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Review

Oxidative Stress and Its Mitigation in Buffalo Sperm Cryopreservation - Mechanisms and Strategies

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Abstract

Cryopreservation is integral to artificial insemination, facilitating the global dissemination of superior genetic material in livestock. However, the process induces oxidative stress (OS), significantly impairing sperm viability and fertility. Excessive reactive oxygen species (ROS) generated during freeze – thaw cycles cause lipid peroxidation, DNA damage, protein denaturation, and mitochondrial dysfunction, particularly in buffalo sperm, which are rich in polyunsaturated fatty acids and low in cholesterol. This results in compromised membrane integrity, reduced motility, and decreased fertilisation capacity. Strategies to mitigate OS include antioxidant supplementation, both enzymatic (e.g., catalase, SOD, GPx) and non-enzymatic (e.g., vitamins C and E, glutathione, trehalose), which enhance cryosurvivability and reduce ROS-induced damage. Additional interventions such as partial deoxygenation of extenders and removal of dead or damaged sperm via filtration, swim-up, density gradient separation, and antibody-conjugated nanoparticles further improve post-thaw semen quality. Despite significant progress, sperm cryopreservation still faces challenges due to reactive oxygen species (ROS) - related damage and variability in response among bulls, highlighting the need for continued research to refine cryopreservation techniques and improve reproductive outcomes in buffalo.

Keywords: Antioxidant Supplementation, Buffalo Spermatozoa, Cryopreservation, Oxidative Stress, Reactive Oxygen Species (ROS).

Introduction

Cryopreservation plays a vital role in artificial insemination (AI), enabling the widespread use of semen from genetically superior bulls and thereby enhancing productivity in the bovine industry (Layek *et al*., 2016). Effective cryopreservation preserves sperm fertility over extended periods by reducing metabolic activity, minimizing toxin accumulation, and conserving substrates (Lemma, 2011). Ensuring the structural and functional integrity of buffalo spermatozoa during freezing is essential to maintain fertility, freezability, and conception rates. AI and semen cryopreservation are the most commonly applied biotechnological tools in animal reproduction (Pesch and Hoffman, 2007), facilitating the large-scale distribution of valuable genetics. However, cryopreservation of bovine sperm faces challenges, including decreased motility, mitochondrial membrane potential, and plasma membrane integrity, as well as increased lipid peroxidation post-thaw (Januskauskas *et al*., 2003; Khumran *et al*., 2015; Korkmaz *et al*., 2017). Factors including temperature, cooling rate, extender composition, cryoprotectant concentration, reactive oxygen species, seminal plasma composition, and hygienic conditions significantly influence sperm longevity and quality (Barbas and Mascarenhas, 2009; Kumar *et al*., 2025). Semen extenders are crucial in maintaining post-thaw sperm viability, underscoring the need for ongoing improvement in extender formulations and freezing protocols (El-Sheshtawy *et al*., 2015; Blackburn, 2004). Despite advances in bovine sperm cryopreservation, post-thaw viability remains suboptimal and highly variable among bulls, even following stringent breeding soundness evaluations (Ugur *et al*., 2019; Kumaresan *et al*., 2012; Gopinathan *et al*., 2016), indicating persisting gaps in knowledge and technology that warrant further research.

Oxidative Stress and Impact on Sperm during Cryopreservation

Oxidative stress (OS) plays a pivotal role in impairing sperm function during cryopreservation by disrupting the cellular structure and biochemical integrity of spermatozoa. Excessive reactive oxygen species (ROS), including superoxide, hydrogen peroxide, hydroxyl radicals, and singlet oxygen, are generated both enzymatically and non-enzymatically within sperm cells and from external sources, such as dead or abnormal sperm and leukocytes (Agarwal and Saleh, 2002). While low ROS levels are necessary for key processes such as capacitation and hyperactivation (Bollwein and Bittner, 2018), cryopreservation significantly increases ROS, leading to oxidative damage to DNA, membranes, and mitochondria (Meyers, 2005; Aitken and Drevet, 2020). The buffalo sperm membrane's high content of polyunsaturated fatty acids (PUFAs) and low cholesterol makes it especially vulnerable to lipid peroxidation, producing harmful metabolites such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), which compromise membrane integrity and fluidity (Bailey *et al*., 2003). Lipid peroxidation disrupts the semi-permeable nature of the plasma membrane, impairing sperm motility and fertilizing ability (Chatterjee and Gagnon, 2001; Khalil *et al*., 2018). Furthermore, oxidative damage alters protein structures through thiol oxidation and peptide bond cleavage, affecting key enzymes like tyrosine phosphatase and reducing motility and fertility. The freeze-thaw process also leads to lipid phase separation and the redistribution or loss of membrane proteins, weakening sperm resistance to cold shock, lipid peroxidation, and apoptosis (Bailey *et al*., 2000; Lemma, 2011; Peris-Frau *et al*., 2019). Reduced antioxidant activity in frozen-thawed semen, particularly the depletion of seminal plasma antioxidants such as glutathione peroxidase (GPx) and superoxide dismutase (SOD), further exacerbates oxidative stress (Bilodeau *et al*., 2000; Marti *et al*., 2008), making sperm more susceptible to ROS-induced damage.

Impacts of Oxidative Stress on Sperm

Oxidative stress significantly impairs sperm function, particularly during cryopreservation, due to alterations in the sperm glycocalyx and membrane phospholipid peroxidation caused by the elevated generation of reactive oxygen species (ROS) (Chatterjee *et al*., 2001; Pini *et al*., 2018). Ruminant sperm are especially vulnerable to lipid peroxidation because of their high polyunsaturated phospholipid content (Bailey *et al*., 2003), leading to the formation of toxic by-products like lipid alkoxyl and peroxyl radicals, malondialdehyde (MDA), acrolein, and 4-hydroxynonenal (4-HNE), which compromise membrane fluidity and integrity (Storey, 1997; Niki *et al*., 2005). 4-HNE, a highly reactive aldehyde, can form adducts with DNA and proteins, exacerbating cellular damage (Bailey *et al*., 2003). Lipid hydroperoxides and hydroxides further perpetuate oxidative damage, weakening the membrane's semi-permeable nature (Pons-Rejraji *et al*., 2009). This lipid peroxidation also releases phosphatidylethanolamine and phosphatidylcholine (Pini *et al*., 2018). MDA and 4-HNE, measured via thiobarbituric acid reactive substances (TBARS), serve as biomarkers of lipid peroxidation, with TBARS levels significantly increasing post-thaw in bull sperm compared to chilled semen (Chatterjee and Gagnon, 2001). In parallel, ROS-induced protein modifications, including oxidation of thiol groups, peptide bond cleavage, and formation of covalent crosslinks, lead to functional

impairments (Schuessler and Schilling, 1984). Proteins containing cysteine, particularly tyrosine phosphatases essential for sperm motility and fertilization, are highly susceptible to oxidative damage (de Lamirande *et al*., 1997; Mammoto *et al*., 1996). There is an inverse relationship between thiol oxidation and sperm motility, as well as fertilisation potential. Additionally, cryopreservation disrupts the plasmalemma—one of the most sensitive sperm structures—via phospholipid phase separation, causing loss or relocation of membrane proteins and lipid–protein interactions necessary for membrane function (Bailey *et al*., 2000; Grötter *et al*., 2019; Lemma, 2011). Proteomic analyses of cryopreserved ram and bull sperm have shown reductions in proteins associated with decapacitation, anti-apoptotic activity, membrane stabilization, cold-shock resistance, gamete fusion, and oocyte interaction (Wojtusik *et al*., 2018; Peris-Frau *et al*., 2019), further underlining the detrimental impact of oxidative stress on sperm viability and fertility.

Strategies to Improve Buffalo Semen Freezability

Significant advancements have been achieved in enhancing the freezability of buffalo semen, with a primary focus on mitigating the harmful effects of reactive oxygen species (ROS) during the cryopreservation process. Over recent decades, research efforts have sing plasma membrane integrity and preventing ROS-induced damage, such as lipid peroxidation (LPO) and apoptosis. Due to eliminating most cytoplasm during the final stages of spermiogenesis, spermatozoa possess limited cytoplasmic antioxidant defenses, rendering them particularly susceptible to oxidative stress (Bucak *et al*., 2007). Furthermore, semen dilution during processing further reduces the antioxidant concentration, thereby increasing the sperm's vulnerability to reactive oxygen species (ROS) (Agarwal *et al*., 2004). , including antioxidant supplementation, apoptosis inhibition, partial deoxygenation of extenders, and the removal of dead or damaged spermatozoa.

Supplementation of Antioxidants

A variety of antioxidants found in seminal plasma and sperm provide defense against oxidative stress (Kim and Parthasarathy, 1998). These antioxidants act as chain-breaking agents, interrupting oxidative chain reactions and thereby shielding cells from damage caused by free radicals (da Silva *et al*., 2011). Antioxidants are broadly classified into two categories: enzymatic and non-enzymatic.

Enzymatic Antioxidants

Enzymatic antioxidants, also known as natural antioxidants, are inherently present within the body. These include catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPX), all of which play crucial roles in preserving cellular integrity by neutralizing excessive reactive oxygen species (ROS). Catalase functions as a positive catalyst, converting hydrogen peroxide (H_2O_2) into water and oxygen. Supplementation of CAT in buffalo semen has been shown to enhance motility and viability, reduce lipid peroxidation (LPO), and increase SOD activity during cryopreservation (Bansal and Cheema, 2016). Similarly, Kumar and Atreja (2012) reported that CAT supplementation in Murrah bulls led to decreased protein tyrosine phosphorylation and improvements in post-thaw motility, acrosomal integrity, viability, and plasma membrane integrity. SOD facilitates the dismutation of superoxide radicals, thereby protecting spermatozoa from lipid peroxidation (LPO) (Sikka, 1996). El-Sisy *et al*. (2008) also noted improved semen quality parameters in buffalo bulls following SOD supplementation. GPX contributes by reducing H_2O_2 to water and converting lipoperoxides into less harmful alkyl alcohols (Amidi *et al*., 2016). Kadirvel *et al*. (2014) reported a strong positive correlation between GPX levels and sperm motility and membrane integrity, along with a negative association with lipid peroxidation.

Non-enzymatic Antioxidants

Non-enzymatic antioxidants, often referred to as synthetic antioxidants, are typically supplemented through diet and include compounds such as vitamins C and E, trehalose, taurine, glutathione (GSH), and cysteine (Agarwal *et al*., 2005). Vitamin C (ascorbic acid) is a key component of the seminal plasma's antioxidant defense system (Agarwal and Prabakaran, 2005). Sandeep *et al*. (2015) reported enhanced freezability and reduced oxidative stress markers following vitamin C supplementation in buffalo semen. Similarly, Patel *et al*. (2016) observed that vitamin C helps prevent enzyme leakage and improves the post-thaw quality of buffalo spermatozoa. Vitamin E (alpha-tocopherol), a fat-soluble antioxidant, maintains cellular integrity by inhibiting lipid peroxidation through scavenging of free radicals. Its supplementation has been shown to enhance the cryopreservability of buffalo semen (Raina *et al*., 2002)

and protect the sperm plasma membrane from oxidative damage while improving sperm kinematics (Kumar *et al*., 2018). GSH, an important antioxidant, neutralizes ROS and maintains disulfide bond stability, thereby safeguarding the sperm plasma membrane from lipid peroxidation (Hu *et al*., 2016). Gangwar *et al*. (2018) reported reduced sperm apoptosis in Murrah buffalo bull semen following GSH supplementation. Trehalose, a non-permeating sugar, prevents cellular dehydration and offers protection against ROS-induced damage. Supplementation with trehalose and taurine has been found to preserve the localization of tyrosine-phosphorylated proteins in cryopreserved buffalo spermatozoa, resembling the patterns seen in fresh semen (Kumar *et al*., 2012). Additionally, membrane stabilizers such as cholesterol-loaded cyclodextrins have been explored to enhance the freezability and cryosurvivability of buffalo sperm, with studies reporting decreased levels of lipid peroxidation and ROS post-cryopreservation (Rajoriya, 2014; Lone, 2015). Notably, the role of humanin in semen cryopreservation was first identified by Yang *et al*. (2019), marking a novel development in the field. Recently an antioxidant, crocin used on frozen-thawed semen decreased membrane lipid peroxidation, protamine deficiency, apoptosis (Lv *et al*., 2020). HNG (S-14 humanin analogue) was found to mitigate GPx4 downregulation in a research, hence reducing ferroptosis in endometrial epithelial cells (Zou *et al*., 2023).

Partial Deoxygenation of Extender

Partial deoxygenation of semen extenders represents a promising strategy developed in our laboratory to enhance the freezability of buffalo spermatozoa. This approach is based on the principle of lowering the dissolved oxygen (DO) concentration to optimal levels to minimize oxidative damage to sperm cells. DO reduction can be achieved through various methods, including mechanical techniques using negative pressure, flash-freezing with liquid nitrogen (LN2) (Balamurugan *et al*., 2018), nitrogen gassing (Mustapha, 2017; Bhutia, 2018), or the use of the Oxyrase enzyme (Ngou *et al*., 2020). Balamurugan *et al*. (2018) reported improved semen parameters and decreased oxidative stress following partial deoxygenation of the extender and advocated the use of LN2 flushing to enhance post-thaw semen quality in buffalo. Similarly, Kumar *et al*. (2018) observed significant improvements in semen quality when nitrogen gassing reduced DO levels to approximately 4 ppm. The Oxyrase enzyme has also shown beneficial effects on semen quality in equine (Darr *et al*., 2016) and bovine species (Ngou *et al*., 2020) by partially removing oxygen from the extender. However, its efficacy in buffalo semen has yet to be established.

Removal of Dead and Damaged Spermatozoa from Fresh Semen

Eliminating dead and damaged spermatozoa from fresh semen is crucial, as they are a significant source of reactive oxygen species (ROS) production. Roca *et al*. (2013) demonstrated the detrimental impact of dead sperm on viable sperm cells, leading to reduced cryosurvivability, and recommended their removal prior to semen processing. Techniques such as Sephadex and glass-wool filtration, swim-up, and density gradient separation can eliminate these non-viable spermatozoa. However, due to their time-consuming nature, these methods are impractical for routine use in semen processing laboratories.

Filtration Technique

The filtration technique operates on the principle that spermatozoa can interact with materials such as glass fibres or Sephadex beads, allowing motile sperm to pass through while retaining dead and damaged cells (Mogas *et al*., 1998). These non-viable spermatozoa tend to adhere to the filter matrix, whereas live, motile sperm remain unbound and can move forward (Bussalleau *et al*., 2008). This method also effectively removes leukocytes, another significant source of ROS (Morrell and Rodriguez-Martinez, 2011). The glass-wool filtration technique efficiently separates live from dead spermatozoa, based on the sticky nature of non-viable cells, which causes them to become trapped in the glass fibers, while viable sperm pass through using their own motility (Mortimer and Mortimer, 1992). A sperm recovery rate of 79.67 ± 1.67% was reported in buffalo semen using this technique (Husna *et al*., 2016). In contrast, the Sephadex filtration method relies on interactions between the sperm plasma membrane and Sephadex particles (Samper *et al*., 1995). It is believed that live sperm possess a net negative surface charge, while dead sperm have a net positive charge. The positively charged dead sperm interact with the negatively charged cellulose in Sephadex, resulting in their agglomeration and subsequent removal (Ahmad *et al*., 2003). This method yielded a total sperm recovery rate of 63.0 ± 1.53% in buffalo semen (Husna *et al*., 2016).

Swim-Up Technique

The swim-up technique is based on the principle that motile spermatozoa actively swim upward into a culture medium layered over the semen sample while dead, damaged, and morphologically abnormal sperm remain at the bottom (Morrell, 2006). This method takes advantage of the ability of viable, morphologically normal sperm to migrate against gravity. Although the technique selectively isolates highly motile sperm, it is associated with a notably low recovery rate. In frozen-thawed buffalo semen, Mehmood *et al*. (2009) reported a recovery rate of only $4.1 \pm 2.8\%$. Additionally, the extended incubation time and the requirement for centrifugation during processing may increase the risk of inducing sperm damage (Henkel, 2013).

Density Gradient Separation Method

This technique operates on the principle that. Motile spermatozoa align with the centrifugal force and settle at the bottom of the tube, forming a pellet, while dead and damaged sperm cannot penetrate the gradient layers and remain trapped (Morrell, 2006). The method commonly employs colloidal silica particles coated with polyvinylpyrrolidone (PVP) (Oshio, 1988). However, PVP has been reported to affect spermatozoa, compromising the integrity of the plasma membrane, mitochondrial membrane, and acrosome (Strehler *et al*., 1998; Avery and Greve, 1995). Additionally, the technique yields a low sperm recovery rate, with Mehmood *et al*. (2009) reporting a rate of only $6.0 \pm 3.0\%$ in buffalo semen.

Antibody Conjugated Nanoparticles (Ab-NP)

The use of antibody-conjugated nanoparticles (Ab-NP) for the removal of dead and damaged spermatozoa has been documented. This technique is based on the principle that non-viable sperm cells express specific ligands on their plasma membrane surface. Antibodies targeting these ligands are conjugated with magnetic nanoparticles, enabling selective binding to dead and damaged sperm. When an external magnetic field is applied, these bound sperm cells are drawn to the bottom of the container, while the supernatant contains live, morphologically normal spermatozoa (Odhiambo *et al*., 2014). This nano-purification approach has been shown to enhance semen quality parameters in bulls (Odhiambo *et al*., 2014), boars (Feugang *et al*., 2015), and buffaloes (Omerdin, 2017). Furthermore, Amarjeet (2019) reported a decrease in malondialdehyde (MDA) levels and an increase in total antioxidant capacity (TAC) in cryopreserved buffalo semen treated with this method.

Challenges in Routine Application of Advanced Semen Processing Techniques

Routine application of advanced semen processing techniques, such as antioxidant supplementation, partial deoxygenation of extenders, and removal of dead or damaged spermatozoa faces significant challenges. Antioxidant supplementation, while beneficial, is hampered by issues of cost, lack of standardization, and the risk of improper dosing, which can negatively impact sperm viability. Partial deoxygenation methods require specialized equipment and precise process control, making them difficult to implement consistently in high-throughput or resource-limited laboratories. Techniques for removing non-viable sperm, including filtration, swim-up, density gradient separation, and antibody-conjugated nanoparticles, are often labor-intensive, costly, and result in substantial sperm loss, limiting their practicality for routine use. Additionally, some methods introduce concerns about chemical toxicity or regulatory approval, further restricting their widespread adoption. These operational and logistical barriers mean that, despite their proven efficacy in improving post-thaw semen quality, such techniques are not easily integrated into everyday semen processing workflows, especially in commercial or field settings where efficiency and scalability are paramount.

Conclusion

In conclusion, while cryopreservation remains a cornerstone of reproductive biotechnology in livestock, particularly for the dissemination of superior buffalo genetics, it is invariably associated with oxidative stress that compromises sperm function and fertility. The excessive generation of reactive oxygen species (ROS) during freezing and thawing disrupts membrane integrity, induces lipid peroxidation, damages DNA and proteins, and impairs motility. These detrimental effects can be mitigated through the strategic use of both enzymatic and non-enzymatic antioxidants and advanced sperm selection and extender deoxygenation techniques. Although these interventions have shown promise in enhancing post-thaw semen quality, the inherent variability among individual bulls and limitations in current methodologies necessitate continued exploration of molecular and nanotechnological approaches to improve semen quality. Advancing our understanding of oxidative mechanisms and refining semen preservation protocols

will be pivotal for cryosurvivability, fertilisation potential, and overall success rates of artificial insemination in buffalo. Ready-to-use semen dilutors with various additives are essential in animal artificial insemination (AI) and semen preservation for maximizing the use of superior male germplasm and enhancing reproductive efficiency.

Contribution by Authors

All the authors contributed equally to writing the manuscript. The final manuscript was read by all authors and consented to publication.

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Conflict of Interests

There is no conflict of interest.

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