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# Mitochondrial specific antioxidant MitoPBN mitigates oxidative stress and improves mitochondrial function in cryopreserved ram sperm

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**Oxidative stress impairs sperm function and fertility in rams, most notably during cryopreservation, and is characterized by compromised sperm function and motility. Mitochondria contribute immensely towards sperm function, such as mitochondrial membrane potential (MMP) which is crucial for ATP production. The purpose of this study was to assess the role of MitoPBN supplementation on cryopreserved sperm quality in mature Ghezel ram sperm. Semen samples were treated with 0 (control), 100, 150, 200, and 250 µmol/L MitoPBN and then analyzed for key sperm factors. Results indicated that supplementation with 100 and 150 µmol/L MitoPBN significantly enhanced total motility, increasing to 52.36 ± 4.26% and 54.16 ± 3.19%, respectively. Progressive motility similarly improved to 24.82 ± 3.27% and 26.77 ± 3.46%, respectively. Additionally, sperm membrane integrity was markedly increased to 50.01 ± 4.22% and 52.54 ± 2.24%, while mitochondrial activity was significantly augmented from 35.14 ± 4.09% to 46.16 ± 4.02% and 50.26 ± 6.69%. Sperm viability also improved, rising to 48.99 ± 3.98% and 52.20 ± 3.17%, respectively. Notably, reactive oxygen species (ROS) levels were reduced to 2.95 ± 0.16% and 2.80 ± 0.11%, respectively, paralleled by enhancements in total antioxidant capacity (TAC; 1.85 ± 0.21% and 1.93 ± 0.16%, respectively) and glutathione peroxidase (GPx) activity (61.16 ± 4.77% and 63.36 ± 4.95%, respectively). Moreover, ATP content reached a peak of 116.29 ± 5.83 in the 150 µmol/L group. In conclusion, our results evidence that oxidative stress during ram sperm cryopreservation is effectively countered by MitoPBN, improving sperm quality.**

**Keywords** Sperm, Cryopreservation, MitoPBN, Mitochondrial, Ram

Successful reproduction in livestock depends on sperm quality, as fertility rates directly influence genetic progress and breeding efficiency. Oxidative stress is a variable that affects the integrity and functioning of spermatozoa and can cause serious cellular damage[1]. Oxidative stress is defined as an imbalance between the formation of reactive oxygen species (ROS) and antioxidant defenses inside sperm cells, which has a negative impact on sperm motility, viability, and overall fertilization capability[2].

In addition to ATP production, mitochondria are essential organelles for a number of cellular processes, including the production of reactive oxygen species (ROS), calcium homeostasis and the intrinsic apoptotic pathway[3]. Some mitochondria are retained in mammalian sperm which reorganize in a tubular shape at the level of the sperm flagellum midpiece, even if the majority of the cytoplasm is lost during spermatogenesis[4]. The activity of sperm mitochondria appears to be crucial for fertilization and embryo development, despite the fact that these organelles are destroyed inside the zygote. Thus, maintaining mitochondrial activity is crucial for maintaining sperm quality[5].

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Recent advances in reproductive biology have emphasized the potential of mitochondria-targeted antioxidants to alleviate oxidative stress in sperm cells[6,7]. One such compound, Mito PBN (mitochondria-targeted phenolic antioxidant), has shown promise in enhancing mitochondrial function and reducing oxidative damage in the treatment of diabetes mellitus[8]. MitoPBN is a mitochondria-targeted antioxidant created to efficiently scavenge reactive oxygen species (ROS) inside the mitochondria, shielding against damage caused by oxidative stress. Because of the mitochondrial membrane potential, it accumulates in the mitochondria and has been demonstrated to trap hydroxyl radicals while preventing superoxide from activating uncoupling proteins. Recent investigations have emphasized its potential therapeutic implications, particularly in the treatment of diabetes, by increasing mitochondrial function and glucose metabolism. MitoPBN importance in research and possible clinical settings for oxidative stress-related diseases is demonstrated by the ability of a liver-targeted formulation called Nano-MitoPBN to normalize glucose levels in diabetic models by increasing mitochondrial respiration and ATP synthesis[8].

Although cryopreservation is a popular method for preserving fresh ram semen, freezing and thawing frequently lead to increased oxidative stress. Sperm quality may be harmed by oxidative damage, which could result in decreased motility and viability after thawing[9]. Several research have examined the effects of antioxidants on ram sperm quality during cryopreservation[10–14]. For example, studies have shown that supplementation with Mito-TEMPO, another mitochondria-targeted antioxidant, enhances post-thaw motility and overall sperm quality in different species. They discovered that Mito-TEMPO improved antioxidant capacity and glucose transporter abundance in frozen-thawed sperm, in addition to increasing mitochondrial membrane potential[15–17]. Likewise, it has been demonstrated that other antioxidants, such as astaxanthin, improve sperm quality by increasing mitochondrial potential and overall antioxidant capacity, while lowering ROS levels during storage[18].

The primary objective of this study was to investigate an innovative application of the mitochondrial antioxidant MitoPBN in enhancing cryopreserved semen quality in Ghezel rams. By comparing variable concentrations of MitoPBN with its impact on sperm motility, membrane integrity, mitochondrial function, and oxidative markers, this study aimed to evaluate whether mitochondrial-targeted antioxidants can reverse oxidative stress during cryopreservation.

## Materials and methods

### Chemicals

All the chemicals were purchased from Merck (Darmstadt, Germany).

### Animal ethics

This study was conducted in accordance with ARRIVE guidelines 2.0[19]. All animal care procedures were approved by the University Ethical Committee of the University of Tabriz (IR. REC.1401.580). In addition, all methods used in the current study were performed under the University Review Board and University Ethical Committee of the University of Tabriz guidelines and regulations.

### Semen collection

Semen was collected from four sexually mature Ghezel rams (farm at the University of Tabriz) using an artificial vagina twice a week for four consecutive weeks. On each collection day, ejaculates from all rams were pooled to create one replicate, resulting in a total of eight replicates[20,21]. Preliminary analyses confirmed minimal intra-individual variability, thereby justifying the pooling strategy.

### Semen processing and freezing

Immediately after semen collection, the collected samples were evaluated for parameters containing volume (ml), concentration (sperm/mL), and progressive motility (%). Semen samples with a volume of 0.5–1.5 ml, motility above 80%, abnormal morphology less than 10%, and sperm concentration above $3 \times 10^9$ were used for the experiment. The pooled semen was then divided into five parts for dilution of the respective treatments. The volume of pooled semen was diluted at 37 °C with tris extender containing 7% (v/v) glycerol and supplemented with MitoPBN at concentrations of 0 (control), 100, 150, 200, and 250 µmol/L. These concentrations were selected based on preliminary dose–response experiments and supported by the literature on mitochondrial-targeted antioxidants. Following dilution with tris extender-containing treatments, the samples were incubated at 4 °C for two hours. Then, the samples were loaded into 0.25 ml straws and positioned 4 cm above the surface of liquid nitrogen for 7 min before being submerged. They were merged in liquid nitrogen (-196 °C), and the straws were stored in liquid nitrogen for a minimum period of 7 days prior to thawing. The cryopreserved straws were thawed for 30 s at 37 °C in a water bath for evaluation.

### Sperm motility

A sperm analyzer (CASA) system IVOS 12 (Hamilton Thorne Inc., Beverly, MA, USA) was used to examine motility parameters. First, frozen sperm were diluted in PBS buffer at 37 °C (Leja 4; 20 µm height; Leja Products, Luzernestraat B.V., Holland) before being placed on a preheated chamber slide. At least 200 cells were examined for each sample. The following parameters were assessed: linearity (LIN; %), curvilinear velocity (VCL; µm/s), average path velocity (VAP; µm/s), straight line velocity (VSL; µm/s), total motility (TM; %), and progressive motility (PM; %).

### TAC, SOD and GPx evaluation

The TEAC Randox kit (RANDOX Laboratories Ltd.) was used to test total antioxidant capacity (TAC). This technique relies on the use of antioxidants that scavenge ABTS cation radicals. The reaction was initiated by

adding 200 µl of $H_2O_2$ after mixing 20 µl of the samples and 1 ml of chromogen (ABTS reagent) had been mixed. This method produces a stable blue-green color with a maximum light absorption at 600 nm, which can be measured using spectrophotometry. We used the Ransox kit (RANDOX Laboratories Ltd.) to evaluate the GPx enzyme activity. Briefly, 10 µl of sample was mixed with 500 µL of GPx Reagent (glutathione, glutathione Reductase, and NADPH) and 10 µL of buffer (EDTA and Phosphate Buffer), followed by the addition of 4 µL of cumene hydroperoxide. Absorbance was measured at a wavelength of 340 nm. Superoxide dismutase (SOD) (RANDOX Laboratories Ltd.) activity was evaluated by spectrophotometry. After mixing 50 µl of the samples with 1.7 mL of a mixed substrate (xanthine and Int), 500 µl of Xanthine Oxidase was added to the solution. The absorbance was measured at a wavelength of 505 nm[22].

## Membrane functionality

The Najafi et al.[23] method was used to conduct the test in the current experiment. One hundred µL of hypoosmotic medium made up of fructose and sodium citrate was added to 10 µL semen. It was incubated at a 37 °C temperature for 30 min. At least 10 µL of the resulting incubated materials was observed through the optics on the contrast phase microscope fitted hot plate at a temperature of 37 °C and 400× magnification. At least 200 sperm were counted for each treatment group, and the proportion with a tied tail was determined.

## Abnormal sperm

To evaluate abnormal sperm, at least three drops of each sample were added to microtubes containing one milliliter of Hancock solution. One drop of the solution was then spotted on a slide and covered with a slide. The percentage of abnormal sperm was measured by counting at least 200 sperm under a microscope with a contrast phase at 400× magnification[24].

## Flow cytometry analyses

Flow cytometry analyses were carried out using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with a 488 nm Argon ion laser. Sperm cells were identified and separated from debris based on forward and side scatter gating. Fluorescence signals were captured using a 530/30 nm band-pass filter for green fluorescence (Rhodamine-123 and Annexin-V) and a 610 nm long-pass filter for red fluorescence (propidium iodide). Data acquisition was performed using CellQuest 3.3 software (Becton Dickinson) with at least 10,000 events recorded per sample to ensure statistical reliability and accuracy.

## Sperm viability and apoptosis assay

The annexin kit was used to define the percentages of viable, apoptotic, and dead sperm. To accomplish these measurements, samples were thawed at room temperature for 30 min; next, 500 µL of buffer was added; subsequently, after being centrifugated (1200 rpm for 10 min), it was added into 500 µL buffer. Annexin was mixed with 100 µL of calcium buffer, and 10 µL of annexin was added and incubated in the dark for 20 min. Then, 10 µl of propidium iodide (PI) was added to the sample and incubated for 15 min. A flow cytometer was used to measure the amount of phosphatidylserine in sperm membranes. If the samples were PI- and annexin-negative, they were considered live sperm in the flow chart of the flow cytometer. The sample was alive but with primary apoptosis if the annexin test was positive, whereas the PI test was negative. It was considered dead sperm with secondary apoptosis if it was both PI-and annexin positive. PI-positive and annexin-negative were considered necrotic sperm[25].

## Assessment of mitochondrial activity

Rhodamine was used as an indicator of mitochondrial activity. First, 500 µL buffer was added to the sperm samples. Then, 10 µL of rhodamine was added and the samples were incubated in the dark at room temperature for 20 min. Next, 10 µL of PI was added, and mitochondrial activity was analyzed by flow cytometry. The rhodamine-positive and PI-negative samples in the diagram were considered active mitochondrial samples by flow cytometry. When both rhodamine and PI are positive, they are considered inactive mitochondria[24].

## Determination of ATP in sperm

ATP was quantified using the method described by Mehdipour et al.[26]. method. Five microliters of each sample was pipetted into 190 µL of perchloric acid, previously diluted in 750 µL of buffer. This was followed by centrifugation process for two minutes at 12,600×g. To the upper phase, which had been transferred into a new tube, 10.7 µL of 2 M KCl, 58.7 µL of 1 M KOH, 10.7 of saturated Tris, and 1 µg/mL of red phenol was added. Finally, 100 µL of reconstituted luciferin–luciferase reagent was added. The ATP standard was serially diluted to obtain standards with concentrations between $10^{-7}$ and $10^{-12}$ M. The amount of ATP was measured in pmol ATP per $10^6$ sperm.

## Measurement of ROS

The ROS levels were measured using the method described by Mehdipour et al.[26]. After incubation at 37 °C in 250 µL PBS for 20-minute incubation of samples, the samples were centrifuged at 300×g for 7 min to extract the supernatant. PBS (3 mL) was then re-centrifuged under the same centrifugal conditions at an interval of 7 min. After adding 10 µL of luminol to a 400 µL sample, the tubes were placed in an Orion II Microplate Luminometer. The results obtained were expressed as $10^3$ photons per minute per $10^6$ spermatozoa.

## Statistical analysis

Pooled semen was cryopreserved in eight replicates, and sperm analysis was performed according to the protocols described in detail in this manuscript. Statistical analyses were conducted using R statistical environment

(version 4.4.0; R Core Team, 2024). Linear mixed-effects models (LME) were applied using the nlme package (version 3.1–162), with treatment as a fixed effect and repeated as a random effect. Results are reported as mean ± SD, with statistical significance denoted as $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

## Results

The effect of MitoPBN on sperm motility and kinematic parameters is illustrated in Figs. 1 and 2. Spermatozoa treated with 100 and 150 µmol/L MitoPBN exhibited significantly higher total motility (TM) (52.36 ± 4.26% and 54.16 ± 3.19%, respectively) compared to the control (41.89 ± 5.31%) ($P < 0.05$). Both the average path velocity (VAP) and straight-line velocity (VSL) peaked at 150 µmol/L, reaching 68.82 ± 2.56% and 55.24 ± 5.35%, respectively. No statistically significant differences were observed between STR and LIN treatment groups. Similarly, ALH and BCF remained stable at all concentrations, confirming that MitoPBN did not negatively influence the dynamics of sperm motion.

As depicted in Fig. 3, membrane integrity was significantly improved in the 100 and 150 µmol/L groups (50.01 ± 4.22% and 52.54 ± 2.24%, respectively) ($P < 0.05$), and the lowest percentage of abnormal spermatozoa was recorded in these groups (19.23 ± 4.08% and 18.82 ± 5.76%, respectively). Mitochondrial activity was highest at 150 µmol/L (50.26 ± 6.69%), followed by 100 µmol/L (46.16 ± 4.02%).

Figure 4 shows that sperm viability was significantly increased in the 100 and 150 µmol/L groups (48.99 ± 3.98% and 52.20 ± 3.17%, respectively) relative to the control (36.08 ± 5.69%) ($P < 0.05$), with the lowest percentages of apoptotic and dead sperm observed at these concentrations (20.10 ± 3.08% and 18.70 ± 2.89%).

The oxidative stress markers and antioxidant enzyme activities are summarized in Fig. 5. The 100 and 150 µmol/L treatments resulted in the lowest reactive oxygen species (ROS) levels (2.95 ± 0.16% and 2.80 ± 0.11%,

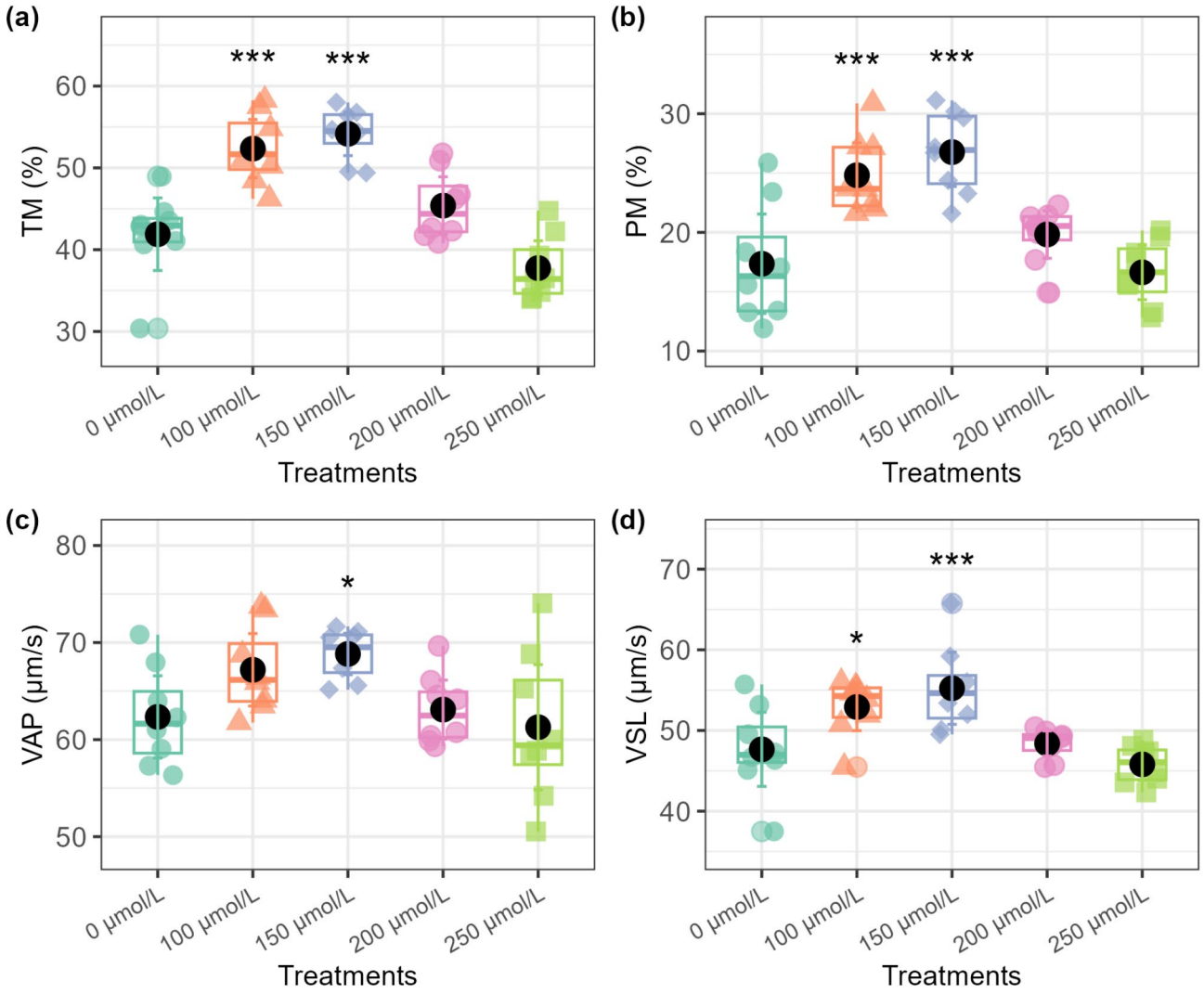


**Fig. 1**. TM: total motility (%) (**a**), PM: progressive motility (%) (**b**), VAP: average path velocity (µm/s) (**c**) and VSL: straight – line velocity (µm/s) (**d**) after thawing samples cryopreserved with different levels of MitoPBN. Insets show P values for the effects of the treatments respect to the control (0 µmol/L of MitoPBN) (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). Data are presented as mean ± SD ($n = 8$ replicates per treatment).

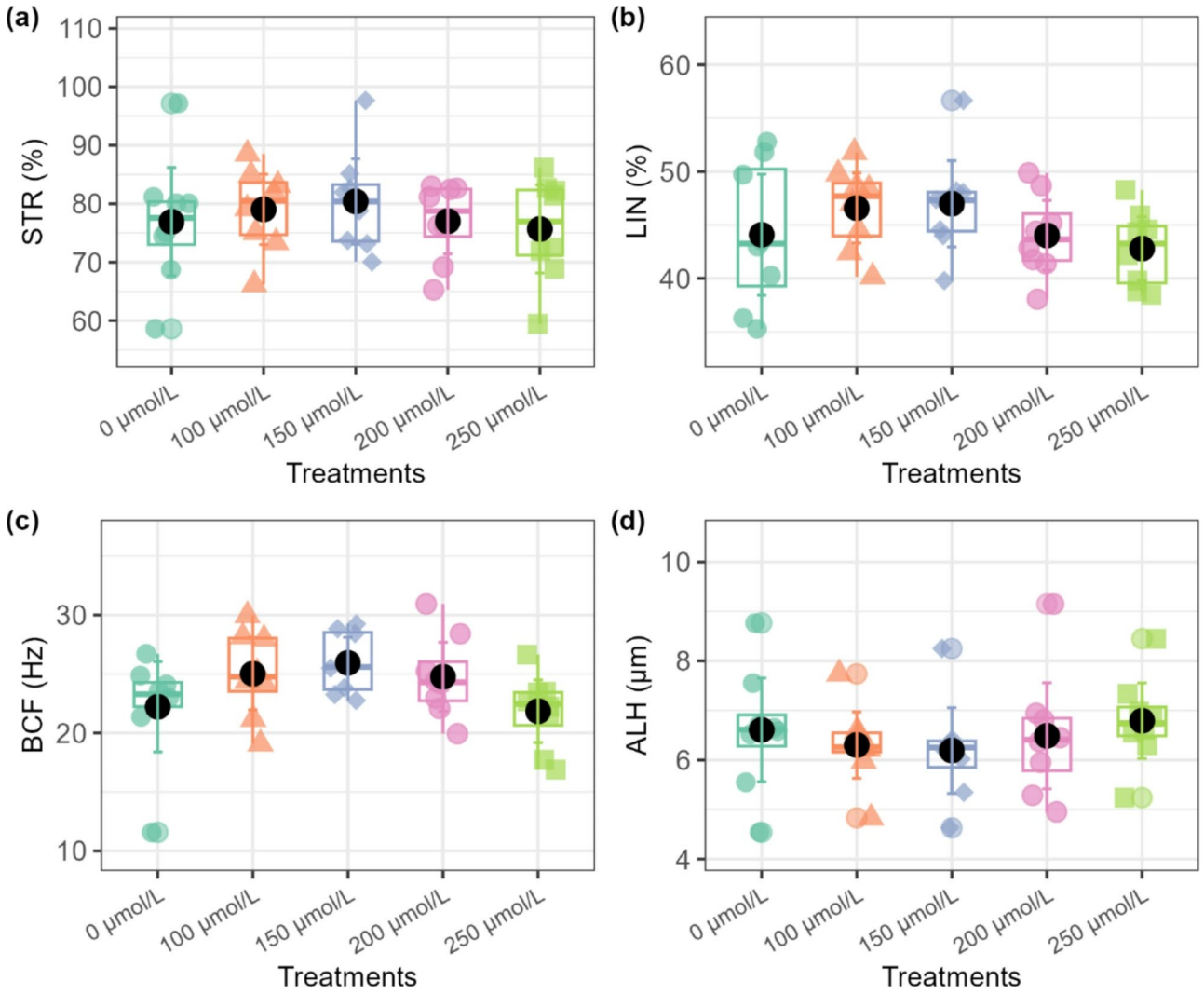


**Fig. 2**. STR: straightness (%) (**a**), LIN: linearity (%) (**b**), ALH: amplitude of the lateral head displacement (µm) (**c**) and BCF: beat cross frequency) Hz (**d**) after thawing samples cryopreserved with different levels of MitoPBN. Insets show P values for the effects of the treatments respect to the control (0 µmol/L of MitoPBN) (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). Data are presented as mean ± SD ($n = 8$ replicates per treatment).

respectively), while total antioxidant capacity (TAC) increased to 1.85 ± 0.21% and 1.93 ± 0.16%, and glutathione peroxidase (GPx) activity reached 61.16 ± 4.77% and 63.36 ± 4.95% ($P < 0.05$).

## Discussion

Cryopreservation is an essential technique for preserving the genetic potential of valuable livestock, particularly rams; however, freezing and thawing expose spermatozoa to a wide range of stress factors, with oxidative stress being a principal contributor that jeopardizes cellular function and integrity[27]. In this study, supplementation with moderate concentrations of MitoPBN improved sperm motility, membrane integrity, mitochondrial function, and viability. In contrast, 250 µmol/L did not further enhance these parameters, which may be attributable to the induction of reductive stress.

Sperm motility is one of the most important factors determining the fertilization potential. Our results show a significant increase in motility parameters like total motility (TM), progressive motility. Motility is an essential attribute in determining the fertilizing potential of sperm and is a key target in cryopreservation. MitoPBN supplementation thus offers cryoprotective benefits in maintaining sperm function due to a decline in oxidative stress. This agrees with observations in related studies using antioxidants to enhance sperm motility and viability post-thawing[28]. Agarwal et al.[29] indicated that supplementing media containing antioxidants leads to a decrease in the level of ROS; this way, motility was preserved. Similarly, Jannatifar et al.[30] reported that NAC improves sperm motility by reducing oxidative damage during cryopreservation.

MitoPBN is a mitochondria-targeted antioxidant designed to scavenge reactive oxygen species (ROS) directly in the mitochondria and preserve mitochondrial membrane potential and ATP production[8]. Sperm motility is heavily dependent on ATP generated by the mitochondria, which is used to power flagellate

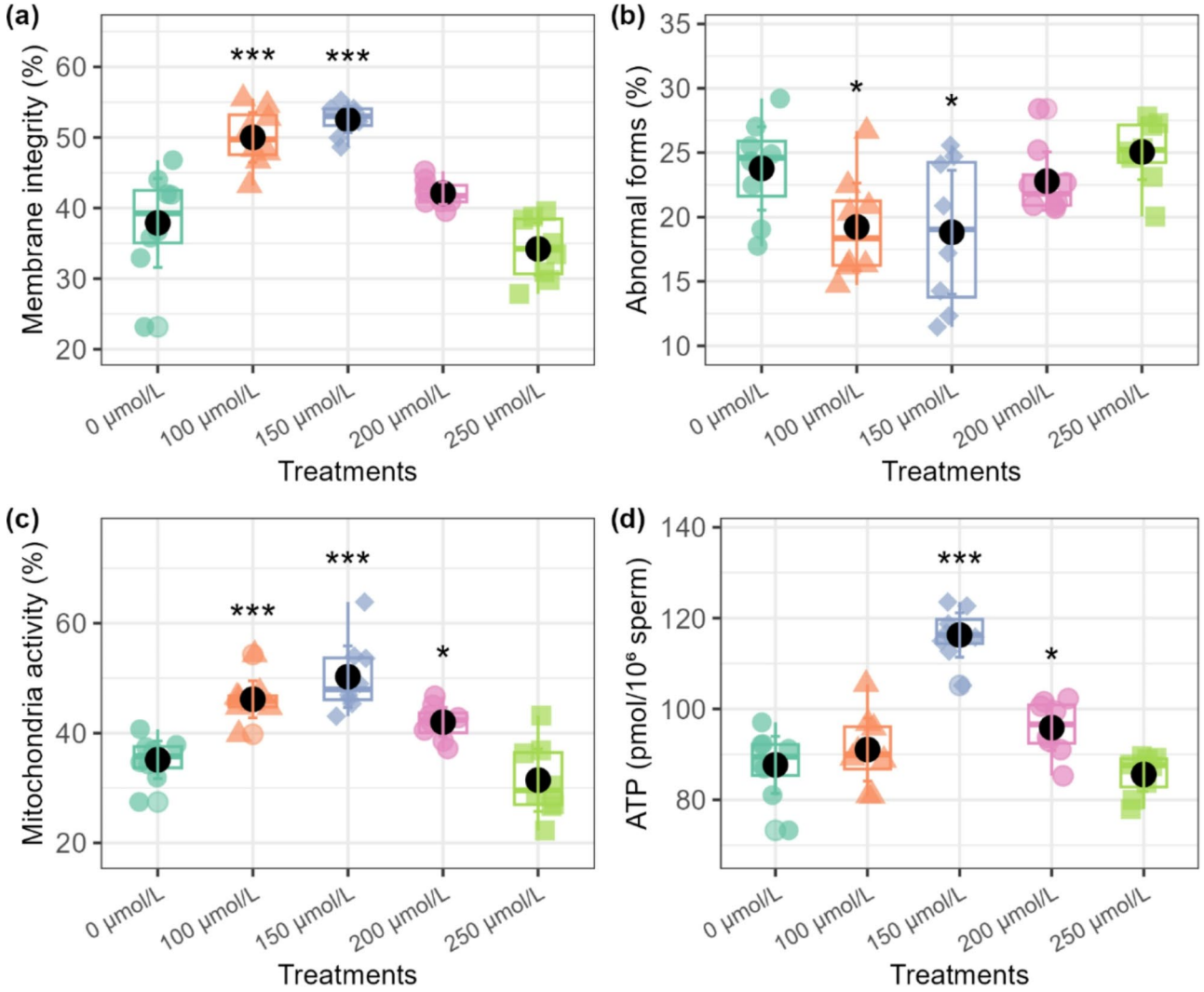


**Fig. 3**. Membrane integrity (%) (**a**), total abnormality (%) (**b**), mitochondrial activity (%) (**c**) and ATP (pmol/$10^6$ sperm) (**d**) after thawing samples cryopreserved with different levels of MitoPBN. Insets show P values for the effects of the treatments respect to the control (0 µmol/L of MitoPBN) (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). Data are presented as mean ± SD ($n = 8$ replicates per treatment).

movement for successful fertilization[31]. Our results demonstrated that at concentrations of 100 and 150 µmol/L, MitoPBN effectively reduced ROS, as evidenced by decreased lipid peroxidation and improved membrane stability. Preservation of mitochondrial function not only ensures enhanced motility but also ensures metabolic competency for successful fertilization[32]. Similar protective effects have been found in studies of other mitochondria-targeted antioxidants, such as MitoTEMPO and MitoQ, which have been employed to preserve sperm function during cryopreservation[15,33]. In this study, optimal concentrations of MitoPBN were associated with improved mitochondrial function, as evidenced by improved ATP production and preservation of the mitochondrial membrane potential. These findings are in accordance with previous research that reported that mitochondria-targeted antioxidants preserve mitochondrial function under stress[34–36]. However, at higher concentrations, potential reductive stress induction can inhibit mitochondrial respiration, thereby limiting ATP production, and ultimately viability and motility[37,38]. The role of MitoPBN is to protect mitochondria from oxidative stress and yet at higher concentrations, it poses the threat of inducing reductive stress, emphasizing the need for careful dose optimization in antioxidant therapy.

Oxidative stress, resulting in an imbalance between ROS production and antioxidant defense systems, damages sperm function by impairing lipids in the membrane, proteins, and nucleic acids[39]. ROS tend to accumulate rapidly during cryopreservation and inflict extensive oxidative damage on spermatozoa by breaking their structural and functional integrity. Moreover, regulation of antioxidant enzyme activity is a major element of the protective mechanism of MitoPBN. The increases in TAC and GPx activity observed in this study indicate that MitoPBN enhances the intrinsic antioxidant defense of sperm. This dual mechanism, where direct scavenging of ROS is supplemented by antioxidant enzyme upregulation, could have a more effective protective effect during freeze–thaw because immediate and effective reduction of oxidative stress is critical.

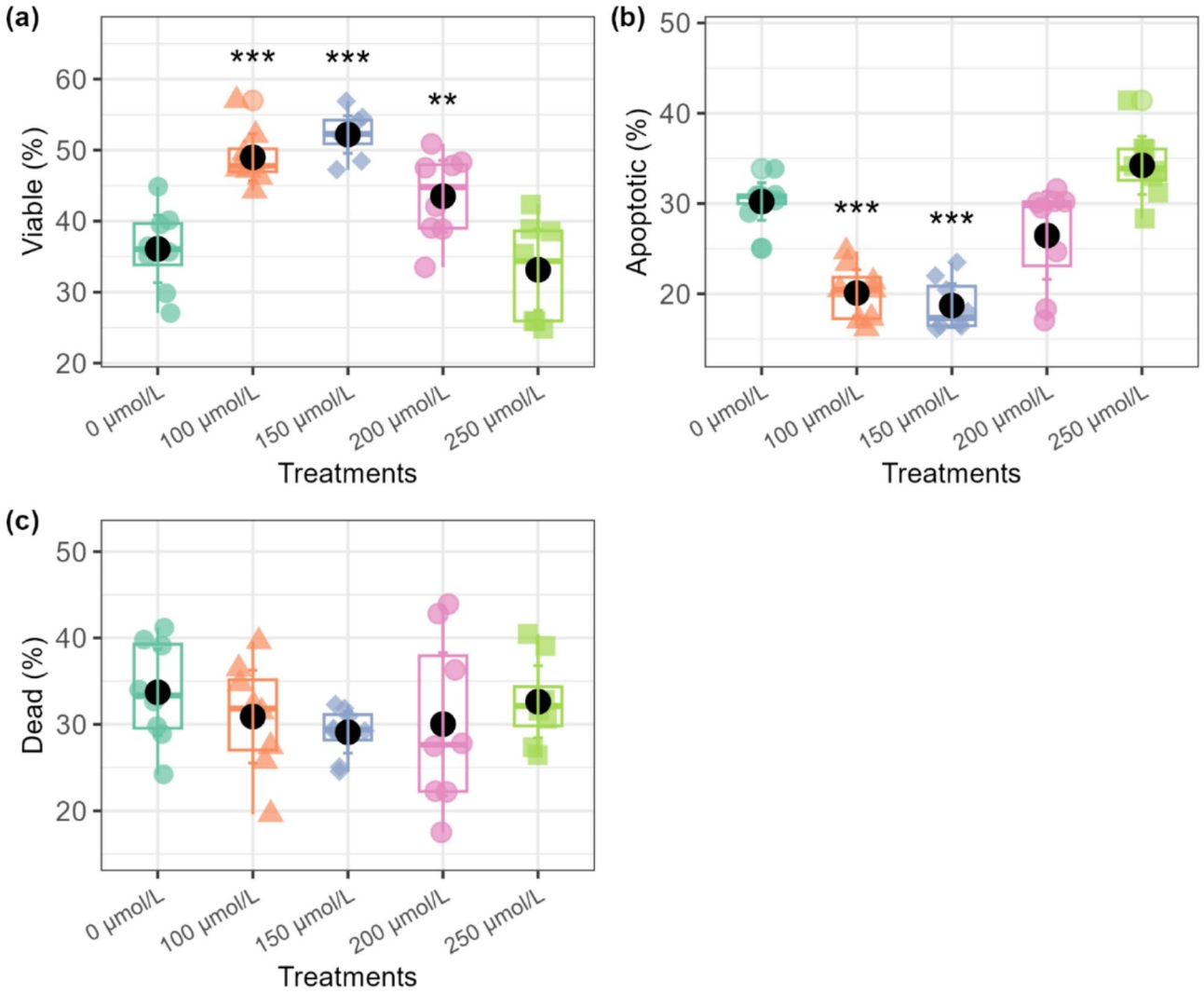


**Fig. 4**. Viability (%) (**a**), apoptotic (%) (**b**) and dead sperm (%) (**c**) after thawing samples cryopreserved with different levels of MitoPBN. Insets show P values for the effects of the treatments respect to the control (0 µmol/L of MitoPBN) (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). Data are presented as mean ± SD ($n = 8$ replicates per treatment).

Notably, moderate concentrations of MitoPBN significantly improved sperm parameters, whereas higher concentrations had no further beneficial effects. This paradoxical observation indicated that a surplus of MitoPBN might lead to reductive stress. While harmful in excess, ROS are also necessary for redox-sensitive signaling that regulates critical functions such as capacitation, acrosomal reaction, and other sperm functions[40]. Over-quenching of ROS by a surplus of antioxidants might therefore eliminate these critical signaling mechanisms and lead to reductive stress that is harmful to cellular function[41]. Optimal use of mitochondrial-targeted antioxidant agents, such as MitoPBN, is therefore not a matter of maximizing ROS scavenging, but of maintaining that fine balance is essential for redox signaling.

A principal cause of sperm damage during cryopreservation is oxidative stress, which can induce lipid peroxidation, DNA fragmentation, and loss of viability in the sperm. Mitochondrial function is essential for sperm viability because not only are mitochondria responsible for producing ATP but they are also involved in apoptosis and intracellular calcium homeostasis[42,43]. The viability of sperm was also increased by MitoPBN treatment and the level of apoptosis was reduced. Kuželová, et al.[17] documented similar findings, with antioxidants such as Mito-Tempo decreasing apoptosis and improving viability in cryopreserved sperm[44]. In our study, mitochondrial function, with its protective function, could have reduced apoptosis, with mitochondrial impairment having a propensity to act as a key inducer of apoptosis in sperm.

Our observations of improved membrane integrity at moderate concentrations of MitoPBN indicate that this antioxidant is effective in preventing oxidative degradation of lipids and proteins in the membranes. Integrity of the membranes is critical for maintaining ion gradients and proper signal transduction, both of which are critical for sperm motility and fertilization potential[31,45]. These observations are in agreement with earlier research,

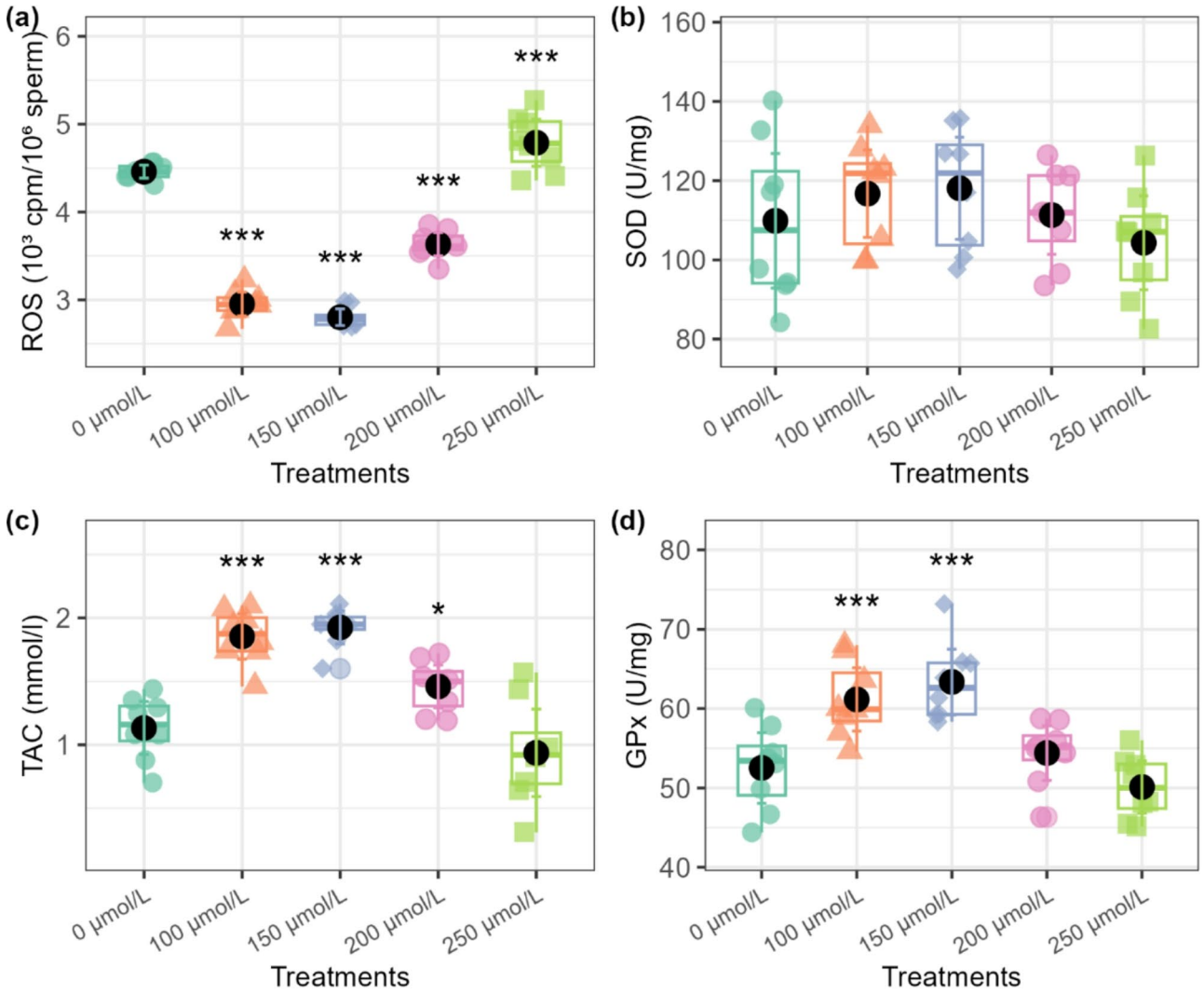


**Fig. 5**. ROS ($10^3$ cpm/$10^6$ sperm) (**a**), SOD (U/mg) (**b**), TAC (mmol/l) (**c**), and GPx (U/mg) (**d**) after thawing samples cryopreserved with different levels of MitoPBN. Insets show P values for the effects of the treatments respect to the control (0 µmol/L of MitoPBN)) (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). Data are presented as mean ± SD ($n = 8$ replicates per treatment).

where antioxidant supplementation was found to reduce lipid peroxidation in the membranes and enhance the quality of spermatozoa after thawing[46–48].

One of the significant results of the current study was the significant reduction in the levels of abnormal spermatozoa. These results suggest that MitoPBN supplementation protects sperm morphology, which is a key determinant of fertilizing capacity. As structural abnormalities have the potential to interfere with sperm motility, acrosomal function, and contact with the oocyte, a smaller number of abnormal spermatozoa indicates better sperm quality[49]. The reduction in sperm abnormalities suggests that MitoPBN is involved in the structural integrity of sperm. One possible explanation is that MitoPBN averts oxidative stress, which leads to morphological abnormalities by disturbing the cellular membranes. Through the stabilization of the plasma membrane and the preservation of the of sperm cells integrity, MitoPBN could avoid the occurrence of morphological abnormalities that occur during cryopreservation or other stressful conditions. Morphological defects in sperm can severely impair sperm function, even with high viability and mitochondrial function[50]. Decreasing abnormal spermatozoa is another evidence that MitoPBN enhances the integrity of sperm at different levels, attesting to its value in the conservation of fertility.

## Conclusion

The addition of MitoPBN during cryopreservation of ram sperm offers significant improvements in sperm motility, mitochondrial activity, oxidative stress reduction, membrane integrity, and sperm viability. The optimal concentration appears to be 150 µmol/L, which balances the antioxidant and mitochondria-enhancing effects without causing toxicity. These findings not only contribute to the existing literature on cryopreservation but also suggest that MitoPBN may be a promising agent for improving the quality of cryopreserved sperm.

## Data availability

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

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## References

1. Rizkallah, N., Chambers, C. G., de Graaf, S. P. & Rickard, J. P. Factors affecting the survival of Ram spermatozoa during liquid storage and options for improvement. *Animals* **12**, 244 (2022).
2. Kaltsas, A. Oxidative stress and male infertility: The protective role of antioxidants. *Medicina* **59**, 1769 (2023).
3. Napolitano, G., Fasciolo, G. & Venditti, P. Mitochondrial management of reactive oxygen species. *Antioxidants* **10**, 1824 (2021).
4. Chianese, R. & Pierantoni, R. Mitochondrial reactive oxygen species (ROS) production alters sperm quality. *Antioxidants* **10**, 92 (2021).
5. Amaral, A., Lourenço, B., Marques, M. & Ramalho-Santos, J. Mitochondria functionality and sperm quality. *Reproduction* **146**, R163–R174 (2013).
6. Tiwari, S., Dewry, R., Srivastava, R., Nath, S. & Mohanty, T. Targeted antioxidant delivery modulates mitochondrial functions, ameliorates oxidative stress and preserve sperm quality during cryopreservation. *Theriogenology* **179**, 22–31 (2022).
7. Escada-Rebelo, S., Cristo, M. I., Ramalho-Santos, J. & Amaral, S. Mitochondria-targeted compounds to assess and improve human sperm function. *Antioxid. Redox. Signal.* **37**, 451–480 (2022).
8. Wu, M. et al. Liver-targeted Nano-MitoPBN normalizes glucose metabolism by improving mitochondrial redox balance. *Biomaterials* **222**, 119457 (2019).
9. Saha, A., Asaduzzaman, M. & Bari, F. Y. Cryopreservation techniques for ram sperm. *Vet. Med. Int.* **2022**, 7378379 (2022).
10. Mehdipour, M., Kia, H. D., Najafi, A., Dodaran, H. V. & García-Álvarez, O. Effect of green tea (*Camellia sinensis*) extract and pre-freezing equilibration time on the post-thawing quality of Ram semen cryopreserved in a soybean lecithin-based extender. *Cryobiology* **73**, 297–303 (2016).
11. Riesco, M. F. et al. Multiparametric study of antioxidant effect on Ram sperm cryopreservation—from field trials to research bench. *Animals* **11**, 283 (2021).
12. Shehab-El-Deen, M., Ali, M. & Al-Sharari, M. Effects of extenders supplementation with gum Arabic and antioxidants on Ram spermatozoa quality after cryopreservation. *Animals* **13**, 111 (2022).
13. Berean, D. I., Bogdan, L. M. & Cimpean, R. Advancements in Understanding and enhancing Antioxidant-Mediated sperm cryopreservation in small ruminants: Challenges and perspectives. *Antioxidants* **13**, 624 (2024).
14. Khalil, W. A. et al. The beneficial effects of Quinoa seed extract supplementation on Ram sperm quality following cryopreservation. *Anim. Reprod. Sci.* **264**, 107472 (2024).
15. Li, C. Y. et al. The effect of the mitochondria-targeted antioxidant Mito-tempo during sperm ultra-rapid freezing. *Cryobiology* **114**, 104860 (2024).
16. Shi, L., Shi, J., Feng, J., Zhang, P. & Ren, Y. Proteomic analysis reveals the potential positive effects of Mito-TEMPO on Ram sperm motility and fertility during cryopreservation. *Theriogenology* **205**, 27–39 (2023).
17. Kuželová, L. et al. Enhancing of rabbit sperm cryopreservation with antioxidants Mito-Tempo and Berberine. *Antioxidants* **13**, 1360 (2024).
18. Sohail, T. et al. Astaxanthin improved the quality of Hu Ram semen by increasing the antioxidant capacity and mitochondrial potential and mitigating free Radicals-Induced oxidative damage. *Animals* **14**, 319 (2024).
19. Du Sert, N. P. et al. Reporting animal research: Explanation and elaboration for the ARRIVE guidelines 2.0. *PLoS Biol.* **18**, e3000411 (2020).
20. Zhang, L. et al. Effect of fumigation height and time on cryopreservation of Ram semen. *Sci. Rep.* **14**, 10944 (2024).
21. Mousavi, S. M. et al. Comparison of two different antioxidants in a nano lecithin-based extender for bull sperm cryopreservation. *Anim. Reprod. Sci.* **209**, 106171 (2019).
22. Najafi, A., Kia, H. D., Mehdipour, M., Hamishehkar, H. & Alvarez-Rodriguez, M. Effect of Quercetin loaded liposomes or nanostructured lipid carrier (NLC) on post-thawed sperm quality and fertility of rooster sperm. *Theriogenology* **152**, 122–128. https://doi.org/10.1016/j.theriogenology.2020.04.033 (2020).
23. Najafi, A., Daghigh-Kia, H., Dodaran, H. V., Mehdipour, M. & Alvarez-Rodriguez, M. Ethylene glycol, but not DMSO, could replace glycerol inclusion in soybean lecithin-based extenders in Ram sperm cryopreservation. *Anim. Reprod. Sci.* **177**, 35–41. https://doi.org/10.1016/j.anireprosci.2016.12.004 (2017).
24. Najafi, A. et al. Effect of tempol and straw size on rooster sperm quality and fertility after post-thawing. *Sci. Rep.* **12**, 12192. https://doi.org/10.1038/s41598-022-16507-6 (2022).
25. Najafi, A., Mohammadi, H., Sharifi, S. D. & Rahimi, A. Apigenin supplementation substantially improves rooster sperm freezability and post-thaw function. *Sci. Rep.* **14**, 4527 (2024).
26. Mehdipour, M., Daghigh Kia, H. & Martinez-Pastor, F. Poloxamer 188 exerts a cryoprotective effect on rooster sperm and allows decreasing glycerol concentration in the freezing extender. *Poult. Sci.* **99**, 6212–6220. https://doi.org/10.1016/j.psj.2020.08.041 (2020).
27. Juyena, N. S. Protein profiles and biochemical characteristics of semen: influence on frozen-thawed spermatozoal quality in Rams (Ovies aries) and alpacas (Vicugna pacos) (2011).
28. Najafi, D., Taheri, R. A., Najafi, A., Rouhollahi, A. A. & Alvarez-Rodriguez, M. Effect of Achillea millefolium-loaded nanophytosome in the post-thawing sperm quality and oxidative status of rooster semen. *Cryobiology* **82**, 37–42. https://doi.org/10.1016/j.cryobiol.2018.04.011 (2018).
29. Agarwal, A., Makker, K. & Sharma, R. Clinical relevance of oxidative stress in male factor infertility: An update. *Am. J. Reprod. Immunol.* **59**, 2–11 (2008).
30. Jannatifar, R., Parivar, K., Roodbari, N. H. & Nasr-Esfahani, M. H. Effects of N-acetyl-cysteine supplementation on sperm quality, chromatin integrity and level of oxidative stress in infertile men. *Reprod. Biol. Endocrinol.* **17**, 1–9 (2019).
31. Freitas, M. J., Vijayaraghavan, S. & Fardilha, M. Signaling mechanisms in mammalian sperm motility. *Biol. Reprod.* **96**, 2–12 (2017).
32. Jansen, R. P. & Burton, G. J. Mitochondrial dysfunction in reproduction. *Mitochondrion* **4**, 577–600 (2004).
33. Shi, L. et al. Effects of mitoquinone (MitoQ) supplementation during Boar semen cryopreservation on sperm quality, antioxidant status and mitochondrial proteomics. *Anim. Reprod. Sci.* **247**, 107099 (2022).
34. Lu, X. et al. Mitochondria-targeted antioxidant MitoTEMPO improves the post-thaw sperm quality. *Cryobiology* **80**, 26–29 (2018).
35. Zhu, Y. et al. Mitochondria-targeted antioxidant MitoQ improves the quality of low temperature-preserved Yak semen via alleviating oxidative stress. *Anim. Reprod. Sci.* **273**, 107680 (2025).
36. Sun, L. et al. Does antioxidant mitoquinone (MitoQ) ameliorate oxidative stress in frozen–thawed rooster sperm? *Animals* **12**, 3181 (2022).

37. Fang, L. et al. Inhibition of ROS production through mitochondria-targeted antioxidant and mitochondrial uncoupling increases post-thaw sperm viability in yellow catfish. *Cryobiology* **69**, 386–393 (2014).
38. Irigoyen, P., Mansilla, S., Castro, L., Cassina, A. & Sapiro, R. Mitochondrial function and reactive oxygen species production during human sperm capacitation: unraveling key players. *FASEB J.* **38**, e23486 (2024).
39. Dutta, S., Majzoub, A. & Agarwal, A. Oxidative stress and sperm function: A systematic review on evaluation and management. *Arab. J. Urol.* **17**, 87–97 (2019).
40. Fatima, S. Role of reactive oxygen species in male reproduction. *Novel Prospects Oxidative Nitrosative Stress* (2018).
41. Espinosa, A., Henríquez-Olguín, C. & Jaimovich, E. Reactive oxygen species and calcium signals in skeletal muscle: A crosstalk involved in both normal signaling and disease. *Cell. Calcium* **60**, 172–179 (2016).
42. Costa, J., Braga, P. C., Rebelo, I., Oliveira, P. F. & Alves, M. G. Mitochondria quality control and male fertility. *Biology* **12**, 827 (2023).
43. Durairajanayagam, D., Singh, D., Agarwal, A. & Henkel, R. Causes and consequences of sperm mitochondrial dysfunction. *Andrologia* **53**, e13666 (2021).
44. Esmaeilkhanian, S., Asadzadeh, N. & Masoudi, R. Flow cytometry study of post-thawed bulk spermatozoa: Mito-TEMPO improves cryopreservation performance by controlling apoptosis rate, DNA fragmentation and ROS production. *Cryobiology* **103**, 147–149 (2021).
45. Swain, D. K. et al. Introduction to the pathways involved in the activation and regulation of sperm motility: A review of the relevance of ion channels. *Anim. Reprod. Sci.* **246**, 107052 (2022).
46. Banday, M. N., Lone, F. A., Rasool, F., Rashid, M. & Shikari, A. Use of antioxidants reduce lipid peroxidation and improve quality of crossbred Ram sperm during its cryopreservation. *Cryobiology* **74**, 25–30 (2017).
47. Bucak, M. N. et al. The effect of antioxidants on post-thawed Angora goat (*Capra hircus ancryrensis*) sperm parameters, lipid peroxidation and antioxidant activities. *Small Ruminant Res.* **89**, 24–30 (2010).
48. Hu, J. H. et al. Effects of Trehalose supplementation on semen quality and oxidative stress variables in frozen-thawed bovine semen. *J. Anim. Sci.* **88**, 1657–1662 (2010).
49. Boe-Hansen, G. B., Fortes, M. S. & Satake, N. Morphological defects, sperm DNA integrity, and protamination of bovine spermatozoa. *Andrology* **6**, 627–633 (2018).
50. Barbagallo, F. et al. Evaluation of sperm mitochondrial function: A key organelle for sperm motility. *J. Clin. Med.* **9**, 363 (2020).


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## Author contributions

M.M.: Conceptualization, Writing – original draft, Writing – review & editing, Supervision. H.M. and M.M.: Carrying out the experiments and collecting the data. S.A.S. and A.R.: Writing–original draft, Conceptualization.

## Declarations

## Competing interests

The authors declare no competing interests.

## Ethics approval and consent to participate

This study is in accordance with the ARRIVE guidelines 2.0. All animal care and procedures were approved by the University Ethical Committee of the University of Tabriz (IR. REC.1401.580). In addition, all methods used in the current study were carried out under the University Review Board and University Ethical Committee of the University of Tabriz guidelines and regulations.

## Additional information

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-95820-2.

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