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Implications of cryopreservation on structural and functional attributes of bovine spermatozoa: An overview

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Abstract

Sperm cryopreservation is an important adjunct to assisted reproduction techniques (ART) for improving the reproductive efficiency of dairy cattle and buffaloes. Improved understanding of mechanisms and challenges of bovine semen cryopreservation is vital for artificial insemination on a commercial basis. Although cryopreservation of bovine spermatozoa is widely practiced and advanced beyond that of other species, there are still major gaps in the knowledge and technology. Upon cryopreservation, disruption of spermatozoal plasma membrane configuration due to alterations in metabolic pathways, enzymes and antioxidants activity add to lower efficiency with loss of sperm longevity and fertilising ability. Therefore, the effective amalgamation of cryo-variables like ambient temperature, cooling and thawing rates, nucleation temperature, type and concentration of the cryoprotectant, seminal plasma composition, free radicals and antioxidant status are required to optimise cryopreservation. Novel strategies like supplementation of cholesterol-loaded cyclodextrins (CLC), nanovesicles, osteopontin, antioxidants, etc., in an extender and recent techniques like nano-purification and modified packaging have to be optimised to ameliorate the cryodamage. This article is intended to describe the basic facts about the sperm cryopreservation process in bovine and the associated biochemical, biophysical, ultra-structural, molecular and functional alterations.

KEYWORDS

antioxidants, cryopreservation, free radicals, sperm, thawing

1 | INTRODUCTION

The developing countries are rich in livestock genetic resources; however, the productivity per se is very poor. This needs to be accelerated to uphold successful dairying and provide food security to the raising population. The best method is by the propagation of elite germplasm and by relying on the concept of achieving 'a calf per cow per year' through timely insemination and successful conception. Cryopreservation of spermatozoa is a milestone in this direction and has led to the widespread use of cryopreserved spermatozoa through the artificial insemination (AI) technique (Ugur et al., 2019). But insemination using cryoinjured spermatozoa with low fertilising capacity accounts for a significant loss to the dairy industry (Chung et al., 2019; Dewry et al., 2020; Singh et al., 2020). The cryo-injury is a consequence of temperature change, induction of osmotic stress, ice crystals formation which serves as the biggest determinant causing irreparable damage by altering the structure and physiology of the sperm cell (Ahmed et al., 2019; Amidi et al., 2016). Although cryopreservation prolongs the storage of spermatozoa, associated cryo-injury may cause loss of sperm motility, and induce acrosomal damage, mitochondrial membrane depolarisation, cryocapacitation and alteration in plasma membrane permeability (Bailey et al., 2000). These factors contribute to the loss of the ability of spermatozoa to interact with the female tract and thus fertilisation

failure (Chen et al., 2015; Ezzati et al., 2020). The freezing-thawing process changes the physiology and deforms the structure of spermatozoa due to changes in osmotic balance, generation of excessive reactive oxidants, and formation of intracellular ice crystals (Kumar et al., 2018; Ugur et al., 2019). Therefore, a thorough understanding is vital to improve cryopreservation and AI programme effectively (Andrabi & Maxwell, 2007; Mughal et al., 2017). Moreover, there is a need for a comprehensive assessment of different aspects including cellular and subcellular alterations of spermatozoa that can precisely identify the alterations during the freezing-thawing process. Therefore, the objective of this article is to provide a concise review of damages and alterations encountered in bovine spermatozoa during and after cryopreservation and to discuss the wide range of deterioration in sperm parameters.

2 | CRYOPRESERVATION—BASIC FACTS AND PROCESS

The process of cryopreservation involves sequential events of temperature reduction, cellular dehydration, freezing and thawing. Cooling of spermatozoa is the simplest method and the core principle behind the cryopreservation process that can successfully lower the sperm metabolic rate and prolong its survival. Freezing of semen at −196°C is found to be the most desirable and commonly used method for preserving the fertilising ability of spermatozoa (Kumar et al., 2019). Different diluent solutions are widely used for preserving the semen straws at −196°C, such as glycerol–egg yolk–citrate, milk glycerol and glycerolated-egg yolk-TRIS. Conventional slow freezing or programmable freezing and vitrification are two major cryopreservation techniques for preserving the semen (Chatterjee et al., 2017). Slow freezing is the most commonly used technique which involves cooling spermatozoa at rates of 1–2°C/min in steps up to −196°C. The drawbacks associated with this traditional method of freezing are primarily due to heterogeneous ice nucleation or uncontrolled growth of ice crystals that disrupt sperm cells, and secondarily due to inadequate latent heat dissipation, that is repetitive freeze-thaw cycles that cause mechanical damage to cells (Kumar et al., 2019). Intracellular ice crystal disrupts cytoskeleton, whereas the extracellular ice crystal increases solute concentration and increases osmolarity, triggering loss of water from cells (Holt et al., 2014; Morris et al., 2012). Cooling rates have a profound effect on various seminal attributes; a slow rate of cooling followed by four hours of equilibration and freezing is reported as a better protocol for cryopreservation of Hariana bull semen (Kumar et al., 2018). Thus, the equilibration time is important for spermatozoa to adapt to low temperatures and to enable the translocation of water, decreasing the damage by ice nucleation during freezing-thawing (Fleisch et al., 2017; Leite et al., 2010).

The efficient cryopreservation depends upon numerous factors such as type of extenders, cryoprotectant, equilibration, cooling rate, packaging and thawing rate as well as the individual animals, breed, and species (Andrabi, 2007; Barbas & Mascarenhas, 2009; Clulow et al., 2008; Cotter et al., 2005). Various researchers summarised that stressors like ice formation, solution effects, and osmotic modifications during cooling and freezing (Chatterjee et al., 2017) cause structural changes in the organisation, fluidity, permeability and lipid composition of the sperm membrane. During cooling, sperm cells are predisposed to numerous detrimental effects including ionic imbalance, metabolic decoupling, cellular acidosis, activation of proteases, membrane phase transition, deprivation of energy, destabilisation of the cytoskeleton, and produce reactive oxygen species (ROS) and reactive nitrogen species (RNS). During freezing, harmful effects of ice crystal formation, hyper-osmolarity, alterations in the cell volume and protein denaturation are commonly perceived (Chatterjee et al., 2017; Srivastava & Pande, 2017). In addition to cooling and freezing stressors, thawing also affects semen quality (Bagchi et al., 2008). The researchers tried to find an optimum combination of time and temperature for thawing and found thawing at 70°C for 5 s is superior to thawing at 37°C for 20 s; however, the latter procedure is usually implemented for practical reasons (Pesch & Hoffmann, 2007). The following sections discuss the effects of freezing on deteriorations in the physical and physiological attributes of spermatozoa.

3 | OSMOTIC STRESS

Cryopreservation induces cooling stress which alters osmotic pressure inside the spermatozoa. This transmuted osmotic pressure plays a critical role during cryopreservation of the spermatozoa and ultimately affects the quality of frozen semen (Khan & Ijaz, 2008). Spermatozoa are of small size with large surface area, their viscosity and glass transition temperature interfere during cryopreservation (Isachenko et al., 2003; Morris et al., 2012) resulting in ice nucleation in the extracellular space and osmotic gradient across the plasma membrane (Devireddy et al., 2002; Li et al., 2010). Additionally, cryopreservation brings about dilution or washing of seminal plasma proteins from the sperm cell milieu, deteriorating the normal seminal parameters. The 'dilution effect' damages the spermatozoal membrane, eventually leading to reduced fertilising ability (Muiño-Blanco et al., 2008) that gets partially repaired by adsorption of seminal plasma proteins on to the sperm surface (Rodríguez-Martínez et al., 2011). The temperature drop during supercooling (0°C to −5°C) prompts several biophysical changes and beyond supercooling (−6°C to −15°C), extracellular ice crystals begin to form (Lemma, 2011). The ice formation in the surrounding medium increases the solute concentration (sugars, salts and proteins). Due to an osmotic pressure gradient, water within the spermatozoa passes out in the surrounding medium (Pesch & Hoffmann, 2007). The exosmosis is particularly high from the head of the spermatozoa and across the semi-permeable plasma membrane. Consequently, spermatozoa become increasingly dehydrated (Andrabi, 2007; Watson, 2000). The severity of dehydration depends upon the rate of efflux of water from the spermatozoa and associated damage depends upon the speed of the temperature drop/cooling rate

(Ashrafi et al., 2011; Pereira et al., 2010). The slow drop in temperature results in the excess efflux of water and greater dehydration. Conversely, rapid temperature drop forms large intracellular ice crystals due to lower efflux of water, causing physical damage to cell membranes and other intracellular components and cell death (Lemma , 2011; Watson, 1995).

4 | OXIDATIVE AND NITROSATIVE STRESS

During cryopreservation, any changes in mitochondrial membrane fluidity and phospholipid configuration of the spermatozoal membrane release ROS which overwhelms the available antioxidant (Lone et al., 2017; Said et al., 2010). During metabolism at the supra-physiological level, free radicals are highly unstable and reactive. The cascade of chain reactions results in oxidative and nitrosative stress (Figure 1.) damaging carbohydrates, lipids, proteins and nucleic acids (Agarwal et al., 2005; Ezzati et al., 2020; Nash et al., 2012). The extreme concentrations of ROS will affect the survivability of the spermatozoa via an intrinsic apoptotic pathway characterised by mitochondrial ROS generation, lipid peroxidation of PUFAs (Polyunsaturated fatty acids)-rich sperm membrane, DNA damage and loss of ATP (Aitken et al., 2016; Lone et al., 2017; Moustafa et al., 2004). ROS such as singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl radicals (OH^-) have been recently implicated in inducing poor sperm function and sperm fertilising ability in bovines (Lone et al., 2018). Some studies reported that as functional DNA repair mechanisms are absent during cryopreservation, frozen-thawed spermatozoa are more prone to attack by oxygen-derived oxidants (Peris et al., 2007; Rath et al., 2009). In bovine, mitochondria of dead and abnormal spermatozoa, leukocytes during inflammation of the male genital tract are the main source of ROS generation via an aromatic amino acid oxidase catalysed reaction (Agarwal et al., 2014; Sapanidou et al., 2015; Sariözkan et al., 2009). The potentially toxic substances released from dead spermatozoa, neutrophils and macrophages affect the cryosurviving spermatozoa, further increasing their level and eventually lead to sperm dysfunction (Gomes et al., 1998). The H_2O_2 induces greater DNA damage and genome alteration during cryopreservation, and the loss of sperm viability by degradation and enlarging of the spermatozoa during storage and post-thaw (Gurler et al., 2016; Guthrie et al., 2008). The absence of sufficient protective antioxidant armoury due to extension of semen volume, cryo-injury and associated toxin production contributes to the accumulation of ROS (du Plessis et al., 2008; Saraswat et al., 2012), impair freezability and fertility of spermatozoa (Chatterjee & Gagnon, 2001).

Nitrosative stress is a subset of oxidative stress that arises from an overproduction of reactive nitrogen species (Uribe et al., 2015,

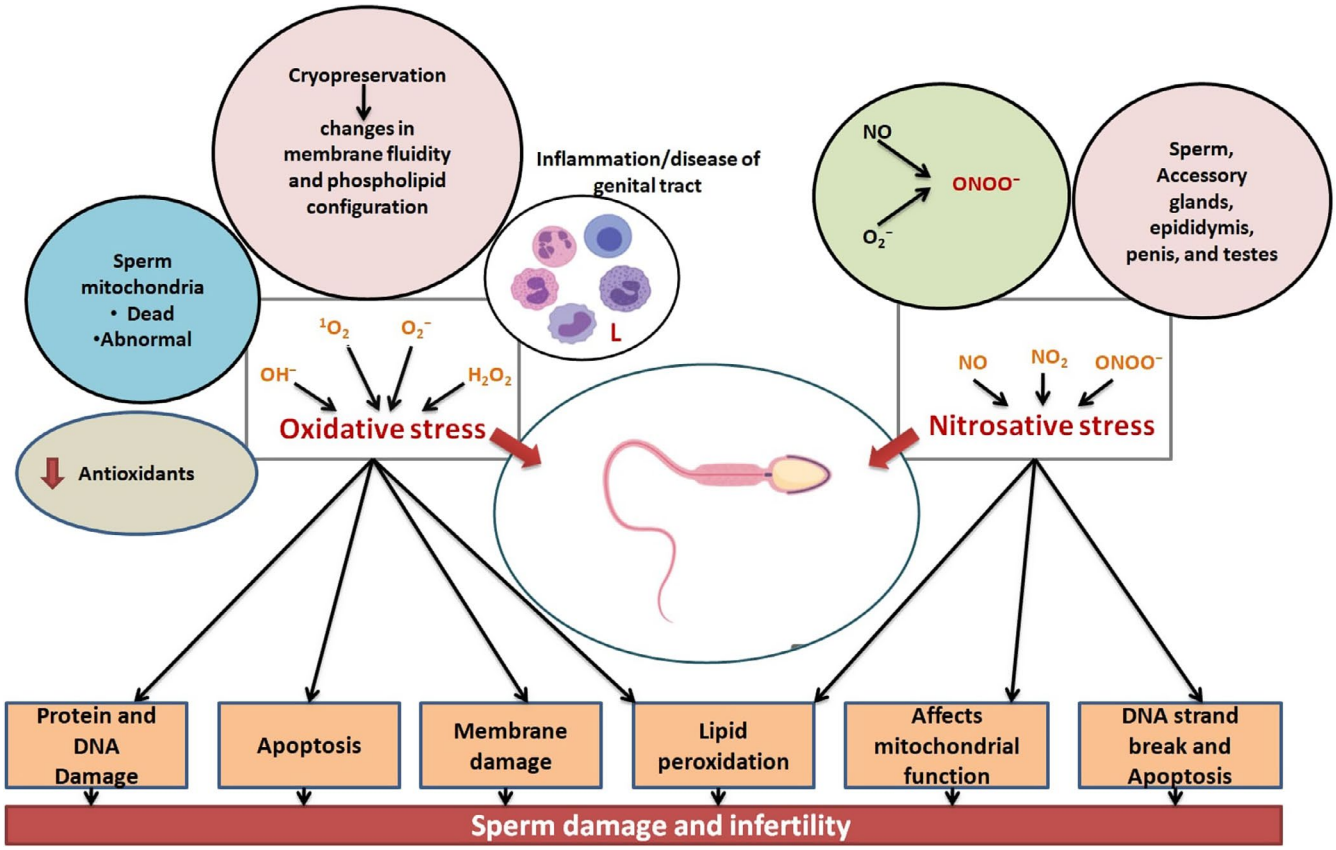

FIGURE 1 Association of Oxidative stress and Nitrsosative stress with sperm damage and infertility. Note: H_2O_2, Hydrogen peroxide; L, Leucocytes; NO, Nitric oxide; NO_2, Nitrogen dioxide; OH^-, Hydroxyl ion; $^1O_2$, Singlet oxygen; O_2^-, Superoxide anion; $ONOO^-$, Peroxynitrite (Created with BioRender.com)

2017). It hampers bull fertility, particularly when cryopreserved (Doshi et al., 2012). RNS includes nitrogen dioxide (NO_2), peroxynitrite ($ONOO^-$), and nitric oxide (NO), and all those products that are formed by the interaction of nitric oxide with oxygen-derived oxidants (Nash et al., 2012). The accessory glands, epididymis, penis and testes are prominent in the production of RNS (Doshi et al., 2012) and are known for their ability to elicit modifications in several biomolecules through oxidation, nitrosylation and nitration (Nash et al., 2012). The mitochondria are the prime site for the formation and reactions related to nitric oxide and peroxynitrite (Vinten-Johansen, 2000), and much of the cytotoxicity of NO and O_2^- radical is mainly mediated by the formation of $ONOO^-$ (Pacher et al., 2007). The $ONOO^-$ further reacts with other molecules and forms nitrogen dioxide and di-nitrogen trioxide, etc., that affects mitochondrial function and triggers the pathogenic mechanism of cell death via nitration and oxidation (Radi, 2013). Increased levels of $ONOO^-$ inhibit ATP production by affecting glycolysis and oxidative phosphorylation (Uribe et al., 2015), break DNA strands, apoptosis and necrosis of spermatozoa.

It is implicated that nitrogen-derived reactive oxidants support several sperm functions in bovines at physiological levels (Miraglia et al., 2011; Naskar, 2018; Upadhyay, 2019); however, at disproportionate levels, it impairs semen quality and sperm fertilising ability (Jalmeria, 2017; Kshetrimayum, 2019). The low concentration of ROS and RNS is mediators of normal sperm functioning and maintains reproductive functions such as cell signalling, tight junction regulation, capacitation, acrosomal reaction, sperm motility and zona binding. In vivo addition of optimised concentration of nitric oxide compounds in cryopreservation media improves various seminal attributes of Murrah buffaloes (Upadhyay, 2019). A balance of free radicals and antioxidants is required to nullify the free radicals during cryopreservation.

5 | BIO-PHYSICAL AND ULTRA-STRUCTURAL ALTERATIONS

The PUFA-rich sperm plasma membrane is the primary site of damage during cryopreservation (Sarlos et al., 2002; Sion et al., 2004; Srivastava & Pande, 2017). During the freeze-thaw process, spermatozoa are susceptible to lipid peroxidation, ice nucleation and osmotic gradient (Li et al., 2010) that seed irreversible damage to the cell membrane (Pommer et al., 2002; Yousef et al., 2003). The temperature and osmotic effects in both freezing and thawing alter sperm water volume and exposed to a high salt concentration, which confers considerable mechanical stress and responsible for asymmetry in the plasma membrane integrity (Noiles et al., 1995). In the course of the cooling process, restrictions of phospholipid lateral movement induce a phase transition and lipid aggregation, triggering irreversible clustering of proteins altering structure (Castro et al., 2016). Furthermore, significant structural reorganisation of the sperm head plasma membrane (Lessard et al., 2000; Medeiros et al., 2002) and scrambling of phospholipids induce the externalisation of phosphatidylserine and phosphatidylethanolamine due to cryocapacitation (Anzar et al., 2002; De Vries et al., 2003). The scrambling is associated with misconfiguration of protein and lipids; morphological changes in the organisation, fluidity, permeability of the sperm membranes and disruption of protein–lipid interactions (Gadella & Harrison, 2002) altering sperm physiological activity (Medeiros et al., 2002). The cryotolerance in bulls spermatozoa depends on several structural characteristics of membranes, like cholesterol/phospholipid molar ratio, (0.45 mol/mol), different fatty acids mapping, degree of hydrocarbon chain saturation, protein/phospholipid ratio (0.80 w/w), protein composition and their expression levels in seminal plasma (Esmaeili et al., 2015; Parks & Lynch, 1992; Ugur et al., 2019). Additionally, during cryopreservation, there is prolonged exposure of spermatozoa to certain seminal plasma proteins causing an efflux of membrane cholesterol (Srivastava et al., 2012), and reduction in phospholipid levels from pre-freeze to the post-thaw stage. Cryopreservation also induces ultra-structural damage to organelles predisposing the spermatozoa to gross morphologic defects, abnormal acrosomes, altered mitochondria, decreased ATP production, cellular integrity, viability, motility and fertilising ability (Dziekońska et al., 2009; Gillan et al., 2005; Nishizono et al., 2004). The biophysical alterations turn the spermatozoa structure and function differently from spermatozoa before cryopreservation.

6 | MOLECULAR ALTERATIONS

The osmotic and temperature changes induce alterations in both lipid and protein framework of spermatozoa reducing fertilising ability (Morris et al., 2012; Pini et al., 2018). The epigenetic factors such as DNA methylation and histone modifications regulate multiple cellular processes and influence gene expression that is dynamically regulated during cryopreservation (Chatterjee et al., 2017; Zeng et al., 2014). Due to the presence of sperm transcripts in bulls (Card et al., 2013), there is transcriptional and post-transcriptional regulation of spermatogenesis (Guo et al., 2017; Luo et al., 2016; Teperek et al., 2016). However, freeze-thaw cycles before AI changes the transcriptomic profiles and related intricate physiology (Card et al., 2013; Chen et al., 2015; Valcarce et al., 2013). The above factors cause altered noncoding RNAs in cryodamage spermatozoa (Capra et al., 2017; Dai et al., 2019; Zhang et al., 2017) and are known to induce apoptosis and differential remodelling of the proteome (Perez-Patiño et al., 2019). The changes in miRNAs expression may be linked with alterations in seminal attributes, and fertilisation rates of spermatozoa impair embryo development (Shangguan et al., 2020). A recent study conducted by Dai et al., (2019) showed that 135 miRNAs were differentially expressed in fresh and frozen-thawed boar spermatozoa reflecting the various structural and functional changes in spermatozoa during cryopreservation. Additionally, altered chromatin structure (Evenson, 2016), disruption of glycocalyx components and denaturation of peripheral proteins are evident (Lemma , 2011).

As per the evidence, cooling stress (Love et al., 2005), cell shrinkage (Kopeika et al., 2015), oxidative stress (McCarthy &

Meyers, 2009; Shaman et al., 2006), osmotic changes (Johnston et al., 2012) and loss of DNA repair enzymes (Bogle et al., 2017) may cause instability of the nuclear DNA, provoke chromatin relaxation and DNA fragmentation during cryopreservation of bovine semen. The sperm DNA fragmentation index (DFI) is considered an essential biomarker for evaluating the relationship between DNA fragmentation and male fertility (Esteves et al., 2014). Spermatozoa provide the zygote with specific mRNA; mRNA transcription is requisite for early embryo development (Ostermeier et al., 2005). Tearing of the tertiary structure of the DNA–protamine complex and degradation of mRNAs (Giaretta et al., 2017) leads to the inaccurate transmission of paternal genetic information and expression of fertility-related proteins (Kashir et al., 2011; Wang et al., 2014), alter transcriptomic profiles of embryos (Ortiz-Rodriguez et al., 2019). The poor quality embryo, impaired preimplantation and poor outcomes of pregnancy are the consequences of DNA fragmentation (de la Calle et al., 2008; Niu et al., 2011), terminating with adverse ART outcome (Oleszczuk et al., 2016; Simon et al., 2010). The retrospective cohort studies carried out by Deng et al., (2019) showed that high DFI reduces good quality embryo recovery rate, while not affecting the live birth rate, miscarriage rate or clinical pregnancy rate. Furthermore, DNA methylation which is part of the epigenetic mechanism is correlated with sperm parameters and found to be important for embryogenesis. However, as a result of cryopreservation, there is an aberrant DNA sperm methylation which is linked to infertility and impaired post-fertilisation development in human and bovine (Aurich et al., 2016; Kropp et al., 2017). The sperm RNA quality and quantity will be affected during cryopreservation, variation in gene expression which is ultimately associated with decreased fertilising potency (Anzar et al., 2002; Ostermeier et al., 2004; Ugur et al., 2019).

7 | SPERM APOPTOSIS

Cryopreservation triggers an apoptotic mechanism (Zeng et al., 2014). The membrane phosphatidylserine translocation occurs in human (Duru et al., 2001), boar (Peña et al., 2003) and bull (Anzar et al., 2002) sperm cells during cryopreservation. The main apoptotic features of sperm cells are decreased mitochondrial membrane potential, caspase activation, reduced cell volume, DNA fragmentation and increased membrane permeability (Di Santo et al., 2011; Paasch et al., 2004; Said et al., 2010). There are significant changes in apoptotic markers and drop in mitochondrial membrane potential after freezing and thawing in living bull sperm cells (Medeiros et al., 2002). The different manifestations of apoptosis are illustrated (Figure 2.).

8 | CRYOCAPACITATION DAMAGES

Cryocapacitation occurs as a result of the production of ROS and RNS during the freeze-thaw procedure (Amin et al., 2018; Bailey et al., 2000; Rodriguez & Beconi, 2009). The adenyl cyclase and

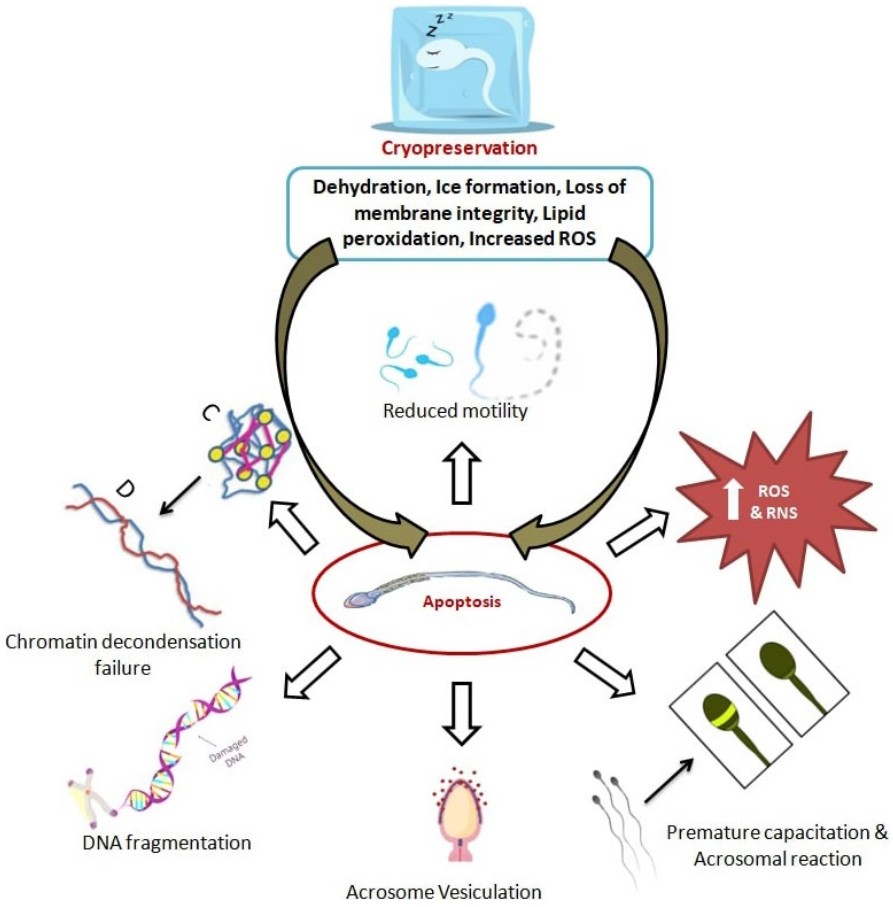

FIGURE 2 Effect of cryopreservation induced apoptosis on bovine spermatozoa. Note: ; C, Chromatin condensation; D, Chromatin decondensation RNS, Reactive nitrogen species; ROS, Reactive oxygen species

protein kinases are activated by reactive oxidants elevating intracellular cAMP sufficient to trigger the mechanisms involved in sperm capacitation. The capacitation-like changes in spermatozoa are induced due to the loss of membrane cholesterol (Neild et al., 2003) and increased intracellular calcium levels during cryopreservation (Longobardi et al., 2017) (Figure 3.). The cold shock alters the selective permeability of the sperm membrane to calcium raising intracellular calcium level during capacitation (Parrish et al., 1999), hyperactivation (Suarez et al., 1993) and acrosomal reaction (Bailey & Storey, 1994). Distorted membrane and lipid–protein interaction are believed to favour further calcium ion influx to trigger intracellular signalling through which spermatozoa enter a partially capacitated state. Cryocapacitation-induced modifications in the sperm membrane make sperm cells more responsive to their surroundings and are associated with reduced longevity and poor survivability in the female reproductive tract (Lemma, 2011; Watson, 2000).

9 | METABOLIC AND FUNCTIONAL ALTERATIONS

The biophysical changes in the post-thaw sperm cells influence both cellular metabolism and other sperm functions (Dziekońska et al., 2009; Gillan et al., 2004). The freeze-thaw injury changes energy sourcing and the accomplishment of physiological functions. The sperm cells require a constant supply of energy from the diluent, and the requirement increases significantly with the hyperactivated motility (Ho et al., 2002; Varner & Johnson, 2007). The nutrients are metabolised intracellularly, resulting in the production of energy-rich ATP via glycolysis, citric acid cycle and oxidative phosphorylation (Dziekońska et al., 2009; Januskauskas & Zilinskas, 2002) which is reported to get hampered by various cooling stressors. Storage at cold shock temperature is linked to alterations in the activity of the glucose-6-phosphate-dehydrogenase, a key enzyme in the process of glycolysis (Lemma , 2011). The diminished intracellular concentration of ATP, with an increased conversion rate of AMP/ADP due to the criticality of the glycolysis process, compromises essential sperm functions (Turner, 2003).

The bovine sperm plasma membrane by its rich PUFA levels is highly susceptible to peroxidation and is considered to be the primary site of cryo-injury. The increase in malondialdehyde (MDA) concentration by lipid peroxidation is linked with deteriorations in seminal parameters (Muzafer et al., 2012). Moreover, the structural reorganisation of sperm head plasma membranes disrupts the ability of the bull spermatozoa (Goldman et al., 1998) to fuse normally with oocyte in the female reproductive tract. Cryopreservation is also associated with premature capacitation and acrosomal reaction which accounts for most of the fertility failure without affecting functionality parameters of spermatozoa (Silva & Gadella, 2006). Upadhyay (2019) reported a sharp reduction in the viability, motility, normal morphology, acrosome integrity and HOST positive spermatozoa after cryopreservation, no variation in the sperm functions was found at 1or 2 months of storage. Cryo-injury-induced thiol oxidation in sperm proteins by ROS (O_2^- and $H_2O_{2)}$ and alteration in their protein profiles are associated with the inhibition of sperm motility and fertilising ability (Casas et al., 2010; Flores et al., 2010).

10 | SPERM ANTIGENICITY

Certain sperm surface antigens play a positive role in fertility (Sprott et al., 2000) through modulation pathways (Hirano et al., 1996), sperm-zona binding (Rajeev & Reddy, 2004) and embryo survival (Jin & Yang, 2017), suggesting that some antigens have a protective role and maintain the seminal attributes during cryopreservation. In contrast, most antigenic proteins have been documented to have adverse effects on sperm function by generating anti-sperm antibodies (ASAs) (IgG and IgA) in reproductive tract secretions, the possible cause of immune infertility in males (Archana et al., 2020). The difference in the effect of antigenicity between the functional attributes of fresh and frozen-thawed spermatozoa could be due to degradation (Meggiolaro et al., 2003) or changes in the antigenic properties of proteins during cryopreservation (Lessard et al., 2000), or due to binding of secretory antibodies to surface antigens causing spermatozoa agglutination (Barbonetti et al., 2019; Srivastava et al., 2017). The ASAs block the sperm capacitation and zona binding by inhibiting the fluidity of the functional membrane (Ferrer et al., 2017) and tyrosine phosphorylation (Pujianto et al., 2018). The antigenic potential of spermatozoa membrane proteins decreases the capacity of mucous penetration, interferes in motility, oocyte binding capacity and fertility in bovines (Kuntareddi et al., 2020). Further, higher antigenic potential can increase spermatozoa's susceptibility to stress and apoptosis, lower mitochondrial membrane potential, and induce acrosome reaction during cryopreservation (Archana et al., 2020).

11 | ASPECTS AND PROSPECTS TO COUNTER CRYODAMAGE

The disadvantages of conventional freezing led to the advent of new cryopreservation techniques, such as directional freezing technique using a multi-thermal gradient system (Arav, 1999) and vitrification (O'Neill et al., 2019) that involves short equilibration time, fast cooling rates and least expensive equipment. In vitrification, the entire solution with spermatozoa is rapidly solidified without crystallisation of ice (Bagchi et al., 2008), achieved by ultra-rapid cooling of the solution where semen samples are transitioned from 37°C to −196°C in less than 1 s. The high solute concentration in this method increases viscosity which prevents nucleation and growth of ice crystals, and ultimately, solution enters a 'glassy-state' (Hunt, 2011). It results in faster cooling rates, superior post-thaw motility and cryotolerance than slow programme freezing (Li et al., 2019). These techniques are found to be superior to conventional freezing and simpler in application but unfortunately have less applicability as encouraging results are only reported in limited species (Kumar et al., 2019). The conventional freezing technique is still most widely

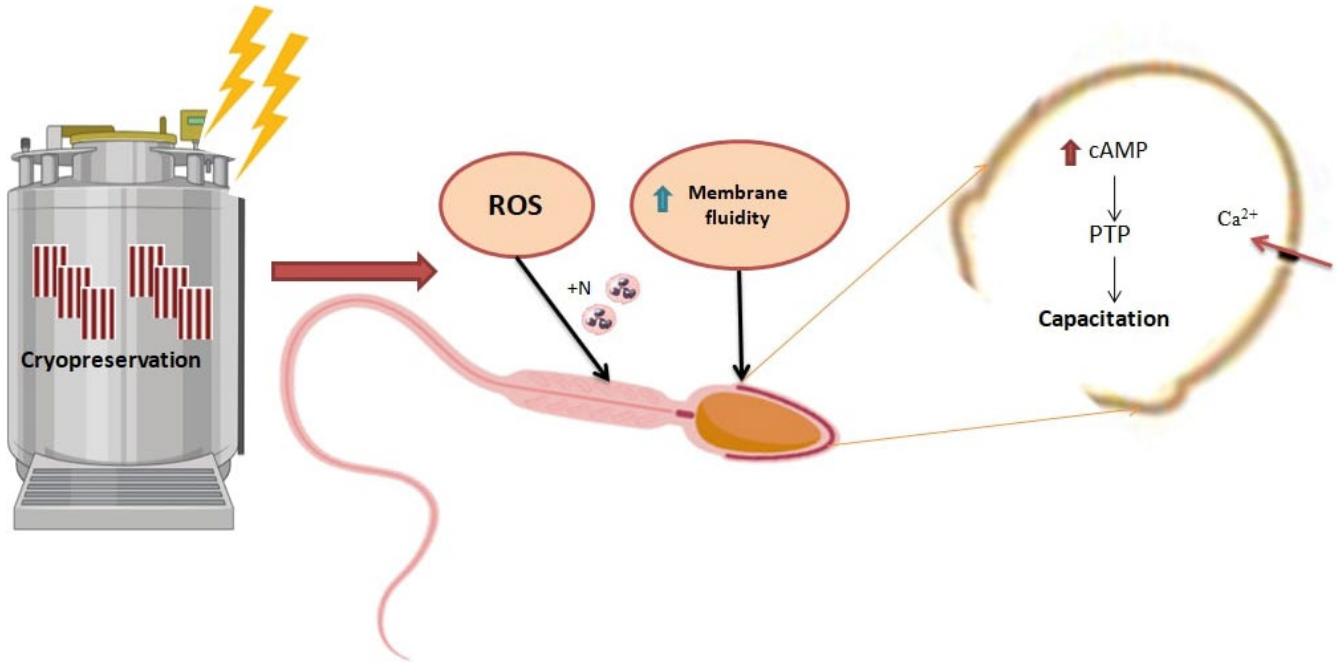

FIGURE 3 The cryopreservation induced capacitation of spermatozoa. Note: Ca^{2+}, Calcium ions; cAMP, Cyclic adenosine monophosphate; N, Neutrophils; PTP, Protein Tyrosine Poshorylation; ROS, Reactive oxygen species (Created with BioRender.com)

used, and the associated cryo-injury can be minimised by altering the type of freezing media, cryoprotectants, antioxidants, protein source, and reducing the mechanical and oxidative stresses involved in the semen processing, storage and transfer. In this context, various concentrations of cholesterol-loaded cyclodextrins (CLC) have been used to prevent cold shock and to enhance cryosurvivability of spermatozoa in cattle (Purdy & Graham, 2004; Yadav et al., 2017) and buffalo bulls (Ejaz et al., 2016; Rajoriya et al., 2016). The addition of CLC at the rate of 3 mg/120 × 10^6 spermatozoa in bubaline species protects sperm progressive motility, membrane fluidity, and enhances in vitro fertility rate (40% more zona binding and 35% higher cleavage rate) (Rajoriya et al., 2020). The cholesterol at low temperatures increases membrane fluidity and prevents membrane damage due to phase separation events from fluid to the gel state (De Leeuw et al., 1990). The addition of antifreeze protein (AFP) to the semen extender of sheep, cattle and buffalo bulls (Prathalingam et al., 2006; Qadeer et al., 2015, 2016) reduces freeze-thaw damage, ice nucleation and ice recrystallisation. The antifreeze protein Type I at the rate of 0.1 μg/ml resulted in greater sperm kinetics, better plasma integrity, and a higher % of normal sperm cells in ram, with no adverse effect on potential fertilisation capacity (Correia et al., 2021). The supplementation of 10 μg/ml of recombinant AFPs in the diluent promotes cryosurvivability yield higher in vitro cleavage and in vivo fertility rate in Nili Ravi buffalo bulls (Qadeer et al., 2016). Nanotechnology provides new opportunities for sperm manipulation, nanoparticles are used to defend against free radicals and concomitant oxidative stress generated during the freeze-thaw procedure (Saadeldin et al., 2020). In Holstein bulls, supplementation of selenium nanoparticles (Se-NPs) at the rate of 1.0 μg/ml in diluents significantly improved sperm characteristics, intact sperm %, and significantly decreased % of apoptotic and necrotic spermatozoa in frozen-thawed semen (Khalil et al., 2019). The extender containing 1.0 mg/ml Se-NPs had a higher conception rate (90%) as compared to the control (59%). The addition of nano-lecithin-based extender with glutathione peroxidase improved bovine sperm plasma membrane integrity, decreased MDA concentration with greater in vitro embryo production capacity (Mousavi et al., 2019). Artificial or natural nanovesicles, including liposomes and exosomes, have recently demonstrated regenerative capabilities to repair damaged spermatozoa and have been found to ameliorate the adverse effects of cryopreservation (Saadeldin et al., 2020). Liposomes may replace damaged lipid skeletons of frozen/thawed spermatozoa with their phospholipid and lipid chain contents and serve as a vehicle for the delivery of antioxidants (Najafi et al., ,,2018, 2020), lecithin (Mehdipour et al., 2017), nutrients and drugs to target tissues (Antimisiaris et al., 2018; Mortazavi et al., 2020). Similarly, exosomes have a possible pleiotropic impact since they contain antioxidants, lipids and other bioactive molecules that repair spermatozoa, increase the efficacy of freezing procedures and improve fertility (Saadeldin et al., 2020). Osteopontin (OPN) is a highly abundant multifunctional phosphoprotein in the seminal plasma of bull with high semen freezability (Rego et al., 2016) and interacts with fertility-related proteins such as CD44 and integrin complexes (Cunha Bustamante-Filho et al., 2020). In vitro treatment of frozen bovine spermatozoa (Gonçalves et al., 2008; Monaco et al., 2009) and buffalo spermatozoa (Boccia et al., 2013) with OPN improves sperm cell viability, increases the number of capacitated spermatozoa, increased fertilisation rate and embryo quality. The addition of

OPN at different concentrations (1 μg/ml and 10 μg/ml) improves in vitro embryo production efficiency by enhancing in vitro sperm capacitation and blastocyst yields in buffalo (Boccia et al., 2013). These findings support the hypothesis that OPN is an essential protein for the interaction of gametes, fertilisation and early embryo development; thus, the addition of OPN isolated from bovine milk in semen and IFV media can be a novel approach (Cunha Bustamante-Filho et al., 2020). Since cryopreservation changes miRNAs regulation during the freeze-thaw process with differential expression in low versus high motile spermatozoa indicating a functional redundancy and possible role in male fertility (Capra et al., 2017). Cold modulated miRNAs (cryomiRs) belonging to freeze related miRNAs family can modulate cold tolerance in mammals and aid the reorganisation of metabolic priorities for freezing survival (Biggar et al., 2009).

12 | ANTIOXIDANT STATUS AND COUNTERACTING OXIDATIVE STRESS

Seminal antioxidants (superoxide dismutase and catalase) vary among bulls from fresh to post-thaw. A drastic decrease was observed in the activity of enzymatic antioxidants and total antioxidant capacity which is known to ameliorate lipid peroxidation and optimise free radicals production (Lone et al., 2016). The decrease in seminal antioxidants level during freezing is due to the extension of semen and associated cryo-injury during the entire process (Castro et al., 2016). Cryopreservation of bull spermatozoa in egg yolk-based extenders significantly reduces the intracellular level of thiols and post-thaw treatment of frozen semen with thiols and antioxidants minimise H_2O_2-mediated loss of sperm motility (Bilodeau et al., 2001). Therefore, antioxidants manifest important mechanisms in vitro and in vivo to minimise oxidative damage to biomolecules and maintain cellular equilibrium (Meamar et al., 2012; Silva et al., 2013; Taylor et al., 2009; Thomson et al., 2009; Thuwanut et al., 2010). The citrate–egg yolk extender containing SOD and butylated hydroxytoluene reduce the production of MDA in bovine semen post-cryopreservation (Asadpour et al., 2012). Fresh bull semen supplemented with curcumin significantly increases GSH content post-thawing (Shokry et al., 2020), its nanoformulations potentiate sperm function, decrease sperm apoptosis and chromatin decondensation of post-thawed buck semen (Ismail et al., 2020). Further, the high content of antioxidants, flavonoids, vitamins, organic acids, pollen grains and honey protects bull semen (El-Sheshtawy et al., 2014; El-Sheshtawy et al., 2014). Some plant extracts (El-Sheshtawy & El-Nattat, 2017; Uysal & Bucak, 2007; Zhao et al., 2009) and olive oil (Krishnappa et al., 2018) have antioxidant and medicinal properties that suppress lipid peroxidation and have been used in semen extenders to boost the quality of mammalian spermatozoa post-thawing (Santos et al., 2018). Supplementation of aqueous leaf extract of *Moringa oleifera* in extender has a profound effect due to its antioxidant and antimicrobial properties improving semen quality in Murrah bulls (Patoliya, 2019). The clove oil has sperm protective activity and has shown good ROS-sequestering activity in both biochemical and cellular assays (Baghshahi et al., 2014). The essential oils such as olive and clove oil may therefore be an agent of interest for the maintenance of sperm quality during in vitro reproductive biotechnology (Santos et al., 2019).

The ROS production is mitigated by reducing leukocyte count or dead spermatozoa or oxygen tension throughout the sperm microenvironment and semen radiation exposure. The already produced ROS is neutralised by enzymatic, nonenzymatic or plant-based antioxidants method (Kumar et al., 2019). The O_2 is reportedly linked with ROS production; therefore, partial deoxygenation of semen extender is done with N_2 gas or by incorporating a commercially available bacterial membrane preparation like oxyrase (Darr et al., 2016; London et al., 2017) into the cryomedium. Further, the use of ROS scavengers enhances the functionality of spermatozoa and protects spermatozoa from the deleterious effects of cryopreservation (Varnet & Aitken, 2004; Watson, 2000). The exogenous addition of enzymes such as catalase, GSH, superoxide dismutase (SOD), other antioxidants like taurine neutralises ROS and maintains the motility of bovine spermatozoa (Bilodeau et al., 2001). Nonenzymatic antioxidants such as vitamin C and vitamin E (Mittal et al., 2014), ferrous sulphate and ascorbic acid (Bansal & Bilaspuri, 2008), vitamin B12 (Hu et al., 2011) and astaxanthin (Lee & Kim, 2018) have been incorporated into the diluent before freezing for improving sperm quality.

The dead and defective spermatozoa (source of ROS) are removed either by sedimentation or migration methods to separate fertile spermatozoa based on their density or motility (Singh et al., 2019). These methods include various density gradient filtration strategies (Morrell & Rodriguez-Martinez, 2016; Oliveira et al., 2012), swim-up assay (Arias et al., 2017), single layer centrifugation (Nongbua et al., 2017), Sephadex column filtration (Galarza et al., 2018; Maurya & Tuli, 2003) and glass wool filtration (Husna et al., 2016) with varying success rate. Raval (2019) observed that in both fresh and frozen-thawed semen, motility, live and HOST positive spermatozoa were significantly increased after filtration, while membrane damage, protamine deficient and cryo-capacitated spermatozoa were significantly lowered in filtered spermatozoa than that to nonfiltered. The nano-purification of semen was documented as a novel technique because of its improved efficiency and practical applicability in mass production (Štiavnická et al., 2017). The semen nano-purification by a nanoparticle-based magnetic purification method is a noninvasive approach to epigenetic-based selection (Feugang et al., 2015). In particular, iron oxide nanoparticles (IONPs) are known for their magnetic properties, biocompatibility and biofunctional properties (Huang & Tang, 2004) since they are coated with ubiquitin antibodies (present on the surface of defective spermatozoa) and with lectins to bind to glycans exposed to acrosomal damage in defective spermatozoa (Odhiambo et al., 2014). The magnetic NPs did not impair the spermatozoa motility and their ability to fertilise oocytes (Ben-David Makhluf et al., 2006). The IONPs combined with 2.0 μg/ml anti-ubiquitin antibodies can be an efficient dose to decimate dead and impaired spermatozoa, reduce oxidative stress and increase the quality of post-thaw buffalo spermatozoa (Bisla et al., 2020). Understanding reactive oxidants and antioxidant

status in extended semen and analysing their concentrations help in the in vitro manipulation of semen for ART (Aliakbari et al., 2016; Najafi, Adutwum, et al., 2018).

A recent approach in low-dose packaging dilutes semen, lowers seminal proteins and spermatozoa per unit volume which consequently lowers semen quality (Karan et al., 2018). The other possible reason is the presence of larger intercellular space between spermatozoa by higher dilution rate that reportedly changes the parameter of freezing and thawing and alters the osmotic effect on the spermatozoa (Raval, 2019). It was hypothesised that the lack of some protective factors from spermatozoa and essential seminal plasma components like proteins, antioxidant enzymes and ions may destabilise the membrane of spermatozoa (Leahy & de Graaf, 2012) and inhibit capacitation (Moura et al., 2007) and acrosome status (Karan et al., 2018) during the freeze-thaw process. Considering the requirement for low-dose packaging, modified packaging (low volume packaging with a small gap between the diluent and diluted semen) is an effective method for low-dose cryopreservation with acceptable post-thaw semen quality. Modified packaging by filling 62.5 μl of diluted semen (5 million spermatozoa) in french mini straws had similar post-thaw semen quality to 250 μl (20 million) conventional packagings (Karan et al., 2018).

13 | CONCLUSION

In recent years, our understanding of the molecular and genetic makeup of spermatozoa has been escalated, and it is the right time to do parallel efforts to understand the overall effect of cryopreservation on spermatozoa. The underlying mechanisms of the cryopreservation process at various stages of processing and post-thaw are associated with interference of one or more orchestrated biological cascades and alterations in ultra-structure. Conventional tests like viability and motility analysis are not a true reflection of the alteration in fertilising ability and abnormal fertilisation. Therefore, a comprehensive assessment of different aspects of cryopreservation such as cryo-variables, freezing protocols, molecular, biochemical and genetic determinants along with the application of an advanced battery of tests is necessary to identify and counter such deterioration. The latest promising technique like sexed semen also employs frozen semen; therefore, researchers need to optimise effective extenders and freezing protocols for a more viable and sustainable dairy sector. Further, modified packaging is capable of delivering all the spermatozoa in a small volume without any loss and could be utilised for commercial purposes in AI. Similarly, nanoparticles and nanoformulations should be widely commercialised with appropriate manufacturing practices that can serve as innovative methods in preserving the fertility of bovine semen.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest regarding authorship of this article.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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