Colegio de Postgraduados

MitoTEMPO® is an antioxidant that protects the bovine sperm acrosome during cryopreservation

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ABSTRACT

Objective: To evaluate the addition of different concentrations of the mitochondrial-targeted antioxidant MitoTEMPO® in a commercial egg yolk-free diluent.

Design/Methods/Approach: Reactive oxygen species (ROS) and ATP production were measured post-thawing by fluorescence, while sperm viability, as well as membrane and acrosome integrity, were assessed using eosin-nigrosin and hypoosmotic Coomassie brilliant blue (HOST/Coomassie) staining of cryopreserved bovine spermatozoa in the commercial medium AndroMed®. Three healthy, fertile 3-year-old Brangus bulls were used, with three ejaculates collected from each bull using an artificial vagina. Ejaculates were divided into three groups and frozen: Group 1 (control), Group 2 (25 μM MitoTEMPO®), and Group 3 (50 μM MitoTEMPO®), with the antioxidant added at the time of strawing. One-way ANOVA (Tukey's multiple comparison test) was used to compare the means of motility, live vs. dead sperm, membrane integrity, and acrosome integrity between treatments, with statistical analysis performed using GraphPad Prism Version 5 (GraphPad Software, Inc., La Jolla, CA, USA) at a significance level of $p<0.05$.

Results: The study demonstrated that the 50 μM concentration improved sperm motility compared to the 25 μM concentration (81.6% *vs.* 76.6%). Coomassie blue staining revealed that the 25 μM group had a higher percentage of sperm with intact acrosomes compared to both the control and the 50 μM groups (58.88% *vs.* 36.21% and 36.34%, respectively). No statistically significant differences were observed in eosin-nigrosin staining, the HOST test, ROS production, or ATP levels between the groups.

Findings/Conclusions: It is concluded that the 25 μM concentration of MitoTEMPO® helps preserve the acrosomal integrity of spermatozoa after the freeze-thaw process.

Keywords: Acrosome, antioxidant, ROS, MitoTEMPO, bovine spermatozoa.

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INTRODUCTION

During sperm cryopreservation, damage often occurs due to ice crystal formation, osmotic shock, and oxidative stress, leading to decreased sperm motility, impaired viability, and poor membrane stability (Gómez-Torres *et al.*, 2017; O'Connell *et al.*, 2002). Oxidative stress reduces sperm quality by generating reactive oxygen species (ROS) and promoting lipid peroxidation during cryopreservation (O'Connell *et al.*, 2002). Antioxidant supplementation during semen processing has been suggested to improve the quality of frozen-thawed sperm (Banihani *et al.*, 2014; Fontoura *et al.*, 2017; Ghorbani *et al.*, 2016; Kalthur *et al.*, 2011; Karimfar *et al.*, 2015), yet the need for an effective antioxidant

remains. MitoTEMPO® (MT) is a novel, cell-permeable ROS scavenger composed of the piperidine nitroxide unit TEMPOL and the lipophilic triphenylphosphonium (TPP) moiety. TPP allows MitoTEMPOL to cross lipid bilayer membranes and accumulate in energized mitochondria, the primary site of ROS generation (Jiang *et al.*, 2015). TEMPOL serves as an effective intracellular ROS scavenger, reducing stress-induced apoptosis and necrosis (Hu & Li, 2016; Trnka *et al.*, 2009). Freezing and thawing cause significant cellular damage, and various studies have sought to analyze these effects (Nieves-Osorno, 2012). While some researchers have proposed mitochondria, lysosomes, and other organelles as primary damage sites, most studies indicate that the plasma membrane is the most affected structure (Barrientos, 2008). During cryopreservation, spermatozoa experience osmotic fluctuations, dehydration, and ice formation, followed by further stress during thawing.

Factors such as cryoprotectant concentration, cooling rate, and thawing temperature contribute to increased cellular stress (Córdoba *et al.*, 2002; Martínez *et al.*, 2006), resulting in morphological alterations in the plasma membrane, acrosome, mitochondria, cytoskeleton, and nucleus (Petrunkina *et al.*, 2004; Petrunkina *et al.*, 2005). Evaluating the impact of these alterations on sperm function requires physiological assays that accurately assess the extent of damage (Petrunkina *et al.*, 2007). To minimize cryopreservation-induced damage, modifications to freezing protocols have been explored, including adjustments in cooling rates, thawing temperatures, and improvements in freezing diluents, with the latter showing the most significant advancements (Flores-González, 2005). However, few studies have investigated the effects of supplemental antioxidants such as MitoTEMPO® on bovine semen cryopreservation. Therefore, the aim of this study was to evaluate the addition of different concentrations of the mitochondrial-targeted antioxidant MitoTEMPO® in the commercial medium AndroMed®, assessing its potential to improve sperm integrity, ATP production, and ROS levels in cryopreserved bovine sperm.

MATERIALS Y METHODS

Semen Collection and Preparation: Using the artificial vagina method, three ejaculates were collected from each of three healthy, fertile 3-year-old Brangus bulls from the ranch "La Sábila" in Moyotepec, municipality of Plan de Ayala, State of Morelos. Samples were diluted in AndroMed® (80%) and maintained in a water bath at 35 °C for evaluation and eventual strawing. Only ejaculates containing at least 80% live motile spermatozoa were used. Semen evaluation included assessments of progressive motility and sperm concentration. Ejaculates with less than 80% progressive motility or more than 20% morphological abnormalities were discarded. Sperm concentration was determined using a hemocytometer (Neubauer chamber) and calculated with the following formula:

$$\textit{No. of sperm} \times 20 \times 10{,}000 \times 5 = \textit{sperm concentration per } \text{mL}$$

where 20 is the dilution factor (25 μL of semen diluted in 500 μL of 0.1% Triton X-100 in PBS), 10,000 corresponds to the chamber volume, and 5 represents the number of frames counted. The number of abnormal spermatozoa was recorded during the counting process.

Once these parameters were evaluated, semen was frozen. Samples were divided into three groups: Group 1 (control, without antioxidant), Group 2 (25 μM MitoTEMPO®), and Group 3 (50 μM MitoTEMPO®), with the antioxidant added immediately before strawing. The freezing protocol followed the guidelines for AndroMed®, an egg yolk-free medium prepared with Milli-Q water, containing phospholipids, TRIS, citric acid, sugars, antioxidants, buffers, glycerin, high-purity water, and antibiotics (Tylosin, Gentamicin, Spectinomycin, Lincomycin) (Nabiev *et al.*, 2003). Sample strawing was performed using 0.25 mL straws at a concentration of 20×10^6 spermatozoa/mL, equilibrated for 4 h at 4 °C. The samples were then fast-frozen by exposing the straws to nitrogen vapors for 10 minutes before being immersed in liquid nitrogen at −196 °C. The frozen samples were stored in goblets and placed in labeled baskets within liquid nitrogen tanks for at least one month before use.

Thawing and post-thaw sperm evaluation: Thawing was performed by removing the straws, exposing them to room temperature for 10 s, and then placing them in a water bath at 37 °C for 40 s. After thawing, sperm viability was evaluated, and acrosomal integrity was analyzed using non-fluorescent eosin-nigrosin staining and the hypo-osmotic test (HOST) in combination with Coomassie brilliant blue (Mejía *et al.*, 2021). Mitochondrial activity was assessed by measuring ATP production and ROS levels spectrophotometrically. To evaluate post-thaw sperm motility, a 20 μL drop of diluted semen was placed on a pre-warmed slide (37 °C), covered with a coverslip, and examined at 400x magnification using a microscope equipped with a heated stage. The percentage of spermatozoa exhibiting coordinated forward movement was estimated. Three ejaculates were evaluated per bull, with all measurements performed in triplicate.

Measurement of reactive oxygen species (ROS): Reactive oxygen species were measured in spermatozoa upon thawing using Amplex Red (Invitrogen, Molecular Probes) following the protocol described by Guerrero-Castillo *et al.* (2012). Thawed samples were incubated in NaCl at 37 °C, after which × cells were placed into a 96-well microplate containing 20 μL of working solution (10 μM Amplex Red, 0.2 units/mL horseradish peroxidase, and 0.2 units superoxide dismutase/mL in 250 mM sodium phosphate, pH 7.4), with a final volume of 100 μL. Fluorescence was measured after 30 minutes using a POLARstar Omega detector (BGM LABTECH, Offenburg, Germany) set at excitation and emission wavelengths of 571 and 585 nm, respectively. Results were interpolated against a calibration curve.

Measurement of intracellular ATP: ATP concentration was determined by bioluminescence using the ATP-dependent luciferase-catalyzed oxidation of luciferin, which enables the detection of extremely low ATP concentrations (Deluca & McElroy, 1978). Intracellular ATP levels were measured using the ATP Bioluminescence Assay Kit CLS II (Roche), optimized for luminometer-based detection and designed to produce a sustained light signal. To quantify intracellular ATP, a fresh ATP calibration curve was prepared daily following the manufacturer's instructions, using lyophilized luciferase reagent. The procedure was as follows: 8×10^6 cells/mL were resuspended in 100 mM Tris-HCl, 4 mM EDTA, pH 7.8. The samples were then immersed in boiling water for 2 min, followed by centrifugation at 15,000x g in a BenchMark MC-12 centrifuge to remove

cell debris. The resulting supernatants were collected and used to quantify endogenous ATP concentrations. Supernatants were pipetted into a microplate, and luciferase reagent was added to each well (Mendoza-Hoffmann *et al.*, 2018). Fluorescence for both ROS and ATP was measured using a POLARstar Omega detector (BGM LABTECH, Offenburg, Germany) at excitation/emission wavelengths of 571-585 nm. Fluorescence signals were interpolated against a calibration curve, and results were normalized to their non-effector control, which was set at 100% (Mendoza *et al.*, 2018).

Evaluation of sperm viability and acrosomal integrity: Sperm viability and acrosomal integrity were assessed upon thawing using the hypo-osmotic test (HOST) in combination with Coomassie brilliant blue staining. Samples were adjusted to a concentration of 35×10^6 spermatozoa/mL and washed by centrifugation with SSF at 2,500 rpm for 3 min to remove the diluent. A control smear was prepared by mixing 100 μL of semen with 100 μL of 4% paraformaldehyde, incubating for 10 minutes at room temperature, washing twice with PBS at 2,500 rpm for 3 min, reconstituting in 100 μL of ammonium chloride, and then smearing and air-drying the sample. For the test smear, 100 μL of semen was mixed with 100 μL of hypo-osmotic solution (50 μL of 1.46% sodium citrate and 50 μL of 2.7% fructose) and incubated for 60 minutes at 37 °C. Subsequently, 200 μL of 4% paraformaldehyde was added for 10 minutes, followed by two washes with PBS and reconstitution in 100 μL of ammonium chloride. A 20 μL aliquot was then smeared onto a slide and air-dried. Both smears were stained by immersion in Coomassie brilliant blue for 10 to 15 min at room temperature, then rinsed in a Coplin jar with distilled water to remove excess dye. Once dried, the slides were examined under a microscope.

Hypo-Osmotic Swelling (HOST) and coomassie blue staining: The HOST test is used to evaluate sperm membrane functionality. A positive result, indicated by bent or curled flagella, signifies an intact and functional membrane, whereas a negative result, characterized by straight flagella, suggests membrane damage. Coomassie blue staining is employed to assess the presence of the acrosome in spermatozoa. A well-defined, deep blue-stained acrosome indicates acrosomal integrity, whereas a light blue or poorly defined staining pattern suggests acrosome loss or damage (Mejía *et al.*, 2021).

Eosin-Nigrosin staining for sperm viability: After thawing the semen straws, 5 μL of semen was mixed with 2.5 μL of dye and incubated for 5 min at 37 °C. Smears were then prepared on defatted slides, air-dried, and mounted with resin before placing the coverslip. Finally, samples were examined under a microscope. Live spermatozoa, having intact membranes, remained unstained, while dead spermatozoa exhibited staining due to plasma membrane damage (Mejía *et al.*, 2021).

Statistical analysis: Two-way ANOVA (Tukey's multiple comparison test), was employed to compare the means of ROS and ATP production between treatments with two concentrations MitoTempo (MT), the Tx 2 (25 μM) and Tx 3 (50 μM) as well as evaluation with eosin-nigrosine and Coomassie staining in combination with the HOST test, using GraphPad Prism Version 5 (GraphPad Software, Inc., La Jolla, CA, USA) with a value of $P\leq0.05$.

RESULTS AND DISCUSSION

Production of Reactive Oxygen Reactive Species (ROS)

No significant differences were observed in ROS concentrations among the groups (Figure 1A). The mean ±SE values were as follows: control group (184.6 nM ±53.70), 25 μM MitoTEMPO® group (157.6 nM ±44.17), and 50 μM MitoTEMPO® group (187.3 nM ±59.36). The results of this study indicate no significant variation in ROS production between the bovine groups. However, a previous study in buffalo reported by Kumar *et al.* (2022) found a reduction in ROS concentration when MitoTEMPO® was added at a specific concentration. This discrepancy may be attributed to the unique characteristics of buffalo semen, which contains higher levels of polyunsaturated fatty acids and lower antioxidant concentrations compared to bull semen (Kumar *et al.*, 2022).

ATP production after thawing

No significant differences were observed in ATP production among the three groups (Mean ±SE): control group (27.62 nM ±7.30), 25 μM MitoTEMPO® group (29.95 nM ±2.50), and 50 μM MitoTEMPO® group (28.44 nM ±7.55) (Figure 1B). Despite advancements in cattle sperm preservation and continuous efforts to improve sperm quality, oxidative stress remains an unavoidable consequence of the freeze-thaw process. Recently, researchers have focused on mitochondria-targeted antioxidants due to their broad applications, high efficiency, and low toxicity (Zhang *et al.*, 2019). Studies on ram spermatozoa, as reported by Zarei *et al.* (2020), have demonstrated that MitoTEMPO® at concentrations of 5 and 50 μM reduces oxidative stress and mitochondrial damage during temperature fluctuations, thereby enhancing sperm preservation. In this study, no significant differences in ATP production were observed between the groups. Sánchez (2023) investigated whether ATP production could serve as an additional parameter alongside motility and viability to assess bull sperm quality, finding that ATP levels were influenced by the preservation medium, partly independent of motility and viability.

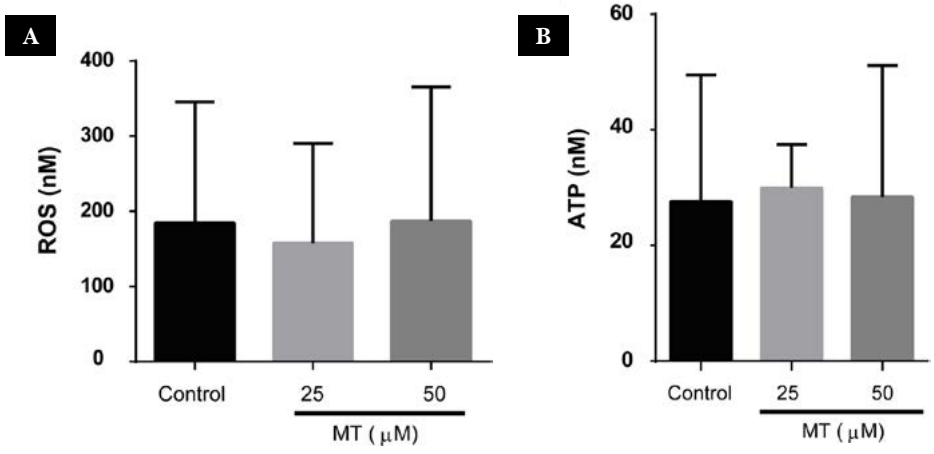

Figure 1. (A) ROS concentration (nM) in bovine spermatozoa; (B) ATP production (nM) in bovine spermatozoa after cryopreservation. Different concentrations of MitoTEMPO® (MT) are indicated. Data represent the average of three biological replicates ± standard deviation ($p \leq 0.05$).

Similarly, Barbosa *et al.* (2011) found no direct correlation between sperm motility and mitochondrial activity.

Sperm viability and acrosomal integrity. Hypo-osmotic test in combination with Coomassie brilliant blue

The effects of MitoTEMPO® supplementation on sperm viability and acrosomal integrity were analyzed, revealing that the percentage of spermatozoa with a positive response to the HOST test was significantly higher in Group 2 (25 μM MT) compared to both the control group and the group supplemented with 50 μM MT (Table 1, Figure 2).

Coomassie brilliant blue staining

The presence of the acrosome in spermatozoa was evaluated using Coomassie brilliant blue staining. The results are presented in Table 2, showing that the group supplemented with 25 μM MitoTEMPO® had a significantly higher percentage of spermatozoa with intact acrosomes compared to the other groups (Figure 3).

Eosine-nigrosine staining

Membrane integrity was evaluated using eosin-nigrosin staining. Although no statistical differences were observed between the groups, semen treated with 25 µM MitoTEMPO® showed a higher percentage of spermatozoa with intact membranes (Table 3, Figure 4).

Table 1. Effect of MitoTEMPO® supplementation at different concentrations on sperm response to the HOST test.

Host	Group 1: without MT	Group 2: 25 μM MT	Group 3: 50 μM MT
Positive to Host	94.33±0.92[a]	95.92±0.92[a]	92.71±0.92[a]
Negative to Host	5.664±0.92[a]	4.080±0.92[a]	7.29±0.92[a]

HOST test results from 3 treatments (Group 1: Control without MitoTEMPO®, Group 2: 25 μM MitoTempo®, Group 3: 50 μM MitoTempo®), showing the proportion of live/dead sperm. Data are shown as average of three biological replicates ± standard deviation, there was no statistical difference between treatments ($p \leq 0.05$).

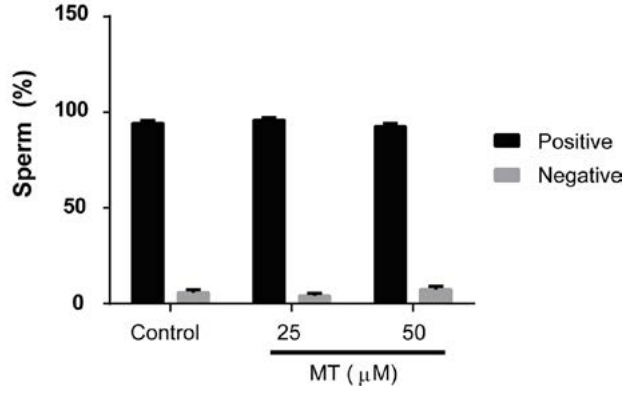

Figure 2. HOST test results for the three treatment groups (Group 1: Control without MitoTEMPO®, Group 2: 25 µM MitoTEMPO®, Group 3: 50 μM MitoTEMPO®), showing the positive/negative sperm ratio. Data represent the average of three biological replicates ± standard deviation ($p \leq 0.05$).

Table 2. Effect of MitoTEMPO® supplementation on acrosomal integrity.

Coomassie stain	Group 1 without MT	Group 2 25 μM MT	Group 3 50 μM MT
Sperm with acrosome	36.21±7.53[a]	58.88±7.53[b]	36.34±7.53[a]
Sperm without acrosome	58.42±6.71[a]	37.77±6.71[b]	37.33±6.71[a]

Coomassie stain results for the three treatment groups (Group 1: Control without MitoTEMPO®, Group 2: 25 μM MitoTEMPO®, Group 3: 50 μM MitoTEMPO®), showing the ratio of sperm with an intact acrosome to sperm without an acrosome. Data represent the average of three biological replicates ± standard deviation. Values with different superscripts indicate significant differences ($p \leq 0.05$).

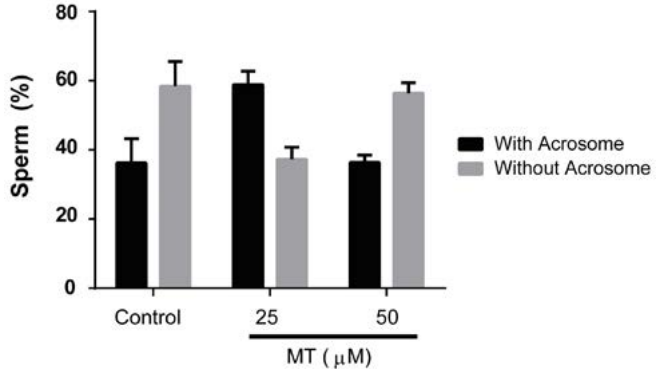

Figure 3. Coomassie staining results showing the ratio of spermatozoa with and without an acrosome after each of the three treatments (Group 1: Control without MitoTEMPO®, Group 2: 25 μM MitoTEMPO®, Group 3: 50 μM MitoTEMPO®). Data represent the average of three biological replicates ± standard deviation ($p \leq 0.05$).

Table 3. Effect of MitoTEMPO® supplementation on sperm membrane integrity.

Eosine-nigrosine stain	Group 1 without MT	Group 2 25 μM MT	Group 3 50 μM MT
Alive sperm	56.46±1.21[a]	60.53±1.21[a]	59.47±1.21[a]
Dead sperm	43.53±1.21[a]	39.48±1.21[a]	40.47±1.21[a]

Eosin-nigrosin staining results for the three treatment groups (Group 1: Control without MitoTEMPO®, Group 2: 25 μM MitoTEMPO®, Group 3: 50 μM MitoTEMPO®), showing the ratio of live to dead sperm. Data represent the average of three biological replicates ± standard deviation. No statistical differences were observed between treatments ($p \leq 0.05$).

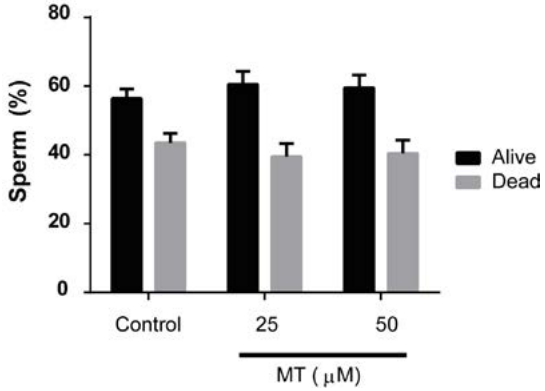

Figure 4. Average eosin-nigrosin staining ratio of live to dead sperm after cryopreservation in the three treatment groups (Group 1: Control without MitoTEMPO®, Group 2: 25 μM MitoTEMPO®, Group 3: 50 μM MitoTEMPO®). Data represent the mean of three biological replicates ± standard deviation ($p \leq 0.05$).

For the evaluation of sperm viability and acrosomal integrity, the HOST test was used. The results showed that spermatozoa treated with 25 μM MitoTEMPO® were better able to compensate for the osmotic imbalance induced by the hypo-osmotic medium compared to both the control group and the 50 μM MitoTEMPO® group. This was evidenced by morphological changes in the flagella, such as dilation and coiling. By combining this test with Coomassie blue staining, the acrosomal state of the spermatozoa was also assessed, revealing that Group 2 (25 μM) had a higher number of spermatozoa with intact acrosomes. These findings represent the first evidence in bovine spermatozoa demonstrating an improvement in acrosomal integrity after the freeze-thawing process. Atuesta *et al.* (2012) reported that cryopreservation negatively affects sperm viability. The acrosome, located in the apical region of the spermatozoon, is essential for fertilization (Pérez *et al.*, 2020), as only spermatozoa capable of undergoing the acrosomal reaction in synchronization with oocyte penetration can successfully complete fertilization (Cox *et al.*, 1998). During the freeze-thaw process, oxidative stress leads to excessive ROS production, triggering lipid peroxidation and causing structural and functional damage to the sperm plasma membrane (Kumar *et al.*, 2021). Zarei *et al.* (2020) explained that MitoTEMPO® not only reduces ROS production but also decreases lipid peroxidation, thereby protecting the sperm plasma membrane. The results of this study differ from those reported by Kumar *et al.* (2021), who found that viability, acrosomal integrity, and the HOST response were improved in buffalo semen with 50 μM MitoTEMPO®. The discrepancy in optimal antioxidant concentration between studies may be attributed to species-specific differences, as buffalo semen has distinct biochemical and physiological properties compared to bovine semen.

Motility

A significant difference was observed in sperm motility between the control group (67.22±1.69) and the groups treated with 25 μM (74.44±1.30) and 50 μM (78.33±1.44) MitoTEMPO®, indicating that the addition of the antioxidant improved motility (Figure 5).

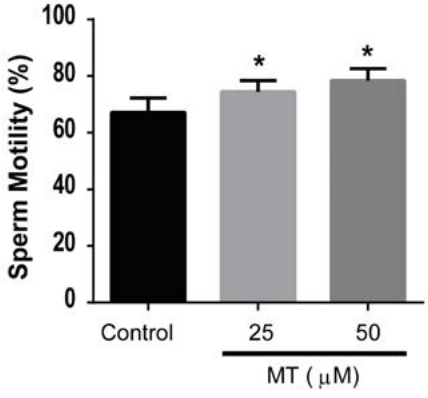

Figure 5. The percentage of sperm motility shows that Group 2 (25 μM MitoTEMPO®) and Group 3 (50 μM MitoTEMPO®) exhibited higher motility after thawing compared to the control group. Values marked with * indicate a statistically significant difference. Data represent the mean of three biological replicates ± standard deviation ($p \leq 0.05$).

The results show a significant improvement ($p \leq 0.05$) in sperm motility in the groups supplemented with MitoTEMPO® (25 and 50 μM) compared to the control group. These findings align with those reported by Valdez (2017), who observed enhanced motility following the addition of natural antioxidants. Similarly, Kumar *et al.* (2021) reported improved progressive motility in buffalo semen treated with 50 μM MitoTEMPO®. However, Foote (2002) found no improvement in motility when Tempo and Tempol were added to bull semen.

CONCLUSIONS

The addition of mitochondria-targeted antioxidants can effectively prevent membrane damage; however, the protection was partial, and only one MitoTEMPO® concentration proved effective. The group supplemented with 25 μM of the antioxidant exhibited a significantly higher percentage of spermatozoa with intact acrosomes compared to both the control and 50 μM groups after the freeze-thaw process. These findings provide valuable insights and highlight the need for further research.

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