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Tribulus terrestris aqueous extract supplementation effects on sperm characteristics and anti-oxidant status during chilled storage of canine semen

Mohammad Moein Sabzeie[1], Esmail Ayen[1]*, Ali Soleimanzadeh[1], Mustafa Numan Bucak[2]

[1] Department of Theriogenology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran; [2] Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Selcuk University, Konya, Türkiye.

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Abstract

The reduction of spermatozoa survival time is a major problem of canine chilled sperm for artificial insemination. The current study looks at the possible advantages of chilling canine sperm to 4.00 ˚C for three days using *Tribulus terrestris* aqueous extract (TTAE). Three mixed-breed dogs were utilized to extract 24 ejaculates, which were then diluted in a Tris-based extender. The ejaculates were then divided into five groups including 20.00, 40.00 and 50.00 µg mL^{-1} of TTAE, sham (distilled water devoid of TTAE) and control (without TTAE) groups. During the three days of experiment, several parameters were measured every 24 hr. It was noticed that after 48 and 72 hr of liquid storage, total and progressive motilities were greater in the group with the 40.00 µg mL^{-1} TTAE concentration than the control group. Compared to the control group, the group with the 40.00 µg mL^{-1} TTAE concentration exhibited superior motility and viability. The percentages obtained from the hypo-osmotic swelling test were much greater. In contrast to the control group, DNA integrity was poorer in the 40.00 µg mL^{-1} TTAE concentration. After 72 hr of storage, the group with 40.00 µg mL^{-1} TTAE concentration had lower malondialdehyde levels but considerably greater total anti-oxidant capacity, superoxide dismutase, glutathione peroxidase and catalase levels than the control groups. The current study found that supplementing the semen extender with 40.00 µg mL^{-1} TTAE improves semen parameters after 72 hr of storage at 4.00 ˚C, and therefore can improve fertilization efficiency.

Introduction

Animals enjoy a wide range of reproduction techniques, but this is not true about dogs, which are limited to reproduction techniques.[1] Chilling the sperm is more manageable, and it doesn't require any special equipment. It is worth noting that chilled sperm has a greater pregnancy rate than frozen-thawed one,[2] which is a strong incentive to use this technique of dog reproduction. However, chilling the sperm can cause damages including making the membrane susceptible to reactive oxygen species (ROS) attacks. Furthermore, it can increase the free radicals' capacity in absorbing electrons. Freezing can also lead to the peroxidation of the DNA by membrane lipids. All these issues can negatively affect sperm motility and fertility. In some cases, cellular death can result from the chilling process.[3]

Various techniques have been used to extend the longevity of spermatozoa, which was lowered owing to sperm freezing at lower temperatures impacting sperm metabolism and motility. Using a diluent made of several components can also help to minimize ROS generation.[4] Researches have looked at the effects of anti-oxidants and discovered that they have varying impacts on the availability of free radicals for molecules.[5] There have been several studies on the role of herbal anti-oxidants.[6-8] *Tribulus terrestris* aqueous extract (TTAE) has been shown to improve libido and spermatogenesis in both people and animals.[9,10] According to previous studies, it can help people with oligozoospermia become more fertile. In certain species, such as rats, rabbits and rams, it also improves spermatogenesis, sexual activity and erection.[11-17] In another research, Mattar and Adday have found that *T. terrestris* extract can increase sperm concentration and motility while decreasing aberrant morphology in mice.[18]

The current study analyses the efficacy of various dosages of TTAE in liquid storage of dog sperm in order to determine the influence of these supplements on sperm

***Correspondence:**

Esmail Ayen. DVM, PhD
Department of Theriogenology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran
E-mail: e.ayen@urmia.ac.ir

parameters such as total motility, progressive motility, motility characteristics and enzyme activity after 72 hr of cooling at 4.00 ˚C.

Materials and Methods

Chemicals. All chemical compounds used in the study were obtained from Sigma-Aldrich (St. Louis, USA).

Plant extract preparation. *T. terrestris* was purchased from a Persian herbal market and its identity was confirmed in Natural Resource Center (Herbarium Number: 6831). The *T. terrestris* was ground to a fine powder and soaked in water for 72 hr. Whatman filter (No.: 40; (Whatman, Pleasanton, USA) was utilized to filter the extract; after that, the extract was concentrated at 50.00 ˚C using a rotary evaporator (Laborota 4,000; Heidolph Instruments GmbH & Co. KG, Schwabach, Germany). The remaining brown pasty extract was dried in an oven at 40.00 ˚C.[19]

Identification of phenolic and flavonoid compounds with high performance liquid chromatography (HPLC) analysis. Based on a modified version of Singleton's method by Dewanto *et al*. the Folin-Ciocalteu reagent was used to analyze the phenolic contents.[20] One aliquot (0.125 mL) of a diluted desirable methanolic extract was poured into 0.50 mL of deionized water containing 0.125 mL of Folin-Ciocalteu reagent. Then, 1.25 mL of 7.00% Na_2CO_3 was added to the mixture, and the solution was shaken and left for 6 min before adding Na_2CO_3. The absorbance was read at 760 nm versus prepared blank following incubation for 90 min at 23.00 ˚C. Reversed-phase liquid chromatograph (model 1100; Agilent Technologies, Santa Clara, USA) coupled with an ultraviolet–visible multi-wavelength detector was adopted to analyze the phenolic compound. With a 250 × 4.60 mm, 4.00 µm Hypersil ODS C18 (Thermo Fisher Scientific, Waltham, USA) reversed-phase column at ambient temperature, the separation was performed. The injected volume was 20.00 µL and prior to injection, the samples were filtered through a 0.45 µm membrane filter and peaks were monitored at 280 nm. The phenolic compounds were distinguished from other components based on their retention duration, spectral features of the peaks versus the standards and transfixing the sample with standards. The analyses were carried out with three replications.

Animals, semen collection and sample preparation. Three mixed-breed mature dogs with an average age of 3 - 5 years were selected for the study, and the semen was collected according to the Kutzler's technique.[21] The dogs were examined for physical health before the study. A Tris-egg-yolk-citric acid extender was used in this investigation.[5] To produce a semen concentration of 200 × 10^6 sperm mL^{-1} (initial dilution), the extender comprised citric acid (1.40 g), Tris (2.40 g), glucose (0.80 g), Na-benzylpenicillin (100,000 IU), streptomycin sulfate (0.10 g), egg yolk (20.00 mL) and distilled water (80.00 mL) at the pH of 6.95. The dogs selected for the research had their sperm collected twice a week for 24 samples.[5] This investigation comprised ejaculates with normal parameters such as spermatozoa concentration of 526 ± 20.00 × 10^6 spermatozoa mL^{-1}, volume of 12.14 ± 0.36 mL, total number of spermatozoa of 1,548 ± 56.00 × 10^6 spermatozoa $pool^{-1}$ and total motility of 79.88 ± 2.14%. Then, the seminal plasma was removed. Before being chilled to 4.00 ˚C, the diluted semen was split into five aliquots including group 1 (control; Tris-yolk extender without TTAE), group 2 (sham; DMSO 5.00% + Tris-yolk extender), group 3 (20.00 µg mL^{-1} TTAE), group 4 (40.00 µg mL^{-1} TTAE) and group 5 (50 µg mL^{-1} TTAE).[19] This study was approved by the Animals Ethics Committee of Urmia University (No. IR-UU-AEC-1861.AD.3).

Sperm motility analysis. Computer-assisted sperm analysis (Test Sperm 3.20; Video test, St. Petersburg, Russia) was used to examine sperm motility in treatment and control groups. It consisted of a phase-contrast microscope (BX41; Olympus, Tokyo, Japan), a digital camera (MD50T, M-shot; Guangzhou, China) and a heating area.[22]

Sperm viability. Nigrosin-eosin staining was used to determine the viability of sperm samples.[23] Using the phase-contrast microscope, 200 cells were counted to determine the sperm viability. The majority of viable sperms were colorless; whereas, those stained partially (light pink head) or entirely (dark pink head) were classified as dead.

Hypo-osmotic swelling test (HOST). The sperm membrane integrity was evaluated using HOST, and the swelled and coiled tails were utilized as a basis of the evaluation. The number of spermatozoa with coiled or swelled tails was recorded.[24]

Sperm DNA damage assessment. The acridine orange staining technique was used to assess DNA integrity. To determine the proportion of spermatozoa with normal DNA, at least 200 spermatozoa were counted at 400× magnification using a fluorescence microscope (model GS7; Nikon, Tokyo, Japan) with excitation at 450 - 490 nm. Double-stranded DNA was green in color; whereas, single-stranded DNA was red or orange and partly stained.[22]

Malondialdehyde (MDA) level determination. A Nalondi™ lipid peroxidation assay kit (Navand Salamat, Urmia, Iran) was used to measure MDA levels using a spectrophotometer. The assessment was based on the reaction of MDA with thiobarbituric acid reactive compounds. The spectrophotometer measured absorbance at 550 nm against a distilled water blank.[22]

Total anti-oxidant capacity (TAC) evaluation. A colorimetric test kit (Navand Salamat) was used to assess the number of anti-oxidants in the sperm. The evaluation was based on the ferric reducing anti-oxidant power test.[25]

Enzyme activity analysis. Superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities were determined using commercial kits (Navand Salamat Co.). Notably, the kits listed were modified to work with the Olympus BX41 system. The GPx activity was measured using NADPH oxidation in the presence of cumene hydroperoxide. The SOD activity was also measured using the xanthine oxidase technique.[26] The hydrogen peroxide technique established by Maehly and Chance was utilized to assess catalase (CAT) activity.[27,28]

Statistical analysis. The experiments were conducted in triplicate, and the resulting data were expressed as mean ± SEM. The SPSS software (version 26.00; IBM Corp., Armonk, USA) was used for statistical analysis. Two-way ANOVA and the Tukey test were the ways for analysis of the statistical significance, and the $p < 0.05$ was the value of significance.

Results

Phytochemical analysis. The phytochemical investigation using reversed-phase HPLC method revealed various phenolic and flavonoid compounds in TTAE being shown in Table 1.

Sperm analysis. The proportion of sperm survival was investigated, and the results revealed that at zero hr, there was no significant difference between groups ($p > 0.05$). This study revealed that the group containing 40.00 µg mL^{-1} *T. terrestris* had the highest proportion of sperm survival (Fig. 1A) and the highest proportion of sperm viability ($p < 0.05$) compared to the other categories at 24 and 72 hr (70.07 ± 1.05 and 39.22 ± 1.88, respectively). In comparison with control groups, the group receiving 40.00 µg mL^{-1} *T. terrestris* had the most significant percentage of survival (63.10 ± 1.72; Table 2).

Sperm plasma membrane integrity evaluation. The membrane continuity of sperm was examined, and it was discovered that the group containing 40.00 µg mL^{-1} of *T. terrestris* had greater membrane continuity than the control groups (75.07 ± 1.52; Fig. 1B). At 48 and 72 hr, there was no significant difference among the control, sham and 20.00 µg mL^{-1} *T. terrestris* groups ($p > 0.05$). At these periods, the percentage of plasma membrane continuity rose substantially ($p < 0.05$) in groups with 40.00 µg mL^{-1} (68.10 ± 1.19) and 50.00 µg mL^{-1} of *T. terrestris* (62.74 ± 1.80 and 50.26 ± 1.39, respectively), with the 40.00 µg mL^{-1} *T. terrestris* group having the greatest value ($p < 0.05$) compared to the other groups (Table 2).

DNA integrity evaluation. The DNA integrity analysis revealed that the group containing 40.00 µg mL^{-1} of *T. terrestris* had the least damage of the DNA ($p < 0.05$) after 24, 48 and 72 hr of storage at 4.00 °C (Fig. 1C). The results revealed that the storage time increase improved DNA integrity ($p < 0.05$; Table 2).

Table 1. The amounts of total phenolic and flavonoid compounds in *Tribulus terrestris* aqueous extract as determined by high performance liquid chromatography analysis.

Compounds	Formula	$[M-H]^-$ Experimental
Quercetin 3-gentiobioside	$C_{27}H_{30}O_{17}$	300.00
Kaempferol 3- gentiobioside	$C_{27}H_{30}O_{16}$	285.00
Tribuluside A	$C_{61}H_{102}O_{31}$	1,197.80; 1,065.80; 903.70
4-O-(4'-O-caffeoyl glucosyl)-5-O-caffeoylquinic acid	$C_{31}H_{34}O_{17}$	255.00
Hypericin	$C_{30}H_{16}O_8$	279.20
Microcephalin I	$C_{28}H_{32}O_{16}$	317.00
Quercetin-3-O-(2,6-α-L-dirhamnopyranosyl-β-D-glucopyranoside)	$C_{33}H_{40}O_{20}$	609.00
Terrestrinin G	$C_{33}H_{52}O_{10}$	295.00
Terrestrinin I	$C_{45}H_{72}O_{20}$	769.60; 607.50

$[M-H]^-$: Deprotonated molecules.

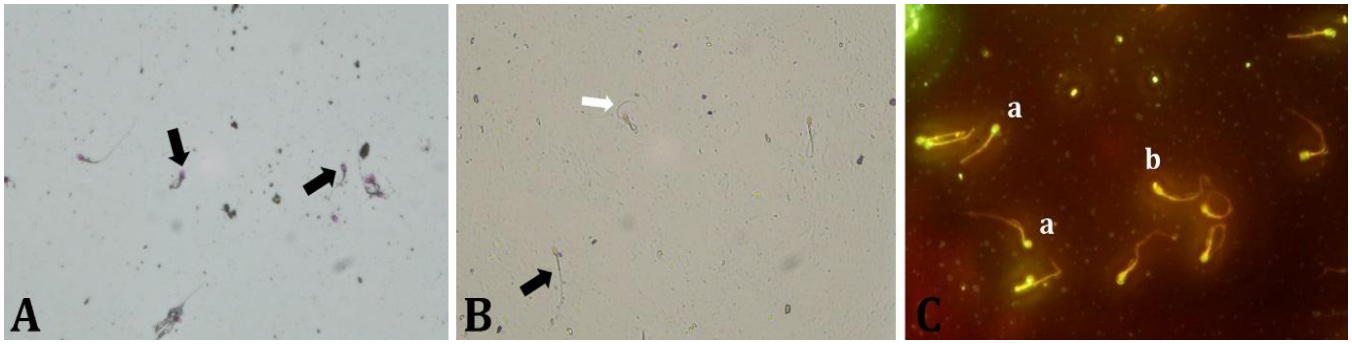

Fig. 1. A) Sperm viability analysis: The dead sperms are shown with black arrows and are red in color (eosin/nigrosine staining). **B)** Sperm membrane integrity analysis: Sperm with plasma membrane integrity (white arrow) and sperm with plasma membrane damage (black arrow) can be observed (hypo-osmotic swelling test). **C)** Sperm DNA damage assessment (acridine orange staining): Normal sperms are green in color (a) and sperm with damaged DNA is yellow-red (b; 400×).

Table 2. Viability, hypo-osmotic swelling test (HOST), DNA integrity, total and progressive motilities and quality characteristics (mean ± SEM), of chilled canine semen after addition of different concentrations of *Tribulus terrestris* aqueous extract in semen extender.

Parameters	Storage time (hr)	Control	Sham	*Tribulus terrestris* aqueous extract		
				20.00 µg mL^{-1}	40.00 µg mL^{-1}	50.00 µg mL^{-1}
Viability (%)	0	75.72 ± 2.49 ^{aA}	75.39 ± 1.11 ^{aA}	77.85 ± 1.26 ^{aA}	75.63 ± 2.34 ^{aA}	75.63 ± 2.34 ^{aA}
	24	61.19 ± 1.84 ^{aB}	60.64 ± 2.38 ^{aB}	63.81 ± 1.47 ^{bB}	70.07 ± 1.05 ^{cA}	66.89 ±1.38 ^{aB}
	48	53.30 ± 1.18 ^{aC}	51.06 ± 1.21 ^{aC}	56.49 ± 1.03 ^{bC}	63.10 ± 1.72 ^{bB}	57.74 ± 1.40 ^{aC}
	72	30.07 ± 1.92 ^{aD}	29.39 ± 1.12 ^{aD}	30.70 ± 1.65 ^{bD}	39.22 ± 1.88 ^{cC}	34.26 ± 1.40 ^{aD}
HOST (%)	0	79.72 ± 2.08 ^{aA}	77.39 ± 2.45 ^{aA}	77.85 ± 1.67 ^{aA}	78.63 ± 2.96 ^{aA}	78.05 ± 1.77 ^{aA}
	24	68.19 ± 1.40 ^{aB}	69.64 ± 1.54 ^{aB}	71.81 ± 2.82 ^{abA}	75.07 ± 1.52 ^{bA}	73.89 ± 1.24 ^{abA}
	48	56.30 ± 1.36 ^{aC}	54.06 ± 1.42 ^{aC}	58.49 ± 1.75 ^{aB}	68.10 ± 1.19 ^{bB}	62.74 ± 1.80 ^{cB}
	72	43.07 ± 1.87 ^{aD}	43.39 ± 1.97 ^{aD}	45.70 ± 1.99 ^{acC}	56.22 ± 1.17 ^{bC}	50.26 ± 1.39 ^{cC}
DNA integrity (%)	0	1.72 ± 0.17 ^{aA}	1.39 ± 0.23 ^{aA}	1.85 ± 0.20 ^{aA}	1.63 ± 0.11 ^{aA}	1.05 ± 0.15 ^{aA}
	24	5.19 ± 0.24 ^{aB}	5.64 ± 0.52 ^{aB}	5.81 ± 0.38 ^{aB}	4.07 ± 0.45 ^{bB}	5.89 ± 0.30 ^{abB}
	48	8.30 ± 0.97 ^{aC}	8.06 ± 0.85 ^{aC}	8.49 ± 0.31 ^{aC}	7.10 ± 0.89 ^{bC}	7.74 ± 0.26 ^{abC}
	72	11.07 ± 0.83 ^{aD}	11.39 ± 0.30 ^{aD}	11.70 ± 0.95 ^{aD}	9.22 ± 0.60 ^{bD}	10.26± 0.34 ^{abD}
Total motility (%)	0	77.72 ± 1.90 ^{aA}	78.39 ± 1.16 ^{aA}	78.85 ± 1.50 ^{aA}	79.63 ± 1.96 ^{aA}	79.05 ± 1.84 ^{aA}
	24	59.72 ± 1.29 ^{abB}	57.39 ± 1.65 ^{aB}	63.85 ± 1.78 ^{bB}	71.63 ± 1.07 ^{cB}	66.05 ± 1.58 ^{bB}
	48	50.19 ± 1.06 ^{aC}	51.64 ± 1.32 ^{acC}	54.81 ± 1.60 ^{acC}	63.07 ± 1.49 ^{bC}	56.89 ± 1.21 ^{cC}
	72	38.30 ± 1.62 ^{abD}	35.06 ± 1.68 ^{aD}	41.49 ± 1.34 ^{bD}	55.10 ± 1.77 ^{cD}	47.74 ± 1.59 ^{dD}
Progressive motility (%)	0	43.07 ± 1.60 ^{aA}	42.39 ± 1.63 ^{aA}	44.70 ± 1.34 ^{aA}	42.22 ± 1.05 ^{aA}	41.26 ± 1.48 ^{aA}
	24	35.72 ± 1.22 ^{aB}	34.39 ± 1.76 ^{aB}	37.85 ± 1.54 ^{abB}	41.63 ± 1.33 ^{bA}	38.05 ± 1.93 ^{abAB}
	48	28.19 ± 1.59 ^{aC}	28.64 ± 1.25 ^{aC}	30.81 ± 0.49 ^{aC}	38.07 ± 1.35 ^{bAB}	33.89 ± 1.42 ^{abB}
	72	14.30 ± 0.66 ^{aD}	15.06 ± 1.84 ^{aD}	18.49 ± 0.91 ^{aD}	34.10 ± 1.50 ^{bB}	25.74 ± 0.77 ^{cC}
VCL (µm sec^{-1})	0	101.07 ± 3.77 ^{aA}	98.39 ± 2.25 ^{aA}	99.70 ± 3.42 ^{aA}	103.22 ± 2.23 ^{aA}	100.26 ± 3.11 ^{aA}
	24	86.72 ± 3.70 ^{aB}	85.39 ± 1.43 ^{aB}	89.85 ± 2.80 ^{aB}	101.63 ± 2.44 ^{bA}	95.05 ± 2.50 ^{abA}
	48	70.19 ± 2.50 ^{aC}	70.64 ± 2.47 ^{aC}	73.81 ± 1.65 ^{aC}	88.07 ± 2.38 ^{bAB}	79.89 ± 1.37 ^{cB}
	72	55.30 ± 1.58 ^{aD}	53.06 ± 1.33 ^{aD}	58.49 ± 2.91 ^{aD}	76.10 ± 1.50 ^{bC}	65.74 ± 1.42 ^{cC}
VSL (µm sec^{-1})	0	71.07 ± 1.33 ^{aA}	70.39 ± 1.85 ^{aA}	71.70 ± 1.31 ^{aA}	70.22 ± 1.50 ^{aA}	72.26 ± 1.79 ^{aA}
	24	60.72 ± 1.91 ^{aB}	57.39 ± 1.16 ^{aB}	62.85 ± 1.93 ^{abB}	68.63 ± 1.41 ^{bA}	64.05 ± 1.38 ^{abAB}
	48	51.19 ± 1.76 ^{aC}	51.64 ± 1.53 ^{aC}	53.81 ± 1.22 ^{aC}	65.07 ± 1.89 ^{bAB}	57.89 ± 1.53 ^{aB}
	72	45.30 ± 1.35 ^{aD}	46.06 ± 1.50 ^{aD}	48.49 ± 1.59 ^{acD}	61.10 ± 1.94 ^{bB}	53.74 ± 1.77 ^{cC}
VAP (µm sec^{-1})	0	83.07 ± 2.67 ^{aA}	83.39 ± 1.74 ^{aA}	81.70 ± 1.36 ^{aA}	84.22 ± 2.0 ^{aA}	82.26 ± 2.45 ^{aA}
	24	75.72 ± 2.09 ^{aB}	72.39 ± 2.55 ^{aB}	78.85 ± 1.39 ^{abB}	82.63 ± 2.28 ^{bA}	80.05 ± 1.54 ^{bA}
	48	66.19 ± 2.50 ^{aC}	65.64 ± 1.27 ^{aC}	66.81 ± 1.93 ^{aC}	79.07 ± 1.90 ^{bA}	69.89 ± 2.39 ^{aB}
	72	53.30 ± 1.67 ^{aD}	51.06 ± 1.19 ^{aD}	57.49 ± 1.42 ^{aD}	70.10 ± 2.45 ^{bB}	63.74 ± 1.72 ^{cB}
LIN (%)	0	59.64 ± 1.61 ^{aA}	58.70 ± 1.44 ^{aA}	59.87 ± 1.94 ^{aA}	58.25 ± 1.91 ^{aA}	59.01 ± 1.38 ^{aA}
	24	57.35 ± 1.40 ^{aAB}	57.48 ± 1.39 ^{aA}	58.30 ± 1.25 ^{aA}	58.92 ± 1.46 ^{aA}	58.65±1.43 ^{aAB}
	48	54.69 ± 1.25 ^{aAB}	53.21 ± 1.72 ^{aA}	55.46 ± 1.90 ^{aAB}	55.80 ± 1.26 ^{aA}	54.12 ± 1.64 ^{aAB}
	72	53.17 ± 1.43 ^{aB}	52.38 ± 1.50 ^{aA}	52.71 ± 1.69 ^{aB}	55.24 ± 1.31 ^{aA}	53.08 ± 1.25 ^{aB}
STR (%)	0	77.46 ± 2.51 ^{aA}	76.70 ± 1.32 ^{aA}	76.87 ± 2.54 ^{aA}	77.25 ± 1.65 ^{aA}	77.01 ± 2.44 ^{aA}
	24	76.35 ± 2.66 ^{aA}	74.48 ± 1.80 ^{aA}	76.30 ± 2.41 ^{aA}	77.92 ± 2.39 ^{aA}	76.65 ± 1.97 ^{aA}
	48	74.69 ± 2.16 ^{aA}	73.21 ± 1.77 ^{aA}	74.46 ± 1.29 ^{aA}	76.80 ± 1.15 ^{aA}	75.12 ± 2.21 ^{aA}
	72	74.17 ± 2.60 ^{aA}	73.38 ± 1.59 ^{aA}	73.71 ± 1.43 ^{aA}	76.24 ± 2.66 ^{aA}	73.08 ± 1.52 ^{aA}
BCF (Hz)	0	25.46 ± 1.68 ^{aA}	24.70 ± 1.55 ^{aA}	23.87 ± 1.84 ^{aA}	24.25 ± 1.49 ^{aA}	23.01 ± 1.12 ^{aA}
	24	23.35 ± 1.81 ^{aA}	21.48 ± 1.35 ^{aAB}	22.30 ± 1.42 ^{aA}	24.92 ± 1.36 ^{aA}	22.65 ± 1.50 ^{aA}
	48	20.69 ± 0.50 ^{aAB}	19.21 ± 1.69 ^{aAB}	20.46 ± 0.66 ^{aA}	23.80 ± 1.44 ^{aA}	22.12 ± 0.96 ^{aA}
	72	16.17 ± 0.89 ^{aB}	16.38 ± 0.82 ^{aB}	18.71 ± 0.58 ^{aA}	23.24 ± 0.43 ^{aA}	21.08 ± 1.24 ^{aA}
ALH (µm)	0	5.91 ± 0.34 ^{aA}	5.87 ± 0.65 ^{aA}	5.90 ± 0.34 ^{aA}	5.94 ± 0.22 ^{aA}	5.92 ± 0.56 ^{Aa}
	24	5.07 ± 0.25 ^{aB}	5.10 ± 0.49 ^{aB}	5.11 ± 0.22 ^{aB}	5.14 ± 0.95 ^{aB}	5.13 ± 0.11 ^{aB}
	48	4.65 ± 0.36 ^{aC}	4.63 ± 0.61 ^{aC}	4.60 ± 0.43 ^{aC}	5.71 ± 0.56 ^{aC}	4.68 ± 0.74 ^{aC}
	72	3.18 ± 0.53 ^{aD}	3.10 ± 0.27 ^{aD}	3.11 ± 0.72 ^{aD}	3.25 ± 0.13 ^{aD}	3.18 ± 0.51 ^{aD}

VCL: Curvilinear velocity; VSL: Straight-line velocity; VAP: Average path velocity; LIN: Linearity; STR: Straightness; BCF: Beat-cross frequency; ALH: Amplitude of lateral head displacement.

^{abcd} Different superscripts within the same row indicate a significant effect of treatment within each duration of storage ($p < 0.05$).

^{ABCD} Different superscripts within the same column indicate a significant effect of storage within each treatment ($p < 0.05$).

Spermatozoa motility characteristics. According to the studies done on distinct groups, increased retention time resulted in a significant reduction in overall motility in several groups ($p < 0.05$). Compared to the other groups, the group with 40.00 µg mL^{-1} of *T. terrestris* exhibited the largest increase in overall mobility throughout varied storage periods ($p < 0.05$). Table 2 shows that after 72 hr of storage, groups containing 40.00 and 50 µg mL^{-1} of *T. terrestris* exhibited the most overall mobility ($p < 0.05$) compared to other groups. When the concentration of *T. terrestris* was increased while the storage duration was maintained, no significant ($p > 0.05$) variation in linearity, beat-cross frequency, straightness or amplitude of lateral head displacement (ALH) was observed (Table 2). Two control groups and the group containing 20.00 µg mL^{-1} of *T. terrestris* had significant lower ($p < 0.05$) curvilinear velocity, straight-line velocity and ALH values (Table 2).

Oxidative stress evaluation. The findings of the zero hr assessment of SOD, CAT, GPx, TAC and MDA revealed that there was no significant difference between the two controls and all treatment groups ($p > 0.05$). Following 24, 48 and 72 hr of maintenance at 4.00 ˚C, SOD levels in the group with 40.00 µg mL^{-1} of *T. terrestris* showed a significant increase compared to the two control groups and the group with 20.00 µg mL^{-1} of extract ($p < 0.05$). After 24 hr of storage at 4.00 ˚C, the group with 40.00 µg mL^{-1} of TTAE had higher CAT values ($p < 0.05$) than the two controls and 20.00 µg mL^{-1} TTAE groups ($p < 0.05$); however, there was no significant difference between the groups with 40.00 and 50.00 µg mL^{-1} of extract after 24 hr of storage ($p > 0.05$). In contrast to other control and treatment groups, it was considerably lower in the 50.00 µg mL^{-1} TTAE group after 48 and 72 hr of storage ($p < 0.05$). After 48 hr of storage, the value in the group with 40.00 µg mL^{-1} of TTAE (37.49 ± 1.86) was substantially greater than the two control groups and the treatment group with 20.00 µg mL^{-1} of TTAE ($p < 0.05$). However, after 72 hr of storage, the quantity was larger than all other groups (33.49 ± 1.40; $p < 0.05$). Compared to two control groups and other treatment groups, semen samples containing 40.00 µg mL^{-1} of *T. terrestris* exhibited the highest TAC values at different storage periods ($p < 0.05$). Furthermore, as seen in Table 3, prolonging the storage period resulted in a significant rise in MDA levels in two of the control groups and the groups receiving 20.00 µg mL^{-1} and 40.00 µg mL^{-1} of TTAE ($p < 0.05$). Following 24, 48 and 72 hr of storage at 4.00 ˚C, MDA levels in groups receiving 40.00 µg mL^{-1} of *T. terrestris* extract significantly decreased compared to two controls and two other treatment groups ($p < 0.05$).

Discussion

The current study looked at how efficiently TTAE prevents oxidative damage in canine sperm during liquid storage. It i worth noting that this study looked at the

Table 3. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), total anti-oxidant capacity (TAC) and malondialdehyde (MDA) levels changes (mean ± SEM), of chilled canine semen after addition of different concentrations of *Tribulus terrestris* aqueous extract in semen extender.

Parameters	Storage time (hr)	Control	Sham	*Tribulus terrestris* aqueous extract		
				20.00 µg mL^{-1}	40.00 µg mL^{-1}	50.00 µg mL^{-1}
SOD (U mL^{-1})	0	117.81 ± 3.18 ^{aA}	120.19 ± 3.71 ^{aA}	119.54 ± 3.49 ^{aA}	120.81 ± 3.40 ^{aA}	121.47 ± 3.66 ^{aA}
	24	101.33 ± 3.20 ^{aB}	103.60 ± 2.56 ^{aB}	107.93 ± 3.25 ^{aB}	117.50 ± 3.89 ^{bA}	114.09 ± 3.34 ^{bB}
	48	93.57 ± 2.76 ^{aC}	90.22 ± 2.92 ^{aC}	95.07 ± 3.28 ^{bC}	110.94 ± 3.53 ^{cB}	103.37 ± 3.20 ^{cC}
	72	77.49 ± 1.91 ^{aD}	77.51 ± 1.68 ^{aD}	81.16 ± 1.15 ^{aD}	104.58 ± 3.07 ^{bB}	95.79 ± 2.81 ^{bD}
CAT (U mL^{-1})	0	1.79 ± 0.08 ^{aA}	1.78 ± 0.13 ^{aA}	1.79 ± 0.21 ^{aA}	1.80 ± 0.10 ^{aA}	1.80 ± 0.10 ^{aA}
	24	1.50 ± 0.11 ^{aB}	1.51 ± 0.19 ^{aB}	1.55 ± 0.16 ^{aB}	1.75 ± 0.19 ^{bA}	1.62 ± 0.10 ^{bB}
	48	1.25 ± 0.17 ^{aC}	1.24 ± 0.07 ^{aC}	1.30 ± 0.19 ^{aC}	1.60 ± 0.24 ^{bB}	1.47 ± 0.10 ^{cC}
	72	0.82 ± 0.10 ^{aD}	0.81 ± 0.14 ^{aD}	0.93 ± 0.12 ^{bD}	1.29 ± 0.15 ^{cC}	1.09 ± 0.10 ^{dD}
GPx (U mL^{-1})	0	41.39 ± 1.39 ^{aA}	41.10 ± 1.52 ^{aA}	41.34 ± 1.91 ^{aA}	41.82 ± 1.64 ^{aA}	41.90 ± 1.52 ^{aA}
	24	35.91 ± 1.77 ^{aB}	35.49 ± 1.14 ^{aB}	37.10 ± 1.46 ^{abB}	39.36 ± 1.71 ^{abB}	37.32 ± 1.86 ^{abB}
	48	30.27 ± 1.56 ^{aC}	29.80 ± 1.03 ^{aC}	32.95 ± 1.61 ^{acC}	37.04 ± 1.86 ^{bcBC}	34.71 ± 1.19 ^{cB}
	72	22.44 ± 1.23 ^{aD}	23.36 ± 1.18 ^{aD}	25.56 ± 1.55 ^{aD}	33.49 ± 1.40 ^{bC}	29.66 ± 1.72 ^{cC}
TAC (mmol mL^{-1})	0	1.65 ± 0.15 ^{aA}	1.63 ± 0.14 ^{aA}	1.64 ± 0.17 ^{aA}	1.67 ± 0.12 ^{aA}	1.66 ± 0.14 ^{aA}
	24	1.22 ± 0.12^{aB}	1.20 ± 0.18 ^{aB}	1.31 ± 0.12 ^{bB}	1.55 ± 0.15 ^{cAB}	1.42 ± 0.19 ^{dB}
	48	0.97 ± 0.09 ^{aC}	0.95 ± 0.12 ^{aC}	1.16 ± 0.14 ^{bC}	1.49 ± 0.18 ^{cBC}	1.35 ± 0.12 ^{dB}
	72	0.66 ± 0.13 ^{aD}	0.65 ± 0.13 ^{aD}	0.78 ± 0.18 ^{bD}	1.31 ± 0.11 ^{cC}	1.12 ± 0.18 ^{dC}
MDA (nmol mL^{-1})	0	1.27 ± 0.14 ^{aA}	1.30 ± 0.18 ^{aA}	1.26 ± 0.12 ^{aA}	1.28 ± 0.18 ^{aA}	1.27 ± 0.14 ^{aA}
	24	2.09 ± 0.42 ^{aB}	2.13 ± 0.24 ^{aB}	1.95 ± 0.19 ^{bB}	1.35 ± 0.15 ^{cA}	1.60 ± 0.12 ^{dB}
	48	4.85 ± 0.70 ^{aC}	4.91 ± 0.31 ^{aC}	4.27 ± 0.43 ^{bC}	3.11 ± 0.17 ^{cB}	3.79 ± 0.14 ^{dC}
	72	7.48 ± 0.54 ^{aD}	7.48 ± 0.47 ^{aD}	7.10 ± 0.65 ^{bD}	5.94 ± 0.27 ^{cC}	6.65 ± 0.17 ^{dD}

^{abcd} Different superscripts within the same row indicate a significant effect of treatment within each duration of storage ($p < 0.05$).
^{ABCD} Different superscripts within the same column indicate a significant effect of storage within each treatment ($p < 0.05$).

impact of different *T. terrestris* concentrations on the liquid storage of canine sperm. It was discovered that adding TTAE at a concentration of 40.00 μg mL^{-1} enhanced sperm parameters. The findings of this study were consistent with those of other *in vitro* experiments done on chilled or frozen human and ram sperm. These studies concluded that TTAE improves sperm motility by decreasing ROS and preserving sperm from oxidative damage.[29,30]

The findings of our study showed that *T. terrestris* concentrations of 40.00 and 50.00 μg mL^{-1} improved the vitality of canine sperm after 72 hr of storage. Accordingly, in comparison with other groups, supplementing the extender with 40 μg mL^{-1} of TTAE substantially preserved plasma membrane integrity.

Another criterion measured and represented as a percentage of DNA fragmentation index is spermatozoa DNA damage.[31,32] In comparison with the control group, adding 40.00 μg mL^{-1} of TTAE improved the percentage of DNA integrity.

The findings of this study demonstrated that sperm is susceptible to oxidation. It was also discovered that adding 40.00 μg mL^{-1} of TTAE to the extender enhances sperm parameters. It was also shown that supplementing the canine semen extender with 40.00 μg mL^{-1} of TTAE increased TAC levels and decreased MDA levels compared to control groups.

In conclusion, the results of the present study clearly indicated that adding TTAE had a significant effect on the sperm characteristics and anti-oxidant status. Further studies using dogs with specific changes in semen quality are necessary to investigate if TTAE addition might be suitable to improve semen quality and/or longevity of chilled dog semen. Also, further studies are needed in order to determine if anti-oxidant supplementation will also improve *in vivo* canine fertility rates, to find other more appropriate anti-oxidants and to define the most effective concentrations, which will improve the quality of chilled semen and increase fertility rates when dog semen is extended.

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Conflict of interest

There are no conflicts of interest to declare for any of the authors.

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