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REVIEW ARTICLE

Functional Additives in Equine Semen Extenders for Cryopreservation: Current Evidence and Future Perspectives

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A B S T R A C T

Cryopreservation of equine semen is an important technique for effective modern breeding programs. Stallion spermatozoa are inherently sensitive to cooling and cryopreservation, during which they are exposed to various stressors that affect both viability and post-thaw functional capabilities. In particular, oxidative stress, osmotic stress, membrane destabilization, and mitochondrial dysfunction are well-established critical issues, and thus numerous compounds have been evaluated as additives in semen extenders to support protective preservation and maintain post-thaw sperm quality and fertility. The current knowledge about protective components in seminal plasma and the effects of several bioactive compounds as alternatives or adjuncts to conventional extenders for equine semen extenders have been discussed in the present review. The main groups of additives for equine semen extenders, their modes of action, biological efficacy, and limitations were also examined. Antioxidants (enzymatic and non-enzymatic), and mitochondrial-targeted antioxidants (MTAs), such as melatonin and MitoQ, provide a recent perspective as antioxidants that protect sperm mitochondria and sustain their bioenergetics. Structural additives such as trehalose, cholesterol, and cholesterol-loaded cyclodextrin have also shown encouraging results. Despite significant advances in seminal extender development, extender additives generally lack consistency in achieving improved sperm characteristics and protection. However, some novel additives are on the horizon with great potential. Recently, advances in nanotechnology have led to the development of novel delivery systems for reproductive additives. Although mostly at experimental stage, these methodologies have the potential for targeted delivery and sustained protection of stallion spermatozoa. Besides optimizing semen storage conditions, further improvement of semen preservation relies on additive optimization of semen extenders and, on the development of well-balanced, species-specific, and individually optimized semen extenders, supplemented by current research methods and delivery systems.

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INTRODUCTION

Horses and other equids have played an important part in the development of civilizations throughout the world over the past few thousand years. In addition to having been ridden or driven for centuries, horses were central to both agriculture and warfare, while donkeys and zebras were both used in agriculture. This contribution continues in sport (horse riding), recreational activities, therapy (equine therapy) and in the field of veterinary medicine as a model species for physiology and reproduction (Klecel and Martyniuk, 2021). In veterinary research, equine orthopedics, reproduction, genetics and health are important for advancing both equine welfare and research in animal science and biotechnologies.

Semen preservation is a critical component of current reproductive biotechnology in livestock breeding including equines, as it allows the distribution of genetics, facilitates breed improvement, and allows long-term storage of valuable stallion genetics. With advances in semen technologies, stallion sperm are becoming increasingly amenable to preservation, particularly

through cooling and cryopreservation methods (Al-Kass and Morrell, 2024). However, there are still inherent limitations associated with the preservation of stallion sperm due to the specialized nature of their membranes, which are inherently deficient in cholesterol and contain high levels of polyunsaturated fatty acids that are susceptible to oxidation and structural damage during preservation (Ullah *et al.,* 2025).

During preservation, spermatozoa encounter many stresses, including osmotic stress, pH stress, energy stress, cold shock, and cryodamage following the process of freezing and thawing (Raheja *et al.,* 2018). These stresses induce membrane fluidization, loss of mitochondrial function and DNA fragmentation, which together result in compromised fertilization capability of spermatozoa (Amrozi *et al.,* 2024). To overcome these adverse effects, semen is diluted using the semen extenders that provide a protective medium and facilitate the maintenance of the viability and functionality of spermatozoa during cryopreservation (Bustani and Baiee, 2021).

In addition to the extender composition used for fresh semen extension, some specific additives are incorporated into the extender during the process of semen cryopreservation (Koca *et al.,* 2024). These are intended to improve post-thaw semen quality by acting through different mechanisms to safeguard sperm against cryoinjury (Sohail *et al.,* 2025). Based on their mechanism of action, these additives are categorized into several groups, including antioxidants, antibiotics, cryoprotectants, energy substrates, membrane stabilizers, osmo-protectants, and others. In recent years, numerous studies have focused on the development and evaluation of novel additives to improve freezing-thawing survivability of stallion spermatozoa. Commonly used additives include antioxidants to counteract oxidative stress damage from ROS (Abdel-Khalek *et al.,* 2022; Gao *et al.,* 2025) and antibiotics to preclude extrinsic bacterial contamination of the sample, as well as potential contamination during collection. A critical class of additives is cryoprotectants that preclude excessive water loss and help maintain the membrane integrity and protect spermatozoa from osmotic stress during freezing and thawing. In addition, they possess protein-stabilizing properties and inhibit the formation of ice crystals in the semen to limit damage to the spermatozoa. Additional additives such as membrane stabilizers are incorporated into the extenders to protect the plasma membrane and preserve physiological functions of spermatozoa (Belala *et al.,* 2026).

At present, the additives used for equine semen preservation have varying levels of success rates (Raheja *et al.,* 2018). As a result of the many variables involved in equine semen preservation, including experimental design, extender components, and stallion biology, there is a need for a detailed understanding about these additives, including their mechanisms of action, limitations, and possible interactions.

This review provides an updated evaluation of the most commonly used classes of additives incorporated in equine semen extenders, with emphasis on the mechanisms involved, the degree to which they increase sperm functionalities, and their limitations. The description also extends to new trends, which include mitochondrial-specific antioxidants and nanostructured delivery systems, with the potential to overcome the issues and problems associated with conventional methods of equine semen cryopreservation and related technologies.

Additives for equine semen extenders: A wide range of additives has been investigated to improve equine semen preservation, targeting oxidative stress, membrane stability and cellular metabolism. These additives can be categorized as: enzymatic antioxidants, non-enzymatic antioxidants, mitochondrial-targeted antioxidants, cryoprotective and structural additives, membrane stabilizers and emerging innovations.

Cryopreservation is a stressful process for sperm, as it is associated with increased production of ROS (Castro *et al.,* 2025). An excess of ROS overwhelms natural antioxidant defenses of the cell, leading to oxidative stress. This damages cellular components through a process known as lipid peroxidation, in which free radicals attack and degrade the polyunsaturated fatty acids (PUFAs) present in the sperm plasma membrane, leading to DNA fragmentation and spermatozoa apoptosis (Mendoza-Viveros *et al.,* 2022; Catalan *et al.*, 2024), as has been shown in Fig. 1.

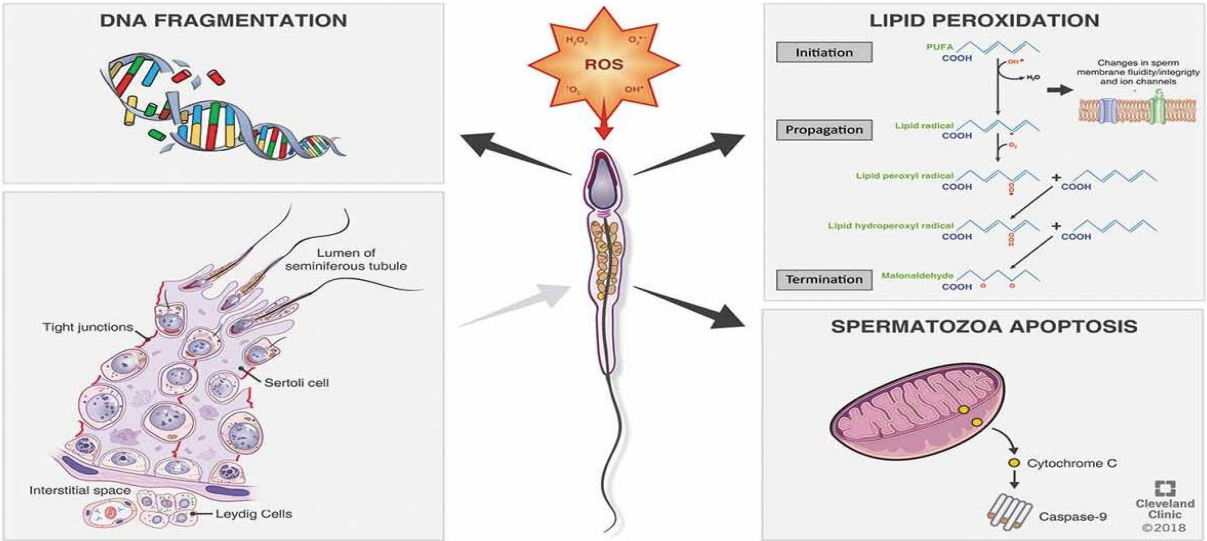

Fig. 1: Illustration of the mechanism of generation of ROS in spermatozoa (Dutta *et al.,* 2019).

Enzymatic Antioxidants

Superoxide dismutase, catalase and glutathione peroxidase enzymatic triad: Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) enzymes naturally exist in the seminal plasma of stallions. They help neutralize reactive ROS by dismutation of superoxide radicals and decomposing hydrogen peroxide and hydroperoxides into harmless molecules (Wang *et al.,* 2025). Their coordinated action provides a line of defense by targeting different ROS molecules. Superoxide dismutase initiates this process by converting superoxide radicals (O_2-) into hydrogen peroxide (H_2O_2) and oxygen (Schanne *et al.,* 2024; Fan *et al.,* 2025). While H_2O_2 is less reactive, it remains harmful if not quickly eliminated. Catalase takes over to prevent H_2O_2 accumulation and its subsequent conversion into the cytotoxic hydroxyl radical (OH) via the Fenton reaction (Wang *et al.,* 2025). It decomposes H_2O_2 into water (H_2O) and molecular oxygen (Benko *et al.,* 2022). The GPX enzyme then uses reduced glutathione (GSH) as an electron donor to reduce not only H_2O_2 but also a broad range of lipid hydroperoxides (LOOH) into their corresponding less harmful alcohols (Kowalczyk, 2022). These LOOHs are key intermediates in the propagation of lipid peroxidation, which induces sperm plasma membrane degradation (Kontogianni and Gerothanassis, 2022).

In stallions, the main isoform of SOD is Copper/Zinc superoxide dismutase (Cu/Zn-SOD or SOD1), which is located in the cytosol (Neuman *et al.,* 2025). However, studies have clearly shown that the amount of SOD1 decreases markedly during cryopreservation (Gaitskell-Phillips *et al.,* 2021). The endogenous CAT, on the other hand, does not appear to change during this process. Among GPX isoforms, GPX4 is considered to be the most important for male fertility due to its unique ability to protect cell membranes from ROS-induced damage (Scarlata and O'Flaherty, 2020). It is present in the mitochondria and helps protect this vital energy-producing organelle, while its presence near the chromatin helps guard sperm DNA. The activity of GPX depends on selenium, an essential cofactor incorporated into this enzyme as selenocysteine, which highlights the importance of selenium in the overall antioxidant strategy for semen preservation (Zečević *et al.,* 2026).

Superoxide dismutase supplementation at 25-50IU/mL in chilled equine semen extenders significantly boosts progressive sperm motility ($P<0.01$) and viability ($P<0.001$) after 24-72h at 5°C, while reducing extracellular signal-regulated kinase (ERK) phosphorylation as an oxidative stress marker. Higher seminal plasma SOD activity correlates with better sperm cryotolerance in stallions, though direct addition to freezing extenders lacks specific equine trials (Papas *et al.,* 2019). However, direct supplementation of purified SOD into equine freezing extenders has not consistently improved post-thaw sperm motility or viability in controlled trials, likely due to stability, penetration and cost issues (Papas *et al.,* 2019).

In chilled equine semen, adding around 15IU/mL of SOD, CAT, and GPX to the semen extender (INRA 96) reduced caspase-3 activity and led to better sperm motility and viability than control samples during refrigerated storage, in which it did not prevent but significantly reduced the DNA damage (Del Prete *et al.,* 2019). In other species including bulls and rams, CAT supplementation of extenders during freezing improved sperm quality, but in equine studies, the effect on cryopreserved sperm remains limited for key parameters such as progressive sperm motility and fertility (Moreira *et al.,* 2024).

MnTBAP: MnTBAP is a synthetic metalloporphyrin that mimics the activity of SOD, an endogenous enzyme that catalyzes the dismutation of superoxide radicals into hydrogen peroxide and oxygen (Uribe *et al.,* 2020; Zheng *et al.,* 2023). By scavenging superoxide, it reduces overall ROS load, thereby limiting lipid peroxidation of sperm membranes, mitochondrial damage, and oxidative DNA breakage in stallion sperm during freezing-thawing and subsequent *in vitro* incubation (Shojaeian *et al.,* 2018; Treulen *et al.,* 2020; Zheng *et al.,* 2023).

In equine sperm, MnTBAP showed effectiveness for its cell permeability; it can get inside the spermatozoon, as well as the extracellular milieu (Shojaeian *et al.,* 2018; Uribe *et al.,* 2020). It is associated with enhanced preservation of sperm plasma membrane integrity and fluidity, which is a crucial condition for equine sperm (Treulen *et al.,* 2020). According to Shojaeian *et al.* (2018), supplementation of semen extender with 20µM of MnTBAP improved total and progressive sperm motility, viability and membrane integrity post-thaw compared to control and lower doses; higher concentrations did not improve the quality but adversely affected sperm quality parameters.

Paraoxonase type-1: Paraoxonase-1 (PON-1), an HDL-associated enzyme, plays a key role in ROS control by hydrolyzing oxidized phospholipids and peroxides (Jakubowski, 2023). In equine reproductive biology, PON-1 seems to be a promising antioxidant, since horse spermatozoa possess membranes rich in polyunsaturated fatty acids (PUFA), making them particularly vulnerable to lipid peroxidation induced by ROS (Rossi *et al.,* 2018). Its mechanism of action relies on degrading oxidized phospholipids and oxidized esters in lipoproteins, thereby preventing propagation of lipid peroxidation chain (García-Heredia *et al.,* 2013). Paraoxonase-1 directly reduces linoleate hydroperoxides and other lipid peroxides through its enzymatic activity. It binds to HDL via hydrophobic N-terminus and ApoA-I interactions. This complex prolongs the lag phase before LDL/LDL oxidation reduces the formation of malondialdehyde and other aldehydes, as well as preservation of membrane fluidity and function (Lucca *et al.,* 2022).

While no study directly evaluated PON-1 as an exogenous additive in equine semen extenders, its natural role suggests its potential benefits if supplemented. According to Catalán *et al.* (2022), PON-1 performed well in combination with other enzymatic antioxidants like SOD and non-enzymatic antioxidants, as measured by Trolox equivalent antioxidant capacity (TEAC). These workers also suggested that PON-1 could be one of the main antioxidant enzymes for supplementation to semen extenders of males of this species.

Non-enzymatic Antioxidants

Vitamin E: Among the non-enzymatic antioxidants investigated for inclusion in semen extenders for various

species, vitamin E is the most studied one. Its role is based on its unique chemical property as a fat-soluble antioxidant, which allows it to integrate directly into the lipid bilayers of cellular membranes, where it acts as a chain breaker (Guseva *et al.,* 2024; Khan *et al.,* 2025). Its primary mechanism of action is the interception of lipid peroxyl radicals (LOO•; Guseva *et al.,* 2024). By donating a hydrogen atom from its phenolic hydroxyl group to the peroxyl radical, vitamin E neutralizes peroxyl radical, forming a stable lipid hydroperoxide (LOOH) and, in turn, generating a tocopheroxyl radical. This tocopheroxyl radical is resonance-stabilized, making it relatively unreactive and unable to abstract a hydrogen atom from another PUFA, thereby effectively terminating the chain reaction and protecting structural integrity of the membrane (Guseva *et al.,* 2024). This mechanism makes it an ideal candidate for defending the lipid-rich plasma membrane against oxidative damage. According to Rossi *et al.* (2016), adding vitamin E to semen cryopreservation extenders generally improves post-thaw sperm motility; however, such results were reported only in other species such as canines and bovines, whereas stallion semen showed non-significant improvement in either sperm total motility, progressive motility, or velocity parameters following supplementation of vitamin E to semen extenders.

The restricted effectiveness of vitamin E relies on the combination of physiological limitations in accessing the sperm membrane and high individual variability among stallions (Ullah *et al.,* 2025). Moreover, abundance of polyunsaturated fatty acids in the plasma membranes of stallion sperm make them prone to lipid peroxidation from oxidative stress during freezing-thawing. Vitamin E alone often proves to be inefficient in stallions because of the fact that equine sperm have elevated mitochondrial activity compared to other species, amplifying ROS production and overwhelming standard vitamin E levels.

Commercial extenders may already contain optimal antioxidants, negating the beneficial effects of added vitamin E. Moreover, benefits of vitamin E supplementation appear to be higher in cooled storage than in cryopreserved semen. Synergistic effects also emerge with combinations like vitamin E + selenium, improving sperm motility in fresh or cooled semen but not always in frozen-thaw semen quality parameters.

Vitamin C: Vitamin C (ascorbic acid) has been studied as an antioxidant additive in equine semen extenders. Still, the results for cryopreservation are mixed and sometimes even negative compared with the control groups. Alamaary *et al.* (2020) added both cysteine and ascorbic acid to a HF-20 extender and recorded decreased post-thaw sperm motility and altered enzyme markers (alkaline phosphatase, lactate dehydrogenase and gamma-glutamyl transferase, suggesting that these antioxidants at the tested doses did not protect stallion sperm and might even be detrimental during cryopreservation. In contrast, a study on cooled equine semen demonstrated that adding ascorbate-based antioxidants reduced lipid peroxidation and improved total sperm motility during 72h of storage at 5°C, indicating a protective effect of ascorbate-based antioxidants on stallion semen stored in liquid form at 5°C (Sampaio *et al.,* 2018).

Ascorbic acid has been shown to lower the levels of enzymes such as alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and gamma-glutamyl transferase (GGT) in frozen-thawed semen, which are associated with sperm motility and viability. This decline in levels of ALP, LDH and GGT signals metabolic interference in sperm, making them more vulnerable to freeze-thaw damage (Alamaary *et al.,* 2020). In contrast, cooled semen stored at 5°C is exposed to a gradual buildup of ROS from ongoing metabolism and seminal plasma effects, where vitamin C effectively neutralizes free radicals and maintains membrane integrity over 24-72 hours (Silvestre *et al.,* 2021). The possible antioxidant defense mechanisms described above have been summarized diagrammatically in Fig. 2.

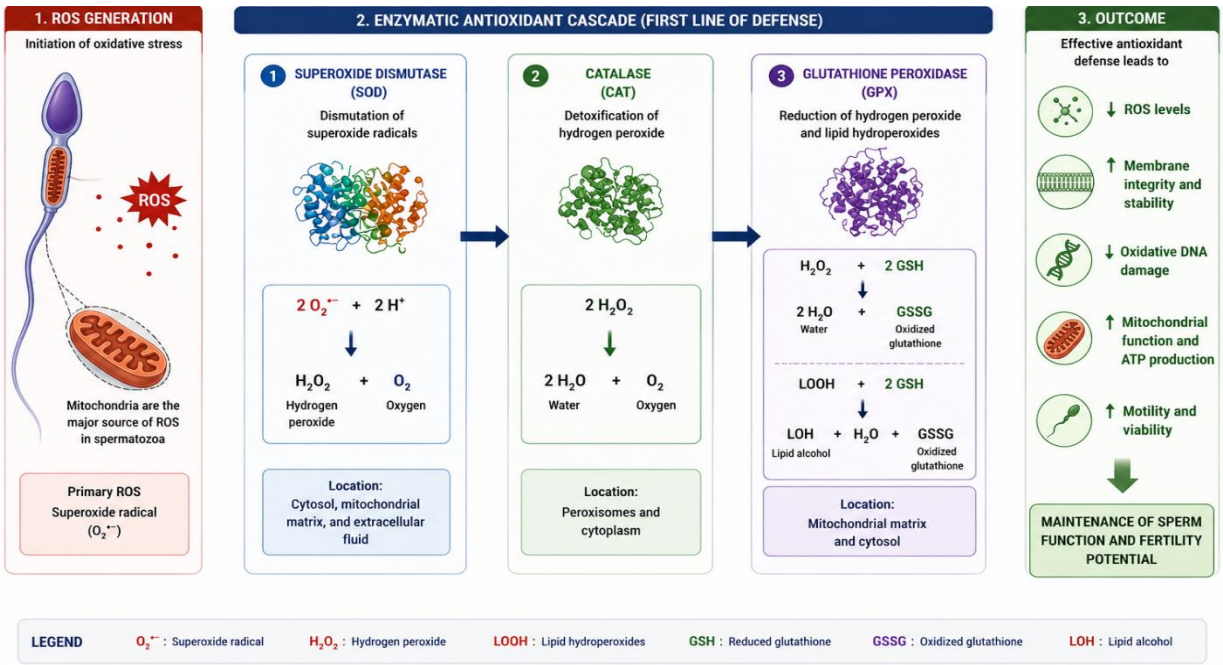

Fig. 2: Diagrammatic illustration of antioxidant defense system in sperm.

Mitochondrial-Targeted Antioxidants: While conventional antioxidants act broadly to neutralize reactive oxygen species, recent strategies focus on targeting their main intracellular source. Mitochondrial-targeted antioxidants have gained attention as a promising approach to directly preserve mitochondrial function and improve sperm energy during cryopreservation. Mitochondrial-targeted antioxidants include melatonin, MitoQ, MitoE/MitoE2 and Mito-TEMPO.

Melatonin: Melatonin is an amphiphilic indoleamine, synthesized in the pineal gland and characterized by its strong antioxidant properties. It is considered as a terminal antioxidant because it irreversibly neutralizes reactive oxygen and nitrogen species (ROS/RNS) through a cascade of reactions, where a single molecule can scavenge multiple radicals. Melatonin provides cyto-protection by a dual-action mechanism: it performs direct radical scavenging while also acting as a molecular regulator that enhances the expression of endogenous antioxidant enzymes. This combined action effectively attenuates oxidative stress-induced cellular damage (Kołodziejska *et al.,* 2025). Melatonin penetrates sperm cells to accumulate in mitochondria, thereby preventing lipid peroxidation and assuring maintenance of membrane fluidity during cooling or freezing. It reduces ROS damage, thereby preserves sperm motility, viability and acrosome integrity in different species including human (Karimfar *et al.,* 2015), buffalo (Inyawilert *et al.,* 2021), giant grouper-*Epinephelus lanceolatus* (Yang *et al.,* 2023) and brown-marbled grouper-*Epinephelus fuscoguttatus* (Zhang *et al.,* 2026).

Its equine semen, the effectiveness of melatonin was recorded by Nogueira *et al.* (2022), who reported an outperformance of the melatonin-supplemented semen samples compared to control samples and those with other additives. In brief, melatonin is highly promising as an extender additive for equine semen preservation, especially in cryopreservation protocols, due to its consistent protection against oxidative stress. Combination of melatonin with Coenzyme Q10 (CoQ10) further improves its effectiveness, suggesting its potential for optimized commercial extenders.

MitoQ: MitoQ is an idebenone derivative covalently linked to a lipophilic triphenyl-phosphonium cation. It has a unique ability for selective accumulation inside sperm mitochondria, where it repeatedly scavenges ROS without being rapidly consumed (Masoudi *et al.,* 2024). When added to semen extender, it acts like a recyclable mitochondrial antioxidant, aiming to protect sperm bioenergetics and motility during cold shock and freezing-thawing by maintaining the stability of the mitochondrial membrane. It can block the mitochondrial permeability transition pores (mPTPs) from opening, and inhibit cytochrome C release and caspase activation. Ultimately, it safeguards mitochondrial DNA while supporting ATP production (Farshad and Wehrend, 2025). A recent study by Elkhawagah *et al.* (2024a) has shown that MitoQ additive at concentrations of 25, 50, and 100nM improved total sperm motility, as well as sperm amplitude of lateral head displacement, while a negative effect was recorded at the highest concentration of 200nM.

In other species, such as male goats, supplementation of semen extender with MitoQ increased total and progressive sperm motility, viability, membrane function and acrosome integrity during cryopreservation (Hatami *et al.,* 2024). In contrast, bull semen showed a slight increase in ROS, suggesting that MitoQ has no beneficial effects on semen quality, and its application may not be of any use for semen preservation in this species (Farshad and Wehrend, 2025).

MitoE/MitoE2: MitoE is a specialized compound produced by attaching vitamin E (alpha-tocopherol) to a TPP+ molecule. This structure allows it to specifically build up and target inside mitochondria by taking advantage of the negative electrical charge of the inner membrane (Nahar and Sohag, 2025). A related compound, MitoE2, is a phosphate ester that dissolves much more easily in water. Once inside the cell, it is converted into an active form of MitoE. MitoE2 is frequently the top choice for laboratory studies because its high solubility lowers the risk of it accidentally acting as a harmful pro-oxidant at larger doses (Leo *et al.,* 2008).

At physiological doses, both MitoE and MitoE2 safely decrease mitochondrial hydrogen peroxide production and lipid peroxidation without triggering unwanted pro-oxidant effects, which makes them excellent for treating mitochondrial stress (Nahar and Sohag, 2025). However, to date, no specific equine study has documented the use of MitoE or MitoE2 additives in an equine semen extender.

Mito-TEMPO: Mito-TEMPO is a specialized piperidine nitroxide linked to a lipophilic triphenyl-phosphonium cation; its structure enables its accumulation within sperm mitochondria. By functioning as a superoxide dismutase (SOD) mimetic, it neutralizes mitochondrial superoxide, thereby mitigating lipid peroxidation, genomic damage, and the dissipation of mitochondrial membrane potential (Kumar *et al.,* 2021; Elkhawagah *et al.,* 2024b).

Research in ruminants and bulls indicates that adding 5-50μM Mito-TEMPO to semen extenders consistently bolsters post-thaw sperm motility, membrane stability, and overall fertility while suppressing oxidative stress. Specifically, in beef bulls, Mito-TEMPO concentrations of 10-40μM have been shown to significantly enhance sperm kinematics, vitality, and the integrity of both the acrosome and DNA (Kumar *et al.,* 2021; Kumar *et al.,* 2022; Elkhawagah *et al.,* 2024b). Currently, no full-length equine-only studies reported the inclusion of MitoTEMPO additive in equine extenders.

Biochemical properties and biological effects of the main mitochondrial-targeted antioxidants are summarized in Table 1. Moreover, mechanisms by which mitochondrial-targeted antioxidants mitigate oxidative stress at the mitochondrial level are summarized in Fig. 3.

Beyond oxidative stress, structural destabilization of the sperm membrane represents a major factor limiting cryo-survival. Consequently, a range of cryoprotective and membrane-stabilizing additives have been developed to maintain membrane integrity during thermal stress.

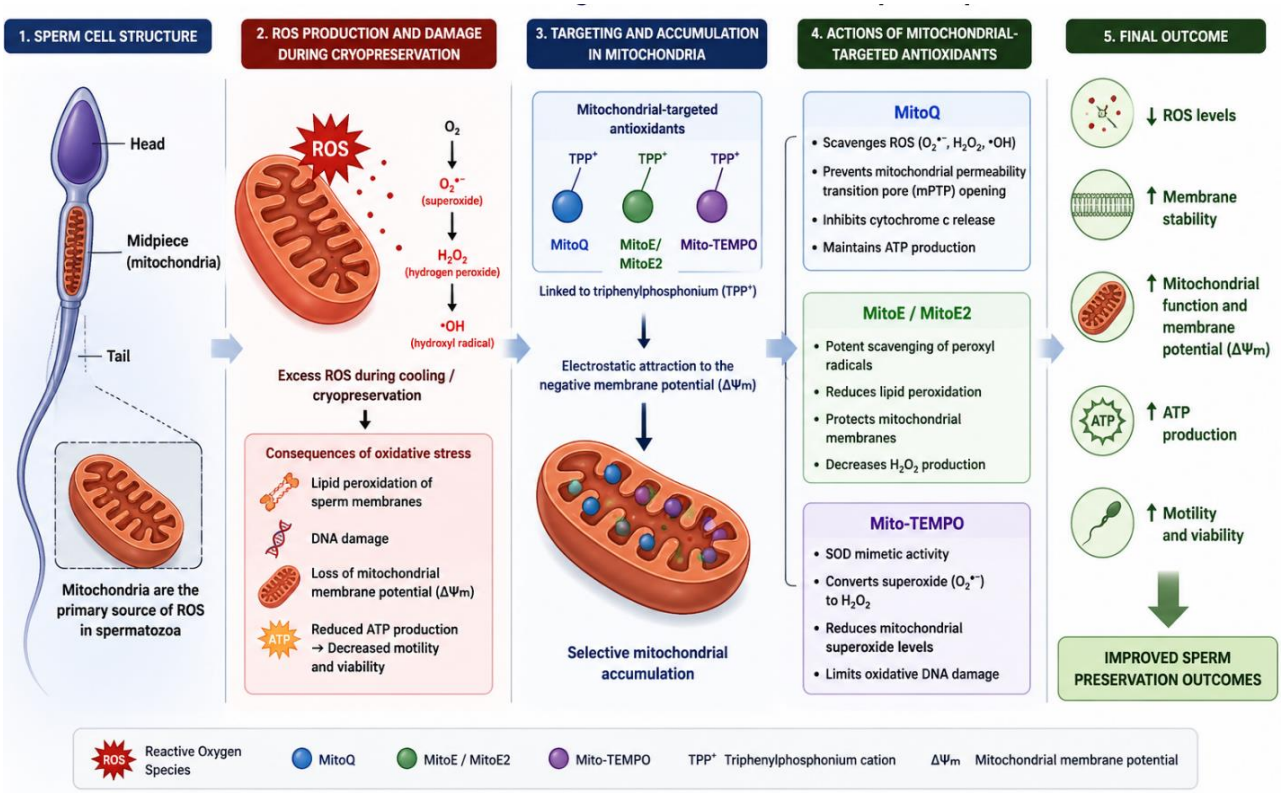

Fig. 3: Diagrammatic illustration of mechanism of actions of mitochondrial-targeted antioxidants.

Table 1: Mitochondrial-targeted antioxidants in semen extenders.

Additives	Chemical nature	Mechanism of action	Reported effects	Limitations/equine data	Sources
MitoQ	Ubiquinone (idebenone) derivative linked to triphenylphosphonium (TPP^+)	Accumulates in mitochondria; scavenges ROS; stabilizes mitochondrial membrane; inhibits mPTP opening and cytochrome C release; supports ATP production	Improves motility and sperm kinematics at 25–100nM; protects mitochondrial function	High doses (200nM) may be detrimental; variable results across species	Masoudi *et al.,* 2024; Elkhawagah *et al.,* 2024a; Hatami *et al.,* 2024; Farshad and Wehrend, 2025.
MitoE/MitoE2	Vitamin E (α-tocopherol) conjugated to TPP^+ (MitoE); phosphate ester form (MitoE2, more water-soluble)	Targets mitochondria; reduces H_2O_2 production and lipid peroxidation; protects mitochondrial membranes	Effective mitochondrial antioxidant in vitro; MitoE2 improves solubility and reduces pro-oxidant risk	No full-length equine-only studies in extenders reported	Leo *et al.,* 2008; Nahar and Sohag, 2025.
Mito-TEMPO	Piperidine nitroxide linked to TPP^+	SOD mimetic; scavenges mitochondrial superoxide; reduces lipid peroxidation and DNA damage; preserves membrane potential	Improves sperm motility, viability, membrane and acrosome integrity in ruminants (5-50μM)	No full-length equine-only studies; evidence extrapolated from other species	Kumar *et al.,* 2021; Kumar *et al.,* 2022; Elkhawagah *et al.,* 2024b.

Cryoprotective and Structural Additives: Beyond oxidative stress, structural destabilization of the sperm membrane represents a major factor limiting cryo-survival. Consequently, a range of cryoprotective and membrane-stabilizing additives has been developed to maintain membrane integrity during thermal stress.

Sugars and osmoprotectants: Trehalose is a non-permeant disaccharide used as a cryoprotectant additive in semen extenders for various species. Its role is not limited to a single pathway, rather it acts through numerous complementary mechanisms, such as membrane stabilization, vitrification, inhibition of ice recrystallization (IRI) and neutralization of ROS.

The main mechanism of trehalose action is explained by the water replacement hypothesis (WRH). In cryopreservation, extracellular ice formation leads to the dehydration of water from around the membrane lipids, consequently disrupting the hydration shell which stabilizes the lipids in a particular phase and increases the space between them, leading to a phase transition and destabilizing the structure (Golovina *et al.,* 2009). Molecular simulation and Nuclear Magnetic Resonance (NMR) experiments have shown that trehalose binds non-randomly to lipid membranes but prefers to associate with the carbonyl group of the SN-2 fatty acids and the phosphate head group. Binding of trehalose to the lipids preferentially maintains the hydrogen bonding network before water is expelled from the cell (Golovina *et al.,* 2010). Additionally, trehalose has been shown to increase the area per lipid (APL), which contributes to a fluidization within the bilayer core of the cell (Liu *et al.,* 2018).

The trehalose also provides vitrification, by enabling a solution to solidify in a glassy, non-crystalline state without any ice damage (Khaydukova *et al.,* 2024). These effects are caused through viscosity enhancement and an appropriate glass transition temperature (Comizzoli *et al.,*

2022). The glass formed by trehalose holds molecular mobility within limits, where cellular contents remain stable and where protein damage during the semen frozen-storage process doesn't occur (Murray *et al.,* 2024).

Trehalose also has another protective effect against the damaging nature of freeze-thawing; ice recrystallisation also seems to contribute, i. e., small crystals can grow to form large ones during thaw, damaging the structure of cells (Chaytor *et al.,* 2012). It is suggested that trehalose works by interacting with the surfaces of ice crystals, and limits their growth or promotes uniform growth of many small ones (Mommaerts *et al.,* 2024). While trehalose is pretty underwhelming as a freeze-point depression substance among the various sugars tested, but it definitely boosts the melting point when put up against saccharose or lactose (Wu *et al.,* 2024). However, despite these limitations, trehalose shows better results in terms of stopping ice growth during the process of both cooling down and reheating of semen compared to other sugars.

Furthermore, apart from the obvious benefits of membrane structural stabilization, trehalose also possesses remarkable antioxidant properties. These properties limit the accumulation of ROS, either via direct action on membrane lipids or through interruption of lipid auto-oxidation processes (Fig. 4).

Although promising, the actual efficacy of trehalose for cryopreservation of equine semen depends to a considerable extent on the optimum trehalose concentration, the stallion breed and semen cryoprotective extender being used. One recent study, that aimed to improve the concentration and role of trehalose in the cryopreservation of Marwari stallion semen, indicated that a 50mM concentration of trehalose could effectively promote the preservation of progressive sperm motility and viability compared with un-supplemented samples, especially when supplemented with lactose (Jhamb *et al.*, 2023).

Ghallab *et al.* (2017) evaluated the impact of various trehalose concentrations (10, 20, 50, 100, 150mM) on Arabian stallion semen cryopreserved using INRA-82 extender and observed that semen samples supplemented with 150mM trehalose showed the highest sperm motility viability, plasma membrane integrity and acrosome status compared to controls and samples supplemented with other concentrations of trehalose. However, despite these innovations in additives, problems remain in obtaining sufficient additive amounts to improve semen quality, concerning bioavailability, stability and a focused application of additives in target cells.

Membrane Stabilizers

cholesterol-loaded cyclodextrin: Cholesterol-loaded cyclodextrin (CLC) is a complex formed between methyl-β-cyclodextrin (a cyclic oligosaccharide) and cholesterol (Batissaco *et al.,* 2020), designed to increase sperm plasma membrane cholesterol: phospholipid ratio (Ahmet *et al.,* 2025). By incorporating itself within the sperm bilayer, CLC can restrict lateral lipid movement whilst avoiding the excessively rigid packed states that occur if it had arrived there in rigid packing. The membrane does not become 'stabler', except that the inherent tendency of the bilayer toward structural disorganization as it cools is delayed (Leahy and Gadella, 2015).

This distinction is important during cooling. When the temperature is lowered, membranes that lack sufficient cholesterol undergo phase transitions, which erratically disrupt the organizational structure of lipids, often resulting in leakage or permanent destruction. Raising the cholesterol-to-phospholipid (C/PL) ratio also changes this response. A gradual thermal transition, which preserves functional integrity of the membrane, directly correlates to enhanced cryotolerance (Cittone *et al.,* 2025). A higher C/PL ratio is a key determinant of cryotolerance. Species with elevated C/PL ratios show significantly greater resistance to cold shock than those with lower C/PL ratios.

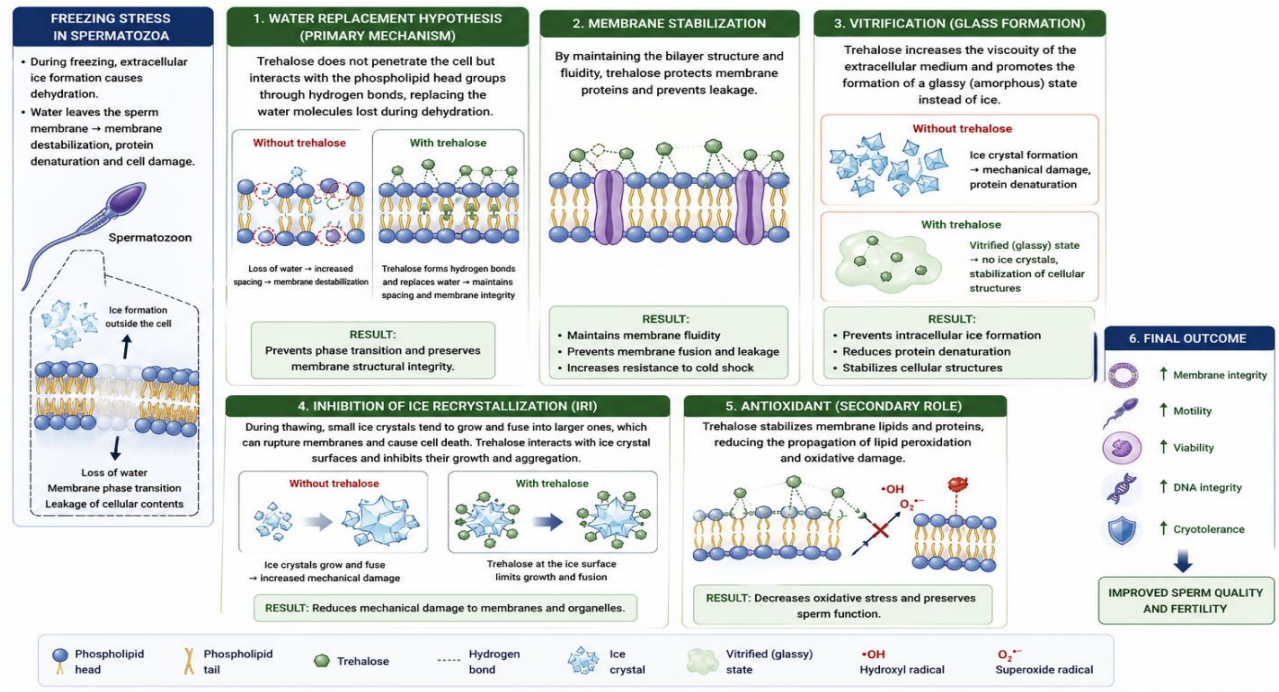

Fig. 4: Diagrammatic illustration of the mechanism of trehalose actions.

Equine sperm are known to have a low C/PL ratio of 0.3, which makes them susceptible to cold shock and the adverse effects of freezing (Ullah *et al.,* 2025). To mitigate these issues, CLC can be added to the semen extender for increasing the C/PL ratio of horse sperm to approximately 0.8. This increased C/PL ratio results in a more resistant membrane configuration of equine sperm which becomes similar to the semen of other animal species and limits the potential of loss of function following freezing (Cittone *et al.,* 2025).

The available experimental data support this mechanistic interpretation. After adding methyl-β-cyclodextrin-cholesterol to stallion semen at 0.75 or 1.5mg/mL, Murphy *et al.* (2014) observed an increase in post-thaw sperm viability in comparison to the untreated controls, correlating this to a decrease in superoxide radical production. Thus, these workers suggested that membrane stabilization not only limits propagation of oxidative stress, but may also play some role in improving post-thaw sperm viability. In contrast, parameters like progressive sperm motility and membrane integrity did not differ between the control and treated samples. Thus, it was concluded that CLC improved post-thaw sperm viability by preserving the membrane, limiting the pathways that lead to some kind of damage. However, CLC did not improve all functions of sperm, an effect that could be seen with the introduction of antioxidants.

A recent study by Cittone *et al.* (2026) explained the magnitude of the effect of CLC on post-thaw sperm quality. The higher ratio of C/PL (2.7:1) exhibited 39-65% sperm motility after a 0°C cold shock, whereas the control group had collapsed motility (~8%). Notably, CLC retained sperm function without egg yolk - based extenders and sperm quality was even further improved when combined with yolk-based extenders. This would suggest some combination of shared and unique responses in semen collected under CLC and egg-yolk-supplemented conditions. A consistent response is never observed, given that the C/PL ratio differs wildly from 0.33-0.79 between individuals, confirming that composition of semen extenders does not show a fixed pattern across stallions and the way they respond to cryopreservation is different. It is well known that CLC works well but it cannot be regarded as the absolute best in just one setting. Cholesterol-loaded cyclodextrins are effective in improving stallion sperm cryotolerance, but only if the cells are properly loaded with cholesterol and even then, it may not be assumed as the final universal sperm supplement, nor it should replace antioxidants/mitochondrial protecting agents which are also used.

Emerging Innovations: Nanotechnology-based Delivery Systems: Recent advancements in animal biotechnology include the development of alternative semen additives, which target and protect the individual sperm cell. New additives such as nanoparticles (NPs) and nanocarriers are applied to semen extenders to improve assisted reproductive technologies (Rautela *et al.,* 2026). These additives are able to protect vital bioactive additives (antioxidants and membrane stabilizers) from damaging effects during cooling/freezing procedures. Nanoparticles and nanocarriers can be designed to improve such semen quality parameters as post - thaw sperm motility and viability, membrane integrity and permeability, sperm nuclear DNA integrity, embryo quality and pregnancy rates, while simultaneously avoiding cytotoxicity associated with normal extender additives (Sindi *et al.,* 2025; Azimi *et al.,* 2026).

Multiple promising nano-cryoprotectants and delivery methods have been introduced on the basis of recent research. In many studies on the post-thaw sperm viability and chromatin integrity, an increase in DNA integrity was found after freezing/thawing when semen extender was supplemented with selenium, copper-zinc oxide or cerium oxide (Saadeldin *et al.* 2020). A technology that involves producing chitosan-selenium nanodots conjugated to an antioxidant enzyme, mimicking the pathways occurring within mitochondria or even within the spermatozoon has also been reported (Sindi *et al.* 2025). Liposomes, exosome-like vesicles and polymeric nanoparticles are used in the organic vehicle category, where anti-apoptotic agents and even RNA and DNA can be administered directly to the cells (Khodayari *et al.,* 2025).

The main advantage of this nanotechnology is controlled local drug delivery at a reduced dose without damaging cells. Furthermore, it helps in maintaining mitochondria and enhances genomic stability by inhibiting lipid peroxidation. Functionalized, as well as magnetic nanotechnology, offers new sperm-sorting mechanisms, damaged sperm are excluded to acquire a pristine and fertile population prior to cryopreservation (Paul *et al.,* 2024; Azimi *et al.,* 2026).

Given this, research is increasingly focusing on vesicular nanocarriers, such as liposomes and niosomes, that can shield sensitive bioactive compounds during semen cryopreservation, processing and artificial insemination in horses. Niosomes encapsulate antioxidants, including artemisinin derivatives, which have shown promising results in increasing post-thaw sperm motility, enhancing sperm membrane stability and restoring the intracellular antioxidant balance of spermatozoa (Abbasi *et al.,* 2025). However, most of these nanocarrier systems relating to horse reproduction, are yet at the experimental phase. Stallion breeding programs currently depend mostly on standard sperm extenders formulated on the basis of additives like egg yolk, skim milk and soybean lecithin. The extreme vulnerability of stallion sperm cells makes them a good target for delivery systems, using nanomaterials for protection. Before these advanced systems can be suggested for commercial practice, further large-scale validation is required to establish standardized dosing protocols, verify long-term biosafety, and ensure consistent *in vivo* fertility rates.

Conclusion: Extenders are primarily responsible for the overall preservation success, rather than anything listed among "adjuvants". Adjuvants are considered important because semen is subjected to thawing after freezing, causing sperm to experience thermal stress. Extenders provide the protection to sperm against thermal damage through scavenging ROS, stabilizing sperm membranes, maintaining sperm metabolism and protecting the cells from cryopreservation stress. The protective ability of extenders, however, depends mostly upon concentration

of cryoprotectants, so it must be accurately calibrated. Extender overuse can induce cytotoxicity.

Limitations in the use of different cryoprotectants do not stop at this level, as sperm from equine males vary individually with different seminal plasma composition and sperm quality, hence giving varied responses to different extenders and extender additives in different stallions. So, finding one universal extender is difficult for semen collected under different conditions; and attempts were made in this review article to tackle these differences and their consequences as a first move towards an individualized breeding-specific extender development.

To overcome these hurdles, pursuing an animal epidemiology project with stronger comparison designs can be suggested to determine specific seminal composition of males of each individual stallion breed. Then, the optimal additives may be compared, especially using nanotechnology-based deliveries in combination with mitochondrial-targeted antioxidants to have greater chemical specificity. The continued reliance on "one-size-fits-all" extender formulations is no longer scientifically justified in equine semen preservation. The existing evidence demonstrates that such generalized approaches fail to account for the marked biological variability among stallions, often resulting in inconsistent and suboptimal outcomes. Future progress in equine reproductive technologies will depend on a transition toward personalized, molecularly guided strategies, in which extender composition is tailored to the specific biochemical and functional characteristics of the stallion semen.

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