)	100
Straightness (STR - %)	75
	Defaults (if < 5 motile cells)
Cell size (pix)	8
Cell intensity	80
	Slow cells
VAP cutoff (µm/s)	9.9
VSL cutoff (µm/s)	20
	Static intensity Ts
Minimum	0.49
Maximum	1.68
	Static size gates
Minimum	0.8
Maximum	4.93
	Static elongation gates
Minimum	22
Maximum	84

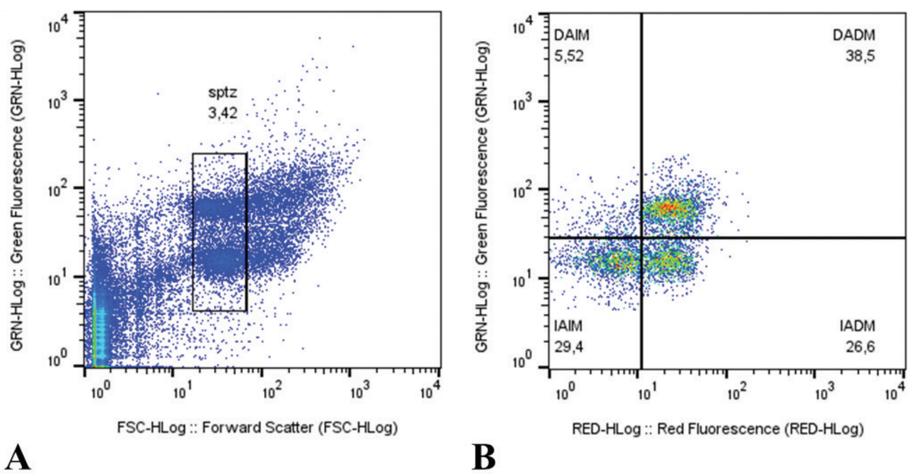

Figure. A gate referred to as 'spitz' was defined based on green fluorescence intensity and particle size, using a logarithmic scale, to encompass the main sperm population (A), when both green and red fluorescence signals were simultaneously applied to the same population 4 distinct subpopulations were defined (B); IAIM (intact acrosome/intact plasma membrane), IADM (intact acrosome/damaged plasma membrane), DAIM (damaged acrosome/intact plasma membrane), and DADM (damaged acrosome/damaged plasma membrane).

Citation: Clinical Theriogenology 2025, 17, 12690, http://dx.doi.org/10.58292/CT.v17.12690