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Original Article

Mito-TEMPO improves preservation of cooled canine semen over a 72-hour period of storage

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Abstract

Background: The positive effect of Mito-TEMPO, as a mitochondria-targeted antioxidant, on semen preservation has been reported in various species; however, little information is available on canine semen in this regard. **Aims:** The present study was conducted to evaluate the effect of Mito-TEMPO on preservation of cooled canine semen over a 72 h period of storage. **Methods:** In study I, the effect of 0 (as control), 25, 50 and 100 µM concentrations of Mito-TEMPO were evaluated on sperm motility, morphology, viability and plasma membrane integrity at time points 0, 24, 48 and 72 h. In study II, the effect of 0 (as control) and 100 µM (as the most effective dose recognized in study I) concentration of Mito-TEMPO was assessed on reactive oxygen species (ROS) and mitochondrial membrane potential at time points 0 and 72 h. **Results:** In study I, motility, velocity, viability, and proportion of sperm with normal morphology and intact plasma membrane decreased in all groups over time ($P<0.05$), but this decrease was slower in sperm treated with different doses of Mito-TEMPO than untreated sperm ($P<0.05$). In this regard, decrease in total and progressive motility observed from 24 h onward in the untreated group; however, such decrease was detected form 48 h onward in groups treated with different doses of Mito-TEMPO ($P<0.05$). Although all doses of Mito-TEMPO were effective, treatment with 100 µM concentration of Mito-TEMPO provided the best preservation as compared with the control group ($P<0.05$). Furthermore, study II revealed that ROS generation increased and mitochondrial membrane potential decreased over time in both groups ($P<0.05$); however, treatment with 100 µM concentration of Mito-TEMPO ameliorated the increase in ROS and the decrease in mitochondrial membrane potential over time ($P<0.05$). **Conclusion:** Mito-TEMPO enhances structural and functional characteristics of canine sperm preserved at 4°C for 72 h, and could serve as an effective antioxidant in canine semen extender.

Key words: Antioxidant, Canine sperm, Cooled preservation, Mito-TEMPO, Mitochondria

Introduction

Insemination using cooled semen is a contemporary popular assisted reproductive technique (ART) in dogs, due to its practical advantages in clinical and breeding settings (Peña *et al*., 2006; Goericke-Pesch *et al*., 2012), including prevention of venereal diseases (Mahiddine and Kim, 2021). In addition, it can be more favorable than insemination using frozen semen because it needs less expensive and sophisticated equipment for processing, preservation and shipping of semen, uses an easier procedure of insemination, and above all, grants higher likelihood of pregnancy (Hollinshead and Hanlon, 2017; Andersen *et al*., 2018; Sugai *et al*., 2024).

Despite these advantages, cooled preservation of canine semen has some limitations, which are mostly related to the limited possible period of storage and deterioration of sperm quality during storage (Andersen *et al*., 2018; Sugai *et al*., 2024). One of the main factors contributing to decreased quality of preserved sperm over time is generation of reactive oxygen species (ROS) in various animals (Aitken, 2017), including canine species (Michael *et al*., 2009; Vieira *et al*., 2018). Excessive ROS can damage sperm plasma membrane, DNA and vital functions through induction of oxidative stress (Aitken, 2017; Gautier and Aurich, 2022). Antioxidants, therefore, have been suggested as supplements to extenders in order to improve cooled preservation of canine semen (Michael *et al*., 2009; Andersen *et al*., 2018; Calabria *et al*., 2023).

One of the main sources of ROS are mitochondria, which use oxidative phosphorylation for energy

production in the sperm (Aitken, 2017; Peña *et al*., 2022). Mitochondria have antioxidant systems, which neutralize the generated ROS and preclude leakage of ROS outside the mitochondria, thereby protecting the sperm from oxidative stress (Napolitano *et al*., 2021; Baek *et al*., 2023). Considering this function of the mitochondria, a class of antioxidants, namely mitochondria-targeted antioxidants, have been developed to mitigate oxidative stress and improve cellular longevity (Jiang *et al*., 2020). Mito-TEMPO is a mitochondria-targeted antioxidant, which is a superoxide dismutase mimetic with effective superoxide scavenging properties (Trnka *et al*., 2008). This antioxidant is conjugated to triphenylphosphonium (TPP), a lipophilic cation, and facilitates targeted transport of the antioxidant into the mitochondria (Jiang *et al*., 2020). Furthermore, Mito-TEMPO has been used to improve preservation of semen in various species including sheep (Asadzadeh *et al*., 2021; Zarei *et al*., 2021; Zarei *et al*., 2022), buffalo (Kumar *et al*., 2022), goat (Esmaeilkhanian *et al*., 2023; Javaheri Barfourooshi *et al*., 2023), cat (Hassan *et al*., 2023), and chicken (Masoudi *et al*., 2020; Masoudi *et al*., 2021). To the best of our knowledge, yet limited information is available on the effect of Mito-TEMPO on cooled canine semen. Based on the results of studies in other species, we hypothesized that addition of Mito-TEMPO to extender could improve cooled preservation of semen in dogs as well. Therefore, the aim of the present study was to examine the impact of Mito-TEMPO on canine semen quality during a 72 h cooled preservation. For this purpose, the effect of different doses of Mito-TEMPO (25, 50 and 100 µM) was evaluated on sperm motility, viability, plasma membrane integrity at different time points, and the effect of 100 µM Mito-TEMPO was evaluated on mitochondrial function and ROS generation.

Materials and Methods

Animals and semen collection

Four intact male mixed breed dogs aged between 3-8 years (body weight = 25-40 kg) were assigned to this study. The semen of three dogs, which were randomly selected from the four available dogs, was pooled for each biological replicate in this research. Semen was collected using the manual technique by stimulating the dog's penis within the prepuce until a partial erection occurred. Then, the prepuce was slid caudally behind the bulbus glandis and the steady pressure on the penis maintained until the dog ejaculated. The collected semen was evaluated using a light microscope for motility and only semen samples with total motility greater than 70% were used for further procedures. Following microscopic evaluation, the semen was centrifuged at 700 ×g, the supernatant was removed and the semen was diluted at a 1:10 ratio using a TRIS-base medium (containing 3.025 g TRIS, 1.7 g citric acid, 1.25 g fructose, 100 IU/ml penicillin, 100 µg /ml streptomycin and 20% egg yolk).

Experimental design

To evaluate the effect of Mito-TEMPO on cooled semen of dogs, two experiments were conducted. In study I, various doses of Mito-TEMPO were added to cooled semen and the effect of various doses of Mito-TEMPO on kinematic variables, viability, morphology, and integrity of plasma membrane were evaluated at time points 0, 24, 48, and 72 h. In study II, the impact of the most effective dose of Mito-TEMPO, which was recognized in study I, was assessed on mitochondrial membrane potential and intracellular ROS at time points 0 and 72 h.

Study I

The diluted semen was divided into four experimental groups, including control (containing no Mito-TEMPO), MT25 (containing Mito-TEMPO at 25 µM concentration), MT50 (containing Mito-TEMPO at 50 µM concentration) and MT100 (containing Mito-TEMPO at 100 µM concentration). The samples, thereafter, were transferred to the laboratory at 4-6°C and were evaluated at the time of arrival at the laboratory (time point 0) as well as 24, 48 and 72 h afterwards. The number of biological replicates for each experimental group was 8 (n=8). In Study I, kinematic variables, morphology, viability and integrity of plasma membrane were assessed using computer-assisted sperm analysis (CASA), Sperm Blue staining, eosin-nigrosin staining and hypo-osmotic swelling test (HOST), respectively.

Study II

The diluted semen samples were divided into two experimental groups, including control (containing no Mito-TEMPO) and MT100 (containing Mito-TEMPO at 100 µM concentration, which was recognized as the most effective dose in study I). The samples, thereafter, were transferred to the laboratory at 4-6°C and were evaluated at the time of arrival at the laboratory (0 h) and 72 h afterwards. The number of biological replicates for each experimental group was 4 (n=4). In study II, mitochondrial membrane potential and intracellular ROS were evaluated by flow cytometry analyses and using tetraethyl benzimidazol-carbocyanine iodide (JC-1) and 2'-7'-dichlorodihydrofluorescein diacetate (DCFH-DA), respectively.

CASA analysis

At the laboratory, semen was diluted to the concentration of 2×10^7 sperm/ml using the TRIS-based medium supplemented with 1% egg yolk, and then, it was analyzed using CASA [Sperm Class Analyzer (SCA) version of 6.3.0.59; Microptic SL, Barcelona, Spain] connected to a microscope (Nikon, Japan) projecting an image on a monitor, and the images were captured using a video camera (Basler A312 digital camera, Germany). The CASA analysis was configured with negative phase-contrast optics and at 50 frames per second, the minimum cell size of 5 μm^2 and the maximum cell size of 80 μm^2. The magnification of

microscope for evaluation of semen samples was ×10 and for each sample more than three fields and at least 200 sperms were analyzed. Total motility was calculated as the proportion of sperms with >10 μm/s curvilinear velocity (VCL). Progressive motility was considered as the proportion of sperms with > 65 μm/s VCL and ≥ 80% straightness (STR). Straightness was calculated by dividing straight line velocity (VSL) by average path velocity (VAP), and linearity (LIN) was determined by dividing VSL by VCL.

Sperm morphology

Sperm morphology was assessed by preparation of a smear slide of semen sample, which was further air-dried, and fixing it by using Sperm Blue fixative for 10 min, and eventually, staining it by Sperm Blue stain (MicropticSL, Barcelona, Spain) for 10 min. Morphological examination was implemented at ×1000-magnification under oil immersion by a light microscope (Nikon, Japan), and a minimum of 200 spermatozoa were counted in each slide. The percentage of sperms with normal morphology was then calculated (Roshan *et al*., 2023).

Sperm viability

Sperm viability was assessed by eosin-nigrosin staining. The sperm smears were prepared by mixing a drop of semen with two drops of eosin-nigrosin stain on a warm slide and spreading the mixture immediately with the aid of a second slide. Viability was assessed by counting 200 spermatozoa by a light microscope (Nikon, Japan) under ×1000-magnification. Sperms that showed partial or complete staining were considered as dead. Only sperms that showed strict exclusion of the stain were considered to be alive. The percentage of viable sperms was considered as sperm viability (Roshan *et al*., 2023).

Sperm plasma membrane integrity

The integrity of sperm plasma membrane was evaluated using a hypo-osmotic swelling test (HOST) solution prepared with 9.0 g fructose and 4.9 g sodium citrate per 1 liter of distilled water. 10 μL of semen was diluted with 100 μL of HOST solution and incubated at 37°C for 60 min. After incubation, 20 μL of diluted semen was spread on a warm slide (37°C) and covered with a cover slip. For evaluation, 200 spermatozoa cells were enumerated under ×1000-magnification using a phase-contrast microscope (Nikon, Japan). Sperms with swollen or coiled tails were recorded as intact and those with curled tails as damaged. The proportion of intact sperms was considered as plasma membrane integrity.

Flow cytometric analyses

Flow cytometry was carried out by FACS Calibur (BD Immunocytometry Systems, San Jose, CA, USA), supplied with 488 nm excitation of an argon laser. In each sample, 10,000 spermatozoa were assessed after gating out debris and aggregates.

Intracellular ROS

Intracellular ROS level was determined by DCFH-DA (25 μM), which was added to 1-2×10^6 sperm/ml fractions and incubated at 25°C for 40 min in dark. Afterwards, 2 μL of propidium iodine (PI) as a counter stain dye was added to samples in order to evaluate intracellular ROS in live cells, and samples were analyzed using the flow cytometer. Green fluorescence of DCFH-DA (500-530 nm) was evaluated by excitation wavelength at 488 nm in the FL-1 channel (Fig. 1A)

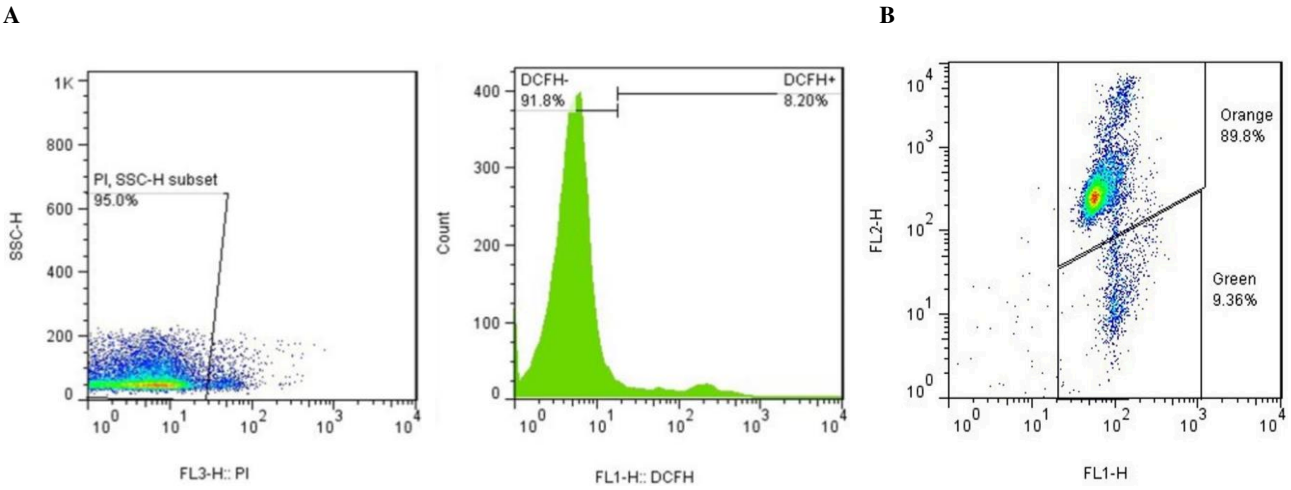


Fig. 1: (**A**) Intracellular ROS level was determined by DCFH-DA and propidium iodine (PI) was used as a counter stain dye in order to evaluate intracellular ROS in live cells. Green fluorescence of DCFH-DA (500-530 nm) was evaluated by excitation wavelength at 488 nm in the FL-1 channel. The PI-negative sperm were considered as live sperm and the proportion of PI-negative and DCFH-DA-positive sperm indicated live sperm with high intracellular ROS level (H_2O_2), and (**B**) Mitochondrial membrane potential (MMP) was evaluated using JC-1 dye as a lipophilic cationic dye remaining as monomers emitting green fluorescence in sperm with low MMP and forming aggregates emitting orange fluorescence in sperm with high MMP. Green and orange fluorescence of JC-1 was monitored by FL1 (530 nm) and FL2 (585 nm) detectors, respectively. The proportion of sperm with orange fluorescent emission (JC-1) were considered as sperm with high mitochondrial membrane potential

(Zarei *et al*., 2022).

Mitochondrial membrane potential

Mitochondrial membrane potential (MMP) was evaluated using JC-1 dye. JC-1 is a lipophilic cationic dye that remains as monomers emitting green fluorescence in sperm with low MMP, whereas it forms aggregates that emit orange fluorescence in sperm with high MMP. Initially, semen samples were centrifuged for 5 min at 500 ×g and the supernatant were removed. Afterwards, the sperm pellet was diluted with PBS at the concentration of 1×10^6 sperm per ml and 1 µL of JC-1 (200 µM dissolved in DMSO; Sigma-Aldrich, MO, USA) was added to 1 ml of the diluted sample, which was further incubated at 38°C for 40 min. At the time of flow cytometry, both JC-1-untreated (negative control) and JC-1-treated aliquots were assessed to better analyze MMP in each semen sample. Both aliquots for each replicate were from the same semen sample. Green and orange fluorescence of JC-1 was monitored by FL1 (530 nm) and FL2 (585 nm) detectors, respectively, and sperms with orange fluorescence emission were considered as having high MMP (Fig. 1B) (Akbarinejad *et al*., 2018, 2020).

Statistical analysis

Data associated with CASA outputs, morphology, viability, plasma membrane integrity, intracellular ROS and mitochondrial membrane potential were analyzed using MIXED procedure and repeated measures models. The LSMEANS statement was used to perform multiple comparisons. All analyses were conducted in SAS version 9.4 (SAS Institute Inc., Cary, NC, USA; SAS, 2013). Differences at $P \leq 0.05$ were considered significant.

Results

CASA analysis

In the control group, total motility decreased between time points 0 h and 24 h, remained unchanged between time points 24 h and 48 h, and decreased between time points 48 h and 72 h ($P \leq 0.05$). In all Mito-TEMPO treated groups (MT25, MT50 and MT100), total motility remained unchanged between time points 0 h and 24 h, decreased at time point 48 h as compared with timepoint 0 h, and at time point 72 h, it remained unchanged as compared with time point 48 h but decreased as compared with time point 24 h ($P \leq 0.05$). Total motility was not different among various experimental groups at time points 0 h, 24 h and 48 h ($P > 0.05$); however, it was higher in MT25, MT50 and MT100 groups than the control group at time point 72 h ($P \leq 0.05$; Fig. 2A).

In the control group, progressive motility decreased constantly from time point 0 h to 72 h ($P < 0.001$). In MT25, MT50 and MT100 groups, progressive motility remained unchanged between time points 0 h and 24 h, but it decreased constantly from time point 24 h to 72 h ($P \leq 0.05$). Progressive motility was not different among various groups at time point 0 h ($P > 0.05$); however, it was higher in MT50 and MT100 groups than the control group at time point 24 h ($P \leq 0.05$), and it was greater in MT25, MT50 and MT100 groups than the control group at timepoint 48 h and 72 h ($P \leq 0.05$; Fig. 2B).

In the control group, VCL decreased progressively from 0 h to 72 h ($P \leq 0.05$). In MT25 and MT50 groups, VCL remained unchanged between 0 h and 24 h, but it decreased constantly from 24 h to 72 h ($P \leq 0.05$). In MT100 group, VCL remained unchanged between 0 h and 24 h, between 24 h and 48 h, and between 48 h and 72 h ($P > 0.05$); however, it was higher at time point 0 h than time points 48 h and 72 h, and it was greater at time point 24 h than time point 72 h ($P \leq 0.05$). At time point 0 h, VCL was greater in MT100 group than the control group ($P = 0.001$). At time points 24 h, 48 h and 72 h, VCL was greater in MT25, MT50 and MT100 groups than the control group ($P < 0.01$; Fig. 2C).

In the control group, VSL decreased progressively from 0 h to 72 h ($P < 0.001$). In MT25 and MT50 groups, VSL remained unchanged between 0 h and 24 h and between 24 h and 48 h, but it was greater at 0 h than 48 h and 72 h, and it was greater at 24 h and 48 h than 72 h ($P < 0.01$). In MT100 group, VSL decreased from 0 h to 24 h ($P = 0.021$), remained unchanged between 24 h and 48 h, and decreased after 48 h ($P \leq 0.05$). At time point 0 h, VSL was greater in MT50 and MT100 groups than the control group ($P \leq 0.05$). At time points 24 h, 48 h and 72 h, VSL was greater in MT25, MT50 and MT100 groups than the control group ($P < 0.001$; Fig. 2D).

In the control group, VAP decreased progressively from 0 h to 72 h ($P \leq 0.05$). In MT25 group, VAP remained unchanged between 0 h and 24 h, and between 24 h and 48 h; however, it was greater at 0 h than 48 h and 72 h ($P < 0.001$), and it was higher at 24 h and 48 h than 72 h ($P \leq 0.05$). In MT50 group, VAP remained unchanged between 0 h and 24 h, and between 48 h and 72 h; however, it was greater at 0 h and 24 h than 48 h and 72 h ($P \leq 0.05$). In MT100 group, VAP remained unchanged between 0 h and 24 h, between 24 h and 48 h, and between 48 h and 72 h ($P > 0.05$); however, it was greater at 0 h than 48 h and 72 h ($P < 0.0001$), and it was greater at 24 h than 72 h ($P = 0.001$). At time point 0 h, VAP did not differ among groups ($P > 0.05$), but at time point 24 h, it was greater in MT100 than the control group ($P = 0.033$), and at time points 48 h and 72 h, it was greater in MT25, MT50 and MT100 groups than the control group ($P \leq 0.05$; Fig. 2E).

In the control group, LIN remained unchanged between 0 h and 24 h, between 24 h and 48 h, and between 48 h and 72 h ($P > 0.05$); however, it was greater at 0 h than 48 h and 72 h ($P < 0.0001$), and it was greater at 24 h than 72 h ($P = 0.020$). In MT25 group, LIN remained unchanged between 0 h and 48 h, but it decreased from 48 h onwards ($P < 0.01$). In MT50 group, LIN remained unchanged between 0 h and 24 h, between 24 h and 48 h, and 48 h and 72 h ($P > 0.05$), but it was greater at 0 h than 72 h ($P = 0.006$). In MT100 group, LIN remained unchanged from 0 h to 72 h ($P > 0.05$). At time point 0 h, LIN was not different among groups ($P > 0.05$). At time points 24 h and 48 h, LIN was greater in MT25,

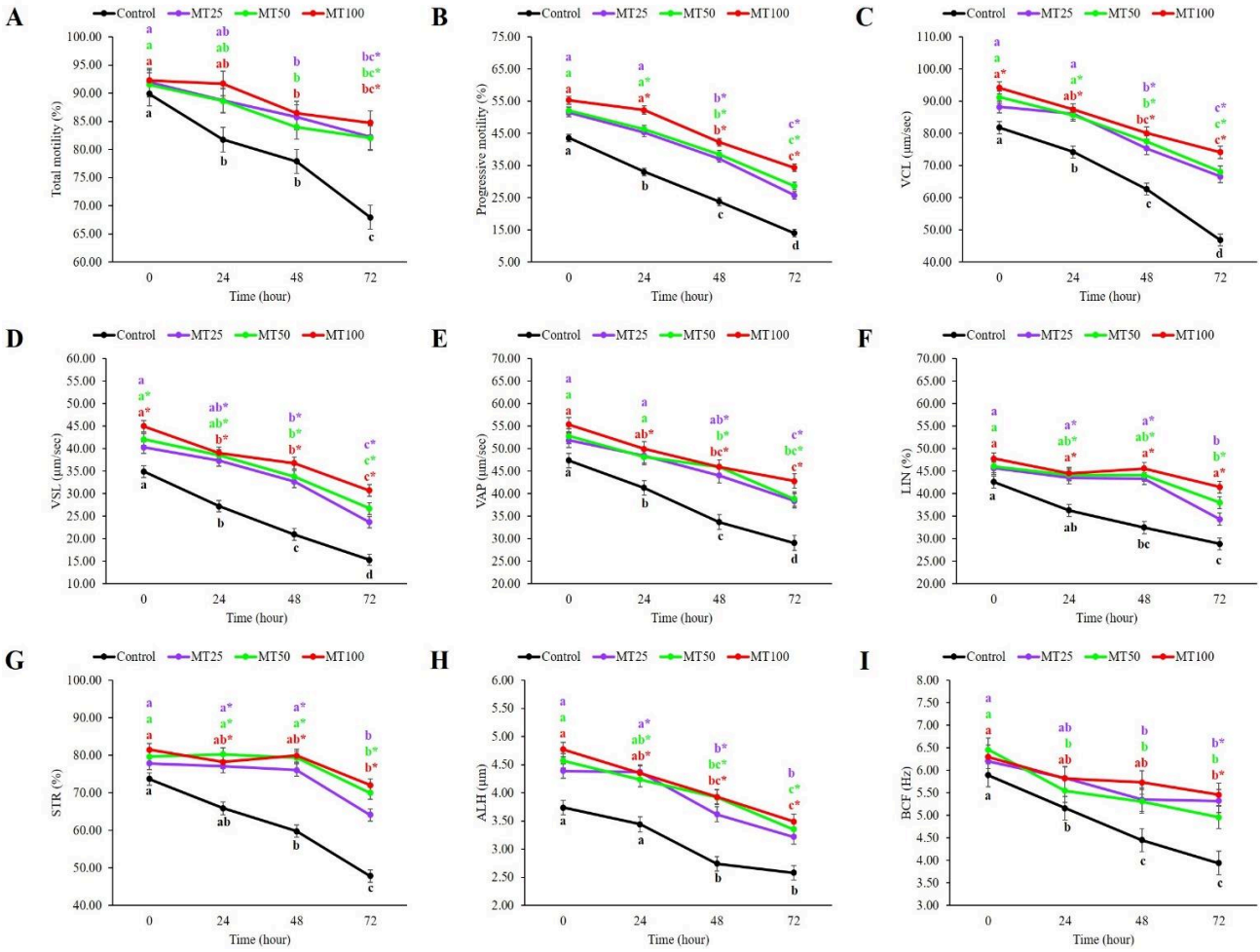


Fig. 2: (**A**) Total motility, (**B**) Progressive motility, (**C**) VCL, (**D**) VSL, (**E**) VAP, (**F**) LIN, (**G**) STR, (**H**) ALH, and (**I**) BCF in the sperm analyzed by CASA in control (no Mito-TEMPO), MT25 (25 µM Mito-TEMPO), MT50 (50 µM Mito-TEMPO) and MT100 (100 µM Mito-TEMPO) groups at time points 0, 24, 48 and 72 h. Data are presented as mean±SEM. Various letters (a, b, c, d) denote significant difference among different time points within each individual experimental group, and the color of letters is based on the color allocated to each individual group (P<0.05). Asterisks (*) indicate significant difference between treated and control groups at the specified time point, and the color of asterisks is based on the color allocated to each individual Mito-TEMPO-treated group (P<0.05)

MT50 and MT100 groups than the control group (P≤0.05). At time point 72 h, LIN was greater in MT50 and MT100 groups than the control group (P<0.001; Fig. 2F).

In the control group, STR remained unchanged between 0 h and 24 h and between 24 h and 48 h (P>0.05); however, it was greater at 0 h than 48 h and 72 h (P<0.0001), and it was greater at 24 h and 48 h than 72 h (P<0.0001). In MT25 and MT50 groups, STR remained unchanged between 0 h and 48 h, but it decreased from 48 h onward (P<0.01). In MT100 group, STR remained unchanged between 0 h and 24 h, between 24 h and 48 h, and between 48 h and 72 h (P>0.05), but it was greater at 0 h than 72 h (P=0.008). At time point 0 h, STR was not different among groups (P>0.05). At time points 24 h, 48 h and 72 h, STR was greater in MT25, MT50 and MT100 groups than the control group (P<0.01; Fig. 2G).

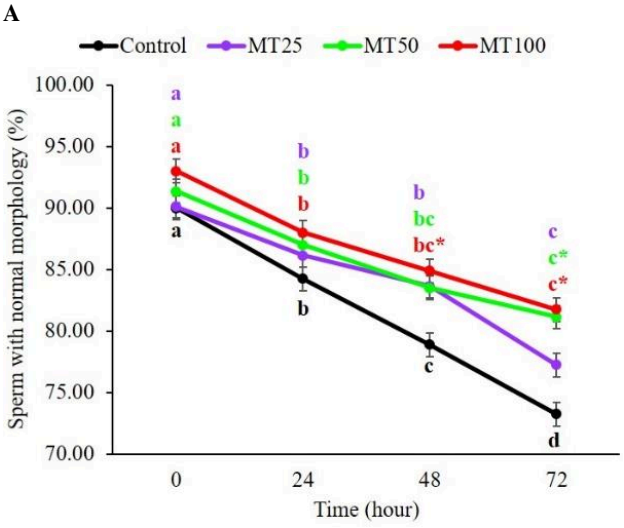
In the control and MT25 groups, ALH remained unchanged between 0 h and 24 h, and between 48 h and 72 h (P>0.05), but it was greater at 0 h and 24 h than 48 h and 72 h (P<0.01). In MT50 and MT100 groups, ALH remained unchanged between 0 h and 24 h, between 24 h and 48 h and between 48 h and 72 h (P>0.05); however, it was greater at 0 h than 48 h and 72 h, and it was greater at 24 h than 72 h (P≤0.05). At time points 0 h and 72 h, ALH was greater in MT50 and MT100 groups than the control group (P<0.01). At time points 24 h and 48 h, ALH was greater in MT25, MT50 and MT100 groups than the control group (P<0.001; Fig. 2H).

In the control group, BCF decreased progressively from 0 h to 48 h (P≤0.05), but it remained unchanged between 48 h and 72 h (P>0.05). In MT25 group, BCF remained unchanged between 0 h and 24 h, between 24 h and 48 h and between 48 h and 72 h (P>0.05), but it was greater at 0 h than 48 h and 72 h (P<0.01). In MT50 group, BCF decreased from 0 h to 24 h (P<0.001), but it remained unchanged afterwards (P>0.05). In MT100 group, BCF remained unchanged between 0 h and 24 h, between 24 h and 48 h and between 48 h and 72 h

(P>0.05), but it was greater at 0 h than 72 h (P=0.002). At time points 0 h, 24 h and 48 h, BCF was not different among groups (P>0.05); however, at time point 72 h, it was greater in MT25 and MT100 groups than the control group (P≤0.05; Fig. 2I).

Sperm morphology

In the control group, proportion of sperms with normal morphology decreased progressively from 0 h to 72 h (P<0.001). In MT25 group, proportion of sperms with normal morphology decreased from 0 h to 24 h (P=0.038), remained unchanged between 24 h and 48 h, and decreased from 48 h to 72 h (P<0.0001). In MT50 and MT100 groups, proportion of sperms with normal morphology decreased from 0 h to 24 h (P≤0.05), remained unchanged between 24 h and 48 h and between 48 h and 72 h (P>0.05), but it was greater at 0 h than 72 h (P<0.0001). At time point 0 h and 24 h, proportion of sperms with normal morphology was not different among groups (P>0.05); however, at time point 48 h, it was greater in MT100 than the control group (P=0.003), and at time point 72 h, it was greater in MT50 and MT100 groups than the control group (P<0.0001; Fig. 3A).



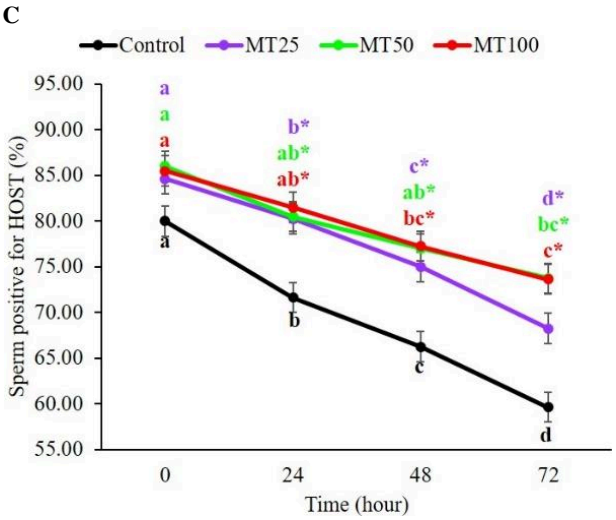


Fig. 3: (**A**) Proportion of sperms with normal morphology determined by Sperm Blue stain in control (containing no Mito-TEMPO), MT25 (containing 25 µM Mito-TEMPO), MT50 (containing 50 µM Mito-TEMPO) and MT100 (containing 100 µM Mito-TEMPO) groups at time points 0, 24, 48 and 72 h, (**B**) Proportion of live sperms determined by eosin-nigrosine stain in control, MT25, MT50 and MT100 groups at time points 0, 24, 48 and 72 h, and (**C**) Plasma membrane integrity of sperms determined by HOST in control, MT25, MT50 and MT100 groups at time points 0, 24, 48 and 72 h. Data are presented as mean±SEM. Various letters (a, b, c, d) denote significant difference among different time points within each individual experimental group, and the color of letters is based on the color allocated to each individual group (P<0.05). Asterisks (*) indicate significant difference between treated and control groups at the specified time point, and the color of asterisks is based on the color allocated to each individual Mito-TEMPO-treated group (P<0.05)

Sperm viability

In the control group, viability decreased progressively from 0 h to 72 h (P<0.0001). In MT25 group, viability remained unchanged between 0 h and 24 h and between 24 h and 48 h (P>0.05), but it decreased from 48 h to 72 h (P=0.002). In MT50 group, viability remained unchanged between 0 h and 24 h, between 24 h and 48 h and between 48 h and 72 h (P>0.05); however, it was greater at 0 h than 48 h and 72 h (P<0.01), and it was greater at 24 h than 72 h (P=0.018). In MT100 group, viability remained unchanged between 0 h and 24 h, between 24 h and 48 h and between 48 h and 72 h (P>0.05); however, it was greater at 0 h and 24 h than 72 h (P<0.001). At time points 0 h and 24 h, viability was not different among groups; however, at time points 48 h and 72 h, it was greater in MT25, MT50 and MT100 groups than the control group (P≤0.05; Fig. 3B).

Sperm plasma membrane integrity

In the control and MT25 groups, proportion of sperms positive for HOST decreased constantly from 0 h to 72 h (P<0.001). In MT50 group, proportion of HOST positive sperms decreased from 0 h to 24 h (P=0.003); however, it remained unchanged between 24 h and 48 h, and between 48 h and 72 h (P>0.05). In MT100 group,

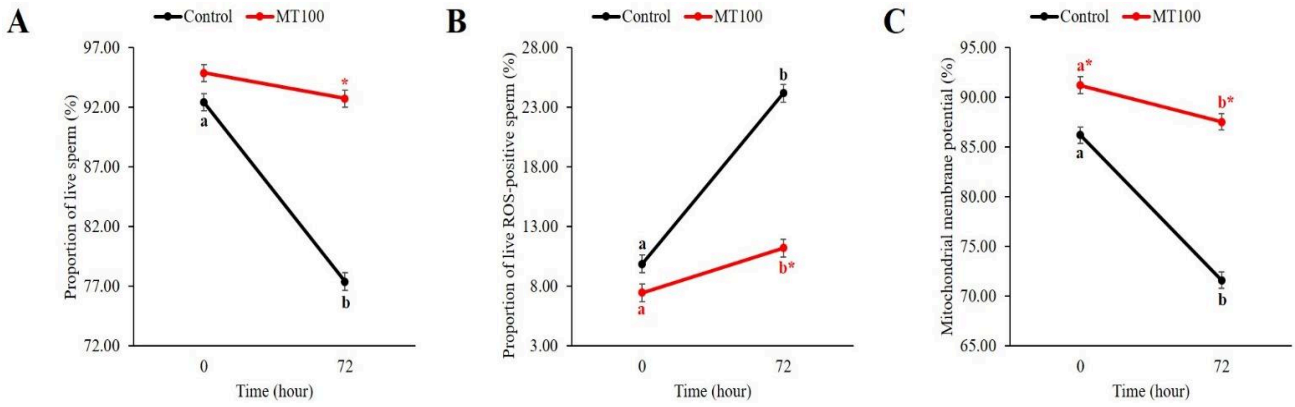


Fig. 4: (**A**) The proportion of sperms negative for PI (live sperm) in control (containing no Mito-TEMPO) and MT100 (containing 100 µM Mito-TEMPO) groups at time points 0 and 72 h, (**B**) The proportion of sperms negative for PI and positive for DCFH-DA (live sperm with high intracellular ROS) in control and MT100 groups at time points 0 and 72 h, and (**C**) Mitochondrial membrane potential of sperm in control and MT100 groups at time points 0 and 72 h. Data are presented as mean±SEM. Various letters (a, b) denote significant difference among different time points within each individual experimental group, and the color of letters is based on the color allocated to each individual group (P<0.05). Asterisks (*) indicate significant difference between MT100 and control groups at the specified time point, and the color of asterisks is based on the color allocated to MT100 group (P<0.05)

proportion of sperms positive for HOST remained unchanged between 0 h and 24 h, between 24 h and 48 h and between 48 h and 72 h (P>0.05); however, it was greater at 0 h than 48 h and 72 h (P<0.0001), and it was greater at 24 h than 72 h (P<0.0001). At time point 0 h, proportion of HOST positive sperms was not different among groups (P>0.05); however, at time points 24 h, 48 h and 72 h, it was greater in MT25, MT50 and MT100 groups than the control group (P≤0.05; Fig. 3C).

Proportion of live and ROS-positive sperms

In the control group, proportion of live (PI-negative) sperm decreased from 0 h to 72 h (P<0.0001); however, in MT100 group, such temporal alteration was not observed (P>0.05). At time point 0 h, proportion of live sperms did not differ between the control and MT100 groups (P>0.05), but at time point 72 h, it was greater in MT100 than the control group (P<0.0001; Fig. 4A).

Proportion of ROS-positive sperms increased form 0 h to 72 h in the control (P<0.0001) and MT100 (P=0.012) groups. At time point 0 h, proportion of live sperms did not differ between the control and MT100 groups (P>0.05); however, at time point 72 h, it was lower in MT100 than the control group (P<0.0001; Fig. 4B).

Mitochondrial membrane potential

Mitochondrial membrane potential decreased form time point 0 h to 72 h in the control (P<0.0001) and MT100 (P=0.026) groups. Mitochondrial membrane potential was greater in MT100 than the control group at time points 0 h (P=0.025) and 72 h (P<0.0001; Fig. 4C).

Discussion

The present study was conducted to evaluate the effect of Mito-TEMPO as a mitochondria-targeted antioxidant on cooled semen in dogs. The results of the present study showed that the most effective dose of Mito-TEMPO (100 µM) significantly mitigates increase in the proportion of ROS-positive sperms over the course of cooled preservation, substantiating that Mito-TEMPO could successfully act as an antioxidant for canine sperm. Mito-TEMPO mimics the activity of superoxide dismutase (Trnka *et al*., 2008), and superoxide dismutase has been recognized as one of the enzymes contributing to endogenous antioxidant defense system in canine sperm (Cassani *et al*., 2005; Koziorowska-Gilun and Strzezek, 2011). However, the results in the control group indicated that the endogenous antioxidant defense system did not suffice to prevent the sharp elevation of ROS in the canine sperm over time. One potential explanation for this phenomenon may be the fact that the capacity of endogenous antioxidant defense system is limited and can be overwhelmed by continuous and/or excessive generation of ROS over time (Dutta *et al*., 2022; Kowalczyk, 2022; O'Flaherty and Scarlata, 2022). Hence, incorporation of Mito-TEMPO in cooled extender in this study might have assisted the endogenous antioxidant defense system to function more efficiently in neutralizing ROS generated by mitochondria. In this context, the positive effect of Mito-TEMPO on alleviation of oxidative stress has been previously reported in sheep (Asadzadeh *et al*., 2021), goats (Esmaeilkhanian *et al*., 2023) and chickens (Masoudi *et al*., 2021).

Another fact is that Mito-TEMPO at concentration of 100 µM enhanced mitochondrial membrane potential and interestingly, this enhancing effect was significant from time point 0 h and continued over the 72 h period of cooled preservation. However, the difference between the control and Mito-TEMPO-treated groups was more pronounced at time point 72 h than 0 h, indicating that Mito-TEMPO not only improves mitochondrial function, but also ameliorates deterioration of mitochondrial activity over time. These positive effects on mitochondrial membrane potential could be attributed to

the neutralizing impact of Mito-TEMPO on ROS. Because elevation of ROS can impair the activity of mitochondria (Vieira *et al*., 2018), which are responsible for generation of energy required for various vital functions of the sperm (Aitken, 2017). Mito-TEMPO has also been reported to improve mitochondrial function in sheep (Asadzadeh *et al*., 2021; Zarei *et al*., 2021; Zarei *et al*., 2022), buffalo (Kumar *et al*., 2022), goat (Esmaeilkhanian *et al*., 2023) and chicken (Masoudi *et al*., 2020; Masoudi *et al*., 2021).

Plasma membrane integrity was also positively influenced by various doses of Mito-TEMPO based on the results of hypo-osmotic swelling test. The reduction of ROS by Mito-TEMPO might have played a role in this regard because ROS can adversely affect plasma membrane of the sperm (Vieira *et al*., 2018). Reaction of ROS with plasma membrane lipids would lead to lipid peroxidation, which disrupts optimal function of sperm plasma membrane (Gautier and Aurich, 2022). Lipid peroxidation, moreover, culminates in formation of some adducts such as aldehydes, which have detrimental effects not only on structural integrity, ion transport, and signal transduction of plasma membrane, but also on function of mitochondria and chemical structure of DNA (Aitken *et al*., 2012; Pizzimenti *et al*., 2013; Moazamian *et al*., 2015). Alternatively, the higher plasma membrane integrity of sperms treated with Mito-TEMPO could be related to more appropriate mitochondrial function. Maintenance of plasma membrane integrity requires energy and it has been shown that ATP synthesized by mitochondria plays a critical role in provision of energy for this purpose (Davila *et al*., 2016). The positive effect of Mito-TEMPO on plasma membrane integrity of sperm has also been indicated in sheep (Asadzadeh *et al*., 2021; Zarei *et al*., 2022), buffalo (Kumar *et al*., 2022), goat (Esmaeilkhanian *et al*., 2023; Javaheri Barfourooshi *et al*., 2023) and chicken (Masoudi *et al*., 2020).

Viability and proportion of sperms with normal morphology were higher in Mito-TEMPO-treated than untreated sperms as well. ROS can trigger apoptotic pathways, and in turn, jeopardize the viability of sperm (Michael *et al*., 2009). One reason for higher viability and better morphology of sperms following treatment with Mito-TEMPO could, therefore, be the diminished level of ROS in treated groups. Viability needs energy like other vital functions of the sperm and ATP produced by mitochondria appeared to be important for survival of sperm (Miki, 2007; Volpe *et al*., 2009; Kumar *et al*., 2016; Peña *et al*., 2022). Hence, the other reason for these findings could be the improved activity of mitochondria. Additionally, possession of an intact plasma membrane is the prerequisite for survival of all cells including sperm (Gautier and Aurich, 2022); as a result, another factor contributing to higher viability and better morphology of sperms in response to Mito-TEMPO could be the result of higher plasma membrane integrity. The beneficial impact of Mito-TEMPO on sperm viability and/or morphology has also been observed in sheep (Asadzadeh *et al*., 2021; Zarei *et al*., 2021; Zarei *et al*., 2022), goat (Javaheri Barfourooshi *et al*., 2023) and chicken (Masoudi *et al*., 2020; Masoudi *et al*., 2021).

Furthermore, Mito-TEMPO mitigated the decline in sperm motility, velocity and progressive motility over time. These better kinematic variables in Mito-TEMPO-treated sperm could partly be attributed to better morphology and plasma membrane integrity. Appropriate sperm morphology as well as composition and structure of plasma membrane are required for the optimal motion trajectory of sperm (García-Vázquez *et al*., 2016; Gaffney *et al*., 2021; Gautier and Aurich, 2022). The positive influence of Mito-TEMPO on mitochondrial function could also contribute to greater motility of Mito-TEMPO-treated sperm since adequate and continuous provision of energy is undeniably required for sperm motility (Miki, 2007; Volpe *et al*., 2009; Kumar *et al*., 2016; Peña *et al*., 2022). Likewise, improvement in sperm motility following inclusion of Mito-TEMPO in semen extender has been shown in sheep (Asadzadeh *et al*., 2021; Zarei *et al*., 2021; Zarei *et al*., 2022), buffalo (Kumar *et al*., 2022), goat (Esmaeilkhanian *et al*., 2023; Javaheri Barfourooshi *et al*., 2023) and chicken (Masoudi *et al*., 2020, 2021).

Collectively, the present study showed that Mito-TEMPO improves the quality of cooled preservation by considering various functional and structural characteristics of canine sperm. Although we could not evaluate fertility of sperms in different groups, there is evidence indicating a positive relationship of sperm motility and morphology with the consequent pregnancy rate (Mickelsen *et al*., 1993; Tesi *et al*., 2018). In this context, Tesi *et al.* (2018) reported that canine semen samples resulting in positive pregnancy test after insemination had 83.9% and 64.9% motile and morphologically normal sperm, respectively, on average whereas semen resulting in negative pregnancy test after insemination had 66.5% and 42.0% motile and morphologically normal sperm, respectively, on average. In the present study, the respective mean percentage of motile and morphologically normal sperm at time point 72 h were 84.74% and 81.75% in MT100 group, and 67.95% and 73.25% in the control group. Hence, it could be surmised that application of Mito-TEMPO in the extender could maintain the motility and morphology of cooled canine sperm at acceptable levels for insemination up to the end of 72 h period of storage. This finding is of importance considering the long period of estrus in the female dog (Peña *et al*., 2006; Goericke-Pesch *et al*., 2012), and in turn, the possibility for repetition of insemination to achieve pregnancy.

In conclusion, the present study showed that addition of Mito-TEMPO as a mitochondria-targeted antioxidant could effectively neutralize ROS and this antioxidant activity was accompanied by improvement in mitochondrial activity, plasma membrane integrity, morphology, viability and motility in the treated sperm. Although these beneficial effects were exerted in response to application of 25, 50 and 100 µM of Mito-TEMPO, the dose of 100 µM Mito-TEMPO appeared to be marginally better than other doses when the treated

groups were compared with the control group over time.

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Conflict of interest

The authors declare no conflict of interests.

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Use of generative AI and AI-assisted technologies

Generative AI and AI-assisted technologies were not used in the present study.

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