Original Article

# Mito-TEMPO Protects Buck's Semen Quality During the Cooling Process

**Fatemeh Zarei[1], Nader Asadzadeh[2], Hassan Khamisabadi[2*], Navid Dadashpour Davachi[2] Reza Masoudi[2]**

*1. Department of Animal Science, Faculty of Agriculture, Tabriz University, Tabriz, Iran.*
*2. Animal Science Research Institute of Iran (ASRI), Agricultural Research Education and Extension Organization (AREEO), Karaj, Iran.*

Use your device to scan and read the article online

**How to Cite This Article** Zarei, F., Asadzadeh, N., Khamisabad, H., Dadashpour Davachi, N., & Masoudi, R. (2026). Mito-TEMPO Protects Buck's Semen Quality During the Cooling Process. *Iranian Journal of Veterinary Medicine, 20*(2), 321-328. http://dx.doi.org/10.32598/ijvm.20.2.1005681

http://dx.doi.org/10.32598/ijvm.20.2.1005681

## ABSTRACT

**Background:** Membrane lipid peroxidation is one of the most significant chemical stresses in sperm. However, the utilization of antioxidants in semen diluent protects cells against oxidative damage, which could be a beneficial way to preserve semen quality during the storage process.

**Objectives:** The current study aimed to assess the effect of Mito-TEMPO on quality parameters of buck semen during the cooling process.

**Methods:** In this experiment, 5 Saanen bucks were used, and their semen samples were collected twice a week. After semen collection and primary evaluation, semen samples were diluted and assigned into 5 equal aliquots. Then, the samples were added to extenders containing 0, 1, 10, 100, and 1000 µM Mito-TEMPO (triphenylphosphonium chloride). The prepared samples were stored at 5 ˚C for up to 60 h. Chilled sperm motility, progressive motility (PM), viability, mitochondrial activity, membrane functionality, and malondialdehyde (MDA) concentration were analyzed during 0, 30, and 60 h.

**Results:** In results at time 0, no difference was observed among groups, but at 30 and 60 h storage, higher ($P \leq 0.05$) sperm total motility (TM), PM, mitochondrial activity, membrane functionality, viability, and lower lipid peroxidation were observed in 10 and 100 µM Mito-TEMPO groups than the others.

**Conclusion:** In conclusion, supplementation of buck cooling medium with Mito-TEMPO as a novel mitochondria-targeted antioxidant could be an impressive method to conserve buck's semen quality during the cooling storage process.

**Keywords:** Buck, Chilling, Mito-TEMPO, Spermatozoa

**Article info:**
**Received:** 15 Nov 2024
**Accepted:** 19 Jan 2025
**Publish:** 01 Mar 2026

*** Corresponding Author:**
*Hassan Khamisabadi, Professor.*
***Address:*** *Animal Science Research Institute of Iran (ASRI), Agricultural Research Education and Extension Organization (AREEO), Karaj, Iran.*
***Phone:*** *+98 (26) 34256001*
***E-mail:*** *h.khamisabadi@areeo.ac.ir*

## Introduction

As one of the key stages of assisted reproductive techniques, artificial insemination plays an important role in the optimization of ruminant reproduction. Success in this method depends on semen quality, which relies on the semen processing and the ability of the extenders to maintain sperm characteristics (Zarei et al., 2022). Although semen liquid storage at 5 ˚C for a short time can prolong the lifespan of sperm via decreasing the cell metabolism rate, it also reduces sperm quality and fertility (Zarei et al., 2022; Stefanov et al., 2015). Essentially, the sperm membrane is rich in polyunsaturated fatty acids, making it highly sensitive to lipid peroxidation by reactive oxygen species (ROS) produced during semen storage, which reduces the functional life of spermatozoa (Aitken et al., 2014). It appears that supplementing extenders with exogenous antioxidants could be an effective method to counteract ROS activities (Masoudi et al., 2022).

Mito-TEMPO, as a novel mitochondria-targeted antioxidant, has been used to reduce sperm storage-associated damage. Mito-TEMPO is a compound with superoxide dismutase (SOD)-like activity, which scavenges the superoxide anion (Dikalova et al., 2010). This substance is combined from piperidine nitroxide TEMPO with antioxidant properties and lipophilic cation triphenylphosphonium (TPP+), which accumulates several hundredfold within mitochondria driven by the membrane potential (Trnka et al., 2008). In this way, a large amount of TEMPO antioxidant, which is conjugated with TPP, accumulates at the mitochondria, the main site of ROS production, and protects the cell against oxidative injury (Zarei et al., 2022). Supplementation of semen extender with Mito-TEMPO has been shown to conserve sperm quality in humans (Lu et al., 2018) and rams (Zarei et al., 2022).

Nowadays, soybean lecithin is used commercially as an alternative to egg yolk in semen extenders (Sharafi et al., 2015). The effective components of soybean lecithin, such as low-density lipoproteins and phospholipids, can protect the sperm plasma membrane from damage during the cooling and freezing process (Bergeron & Manjunath, 2006). This non-animal additive can decrease hygienic risks of disease transmission in international semen transport (Chelucci et al., 2015). No study has yet reported on the effect of Mito-TEMPO on the quality of buck semen chilled in an extender containing soybean lecithin. Therefore, the objective of the current study was to determine the effects of different concentrations of Mito-TEMPO on total motility (TM), progressive motility (PM), viability, mitochondrial activity, membrane functionality, and malondialdehyde (MDA) concentration of buck's cooled semen.

## Materials and Methods

### Ethics and used chemicals

The current study was approved by the Research Ethics Committees of the Animal Science Research Institute of Iran. All chemicals were purchased from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany) companies.

### Semen collection and chilling medium preparation

Five Saanen goats (3-4 years old) were used in this study. Semen samples were collected during the breeding season and twice a week by an artificial vagina. Collected samples were transported to the laboratory in a water bath (38 ˚C) for quality evaluation. Only samples with the following parameters were selected for cooling storage: Sperm concentration $\geq 3\times10^9$ sperm/mL, semen volume 1-2 mL, abnormal morphology ≤15% and total sperm motility ≥70%. After evaluation, selected samples were mixed to omit individual differences and diluted in the extender.

The chilling medium was composed of fructose (1.26 g/100 mL), citric acid (1.64 g/100 mL), soybean lecithin (1.5% w/v), Tris (3.07 g/100 mL), streptomycin (1 mg), penicillin (100 IU), and catalase (200 IU/mL). Osmolarity and pH were set at 425 mOsm/kg and 6.8, respectively. Then, different concentrations of Mito-TEMPO were added to this extender, and five experimental groups were prepared as follows: Medium without Mito-TEMPO (MT0), medium containing 1 µM (MT1), 10 µM (MT10), 100 µM (MT100), and 1000 µM (MT1000) Mito-TEMPO. Sperm TM, PM, viability, membrane functionality, mitochondrial activity, and MDA content were assessed at 0 (start time), 30, and 60 h after cooling.

### Chilled semen quality evaluation

Sperm class analysis software (SCA) (Version 5.1; Microptic, Barcelona, Spain) was used to assess sperm TM and PM during 0, 30, and 60 h chilling times. To determine sperm motility parameters, 5 µL of each semen sample was placed onto a pre-warmed chamber slide. Afterward, for each sample, 6 fields containing a minimum of 400 sperm were counted (Masoudi et al., 2022).

**Table 1.** Effects of Mito-TEMPO on buck's chilled sperm TM and PM during 0, 30, and 60 h of storage at 5 ˚C

| Mito-TEMPO | Mean±SE | | | | | |
|---|---|---|---|---|---|---|
| | TM (%) | | | PM (%) | | |
| Concentrations (µM) | 0 h | 30 h | 60 h | 0 h | 30 h | 60 h |
| MT0 | 85±1 | 56±1.3$^{b}$ | 18.1±1.6$^{b}$ | 65.3±0.9 | 19.4±1.2$^{b}$ | 9.5±1.5$^{b}$ |
| MT1 | 85±1 | 57.9±1.3$^{ab}$ | 19.0±1.6$^{b}$ | 64.8±0.9 | 20.1±1.2$^{b}$ | 10±1.5$^{b}$ |
| MT10 | 84.5±1 | 59±1.3$^{a}$ | 22.4±1.6$^{a}$ | 65.1±0.9 | 24.2±1.2$^{a}$ | 14.1±1.5$^{a}$ |
| MT100 | 84±1 | 60.5±1.3$^{a}$ | 23.6±1.6$^{a}$ | 65±0.9 | 25±1.2$^{a}$ | 14.5±1.5$^{a}$ |
| MT1000 | 86±1 | 55.6±1.3$^{b}$ | 16.4±1.6$^{b}$ | 66.2±0.9 | 18.5±1.2$^{b}$ | 9±1.5$^{b}$ |

Note: Different letters within the same column indicate significant differences among the groups (P≤0.05).

The hypo-osmotic swelling test was used to evaluate membrane functionality. This method depends on the resistance of the sperm membrane to stressful conditions in a hypoosmotic medium (Zarei et al., 2022). In this assessment, 5 µL of buck's chilled semen samples were mixed with 50 µL of hypo-osmotic solution and incubated for 30 min. Then, under a phase contrast microscope, 300 sperm cells were counted, and the percentage of sperm cells with swollen and curved tails was recorded as having an intact membrane.

Rhodamine 123 (Rh123; Invitrogen TM, Eugene, OR, USA) and propidium iodide (PI) as fluorescent dye were used to determine mitochondrial activity during a flow cytometry study. To assess mitochondrial activity, 10 µL of Rh123 solution was added to 300 µL of chilled semen and incubated in the dark room (20 min). Then, the semen sample was centrifuged (500 g for 3 min) and then resuspended in 500 µL of Tris buffer. Next, 10 µL of PI was added to the sperm suspension. Using a FACSCalibur flow cytometer (Becton Dickinson, USA), 10000 events were recorded. Sperm cells with a negative signal for PI and a positive signal for Rh123 (Rh123+/PI-) were recorded as sperm with active mitochondria (Heidari et al., 2022).

Lipid peroxidation in buck's chilled semen was assessed via measurement of MDA concentration by reaction of thiobarbituric acid. The MDA content was determined by absorption using a standard curve of MDA equivalent generated through acid-catalyzed hydrolysis of 1,1,3,3-tetramethoxypropane (Sharafi et al., 2015). Diluted semen sample in an amount of 1 mL ($250\times10^{6}$ sperm cells/mL) was mixed with 1 mL of cold 20% (w/v) trichloroacetic acid to precipitate protein. The precipitate was pelleted via centrifuge (900 g for 15 min), and 1 mL of the supernatant was incubated with 1 mL of 0.67% (w/v) thiobarbituric in a water bath (100 ˚C for 10 min). After cooling, the spectrophotometer (UV-1200, Shimadzu, Japan) recorded the absorbance at 532 nm, and the MDA concentration was expressed as nmol/mL.

Eosin–nigrosine staining was used to assess buck's chilled semen viability. The semen sample was mixed with two drops of eosin–nigrosine dye (1.67 g eosin-Y, 10 g nigrosine, and 2.9 g sodium citrate dissolved in 100 mL of distilled water) on a warm slide. Then, this mixture was spread on a second slide, and 200 sperm cells were counted under a phase contrast microscope (×400 magnification). Sperm cells with an unstained head were recorded as live cells, and dead cells were stained with a head (Masoudi et al., 2022).

## Statistical analysis

The Shapiro-Wilk test was used to examine the data for normality, and data analysis was conducted using Proc GLM of SAS software, version 9.1 (SAS Institute, 2002, USA). Statistical differences between groups were determined by Tukey test. Results are presented as Mean±SE.

# Results

### TM and PM

The effects of Mito-TEMPO on buck's chilled sperm motility parameters at 0, 30, and 60 h after cooling are shown in Table 1. At the storage time of 0 hours, no significant difference (P>0.05) was observed among groups. However, during the 30- and 60-h cooling periods, TM and PM in groups receiving 10 and 100 µM Mito-TEMPO were higher (P≤0.05) than in the other groups.

**Table 2.** Effects of Mito-TEMPO on buck's chilled sperm viability and MDA concentration during 0, 30, and 60 h of storage at 5 ˚C

| Mito-TEMPO | Mean±SE | | | | | |
|---|---|---|---|---|---|---|
| | Viability (%) | | | MDA Concentration (nmol/mL) | | |
| Concentrations (µM) | 0 h | 30 h | 60 h | 0 h | 30 h | 60 h |
| MT0 | 85.8±1.1 | 55.2±1.5$^{b}$ | 16.0±2$^{b}$ | 3.15±0.25 | 5.8±0.35$^{b}$ | 9.95±0.57$^{b}$ |
| MT1 | 86.5±1.1 | 58.4±1.5$^{ab}$ | 16.5±2$^{b}$ | 3.1±0.25 | 5.05±0.35$^{ab}$ | 9±0.57$^{ab}$ |
| MT10 | 86.2±1.1 | 60.1±1.5$^{a}$ | 21.0±2$^{a}$ | 3.45±0.25 | 4.65±0.35$^{a}$ | 8.55±0.57$^{a}$ |
| MT100 | 85±1.1 | 61.3±1.5$^{a}$ | 22.6±2$^{a}$ | 3.3±0.25 | 4.4±0.35$^{a}$ | 8±0.57$^{a}$ |
| MT1000 | 87±1.1 | 55.8±1.5$^{b}$ | 14.6±2$^{b}$ | 3.4±0.25 | 6±0.35$^{b}$ | 10.1±0.57$^{b}$ |

Note: Different letters within the same column indicate significant differences among the groups (P≤0.05).

### Viability and MDA concentration

Table 2 summarizes the effects of Mito-TEMPO on buck's chilled sperm viability and lipid peroxidation during cooling storage in media containing different doses of Mito-TEMPO. At first time (0 h), no significant difference (P>0.05) was observed between treatments for sperm viability and MDA concentration. At 30 and 60 h, 10 and 100 µM Mito-TEMPO presented lower (P≤0.05) MDA concentration in contrast with the other groups, but the difference among the other three groups was not significant (P>0.05). Regarding the viability rate, during the 30- and 60-h cooling periods, 10 and 100 µM Mito-TEMPO showed higher viability (P≤0.05) compared to the others.

### Mitochondrial activity and membrane functionality

The data related to the effects of Mito-TEMPO on buck's chilled sperm mitochondrial activity and membrane functionality are shown in Table 3. In both parameters, there were no statistically significant differences (P>0.05) among groups at 0 h storage. However, during the 30- and 60-h cooling periods, mitochondrial activity and membrane functionality were greater (P≤0.05) in the 10 and 100 µM Mito-TEMPO groups compared to the other groups, and the observed differences among the other groups were not significant (P>0.05).

## Discussion

Semen liquid storage is generally associated with biochemical, osmotic, and thermal changes that lead to a decrease in sperm characteristics and reduced fertility (Gibb et al., 2015; Zarei et al., 2022). Some biochemical changes in sperm can be attributed to oxidative stress

**Table 3.** Effects of Mito-TEMPO on buck's chilled sperm mitochondrial activity and membrane functionality during 0, 30, and 60 h of storage at 5 ˚C

| Mito-TEMPO | Mean±SE | | | | | |
|---|---|---|---|---|---|---|
| | Mitochondrial Activity (%) | | | Membrane Functionality (%) | | |
| Concentrations (µM) | 0 h | 30 h | 60 h | 0 h | 30 h | 60 h |
| MT0 | 87.4±1.1 | 56.2±1.6$^{b}$ | 22.5±1.4$^{b}$ | 85±1 | 56±1.8$^{b}$ | 21.5±2$^{b}$ |
| MT1 | 88.5±1.1 | 58.1±1.6$^{ab}$ | 25±1.4$^{ab}$ | 86±1 | 58.4±1.8$^{ab}$ | 23±2$^{ab}$ |
| MT10 | 88±1.1 | 60±1.6$^{a}$ | 26.5±1.4$^{a}$ | 86±1 | 60±1.8$^{a}$ | 25.9±2$^{a}$ |
| MT100 | 87.2±1.1 | 60.5±1.6$^{a}$ | 26.9±1.4$^{a}$ | 85.5±1 | 60.5±1.8$^{a}$ | 26.8±2$^{a}$ |
| MT1000 | 89±1.1 | 56.9±1.6$^{b}$ | 23.4±1.4$^{b}$ | 86.8±1 | 55.5±1.8$^{b}$ | 21.5±2$^{b}$ |

Note: Different letters within the same column indicate significant differences among the groups (P≤0.05).

because sperm antioxidant enzymes lack the capacity to sustain sperm quality against excessive ROS generation (Kasimanickam et al., 2006). In this situation, using exogenous antioxidants as compounds that suppress the formation of ROS and counteract their actions (Bansal & Bilaspuri, 2011) could be an efficient method to preserve sperm against storage shocks and damage (Asl et al., 2018). ROS scavenging mitochondria-targeted antioxidants, such as Mito-TEMPO, with high efficiency, wide application, and low toxicity are potential candidates to protect sperm from damage caused by storage (Oyewole & Birch-Machin, 2015). There is no information about the effects of Mito-TEMPO on buck semen in medium containing soybean lecithin during liquid storage. Therefore, the current study was conducted to evaluate the impact of this antioxidant on buck's chilled semen quality parameters during 60 hours of storage at 5 °C. Using Mito-TEMPO (10 and 100 µM) effectively protected semen quality, resulting in higher TM, PM, membrane functionality, mitochondrial activity, and viability, and a lower MDA concentration. However, the efficiency of 60 h-stored semen samples was lower than that of 30 h-stored samples. Indeed, with increasing storage time, the sperm quality parameters decreased. The results of this experiment are in agreement with our previous studies on cooling storage of ram (Zarei et al., 2022).

Mitochondria, as an important intracellular organelle, regulate bioenergetic metabolism and provide ATP via oxidative phosphorylation for sperm function (Cabezas-Opazo et al., 2015). This activity correlates with sperm quality parameters and semen storage potential (Chen et al., 2014). The accumulation of toxic products, such as ROS, to high concentrations that are formed from the sperm's metabolism in the cooling process, leads to oxidative stress and mitochondrial inefficiency to supply the required energy for spermatozoa (Agarwal & Prabakaran, 2005).

Mitochondria-targeted antioxidant, Mito-TEMPO, comprised of a lipophilic triphenylphosphonium (TPP) moiety and piperidine nitroxide TEMPO (Lu et al., 2018). This compound is a novel, cell-permeable antioxidant because the TPP unit, with its positive charge, enables Mito-TEMPO to pass through cell lipid bilayer membranes rapidly and accumulate 1000-fold in the mitochondrial matrix, the major site of ROS generation (Jiang et al., 2015). The TEMPO unit catalyzes the dismutation of superoxide and reacts directly with many radicals (Yang et al., 2018). TEMPO, through its hydroxylamine-like structure, inhibits the excessive generation and overflow of oxygen-free radicals by converting superoxide anions into hydrogen peroxide and oxygen. The nitroxide radicals produced act as SOD to maintain the stability of the phospholipid bilayer membrane and the electron transport chain (Du et al., 2017). Therefore, this ROS scavenging property can reduce the rate of lipid peroxidation, helping sperm cells remain safe against oxidative stress (Lu et al., 2018). In the current study, concentrations of 10 and 100 µM Mito-TEMPO decreased the MDA content, an indicator of lipid peroxidation.

Furthermore, Mito-TEMPO exerts a targeted antioxidant effect, as well as regulating antioxidant-related enzyme activities (Trnka et al., 2009). Phosphohexose isomerase (GPI), a ubiquitous cytosolic enzyme, plays a critical role in providing ATP via glycolytic and gluconeogenic pathways and is closely related to sperm quality. This soluble enzyme is loosely bound to the sperm mitochondria (Jiang et al., 2015), so it releases easily into the extracellular space, which may induce a reduction in sperm motility, membrane functionality, and viability (Harrison & White, 1972). One of the possible mechanisms by which Mito-TEMPO affects sperm could be attributed to regulating GPI activity, which is reduced during semen manipulation. However, this reduction would be controlled by Mito-TEMPO, which improves sperm energy metabolism and conserves sperm quality parameters during the cooling process (Jiang et al., 2015). According to our findings in this work, Mito-TEMPO can relieve the decline in buck's sperm motility, viability, membrane functionality, and mitochondrial activity during the cooling process.

## Conclusion

Supplementation of buck's semen extender with 10 and 100 µM Mito-TEMPO by maintenance of mitochondrial activity preserved the sperm quality parameters after the cooling process. Therefore, adding mitochondria-targeted antioxidant Mito-TEMPO to the soybean lecithin-based chilling media is an efficient method for retaining buck semen quality for artificial insemination.

## Ethical Considerations

### Compliance with ethical guidelines

This study was approved by the Research Ethics Committees of the Animal Science Research Institute of Iran (ASRI), Karaj, Iran.

## Funding

This research was supported by a research project, funded by the Animal Science Research Institute of Iran (ASRI), Karaj, Iran (Project No.: 175922).

## Authors' contributions

Conceptualization, supervision, and validation: Nader Asadzadeh and Hassan Khamisabadi; Methodology and data curation: Navid Dadashpour Davachi and Reza Masoudi; Investigation and writing the original draft: Fatemeh Zarei; Review and editing: Nader Asadzadeh and Hassan Khamisabadi, Navid Dadashpour Davachi, and Reza Masoudi.

## Conflict of interest

The authors declared no conflict of interest.

## Acknowledgments

The authors would like to acknowledge Animal Science Research Institute of Iran (ASRI) for supporting this study.

## References

Agarwal, A., & Prabakaran, S. A. (2005). Mechanism, measurement, and prevention of oxidative stress in male reproductive physiology. *Indian Journal of Experimental Biology, 43*(11), 963-974. [PMID]

Aitken, R. J., Smith, T. B., Jobling, M. S., Baker, M. A., & De Iuliis, G. N. (2014). Oxidative stress and male reproductive health. *Asian Journal of Andrology, 16*(1), 31-38. [DOI:10.4103/1008-682X.122203] [PMID]

Bansal, A. K., & Bilaspuri, G. S. (20110). Impacts of oxidative stress and antioxidants on semen functions. *Veterinary Medicine International, 2010,* 686137. [DOI:10.4061/2011/686137] [PMID]

Bergeron, A., & Manjunath, P. (2006). New insights towards understanding the mechanisms of sperm protection by egg yolk and milk. *Molecular Reproduction and Development, 73*(10), 1338-1344. [DOI:10.1002/mrd.20565] [PMID]

Cabezas-Opazo, F. A., Vergara-Pulgar, K., Pérez, M. J., Jara, C., Osorio-Fuentealba, C., & Quintanilla, R. A. (2015). Mitochondrial Dysfunction Contributes to the Pathogenesis of Alzheimer's Disease. *Oxidative Medicine and Cellular Longevity, 2015,* 509654. [DOI:10.1155/2015/509654] [PMID]

Chelucci, S., Pasciu, V., Succu, S., Addis, D., Leoni, G. G., & Manca, M. E., et al. (2015). Soybean lecithin-based extender preserves spermatozoa membrane integrity and fertilizing potential during goat semen cryopreservation. *Theriogenology, 83*(6), 1064-1074. [DOI:10.1016/j.theriogenology.2014.12.012] [PMID]

Chen, P. I., Cao, A., Miyagawa, K., Tojais, N. F., Hennigs, J. K., & Li, C. G., al. (2017). Amphetamines promote mitochondrial dysfunction and DNA damage in pulmonary hypertension. *JCI Insight, 2*(2). [DOI:10.1172/jci.insight.90427]

Dikalova, A. E., Bikineyeva, A. T., Budzyn, K., Nazarewicz, R. R., McCann, L., & Lewis, W., et al. (2010). Therapeutic targeting of mitochondrial superoxide in hypertension. *Circulation Research, 107*(1), 106–116. [DOI:10.1161/CIRCRESAHA.109.214601] [PMID]

Du, K., Farhood, A., & Jaeschke, H. (2017). Mitochondria-targeted antioxidant Mito-Tempo protects against acetaminophen hepatotoxicity. *Archives of Toxicology, 91*(2), 761-773. [DOI:10.1007/s00204-016-1692-0] [PMID]

Esmaeilkhanian, S., Asadzadeh, N., & Masoudi, R. (2023). Flow cytometry study of post-thawed buck spermatozoa: Mito-TEMPO improves cryopreservation performance by controlling apoptosis rate, DNA fragmentation and ROS production. *Cryobiology, 110,* 108-110. [DOI:10.1016/j.cryobiol.2022.11.238]

Gibb, Z., Lambourne, S. R., Quadrelli, J., Smith, N. D., & Aitken, R. J. (2015). L-carnitine and pyruvate are prosurvival factors during the storage of stallion spermatozoa at room temperature. *Biology of Reproduction, 93*(4), 104. [DOI:10.1095/biolreprod.115.131326] [PMID]

Harrison, R. A., & White, I. G. (1972). Glycolytic enzymes in the spermatozoa and cytoplasmic droplets of bull, boar and ram, and their leakage after shock. *Journal of Reproduction and Fertility, 30*(1), 105–115. [DOI:10.1530/jrf.0.0300105] [PMID]

Heidari, M., Qasemi-Panahi, B., Moghaddam, G., Daghigh-Kia, H., & Masoudi, R. (2022). L-carnitine improves quality parameters and epigenetic patterns of buck's frozen-thawed semen. *Animal Reproduction Science, 247,* 107092. [DOI:10.1016/j.anireprosci.2022.107092] [PMID]

Jiang, X. P., Wang, S. Q., Wang, W., Xu, Y., Xu, Z., & Tang, J. Y., et al. (2015). Enolase1 (ENO1) and glucose-6-phosphate isomerase (GPI) are good markers to predict human sperm freezability. *Cryobiology, 71*(1), 141-145. [DOI:10.1016/j.cryobiol.2015.04.006] [PMID]

Kasimanickam, R., Pelzer, K. D., Kasimanickam, V., Swecker, W. S., & Thatcher, C. D. (2006). Association of classical semen parameters, sperm DNA fragmentation index, lipid peroxidation and antioxidant enzymatic activity of semen in ramlambs. *Theriogenology, 65*(7), 1407-1421. [DOI:10.1016/j.theriogenology.2005.05.056] [PMID]

Lu, X., Zhang, Y., Bai, H., Liu, J., Li, J., & Wu, B. (2018). Mitochondria-targeted antioxidant MitoTEMPO improves the post-thaw sperm quality. *Cryobiology, 80,* 26-29. [DOI:10.1016/j.cryobiol.2017.12.009] [PMID]

Masoudi, R., Esmaeilkhanian, S., Sharafi, M., Abdollahi, Z., Jafari, V., & Hatefi, A., et al. (2022). Cysteamine enhances quality and fertility potential of rooster semen in cooled storage. *Theriogenology, 177,* 29-33. [DOI:10.1016/j.theriogenology.2021.09.023] [PMID]

Oyewole, A. O., & Birch-Machin, M. A. (2015). Mitochondria-targeted antioxidants. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology, 29*(12), 4766–4771.[DOI:10.1096/fj.15-275404] [PMID]

Safari Asl, R., Shariatmadari, F., Sharafi, M., Karimi Torshizi, M. A., & Shahverdi, A. (2018). Dietary fish oil supplemented with vitamin E improves quality indicators of rooster cold-stored semen through reducing lipid peroxidation. *Cryobiology, 84,* 15–19. [DOI:10.1016/j.cryobiol.2018.08.008] [PMID]

Sharafi, M., Zhandi, M., & Akbari Sharif, A. (2015). Supplementation of soybean lecithin-based semen extender by antioxidants: complementary flowcytometric study on post-thawed ram spermatozoa. *Cell and Tissue Banking, 16*(2), 261–269. [DOI:10.1007/s10561-014-9458-5] [PMID]

Stefanov, R. G., Anev, G., & Abadjieva, D. V. (2015). Effect of different extenders and storage periods on motility and fertility of ram sperm. *Macedonian Veterinary Review, 38*(1), 85-89. [DOI:10.14432/j.macvetrev.2014.12.036]

Trnka, J., Blaikie, F. H., Logan, A., Smith, R. A., & Murphy, M. P. (2009). Antioxidant properties of MitoTEMPOL and its hydroxylamine. *Free Radical Research, 43*(1), 4-12. [DOI:10.1080/10715760802582183] [PMID]

Yang, S. G., Park, H. J., Kim, J. W., Jung, J. M., Kim, M. J., & Jegal, H. G., et al. (2018). Mito-TEMPO improves development competence by reducing superoxide in preimplantation porcine embryos. *Scientific Reports, 8*(1), 10130. [DOI:10.1038/s41598-018-28497-5] [PMID]

Zarei, F., Daghigh-Kia, H., & Masoudi, R. (2022). Supplementation of ram's semen extender with Mito-TEMPO II: Quality evaluation and flow cytometry study of post-thawed spermatozoa. *Andrologia, 54*(1), e14299. [DOI:10.1111/and.14299] [PMID]

This Page Intentionally Left Blank