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Review article

# Current perspectives on ruminant sperm freezability: Harnessing molecular changes related to semen quality through omics technologies

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## Abstract

The recent advances in sperm cryopreservation transcend cryobanking and other assisted reproductive technologies. Since its discovery, cryopreservation has contributed positive impacts on animal breeding as well as in genetic exchange, improvement, and conservation efforts. However, cryoinjury and variabilities in cryopreservation outcomes remain as key challenges to sperm cryobiology. The present work explored the molecular bases for such freezability differences and freezing-thawing injuries in the ruminant sperm. Relevant biomarkers identified in the seminal plasma and the spermatozoa were highlighted, including lipids, proteins, metabolites, transcripts, and genes. Specific molecular mechanisms concerning sperm structures and functions were also examined relative to their association to cryotolerance, and spermiogram or seminogram modifications following cryopreservation procedures. Current conflicts and gaps in the knowledge base on ruminant spermatozoa were also emphasized. Further investigation of these areas using the available breakthrough molecular tools such as omics technologies is therefore proposed to improve, optimize, or even predict the overall quality of frozen-thawed ruminant semen towards reproductive efficiency.

**Keywords:** Cryopreservation, Freezability, Molecular changes, Ruminant sperm, Semen quality

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## INTRODUCTION

Over the years, substantial progress has been made toward the improvement of assisted reproductive technologies (ARTs) for domestic and wild animals. Sperm cryopreservation allowed for the long-term storage and utilization of male gametes from organisms with superior genetics (Ugur et al., 2019), declining conservation status, or even those at the brink of extinction (Pukazhenthi, 2016). Recently, the ruminant spermatozoa have been vitrified (Arando et al., 2017; Pradieé et al., 2018), and their freezing medium was supplemented with nanoparticles (Hozyen et al., 2020; Khalil et al., 2019) and other novel compounds (Batissaco et al., 2020; Fang et al., 2020). These approaches offer alternatives to conventional techniques and extender components to enhance cryosurvival and fertility in frozen-thawed semen.

Despite these developments, cryopreservation procedures compromise sperm quality and fertilizing ability. Spermatozoa are subjected to chemical, osmotic, mechanical, and thermal stresses during the dilution, cooling, equilibration, freezing, and thawing stages (Andrabi, 2009; Rasul et al., 2001). Even with optimized protocols, it is now generally accepted that only approximately half of the total sperm population (about 40%–50%) endure the freeze-thaw cycles (Grötter et al., 2019). Moreover, the cryosurvivability of spermatozoa differs within and between species and individuals. Cryopreservation outcomes still varied in different males with fundamentally similar spermiogram or seminogram results. Sperm from some animals with outstanding reproductive success in natural mating also exhibited poor freezing resilience (Gomes et al., 2020; Kumar et al., 2019; Rego et al., 2016).

The advent of functional genomics (lipidomics, metabolomics, proteomics, and transcriptomics) and epigenomics (chromatin dynamics and DNA methylation) (Khan et al., 2021; Peris-Frau et al., 2020; Ugur et al., 2019) revolutionized our current understanding of the complexities of reproductive biology, particularly that of sperm physiology. These have brought us closer than ever in elucidating the molecular changes in frozen-thawed spermatozoa relative to semen quality, as well as in unraveling the molecular factors involved in freezability differences, which were the main highlights of this review. Unless there is a compelling need to incorporate accounts from other species, discussions of cryoinjury and freezing resistance have primarily focused on ruminants. Moreover, the association of freezability with sperm structures and functions was also discussed in sections.

## SPERM FREEZABILITY

During cryopreservation, the successive processes of temperature reduction, cell dehydration, freezing, storage, and finally thawing expose sperm to cryoinjuries, which affect cryosurvival (Ugur et al., 2019). These are generated, at least in part, by osmotic shock caused by solution effects on cell volume, mechanical disruptions from intracellular ice crystal formation, and thermal challenges from cold shock (Khan et al., 2021; Khalil et al., 2018). Deleterious changes to sperm architecture and functions (Figure 1) result from

such causes, including a breakdown of membrane permeability and fluidity, free radical production, cytoskeleton destabilization, ionic imbalance, DNA fragmentation, disturbance in macromolecular interactions, and protein, RNA, and epigenetic modifications. The ability of spermatozoa to maintain their structural integrity and functional competence following cryopreservation-induced stresses relates to their freezability, cryotolerance or cryoresistance (Martínez-Fresneda et al., 2021; Peris-Frau et al., 2020). In this context, males were frequently classified as either good or bad freezers. Semen samples were also phenotypically categorized into high and low freezing-resilient groups, using a variety of criteria, as shown in Table 1. These attributes utilized as sperm freezability determinants in ruminants include motility and velocity (Perumal et al., 2014; Rego et al., 2016; Ryu et al., 2019), viability (Gomes et al., 2020; Hitit et al., 2020), and morphometry (Esteso et al., 2006; Ramón et al., 2013; Ros-Santaella et al., 2014), with motility and viability being the most important indices for predicting cryoresistance and fertilizing ability.

Variations in sperm responses during cryopreservation are among the conundrums in sperm cryobiology. Nonetheless, several factors, including sperm source (Martínez-Fresneda et al., 2019a; Martínez-Fresneda et al., 2021; Pini et al., 2016), quantitative proteomic variations in sperm and seminal plasma (Gomes et al., 2020; Morató et al., 2018; Rego et al., 2016; Ryu et al., 2019; Song et al., 2020; Wang et al., 2014), seasonal influences (Martínez-Fresneda et al., 2019b; Westfalewicz et al., 2019), inter-species differences (Dorado et al., 2010; Rickard et al., 2015), and genetic control (Ramón et al., 2013), have already been implicated in ruminant sperm cryotolerance.

A plethora of studies have identified candidate markers for semen freezability and fertility from genes, transcripts, proteins, and metabolites. Tables 2 and 3 listed some differentially represented proteins in the ruminant spermatozoa or semen with high and low freezability or motility, respectively. These proteins are involved with a wide array of cryosurvival functions, for instance, resistance to cold-shock as facilitated by the abundance of heat-shock protein (HSP90) (Wang et al., 2014), or tolerance to cryoprotective agents (CPAs) as enabled by the high expressions of cytosolic 5-nucleotidase 1B (NT5C1B) and fumarate hydratase (FH) (Song et al., 2020).

**Table 1** Criteria used by some studies to define sperm freezability in ruminants

| Freezability Criteria | Animal Species | Reference |
|---|---|---|
| Post-thaw sperm viability greater (high) or lesser (low) than the average of the sample population | Holstein bull | Gomes et al. (2020) |
| Post-thaw motility of >60% (high) or <15% (low) | Native Korean beef bull | Ryu et al. (2019) |
| Post-thaw motility of >50% (high) or <50% (low) | Jersey crossbred bull | Perumal et al. (2014) |
| Post-thaw motility of >30% (high freezability) or <30% (low freezability) and sperm vigor of >3.0 (high) or <3.0 (low) | Guzerat bull | Rego et al. (2016) |
| Pre-freeze and post-thaw motility difference, small (high) or large (low) decrease | Mixed breed ram (Merino, Poll Dorset, Finn X and Coopworth) | Rickard et al. (2015) |
| Post-thaw motility of >40% (high) or <40% (low) | Holstein bull | Wang et al. (2014) |
| | Murrah buffalo bull | Singh et al. (2014) |
| Post-thaw rapid progressive motility of >30% (high) or <30% (low) | Florida buck | Dorado et al. (2010) |
| Post-thaw sperm viability of >90% (high) or <90% (low) of ejaculates during a two-year period<br>*Frozen-thawed ejaculate is considered viable if it has a minimum of 30% motility, 45% acrosomal integrity, 20% major defects, 25% minor defects, and 30% total sperm defects | *Bos taurus indicus* and *Bos taurus taurus bulls* | Jobim et al. (2004) |

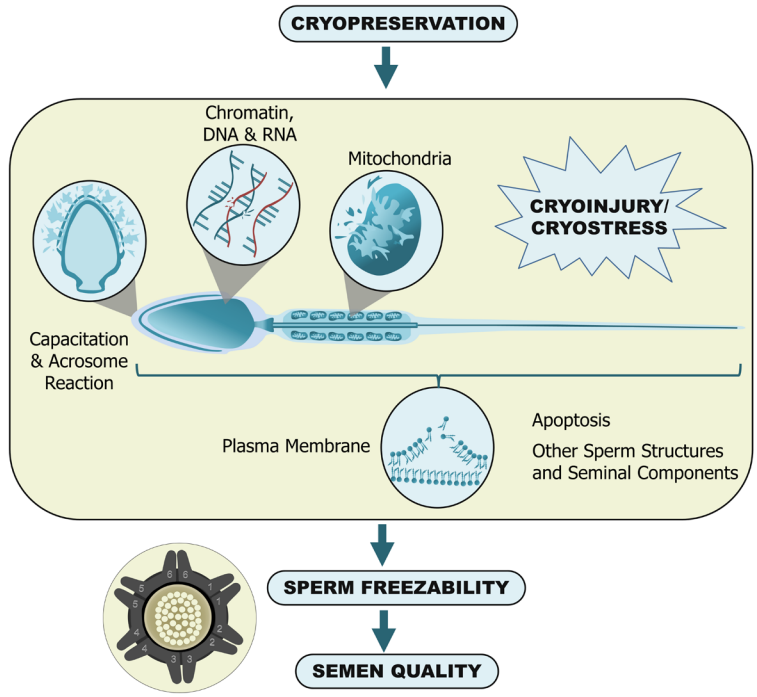


**Figure 1** Sperm structures and functions affected by cryopreservation, which are also associated with freezability phenotypes.

**Table 2** Proteins which were either found in greater abundance, or highly expressed in the spermatozoa or seminal plasma of ruminant species with high freezability (or motility) phenotypes

| Protein | Species and Source | Functional Attributes |
|---|---|---|
| 14-3-3 protein zeta/delta | Murrah buffalo seminal plasma (Codognoto et al., 2018) | Protein phosphatase 1 modulation, protein synthesis, interactions and cellular transport, sperm motility and fertility, spermiation and spermatogenesis |
| 26S proteasome complex (PSMA2, PSMA8, PSMD13) | Lacaune ram seminal plasma (Soleilhavoup et al., 2014) | Capacitation, acrosome reaction, zona pellucida penetration, and sperm cell organisation |
| | Mixed breed (Merino, Poll Dorset, Finn X and Coopworth) ram seminal plasma (Rickard et al., 2015) | |
| Acidic seminal fluid protein (aSFP) | Bos taurus indicus and Bos taurus taurus seminal plasma (Jobim et al., 2004) | Oxidative stress protection, and stimulative effect on ovulation upon insemination |
| Acrosin inhibitor 1 (SPINK2) | Murrah buffalo seminal plasma (Codognoto et al., 2018) | Acrosin release |
| Acrosome formation-associated factor isoform 2 (AFAF) | Guzerat bull sperm (Rego et al., 2016) | Acrosome formation, acrosomal reaction, and fertilization |
| Alpha-enolase (ENO1) | Lacaune ram seminal plasma (Soleilhavoup et al., 2014) | Sperm energy metabolism, and motility |
| Annexin A1 | Guzerat bull sperm and seminal plasma (Rego et al., 2016) | Sperm Ca2+ metabolism, membrane repair, reorganization of the actin cytoskeleton, and inflammatory process regulation |
| ATP synthase beta subunit (ATP5F1B, ATP1B1), F1-ATPase complexed with aurovertin B (F1-ATPase) | Hanwoo bull cauda epididymal sperm (Ryu et al., 2019) | Sperm energy metabolism, and motility |
| Clusterin | Bos taurus indicus and Bos taurus taurus seminal plasma (Jobim et al., 2004) | Sperm membrane protection, mitochondrial membrane integrity, suppression of stress-induced apoptosis, cell adhesion and clustering, and sperm maturation |
| Dihydrolipoyl dehydrogenase | Guzerat bull sperm (Rego et al., 2016) | Sperm energy metabolism, and sperm hyperactivation |
| Disintegrin | Guzerat bull sperm (Rego et al., 2016) | Sperm-egg binding, and fertilization |
| DNase I-like proteins (Desoxyribonuclease γ and Deoxyribonuclease I-Like III (DNASE1L3)) | Guzerat bull seminal plasma (Rego et al., 2016) | Apoptosis, anti-DNA autoimmunity suppression, and fertility |
| Gelsolin (GSN) | Lacaune ram seminal plasma (Soleilhavoup et al., 2014) | Ca2+-mediated events, capacitation, acrosome reaction, and actin polymerization |
| Glucose-6-phosphate isomerase (GPI) | Lacaune ram seminal plasma (Soleilhavoup et al., 2014) | Sperm energy metabolism, and motility |
| Glyceraldehyde-3-phosphate dehydrogenase (GAPDHS, G3PT) | Guzerat bull sperm (Rego et al., 2016) | Sperm energy metabolism, and motility |
| Heat shock protein 90 (HSP90, HSP90AA1), heat shock protein 70 (HSP70) | Lacaune ram seminal plasma (Soleilhavoup et al., 2014) | Protection from stresses, and sperm motility, fertilizing ability, and resistance to cryopreservation |
| | Mixed breed (Merino, Poll Dorset, Finn X and Coopworth) ram seminal plasma (Rickard et al., 2015) | |
| | Holstein bull sperm (Wang et al., 2013) | |

**Table 2** Proteins which were either found in greater abundance, or highly expressed in the spermatozoa or seminal plasma of ruminant species with high freezability (or motility) phenotypes (Cont.)

| Protein | Species and Source | Functional Attributes |
|---|---|---|
| Metalloproteinase domain-containing protein 2 (ADAM2) | Guzerat bull sperm (Rego et al., 2016) | Sperm-egg recognition, fertilization and membrane stabilization |
| Osteopontin-K | Guzerat bull seminal plasma (Rego et al., 2016) | Sperm binding during ejaculation, sperm-oocyte interaction, capacitation, fertilization, cleavage, and embryo development |
| Seminal plasma protein BSP-30 kDa (BSP5) | Holstein bull seminal plasma (Gomes et al., 2020) | Sperm interaction at ejaculation, capacitation, sperm and oviduct epithelia interaction, fertilization, and stability of sperm membrane structure |
| Seminal ribonuclease (SRN) | Holstein bull seminal plasma (Gomes et al., 2020)<br>Murrah buffalo seminal plasma (Codognoto et al., 2018) | Sperm capacitation, catalytic activity, antioxidant function, and suppression of immune reaction |
| Sorbitol dehydrogenase (SORD) | Mixed breed (Merino, Poll Dorset, Finn X and Coopworth) ram seminal plasma (Rickard et al., 2015) | Sperm motility, protein tyrosine phosphorylation, and fructose biosynthetic and glucose metabolic processes |
| Spermadhesin-1 (SPADH1) | Murrah buffalo seminal plasma (Codognoto et al., 2018) | Carbohydrate-binding activity, sperm capacitation, sperm-oviduct interaction, sperm membrane stability, and sperm-egg binding |
| T-complex protein 1 (CCT6A, CCT6A, CCT7) | Lacaune ram seminal plasma (Soleilhavoup et al., 2014)<br>Mixed breed (Merino, Poll Dorset, Finn X and Coopworth) ram seminal plasma (Rickard et al., 2015) | Zona pellucida interactions, capacitation, sperm motility, actin and tubulin folding, and sperm cell organisation |
| Vasolin containing protein (VCP, transitional endoplasmic reticulum ATPase) | Lacaune ram seminal plasma (Soleilhavoup et al., 2014)<br>Mixed breed (Merino, Poll Dorset, Finn X and Coopworth) ram seminal plasma (Rickard et al., 2015) | Sperm capacitation, apoptosis suppression, DNA damage responses, ATP metabolic processes, membrane usion, vesicle-mediated transport, and the cell division cycle |

**Table 3** Proteins which were either found in greater abundance, or highly expressed in the spermatozoa or seminal plasma of ruminant species with low freezability (or motility) phenotypes.

| Protein | Species and Source | Functional Attributes |
|---|---|---|
| Acrosin inhibitor 1 (SPINK2) | Guzerat bull seminal plasma (Rego et al., 2016) | Acrosin release |
| Alpha-enolase (ENO1) | Mixed breed (Merino, Poll Dorset, Finn X and Coopworth) ram seminal plasma (Rickard et al., 2015) | Sperm energy metabolism, and motility |
| Annexin A1 | Guzerat bull sperm and seminal plasma (Rego et al., 2016) | Sperm Ca2+ metabolism, membrane repair, reorganization of the actin cytoskeleton, and inflammatory process regulation |
| ATP synthase beta subunit (ATP5F1B, ATP1B1), F1-ATPase complexed with aurovertin B (F1-ATPase) | Holstein bull seminal plasma (Gomes et al., 2020) | Sperm energy metabolism, and motility |
| Binder of SPerm 1 (BSP1, BSP-A1, BSP-A2, PDC-109) | Nelore bull seminal plasma (Magalhães et al., 2016) | Sperm motility, cholesterol efflux, capacitation, and acrosome reaction |
| | Murrah buffalo bull seminal plasma (Singh et al., 2014) | |
| Calmodulin (CALM) | Holstein bull seminal plasma (Gomes et al., 2020) | Ca2+-mediated events, capacitation, and acrosome reaction |
| Ephrin-A1 (EFNA1) | Guzerat bull seminal plasma (Rego et al., 2016) | Tyrosine phosphorylation, vascular development and remodeling, and inflammatory cell recruitment, migration and proliferation |
| Gelsolin (GSN) | Holstein bull seminal plasma (Gomes et al., 2020) | Ca2+-mediated events, capacitation, acrosome reaction, and actin polymerization |
| Glucose-6-phosphate isomerase (GPI) | Holstein bull seminal plasma (Gomes et al., 2020) | Sperm energy metabolism, and motility |
| Glutathione peroxidase 3 (GPX3) | Guzerat bull seminal plasma (Rego et al., 2016) | Antioxidant function, and membrane protection against lipid peroxidation |
| Glutathione s-transferase mu 5 (GSTM5) | Hanwoo bull cauda epididymal sperm (Ryu et al., 2019) | Oxidative stress protection |
| Glyceraldehyde-3-phosphate dehydrogenase (GAPDHS, G3PT) | Holstein bull seminal plasma (Gomes et al., 2020) | Sperm energy metabolism, and motility |
| Metalloproteinase inhibitor 2 (TIMP-2) | Guzerat bull seminal plasma (Rego et al., 2016) | Membrane destabilization, metalloproteinase regulation, and fertility |
| Prosaposin | Murrah buffalo seminal plasma (Codognoto et al., 2018) | Sperm-oocyte binding, fertilization and embryo development |
| Peroxiredoxin-5 (PRDX5) | Holstein bull seminal plasma (Gomes et al., 2020) | Protection from reactive oxygen species (ROS), and inflammatory and immune response processes |
| Platelet-activating factor acetylhydrolase (PAFA) | Guzerat bull seminal plasma (Rego et al., 2016) | Oxidative stress protection, sperm membrane stabilization and platelet-activating factor (PAF) regulation |
| Peptide YY | Murrah buffalo seminal plasma (Codognoto et al., 2018) | Antimicrobial activity, calcium influx inhibition, sperm motility and acrosome reaction |

**Table 3** Proteins which were either found in greater abundance, or highly expressed in the spermatozoa or seminal plasma of ruminant species with low freezability (or motility) phenotypes. (Cont.)

| Protein | Species and Source | Functional Attributes |
|---|---|---|
| Secretoglobin family 1D member (SCGB1D) | Holstein bull seminal plasma (Gomes et al., 2020) | Steroid binding, and inflammation-related events |
| Spermadhesin-1 (SPADH1) | Holstein bull seminal plasma (Gomes et al., 2020) | Carbohydrate-binding activity, sperm capacitation, sperm-oviduct interaction, sperm membrane stability, and sperm-egg binding |
| Sperm equatorial segment protein 1 (SPESP1) | Holstein bull seminal plasma (Gomes et al., 2020) | Sperm-egg binding, and fertilization |
| Tubulin beta-4B chain<br>Tubulin beta-4A chain<br>Tubulin beta-5 chain<br>Tubulin beta-2B chain | Holstein bull seminal plasma (Gomes et al., 2020) | Sperm motility, and microtubule components |
| Voltage-dependent anion-selective channel protein 2 (VDAC2) | Hanwoo bull cauda epididymal sperm (Ryu et al., 2019) | Metabolite diffusion, and involvement in mitochondrial apoptotic pathway |
| Zinc-alpha-2-glycoprotein-like (ZA2G, AZGP1) | Lacaune ram seminal plasma (Soleilhavoup et al., 2014) | Sperm motility, transmembrane transport and polyunsaturated fatty acid binding |
| | Mixed breed (Merino, Poll Dorset, Finn X and Coopworth) ram seminal plasma (Rickard et al., 2015) | |

## FREEZABILITY AND SPERM PLASMA MEMBRANE

The sperm's ability to survive in the female reproductive tract and subsequently bind and penetrate the outer oocytic investments is dependent on an intact and functional plasma membrane (Martí et al., 2003). However, its composition differs between animals, species, and even fertile and subfertile groups of similar species (Mandal et al., 2014), resulting in variations in sperm sensitivity to cryopreservation. The cholesterol to phospholipid and polyunsaturated to saturated phospholipid-bound fatty acid ratios in the plasma membrane impart for the spermatozoa's susceptibility or resistance to cold-shock through their contributions to membrane fluidity and stability at low temperatures (Mocé et al., 2010). As a result of their high unsaturated phospholipid and low cholesterol contents, the ruminant spermatozoa, therefore, are intrinsically very sensitive to cold shock compared to other animal species and humans (Bailey et al., 2000). Between domestic large ruminants, buffalo spermatozoa have been observed to be more vulnerable to freezing and thawing hazards than cattle spermatozoa (Andrabi, 2009). Contemporary lipidomics has established the possibility of predicting cryopreservation outcomes in the ruminant sperm. Quantities of saturated fatty acids like arachidic acid (22:0) and monounsaturated fatty acids such as oleic acid (18:1 cis9) differ between Holstein bulls of opposite freezabilities (Evans et al., 2020). This may also somewhat explain the higher plasma membrane integrity observed in good freezability phenotypes (Rego et al., 2016; Wang et al., 2014).

Greater degrees of cold shock suffered by the spermatozoa translate to greater damages to their plasma membrane (López Armengol et al., 2012).

These appear as ultrastructural alterations such as breakage or discontinuities, swellings, blebs or vacuolizations, or complete loss (Khalil et al., 2018; Shi et al., 2014). These can be explained by lipid phase separation, redistribution of phospholipids and proteins, and disruption of the interactions of lipid-lipid and lipid-protein components during cryopreservation (Chatterjee et al., 2001a; De Leeuw et al., 1990; Lemma, 2011). The freeze-thaw cycles also promote lipid peroxidation (LPO) in the membranes following the overproduction of reactive oxygen species (ROS) (Kadirvel et al., 2009a; Maia et al., 2010). While there was an inconsequential difference in the LPO status of good and poor freezability bulls (Hitit et al., 2020), its negative association with sperm motility, DNA integrity, and viability was validated in a number of independent studies (Ahmed et al., 2018; Anghel et al., 2010; Lone et al., 2018).

On the other hand, ROS, as redox signaling molecules, mediate reproductive processes such as sperm hyperactivation, capacitation, acrosome response, sperm-oocyte contact, and fertilization at appropriate physiological concentrations (Aitken, 1995; Gonçalves et al., 2010). Despite this, cryopreservation precipitates their elevated or even uncontrolled production in the spermatozoa (Chatterjee et al., 2001b; Gürler et al., 2016; Santiani et al., 2014). Kumaresan and co-workers (2017) employed the sperm ROS status, specifically that of hydrogen peroxide ($H_2O_2$), in their development of a fertility prediction model in bull, taking advantage of the variations in values obtained from above- and below-average fertility animals. Moreover, during an experimental treatment with exogenous $H_2O_2$, motility was the major sperm function affected (Peris et al., 2007). Significant correlations were also demonstrated between the percentage of live $H_2O_2$-positive spermatozoa, and post-thaw viability and freezability in cattle (Hitit et al., 2020); however, more studies for other ruminant species are needed.

Freezing-thawing procedures, likewise, modulate the activity levels and cellular distribution of enzymatic antioxidants like catalase (CAT), glutathione peroxidase (GPX), superoxide dismutase (SOD), and glutathione reductase (GR) (Bilodeau et al., 2000; Martí et al., 2008a). Glutathione peroxidase 3 (GPX3) and peroxiredoxin-5 (PRDX5) were shown to be highly expressed in the seminal plasma of low freezability bulls, probably to protect the sperm from increased ROS release (Gomes et al., 2020; Rego et al., 2016). An overwhelm in antioxidant scavenging activities also trigger other deleterious effects, including premature capacitation and apoptosis, concomitant to peroxidative membrane and DNA damages (Chatterjee et al., 2001b; Riesco et al., 2021; Santiani et al., 2014). Therefore, oxidative stress curtails sperm functionality, vitality and overall quality, with negative consequences to subsequent fertilization and embryonic development (Bollwein et al., 2018).

The stages of cryopreservation may also have an impact on sperm membrane or surface proteins. Dhanju et al. (2001) confirmed the gradual decrease in the sperm membrane protein content and quality, particularly in terms of number and molecular weight, throughout the duration of freezing. In addition, structural conformation, distribution, and abundance of sulf-hydryl-containing surface proteins in the bull sperm were also altered (Chatterjee et al., 2001b). Proteins associated with membrane stabilization and protection from cryostresses such as acidic seminal fluid protein (aSFP) (Jobim et al., 2004), binder of SPerm 1 protein (BSP1) (Magalhães et al., 2016; Singh et al., 2014), seminal plasma protein BSP-30 kDa (BSP5) (Gomes et al., 2020),

metalloproteinase domain-containing protein 2 (ADAM2) and metalloproteinase inhibitor 2 (TIMP-2) (Rego et al., 2016) varied between low- and high- freezing resilient ruminant spermatozoa, and thus may affect the membrane fusion events of fertilization.

## FREEZABILITY, CAPACITATION AND ACROSOME REACTION

Capacitation and acrosome reaction are fundamental events in fertilization. However, the freeze-thaw cycles can induce their precocious initiation (Khalil et al., 2018; Srivastava et al., 2013). Both partially and fully cryopreserved spermatozoa were observed to undergo cryocapacitation and premature acrosome reaction, thus, affecting their longevity, survivability, and fertility (Kumaresan et al., 2017; Talukdar et al., 2016; Watson, 2000). The post-thaw stability of the acrosomal membrane was also found to be correlated with freezability and motility phenotypes, with high freezing-tolerant and motile sperm samples having greater acrosomal integrity (Capra et al., 2017; Hitit et al., 2020; Singh et al., 2014; Wang et al., 2014).

In addition to its role in cryotolerance (Amorim et al., 2009; Rajoriya et al., 2014), cholesterol also plays a pivotal function in membrane stabilization to prevent capacitation-like changes (Longobardi et al., 2017; Rajoriya et al., 2020). Nonetheless, cryopreservation allows frozen-thawed spermatozoa to bypass the true capacitation cascade through the considerable removal of cholesterol from the sperm plasma membrane (Kadirvel et al., 2009b; Rajoriya et al., 2016; Yadav et al., 2017). Greater efflux of cholesterol was noted in non-freezable ejaculates compared to their freezable counterparts (Singh et al., 2014). As a consequence, this process enhances membrane fluidity and permeability and promotes calcium influx (Minervini et al., 2013; Watson, 2000). Because of the difficulty in maintaining and regulating concentration gradients over time, this uncontrolled intracellular calcium surge may be deleterious during cryoinjury, impacting sperm cryosurvival (Bailey et al., 1994; Bailey et al., 2000).

Tyrosine phosphorylation is recognized as the hallmark of sperm capacitation, apart from its function in sperm motility regulation (Naresh et al., 2015; Yadav et al., 2017). Cryocapacitated spermatozoa likewise undergo this same phenomenon. However, there were some reductions in the zona binding ability of frozen-thawed sperm, which could have an impact later in fertilization (Cormier et al., 2003; Kadirvel et al., 2011). Although the exact molecular mechanisms of cryocapacitation have yet to be fully understood (Rajoriya et al., 2016; Talukdar et al., 2016), disparate phosphotyrosine-containing protein profiles of physiologically capacitated and cryocapacitated spermatozoa have been previously described (Cormier et al., 2003). Moreover, cryopreservation enhanced the tyrosine phosphorylation of cattle and buffalo sperm proteins such as glutathione-S-transferase (GST), p20, p30, p32, p38, p49, p56, p59, p72 and p86 (Kumar et al., 2012; Kumar et al., 2011; Kumar et al., 2014; Yadav et al., 2017). Furthermore, capacitation-associated proteins such as actin-related protein M1, actin-related protein T2, capping protein beta 3 isoform, glutathione S-transferase, isocitrate dehydrogenase, NADH dehydrogenase, outer dense fiber protein 2, phosphatidylethanolamine-binding

protein 4, ropporin-1 and triosephosphate isomerase were found to be carbonylated as a result of oxidative modifications in cryopreserved semen (Mostek et al., 2017). These post-translationally modified proteins may be suitable sperm cryotolerance markers (Harayama et al., 2010) that warrant additional investigation. Furthermore, some authors have also identified, characterized, and localized ruminant spermatozoa acrosomal membrane proteins, such as the 64 kDa bovine polypeptide, and the 34 and 39 kDa ovine polypeptides (Harayama et al., 2010; Nagdas et al., 2013; Sukardi et al., 2001). However, their precise roles in vesiculation, membrane fusion, and content release during physiological and cryopreservation-triggered acrosomal reactions, and correlation with freezability and fertility phenotypes remain elusive.

## FREEZABILITY AND SPERM APOPTOSIS

The freeze-thaw cycles also incite apoptosis-like events in spermatozoa. Proportions of apoptotic sperm rose after cryopreservation, though they were already existent before the procedure (Anzar et al., 2002; Khan et al., 2009; Martin et al., 2007; Martin et al., 2004). Nakidkina et al. (2019) underscored the likely involvement of apoptosis in poor quality semen, as it might be an attribute related to low motility, plasma membrane integrity (Khan et al., 2009) and fertilizing ability (Anzar et al., 2002) in ruminants. Nevertheless, the ambiguous role of apoptosis in the ejaculated sperm (Martí et al., 2008b) has been the subject of recent andrological studies in several other domestic animals.

Externalization of phosphatidylserine is a well-known apoptosis-related characteristic. Rather than being sequestered in the inner cytosolic leaflet of the sperm plasma membrane by ATP-dependent aminophospholipid translocases or flippases (Dalal et al., 2016), the phospholipid is translocated and expressed to the outer surface during the dilution, cooling/refrigeration, and freezing-thawing procedures (Ahmad et al., 2015; Del Valle et al., 2010; Mendoza et al., 2013). On the contrary, Januskauskas et al. (2003) and Müller et al. (1994) found that phosphatidylserine remained undisturbed in the cytoplasmic surface, and the stability of the plasma membrane bilayer asymmetry was maintained in intact ram and bull cryopreserved spermatozoa despite diminished aminophospholipid translocase activity, respectively.

Apoptotic spermatozoa, like cryocapacitated sperm populations, have low mitochondrial transmembrane potential (ΔΨM) and structural membrane integrity (SMI) (Varela et al., 2020). These modifications may be related to the opening of the mitochondrial permeability transition pore (mPTP) (Castro et al., 2016; Fang et al., 2020), which initiates the cytosolic release and activation of numerous proapoptotic factors like Bax, Bid, Bim and SMAC proteins (Fang et al., 2020; Martin et al., 2007; Martin et al., 2004). Apoptotic regulators such as cytochrome c, apoptosis-inducing Fas receptor, heat shock proteins (HTRA, HSP60, HSP70), and antiapoptotic factors (Bcl-2, Livin, Survivin, CD40L, insulin-like growth factor-I and -II) had greater signaling in refrigerated ram spermatozoa compared to their fresh swim-up state (Mendoza et al., 2013). In contrast, spermatozoa expressing high-ΔΨM have reduced risks of suffering from the aforementioned injuries, relative to their cryotolerance (Varela et al., 2020).

Another key event in programmed cell death is the activation of caspases (Martí et al., 2008b). These members of the cysteinyl aspartate-specific protease family have also been found in cryopreserved ruminant spermatozoa, mostly in the post-acrosomal region and intermediate piece (Martin et al., 2004; Mendoza et al., 2013). Following refrigeration or cryopreservation of ram and bull spermatozoa, there was a remarkable increment in the quantity of active executioner caspases-3 and -7 (Mendoza et al., 2013) and initiator caspase-9 (Martin et al., 2007), respectively. On the other hand, caspase activity decreased in cold-shocked ram spermatozoa (Martí et al., 2008b). While the above texts highlight the dependence of biochemical and molecular apoptotic cascade to the structural and functional state of the mitochondria (Nakidkina et al., 2019), other cryopreservation-induced alterations to the sperm organelle and their effects on freezability and other semen quality parameters will be discussed in detail in a later section.

## FREEZABILITY AND SPERM CHROMATIN, DNA AND RNA

Because of its direct role in fertilization and subsequent embryonic development via haploid paternal genetic contribution, the integrity of the sperm DNA cannot be undervalued in reproduction. Ultrastructural examination of cryopreserved sperm revealed irreversible nuclear and chromatin damages (Khalil et al., 2018). Spermatozoa exhibiting DNA fragmentation and/or chromatin overcondensation also increased after freezing and thawing (Erickson et al., 2015; Gürler et al., 2016; Kumar et al., 2011; Peris et al., 2007). Correspondingly, abnormal morphology and low motility were linked to DNA and chromatin damage in buffalo and bull sperm, with implications on the cryosurvival of the spermatozoa (Januskauskas et al., 2001; Mahmoud et al., 2015). A high correlation between DNA and chromatin stability and semen fertility has also been proposed (Dogan et al., 2015; Khalifa et al., 2013; Kumaresan et al., 2017; Waterhouse et al., 2006) since DNA-nicked sperm appears to result in poor cleavage and blastocyst turn-over rates (Erickson et al., 2015).

Other than the fact that the specific mechanisms governing sperm DNA injuries are not yet completely elucidated (Gürler et al., 2016; Peris et al., 2007), opposing results from some studies further confound the present knowledge on the effects of cryopreservation on ruminant sperm DNA and chromatin, and their relationships with semen quality. According to Martin et al. (2004), cryopreservation did not affect DNA fragmentation and nucleus condensation, but Kadirvel et al. (2009b) indicated that sperm DNA damage was only influenced by liquid storage rather than by the freezing and thawing procedures. Additionally, Dogan et al. (2013) claimed that the association between fertility and DNA damage in cryopreserved spermatozoa was lacking. Nevertheless, pieces of evidence point to lipid peroxidation, oxidative stress and ROS production as bases for DNA and chromatin integrity loss during cryopreservation (Kumar et al., 2011; Peris et al., 2007), as previously expounded.

With the emerging relevance of epigenetics on sperm functionality, the relationship of aberrant DNA methylation (formation of 5-methylcytosine from

the covalent addition of a methyl group to carbon five of cytosine) with DNA fragmentation has recently been established in ram spermatozoa (Pool et al., 2020). However, when compared to humans and other animal species (Aurich et al., 2016; Khosravizadeh et al., 2020), there is a paucity of information on the role of cryopreservation in irregular DNA methylation in ruminants. Some researchers have associated global methylation to semen parameters such as motility, morphology (Capra et al., 2019; Pool et al., 2020) and fertility (Kropp et al., 2017; Verma et al., 2014), implicating its potential role in ruminant sperm cryosurvival and overall quality. Regarding freezability, Capra et al. (2019) determined several differentially methylated genes functioning in chromatin arrangement in both high and low motility bovine sperm populations. These consist of histone lysine demethylases 2A (KDM2A), histone lysine methyltransferases 2A (KMT2A), telomerase-associated protein 1 (TEP1), telomerase reverse transcriptase (TERT), nuclear receptor-binding SET Domain 2 (NSD2)/ multiple myeloma SET domain (MMSET)/ Wolf-Hirschhorn syndrome candidate-1(WHSC1), among others.

Protamination (the stepwise replacement of nuclear histones into transition proteins, and finally into much smaller and highly basic protamines) is one of the distinct epigenetic facets of sperm cells that act in the well-organized packaging of DNA through adequate chromatin compaction (Champroux et al., 2018; Gosálvez et al., 2011; Jenkins et al., 2012). Freezing and thawing affect sperm protamine levels, leading to reduced PRM2 and PRM3 transcripts and proteins in cryopreserved cattle spermatozoa (Lv et al., 2020). This deficiency could result in DNA fragmentation and low fertility in ruminants (Boe-Hansen et al., 2018; Dogan et al., 2015; Fortes et al., 2014; Pool et al., 2020), aside from its relationship to sperm concentration, mass activity and morphology. Surprisingly, despite comparable protamine deficits in spermatozoa of contrasting freezabilities, a notable association between the variables existed (Hitit et al., 2020). Moreover, spermatozoa of lesser and greater motilities also showed insignificant differences in PRM1 and PRM2 differential expressions. However, PRM2 amino acid sequence changes that result from nucleotide base modifications (G35A, A49G and A64G) had a detrimental impact on sperm motility metrics (mass, initial progressive, and post-thaw) (Yathish et al., 2018).

Single nucleotide polymorphisms (SNPs) have also been examined for their relationships to semen quality. Poor motility and kinetics, and low ATP content in ruminant spermatozoa, which could translate to decreased freezability, were attributed to mutations in certain genes like cation channel of sperm 2 (CatSper2) (Sivakumar et al., 2018), follicle-stimulating hormone β-subunit (FSHB) (Dai et al., 2009), gonadotropin-releasing hormone receptor (GnRHR) (Mahmoud et al., 2021; Yang et al., 2010), glutathione-S-transferase M1 (GSTM1) (Hering et al., 2015), heat shock protein 70 (HSP70) (Nikbin et al., 2014), inhibin β-subunit (INHBA) (Sang et al., 2011), and prion protein testis-specific (PRNT) (Pereira et al., 2018). These genetic substitutions, insertions or deletions were also shown to be responsible for other semen characteristics such as volume, concentration, livability, morphology, acrosome integrity, fertility and ability for embryonic development (Dai et al., 2009; Mahmoud et al., 2021; Nikbin et al., 2014; Pereira et al., 2018; Yang et al., 2010). Hence, robust molecular exploration and analysis of their association with post-thaw semen quality and other reproductive parameters are required.

The sperm's contribution to the oocyte during fertilization includes an RNA pool composed of messenger RNAs (mRNAs), ribosomal RNAs (rRNAs), mitochondrial RNAs (mt-RNAs), transfer RNAs (tRNAs), and noncoding RNAs (ncRNAs) including microRNAs (miRNAs) and Piwi-interacting RNAs (piRNAs) (Capra et al., 2017; Card et al., 2013; Sellappan et al., 2017). Nevertheless, successive freeze-thaw cycles result in the differential expressions of spermatozoal transcripts from fresh and frozen-thawed samples, such as the ribosomal protein L31 (RPL31), protein kinase C epsilon (PRKCE), 3′-phosphoadenosine 5′-phosphosulfate synthase 2 (PAPSS2), proteolipid protein 1 (PLP1), serine/threonine testis-specific protein kinase (TSSK6), protamine 1 (PRM1) and protamine 2 (PRM2) (Chen et al., 2015; Nazari et al., 2020; Shangguan et al., 2020). Expression of mRNAs and miRNAs like PRM1, TSSK6, metalloproteinase non-coding RNA (ADAM5P), cytochrome P450 aromatase (aromP450), and cytochrome oxidase subunit 7C (COX7C) also varied between higher and lower fertility and motility sperm populations (Bissonnette et al., 2009; Capra et al., 2017; Card et al., 2017; Ganguly et al., 2013; Govindaraju et al., 2012; Tiwari et al., 2008), implying RNAs' influence on sperm vitality and over-all quality for later embryogenesis.

## FREEZABILITY AND SPERM MITOCHONDRIA

Mitochondria's central role in the spermatozoa extends beyond motility, with its apparent involvement in fertilization-related events like hyperactivation, capacitation, acrosome reaction, and, ultimately, oocyte penetration (Barbagallo et al., 2020; Moraes et al., 2018). Ultrastructural cryopreservation-induced alterations to the mitochondria encompass vacuolations, rupture, complete loss, cristae distortion, membrane space constrictions, and other structural disorganizations (Khalil et al., 2018; Shi et al., 2014).

The chilling injury to the mitochondria causes mitochondrial permeability transition (MPT), as examined by Treulen and colleagues (2018) using bovine sperm as a model. Dramatic reductions in the sperm mitochondrial membrane potential (MMP) ensue from this change. These then cause detrimental repercussions to the other physiological aspects of the spermatozoa, including decreased motility, viability, and fertility, and thus, may impinge on cryoresistance and semen quality (Castro et al., 2016; Martin et al., 2007; Martin et al., 2004; Shah et al., 2017). The functional state of the mitochondria also differed considerably between high- and low-freezing tolerant spermatozoa, with the former having greater MMP than the latter (Ros-Santaella et al., 2014; Ryu et al., 2019). Moreover, high freezability spermatozoa were identified to be more viable, motile, and rapid than their low freezability counterparts (Rego et al., 2016; Ryu et al., 2019).

Studies on sperm bioenergetics show that oxidative phosphorylation and glycolysis are both essential pathways fueling ruminant spermatozoa energy production (Losano et al., 2017a; Losano et al., 2017b). The cryopreservation method quantitatively altered several mitochondrial proteins involved in such metabolic activities, affecting post-thaw sperm motility. These include aconitate hydratase (ACO2), fructose-bisphosphate aldolase (ALDOA), hexokinasel (HXK1), phosphoglycerate mutase 2 (PGAM2), phosphoglycerate kinase 2

(PGK2), pyruvate kinase M2 (PKM2), nucleoside diphosphate kinase 7 (NDPK7) and NADH dehydrogenase flavoprotein 2 (NDUFV2) (He et al., 2016; Wojtusik et al., 2018; Yoon et al., 2016b). In addition, the abundance of bull and ram enzymes, like alpha-enolase (ENO1) ATP synthase, fructose-1,6-bisphosphatase 1 (FBP1), glucose-6-phosphate isomerase (GPI), mitochondrial isoform 1 of dihydrolipoyl dehydrogenase, testis-specific glyceraldehyde-3-phosphate dehydrogenase (GAPDH5), and triosephosphate isomerase (TPI), also varied between freezability and motility sperm populations (Gomes et al., 2020; Rego et al., 2016; Rickard et al., 2015; Ryu et al., 2019; Soleilhavoup et al., 2014).

## FREEZABILITY AND OTHER SPERM STRUCTURES AND SEMINAL COMPONENTS

Because sperm cytoskeletal elements are susceptible to temperature and osmotic fluctuations, they become unstable, change in distribution and quantity, or worse, are degraded and lost, during chilling and cryopreservation (Naresh, 2016). The freezing-thawing procedure also disrupted the cytoskeletal protein localization patterns and interactions in the bovine and ovine sperm head and flagellum, particularly those of actin, F-actin, and dystrobrevin (Felipe-Pérez et al., 2012; Holt et al., 1991). Actin polymerization was also induced in bubaline-cooled and cryopreserved spermatozoa as a capacitation-like change (Naresh, 2016). Aside from actin (Naresh, 2016; Yoon et al., 2016a), other cytoskeletal proteins such as outer dense fiber 2 (ODF2) (Yoon et al., 2016a; Yoon et al., 2016b), ropporin-1 (ROPN1) (Yoon et al., 2016a), and tektin 4 (TEKT4) (Wojtusik et al., 2018) also showed a marked reduction in expression. As a result, these modifications disturb the cytoskeleton's regulation of sperm cell volume and axoneme integrity, which may impact cryosurvival and post-thaw motility. Comparative proteomics of high- and low-freezing resilient spermatozoa also revealed quantitative differences in actin cytoskeleton-associated proteins like annexin A1 (Rego et al., 2016), gelsolin (GSN) (Gomes et al., 2020; Soleilhavoup et al., 2014), and T-complex protein 1 (CCT6A, CCT6A, CCT7) (Rickard et al., 2015; Soleilhavoup et al., 2014).

The metabolite profiles of the seminal plasma and spermatozoa were also explored to ascertain possible correlations with freezability and fertilizing ability. Such molecular elements may directly contribute to and reliably represent characteristic phenotypes as end-products of complex biochemical cascades (Kumar et al., 2015). The discovery of the varying levels of citrate, isoleucine, leucine, taurine, tryptamine (Kumar et al., 2015), fructose, 2-oxoglutaric acid (Velho et al., 2018), benzoic acid, lactic acid, palmitic acid, carbamate, GABA (Menezes et al., 2019), aspartic acid, iron, zinc (Narud et al., 2020), butyrylcarnitine, glycerophosphocholine, glycine betaine and l-carnitine (Longobardi et al., 2020) in fresh and cryopreserved semen of high- and low-fertility animals offered some promise in accurately predicting reproductive success. Moreover, good and poor freezability bovine groups presented distinct amino acid signatures. The former had more phenylalanine concentrations, which was linked to post-thaw viability and presumably contributed to antioxidant responses (Hitit et al., 2020). While the effects of cryopreservation

on sperm metabolome have already been recognized (Longobardi et al., 2020), similar researches in other ruminant species are required to fill knowledge gaps and generate applications for conventional reproductive techniques.

## CONCLUDING REMARKS

As particular molecules related to various sperm functions can be modified by the freezing-thawing procedures, there has been a growing interest in elucidating the molecular underpinnings of sperm biological response to cryopreservation. Sperm lipids, proteins, metabolites, transcripts, and genes can be harnessed as putative markers to classify and predict freezability and fertility phenotypes (Figure 2), or they can be incorporated as additives to freezing mediums to optimize cryopreserved semen quality. These pieces of information, along with modern molecular biological technologies, open new research horizons for the improvement of reproductive outcomes not only in ruminants but also in other domestic and wild animal species.

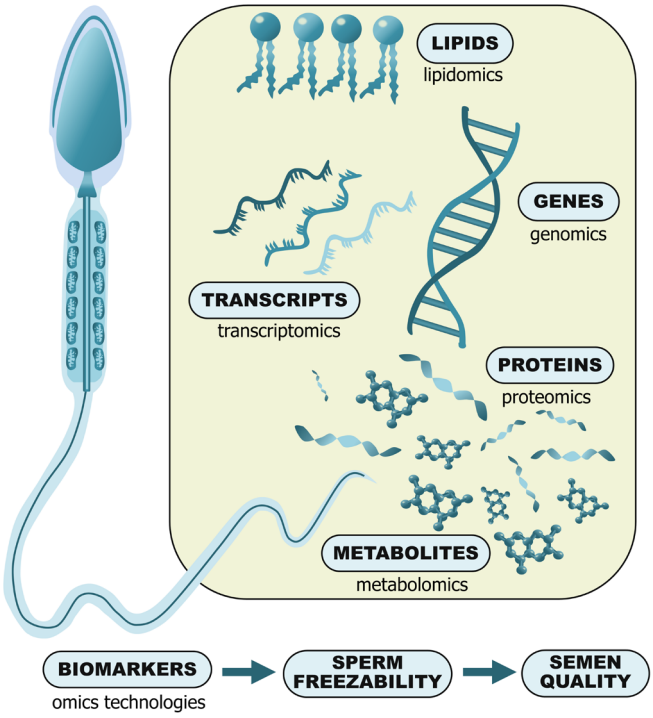


**Figure 2** Putative biomarkers for sperm freezability identified by available omics technologies which can be used to improve semen quality.

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## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

## AUTHOR CONTRIBUTIONS

A.S. conceived the topic, while M.B.S.S. mined and analyzed relevant publications and drafted the article. P.C., K.S., S.B. and A.S. supervised, reviewed, and edited the final paper. All authors have read and agreed to the published version of the manuscript.

## REFERENCES

Ahmad, M., Ahmad, N., Riaz, A., Anzar, M., 2015. Sperm survival kinetics in different types of bull semen: progressive motility, plasma membrane integrity, acrosomal status and reactive oxygen species generation. Reprod. Fertil. Dev., 27(5), 784-793.

Ahmed, S., Khan, M. I.-u.-R., Ahmad, M., Iqbal, S., 2018. Effect of age on lipid peroxidation of fresh and frozen-thawed semen of Nili-Ravi buffalo bulls. Ital. J. Anim. Sci., 17(3), 730-735.

Aitken, R. J., 1995. Free radicals, lipid peroxidation and sperm function. Reprod. Fertil. Dev., 7(4), 659-668.

Amorim, E. A. M., Graham, J. K., Spizziri, B., Meyers, M., Torres, C. A. A., 2009. Effect of cholesterol or cholesteryl conjugates on the cryosurvival of bull sperm. Cryobiology, 58(2), 210-214.

Andrabi, S. M. H., 2009. Factors affecting the quality of cryopreserved buffalo (*Bubalus bubalis*) bull spermatozoa. Reprod. Domest. Anim., 44(3), 552-569.

Anghel, A., Stela, Z., 2010. Role of antioxidant additives in the protection of the cryopreserved semen against free radicals. Rom. Biotechnol. Lett., 15, 33-41.

Anzar, M., He, L., Buhr, M. M., Kroetsch, T. G., Pauls, K. P., 2002. Sperm apoptosis in fresh and cryopreserved bull semen detected by flow cytometry and its relationship with fertility. Biol. Reprod., 66(2), 354-360.

Arando, A., Gonzalez, A., Delgado, J. V., Arrebola, F. A., Perez-Marín, C. C., 2017. Storage temperature and sucrose concentrations affect ram sperm quality after vitrification. Anim. Reprod. Sci., 181, 175-185.

Aurich, C., Schreiner, B., Ille, N., Alvarenga, M., Scarlet, D., 2016. Cytosine methylation of sperm DNA in horse semen after cryopreservation. Theriogenology, 86(5), 1347-1352.

Bailey, J., Buhr, M. J., 1994. Cryopreservation alters the Ca2+ flux of bovine spermatozoa. Can. J. Anim. Sci., 74, 45-51.

Bailey, J. L., Bilodeau, J. F., Cormier, N., 2000. Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. J. Androl., 21(1), 1-7.

Barbagallo, F., La Vignera, S., Cannarella, R., Aversa, A., Calogero, A. E., Condorelli, R. A., 2020. Evaluation of sperm mitochondrial function: A key organelle for sperm motility. J. Clin. Med., 9(2), 363.

Batissaco, L., Arruda, R. P., Alves, M., Torres, M. A., Lemes, K. M., Prado-Filho, R. R., Almeida, T. G., de Andrade, A., Celeghini, E., 2020. Cholesterol-loaded cyclodextrin is efficient in preserving sperm quality of cryopreserved ram semen with low freezability. Reprod. Biol., 20(1), 14-24.

Bilodeau, J. F., Chatterjee, S., Sirard, M. A., Gagnon, C., 2000. Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. Mol. Reprod. Dev., 55(3), 282-288.

Bissonnette, N., Lévesque-Sergerie, J. P., Thibault, C., Boissonneault, G., 2009. Spermatozoal transcriptome profiling for bull sperm motility: a potential tool to evaluate semen quality. Reproduction, 138(1), 65-80.

Boe-Hansen, G. B., Fortes, M. R. S., Satake, N., 2018. Morphological defects, sperm DNA integrity, and protamination of bovine spermatozoa. Andrology, 6(4), 627-633.

Bollwein, H., Bittner, L., 2018. Impacts of oxidative stress on bovine sperm function and subsequent in vitro embryo development. Anim. Reprod., 15, 703-710.

Capra, E., Lazzari, B., Turri, F., Cremonesi, P., Portela, A., Ajmone-Marsan, P., Stella, A., Pizzi, F., 2019. Epigenetic analysis of high and low motile sperm populations reveals methylation variation in satellite regions within the pericentromeric position and in genes functionally related to sperm DNA organization and maintenance in Bos taurus. BMC Genomics, 20(1), 940.

Capra, E., Turri, F., Lazzari, B., Cremonesi, P., Gliozzi, T. M., Fojadelli, I., Stella, A., Pizzi, F., 2017. Small RNA sequencing of cryopreserved semen from single bull revealed altered miRNAs and piRNAs expression between high- and low-motile sperm populations. BMC Genomics, 18(1), 14.

Card, C. J., Anderson, E. J., Zamberlan, S., Krieger, K. E., Kaproth, M., Sartini, B. L., 2013. Cryopreserved bovine spermatozoal transcript profile as revealed by high-throughput ribonucleic acid sequencing. Biol. Reprod., 88(2), 49.

Card, C. J., Krieger, K. E., Kaproth, M., Sartini, B. L., 2017. Oligo-dT selected spermatozoal transcript profiles differ among higher and lower fertility dairy sires. Anim. Reprod. Sci., 177, 105-123.

Castro, L. S., Hamilton, T. R., Mendes, C. M., Nichi, M., Barnabe, V. H., Visintin, J. A., Assumpção, M. E., 2016. Sperm cryodamage occurs after rapid freezing phase: flow cytometry approach and antioxidant enzymes activity at different stages of cryopreservation. J. Anim. Sci. Biotechnol., 7, 17.

Champroux, A., Cocquet, J., Henry-Berger, J., Drevet, J. R., Kocer, A., 2018. A decade of exploring the mammalian sperm epigenome: Paternal epigenetic and transgenerational inheritance. Front. Cell. Dev. Biol., 6, 50.

Chatterjee, S., de Lamirande, E., Gagnon, C., 2001a. Cryopreservation alters membrane sulfhydryl status of bull spermatozoa: protection by oxidized glutathione. Mol. Reprod. Dev., 60(4), 498-506.

Chatterjee, S., Gagnon, C., 2001b. Production of reactive oxygen species by spermatozoa undergoing cooling, freezing, and thawing. Mol. Reprod. Dev., 59(4), 451-458.

Chen, X., Wang, Y., Zhu, H., Hao, H., Zhao, X., Qin, T., Wang, D., 2015. Comparative transcript profiling of gene expression of fresh and frozen-thawed bull sperm. Theriogenology, 83(4), 504-511.

Codognoto, V. M., Yamada, P. H., Schmith, R. A., de Ruediger, F. R., Scott, C., de Faria Lainetti, P., Brochine, S., de Paula Freitas-Dell'Aqua, C., de Souza, F. F., Oba, E., 2018. Functional insights into the role of seminal plasma proteins on sperm motility of buffalo. Anim. Reprod. Sci., 195, 251–258.

Cormier, N., Bailey, J. L., 2003. A differential mechanism is involved during heparin- and cryopreservation-induced capacitation of bovine spermatozoa. Biol. Reprod., 69(1), 177-185.

Dai, L., Zhao, Z., Zhao, R., Xiao, S., Jiang, H., Yue, X., Li, X., Gao, Y., Liu, J., Zhang, J., 2009. Effects of novel single nucleotide polymorphisms of the FSH beta-subunit gene on semen quality and fertility in bulls. Anim. Reprod. Sci., 114(1), 14-22.

Dalal, J., Kumar, A., Honparkhe, M., Deka, D., Singh, N., 2016. Minimization of apoptosis-like changes in cryopreserved buffalo bull sperm by supplementing extender with Bcl-2 protein. Vet. World, 9, 432-436.

De Leeuw, F. E., Chen, H. C., Colenbrander, B., Verkleij, A. J., 1990. Cold-induced ultrastructural changes in bull and boar sperm plasma membranes. Cryobiology, 27(2), 171-183.

Del Valle, I., Mendoza, N., Casao, A., Cebrián-Pérez, J. A., Pérez-Pé, R., Muiño-Blanco, T., 2010. Significance of non-conventional parameters in the evaluation of cooling-induced damage to ram spermatozoa diluted in three different media. Reprod. Domest. Anim., 45(6), e260-e268.

Dhanju, C. K., Cheema, R. S., Kaur, S. P., 2001. Effect of freezing on proteins and protein profiles of sperm membrane extracts and seminal plasma of buffalo bulls. Asian-Australas. J. Anim. Sci., 14(12), 1678-1682.

Dogan, S., Mason, M. C., Govindaraju, A., Belser, L., Kaya, A., Stokes, J., Rowe, D., Memili, E., 2013. Interrelationships between apoptosis and fertility in bull sperm. J. Reprod. Dev., 59(1), 18-26.

Dogan, S., Vargovic, P., Oliveira, R., Belser, L. E., Kaya, A., Moura, A., Sutovsky, P., Parrish, J., Topper, E., Memili, E., 2015. Sperm protamine-status correlates to the fertility of breeding bulls. Biol. Reprod., 92(4), 92.

Dorado, J., Muñoz-Serrano, A., Hidalgo, M., 2010. The effect of cryopreservation on goat semen characteristics related to sperm freezability. Anim. Reprod. Sci., 121(1), 115-123.

Dutta, S., Majzoub, A., Agarwal, A., 2019. Oxidative stress and sperm function: A systematic review on evaluation and management. Arab. J. Urol., 17(2), 87-97.

Erickson, L., Kroetsch, T., Anzar, M., 2015. Relationship between sperm apoptosis and bull fertility: in vivo and in vitro studies. Reprod. Fertil. Dev., 28(9), 1369-1375.

Esteso, M. C., Soler, A. J., Fernández-Santos, M. R., Quintero-Moreno, A. A., Garde, J. J., 2006. Functional significance of the sperm head morphometric size and shape for determining freezability in Iberian red deer (*Cervus elaphus hispanicus*) epididymal sperm samples. J. Androl., 27(5), 662-670.

Evans, H. C., Dinh, T., Ugur, M. R., Hitit, M., Sajeev, D., Kaya, A., Topper, E., Nicodemus, M. C., Smith, G. D., Memili, E., 2020. Lipidomic markers of sperm cryotolerance in cattle. Sci. Rep., 10(1), 20192.

Fang, Y., Zhao, C., Xiang, H., Jia, G., Zhong, R., 2020. Melatonin improves cryopreservation of ram sperm by inhibiting mitochondrial permeability transition pore opening. Reprod. Domest. Anim., 55(9), 1240-1249.

Felipe-Pérez, Y. E., Valencia, J., Juárez-Mosqueda, M. D. L., Pescador, N., Roa-Espitia, A. L., Hernández-González, E. O., 2012. Cytoskeletal proteins F-actin and β-dystrobrevin are altered by the cryopreservation process in bull sperm. Cryobiology, 64(2), 103-109.

Fortes, M. R., Satake, N., Corbet, D. H., Corbet, N. J., Burns, B. M., Moore, S. S., Boe-Hansen, G. B., 2014. Sperm protamine deficiency correlates with sperm DNA damage in Bos indicus bulls. Andrology, 2(3), 370-378.

Ganguly, I., Gaur, G. K., Kumar, S., Mandal, D. K., Kumar, M., Singh, U., Kumar, S., Sharma, A., 2013. Differential expression of protamine 1 and 2 genes in mature spermatozoa of normal and motility impaired semen producing crossbred Frieswal (HF×Sahiwal) bulls. Res. Vet. Sci., 94(2), 256-262.

Gomes, F.P., Park, R., Viana, A.G., Fernández-Costa, C., Topper, E., Kaya, A., Memili, E., Yates, J., Moura, A., 2020. Protein signatures of seminal plasma from bulls with contrasting frozen-thawed sperm viability. Sci. Rep., 10(1), 14661.

Gonçalves, F. S., Barretto, L. S., Arruda, R. P., Perri, S. H., Mingoti, G. Z., 2010. Effect of antioxidants during bovine in vitro fertilization procedures on spermatozoa and embryo development. Reprod. Domest. Anim., 45(1), 129-135.

Gosálvez, J., López-Fernández, C., Fernández, J. L., Gouraud, A., Holt, W. V., 2011. Relationships between the dynamics of iatrogenic DNA damage and genomic design in mammalian spermatozoa from eleven species. Mol. Reprod. Dev., 78(12), 951-961.

Govindaraju, A., Uzun, A., Robertson, L., Atli, M. O., Kaya, A., Topper, E., Crate, E. A., Padbury, J., Perkins, A., Memili, E., 2012. Dynamics of microRNAs in bull spermatozoa. Reprod. Biol. Endocrinol., 10, 82.

Grötter, L. G., Cattaneo, L., Marini, P. E., Kjelland, M. E., Ferré, L. B., 2019. Recent advances in bovine sperm cryopreservation techniques with a focus on sperm post-thaw quality optimization. Reprod. Domest. Anim., 54(4), 655-665.

Gürler, H., Malama, E., Heppelmann, M., Calisici, O., Leiding, C., Kastelic, J. P., Bollwein, H., 2016. Effects of cryopreservation on sperm viability, synthesis of reactive oxygen species, and DNA damage of bovine sperm. Theriogenology, 86(2), 562-571.

Harayama, H., Nishijima, K., Murase, T., Sakase, M., Fukushima, M., 2010. Relationship of protein tyrosine phosphorylation state with tolerance to frozen storage and the potential to undergo cyclic AMP-dependent hyperactivation in the spermatozoa of Japanese Black bulls. Mol. Reprod. Dev., 77(10), 910-921.

He, Y., Wang, K., Zhao, X., Zhang, Y., Ma, Y., Hu, J., 2016. Differential proteome association study of freeze-thaw damage in ram sperm. Cryobiology, 72(1), 60-68.

Hering, D. M., Lecewicz, M., Kordan, W., Majewska, A., Kaminski, S., 2015. Missense mutation in glutathione-S-transferase M1 gene is associated with sperm motility and ATP content in frozen-thawed semen of Holstein-Friesian bulls. Anim. Reprod. Sci., 159, 94-97.

Hitit, M., Ugur, M. R., Dinh, T., Sajeev, D., Kaya, A., Topper, E., Tan, W., Memili, E., 2020. Cellular and functional physiopathology of bull sperm with altered sperm freezability. Front. Vet. Sci., 7, 581137.

Holt, W. V., North, R. D., 1991. Cryopreservation, actin localization and thermotropic phase transitions in ram spermatozoa. J. Reprod. Fertil., 91(2), 451-461.

Hozyen, H., Elshamy, A., Farghali, A., 2020. Supplementation of nano selenium minimizes freeze-thaw induced damage to ram spermatozoa. Int. J. Vet. Sci., 8, 249-254.

Januskauskas, A., Johannisson, A., Rodriguez-Martinez, H., 2001. Assessment of sperm quality through fluorometry and sperm chromatin structure assay in relation to field fertility of frozen-thawed semen from Swedish AI bulls. Theriogenology, 55(4), 947-961.

Januskauskas, A., Johannisson, A., Rodriguez-Martinez, H., 2003. Subtle membrane changes in cryopreserved bull semen in relation with sperm viability, chromatin structure, and field fertility. Theriogenology, 60(4), 743-758.

Jenkins, T. G., Carrell, D. T., 2012. The sperm epigenome and potential implications for the developing embryo. Reproduction, 143(6), 727-734.

Jobim, M. I., Oberst, E. R., Salbego, C. G., Souza, D. O., Wald, V. B., Tramontina, F., Mattos, R. C., 2004. Two-dimensional polyacrylamide gel electrophoresis of bovine seminal plasma proteins and their relation with semen freezability. Theriogenology, 61(2-3), 255-266.

Kadirvel, G., Kathiravan, P., Kumar, S., 2011. Protein tyrosine phosphorylation and zona binding ability of in vitro capacitated and cryopreserved buffalo spermatozoa. Theriogenology, 75(9), 1630-1639.

Kadirvel, G., Kumar, S., Kumaresan, A., 2009a. Lipid peroxidation, mitochondrial membrane potential and DNA integrity of spermatozoa in relation to intracellular reactive oxygen species in liquid and frozen-thawed buffalo semen. Anim. Reprod. Sci., 114(1-3), 125-134.

Kadirvel, G., Kumar, S., Kumaresan, A., Kathiravan, P., 2009b. Capacitation status of fresh and frozen-thawed buffalo spermatozoa in relation to cholesterol level, membrane fluidity and intracellular calcium. Anim. Reprod. Sci., 116(3-4), 244-253.

Khalifa, T., Lymberopoulos, A., 2013. Changeability of sperm chromatin structure during liquid storage of ovine semen in milk-egg yolk- and soybean lecithin-based extenders and their relationships to field-fertility. Cell Tissue Bank., 14(4), 687-698.

Khalil, W. A., El-Harairy, M. A., Zeidan, A. E. B., Hassan, M. A. E., 2019. Impact of selenium nano-particles in semen extender on bull sperm quality after cryopreservation. Theriogenology, 126, 121-127.

Khalil, W. A., El-Harairy, M. A., Zeidan, A. E. B., Hassan, M. A. E., Mohey-Elsaeed, O., 2018. Evaluation of bull spermatozoa during and after cryopreservation: Structural and ultrastructural insights. Int. J. Vet. Sci. Med., 6, S49-S56.

Khan, D. R., Ahmad, N., Anzar, M., Channa, A. A., 2009. Apoptosis in fresh and cryopreserved buffalo sperm. Theriogenology, 71(5), 872-876.

Khan, I. M., Cao, Z., Liu, H., Khan, A., Rahman, S. U., Khan, M. Z., Sathanawongs, A., & Zhang, Y., 2021. Impact of cryopreservation on spermatozoa freeze-thawed traits and relevance OMICS to assess sperm cryo-tolerance in farm animals. Front. Vet. Sci., 8, 609180.

Khosravizadeh, Z., Hassanzadeh, G., Tavakkoly Bazzaz, J., Alizadeh, F., Totonchi, M., Salehi, E., Khodamoradi, K., Khanehzad, M., Hosseini, S. R., Abolhassani, F., 2020. The effect of cryopreservation on DNA methylation patterns of the chromosome 15q11–q13 region in human spermatozoa. Cell Tissue Bank., 21(3), 433-445.

Kropp, J., Carrillo, J. A., Namous, H., Daniels, A., Salih, S. M., Song, J., Khatib, H., 2017. Male fertility status is associated with DNA methylation signatures in sperm and transcriptomic profiles of bovine preimplantation embryos. BMC Genomics, 18(1), 280.

Kumar, A., Kroetsch, T., Blondin, P., Anzar, M., 2015. Fertility-associated metabolites in bull seminal plasma and blood serum: 1H nuclear magnetic resonance analysis. Mol. Reprod. Dev., 82(2), 123-131.

Kumar, A., Prasad, J. K., Srivastava, N., Ghosh, S. K., 2019. Strategies to minimize various stress-related freeze-thaw damages during conventional cryopreservation of mammalian spermatozoa. Biopreserv. Biobank., 17(6), 603-612.

Kumar, R., Atreja, S. K., 2012. Effect of incorporation of additives in tris-based egg yolk extender on buffalo (*Bubalus bubalis*) sperm tyrosine phosphorylation during cryopreservation. Reprod. Domest. Anim., 47(3), 485-490.

Kumar, R., Jagan Mohanarao, G., Arvind, Atreja, S. K., 2011. Freeze–thaw induced genotoxicity in buffalo (*Bubalus bubalis*) spermatozoa in relation to total antioxidant status. Mol. Biol. Rep., 38(3), 1499-1506.

Kumar, R., Singh, V. K., Atreja, S. K., 2014. Glutathione-S-transferase: Role in buffalo (*Bubalus bubalis*) sperm capacitation and cryopreservation. Theriogenology, 81(4), 587-598.

Kumaresan, A., Johannisson, A., Al-Essawe, E. M., Morrell, J. M., 2017. Sperm viability, reactive oxygen species, and DNA fragmentation index combined can discriminate between above- and below-average fertility bulls. J. Dairy. Sci., 100(7), 5824-5836.

Lemma, A., 2011. Effect of cryopreservation on sperm quality and fertility. Artif. Insemin. Farm. Anim.,191–216.

Lone, S. A., Prasad, J. K., Ghosh, S. K., Das, G. K., Balamurugan, B., Verma, M. R., 2018. Study on correlation of sperm quality parameters with antioxidant and oxidant status of buffalo bull semen during various stages of cryopreservation. Andrologia, 50(4), e12970.

Longobardi, V., Albero, G., De Canditiis, C., Salzano, A., Natale, A., Balestrieri, A., Neglia, G., Campanile, G., Gasparrini, B., 2017. Cholesterol-loaded cyclodextrins prevent cryocapacitation damages in buffalo (*Bubalus bubalis*) cryopreserved sperm. Theriogenology, 89, 359-364.

Longobardi, V., Kosior, M. A., Pagano, N., Fatone, G., Staropoli, A., Vassetti, A., Vinale, F., Campanile, G., Gasparrini, B., 2020. Changes in bull semen metabolome in relation to cryopreservation and fertility. Animals, 10(6), 1065.

López Armengol, M. F., Jurado, S. B., Pelufo, V., Aisen, E. G., 2012. A quantitative ultramorphological approach for systematic assessment of sperm head regions: An example in rams. Cryobiology, 64 (3), 223-234.

Losano, J., Angrimani, D., Dalmazzo, A., Rui, B. R., Brito, M. M., Mendes, C. M., Kawai, G., Vannucchi, C. I., Assumpção, M., Barnabe, V. H., Nichi, M., 2017a. Effect of mitochondrial uncoupling and glycolysis inhibition on ram sperm functionality. Reprod. Domest. Anim., 52(2), 289-297.

Losano, J. D. A., Padín, J. F., Méndez-López, I., Angrimani, D. S. R., García, A. G., Barnabe, V. H., Nichi, M., 2017b. The stimulated glycolytic pathway is able to maintain ATP levels and kinetic patterns of bovine epididymal sperm subjected to mitochondrial uncoupling. Oxid. Med. Cell. Longev., 1682393.

Lv, Y., Ji, S., Chen, X., Xu, D., Luo, X.T., Cheng, M., Zhang, Y., Qu, X., Jin, Y., 2020. Effects of crocin on frozen-thawed sperm apoptosis, protamine expression and membrane lipid oxidation in Yanbian yellow cattle. Reprod. Domest. Anim., 55(8), 1011-1020.

Magalhães, M. J., Jr, Martins, L. F., Senra, R. L., Santos, T. F., Okano, D. S., Pereira, P. R., Faria-Campos, A., Campos, S. V., Guimarães, J. D., Baracat-Pereira, M. C., 2016. Differential abundances of four forms of binder of SPerm 1 in the seminal plasma of Bos taurus indicus bulls with different patterns of semen freezability. Theriogenology, 86(3), 766-777.e762.

Mahmoud, K. G., Sakr, A. M., Ibrahim, S. R., Sosa, A. S., Hasanain, M. H., Nawito, M. F., 2021. GnRHR gene polymorphism and its correlation with semen quality in Buffalo bulls (*Bubalus bubalis*). Iraqi J. Vet. Sci., 35(2), 381-386.

Mahmoud, K. G. M., El-Sokary, A. A. E., Abdel-Ghaffar, A. E., Abou El-Roos, M. E. A., Ahmed, Y. F., 2015. Analysis of chromatin integrity and DNA damage of buffalo spermatozoa. Iran. J. Vet. Res., 16(2), 161-166.

Maia, M. d. S., Bicudo, S. D., Sicherle, C. C., Rodello, L., Gallego, I. C. S., 2010. Lipid peroxidation and generation of hydrogen peroxide in frozen-thawed ram semen cryopreserved in extenders with antioxidants. Anim. Reprod. Sci., 122(1), 118-123.

Mandal, R., Badyakar, D., Chakrabarty, J., 2014. Role of membrane lipid fatty acids in sperm cryopreservation. Adv. Androl., 190542.

Martí, E., Marti, J. I., Muiño-Blanco, T., Cebrián-Pérez, J. A., 2008a. Effect of the cryopreservation process on the activity and immunolocalization of antioxidant enzymes in ram spermatozoa. J. Androl., 29(4), 459-467.

Martí, E., Pérez-Pé, R., Colás, C., Muiño-Blanco, T., Cebrián-Pérez, J. A., 2008b. Study of apoptosis-related markers in ram spermatozoa. Anim. Reprod. Sci., 106(1), 113-132.

Martí, J. I., Martí, E., Cebrián-Pérez, J. A., Muiño-Blanco, T., 2003. Survival rate and antioxidant enzyme activity of ram spermatozoa after dilution with different extenders or selection by a dextran swim-up procedure. Theriogenology, 60(6), 1025-1037.

Martin, G., Cagnon, N., Sabido, O., Sion, B., Grizard, G., Durand, P., Levy, R., 2007. Kinetics of occurrence of some features of apoptosis during the cryopreservation process of bovine spermatozoa. Hum. Reprod., 22(2), 380-388.

Martin, G., Sabido, O., Durand, P., Levy, R., 2004. Cryopreservation induces an apoptosis-like mechanism in bull sperm. Biol. Reprod., 71(1), 28-37.

Martínez-Fresneda, L., Castaño, C., Bóveda, P., Tesfaye, D., Schellander, K., Santiago-Moreno, J., García-Vázquez, F. A., 2019a. Epididymal and ejaculated sperm differ on their response to the cryopreservation and capacitation processes in mouflon (*Ovis musimon*). Sci. Rep., 9(1), 15659.

Martínez-Fresneda, L., O Brien, E., Velázquez, R., Toledano-Díaz, A., Martínez-Cáceres, C. M., Tesfaye, D., Schellander, K., García-Vázquez, F. A., Santiago-Moreno, J., 2019b. Seasonal variation in sperm freezability associated with changes in testicular germinal epithelium in domestic (*Ovis aries*) and wild (*Ovis musimon*) sheep. Reprod. Fertil. Dev., 31(10), 1545-1557.

Martínez-Fresneda, L., Sylvester, M., Shakeri, F., Bunes, A., Del Pozo, J. C., García-Vázquez, F. A., Neuhoff, C., Tesfaye, D., Schellander, K., Santiago-Moreno, J., 2021. Differential proteome between ejaculate and epididymal sperm represents a key factor for sperm freezability in wild small ruminants. Cryobiology, 99, 64-77.

Mendoza, N., Casao, A., Pérez-Pé, R., Cebrián-Pérez, J. A., Muiño-Blanco, T., 2013. New insights into the mechanisms of ram sperm protection by seminal plasma proteins. Biol. Reprod., 88(6), 149.

Menezes, E. B., Velho, A. L. C., Santos, F., Dinh, T., Kaya, A., Topper, E., Moura, A. A., Memili, E., 2019. Uncovering sperm metabolome to discover biomarkers for bull fertility. BMC Genomics, 20(1), 714.

Minervini, F., Guastamacchia, R., Pizzi, F., Dell'Aquila, M. E., Barile, V. L., 2013. Assessment of different functional parameters of frozen-thawed buffalo spermatozoa by using cytofluorimetric determinations. Reprod. Domest. Anim., 48(2), 317-324.

Mocé, E., Blanch, E., Tomás, C., Graham, J. K., 2010. Use of cholesterol in sperm cryopreservation: present moment and perspectives to future. Reprod. Domest. Anim., 45 Suppl 2, 57-66.

Moraes, C. R., Meyers, S., 2018. The sperm mitochondrion: Organelle of many functions. Anim. Reprod. Sci., 194, 71-80.

Morató, R., Prieto-Martínez, N., Muiño, R., Hidalgo, C. O., Rodríguez-Gil, J. E., Bonet, S., Yeste, M., 2018. Aquaporin 11 is related to cryotolerance and fertilising ability of frozen-thawed bull spermatozoa. Reprod. Fertil. Dev., 30(8), 1099-1108.

Mostek, A., Dietrich, M. A., Słowińska, M., Ciereszko, A., 2017. Cryopreservation of bull semen is associated with carbonylation of sperm proteins. Theriogenology, 92, 95-102.

Müller, K., Pomorski, T., Müller, P., Zachowski, A., Herrmann, A., 1994. Protein-dependent translocation of aminophospholipids and asymmetric transbilayer distribution of phospholipids in the plasma membrane of ram sperm cells. Biochemistry, 33(33), 9968-9974.

Nagdas, S. K., Buchanan, T., McCaskill, S., Mackey, J., Alvarez, G. E., Raychoudhury, S., 2013. Isolation of a calcium-binding protein of the acrosomal membrane of bovine spermatozoa. Int. J. Biochem. Cell. Biol., 45(4), 876-884.

Nakidkina, A. N., Kuzmina, T., 2019. Apoptosis in spermatozoa and its role in deteriorating semen quality. Russ. J. Dev. Biol., 50, 165 - 172.

Naresh, S., 2016. Effect of cooling (4°C) and cryopreservation on cytoskeleton actin and protein tyrosine phosphorylation in buffalo spermatozoa. Cryobiology, 72(1), 7-13.

Naresh, S., Atreja, S. K., 2015. The protein tyrosine phosphorylation during in vitro capacitation and cryopreservation of mammalian spermatozoa. Cryobiology, 70(3), 211-216.

Narud, B., Klinkenberg, G., Khezri, A., Zeremichael, T. T., Stenseth, E.-B., Nordborg, A., Haukaas, T. H., Morrell, J. M. Heringstad, B., Myromslien, F. D., Kommisrud, E., 2020. Differences in sperm functionality and intracellular metabolites in Norwegian Red bulls of contrasting fertility. Theriogenology, 157, 24-32.

Nazari, H., Ahmadi, E., Hosseini Fahraji, H., Afzali, A., Davoodian, N., 2020. Cryopreservation and its effects on motility and gene expression patterns and fertilizing potential of bovine epididymal sperm. Vet. Med. Sci., 7(1), 127-135.

Nikbin, S., Panandam, J. M., Yaakub, H., Murugaiyah, M., Sazili, A. Q., 2014. Novel SNPs in heat shock protein 70 gene and their association with sperm quality traits of Boer goats and Boer crosses. Anim. Reprod. Sci., 146(3-4), 176-181.

Pereira, R. M., Mesquita, P., Pires, V., Baptista, M. C., Barbas, J. P., Pimenta, J., Horta, A., Prates, J., Marques, C. C., 2018. Prion protein testis specific (PRNT) gene polymorphisms and transcript level in ovine spermatozoa: Implications in freezability, fertilization and em bryo production. Theriogenology, 115, 124-132.

Peris-Frau, P., Soler, A. J., Iniesta-Cuerda, M., Martín-Maestro, A., Sánchez-Ajofrín, I., Medina-Chávez, D. A., Fernández-Santos, M. R., García-Álvarez, O., Maroto-Morales, A., Montoro, V., Garde, J. J. 2020. Sperm cryodamage in ruminants: Understanding the molecular changes induced by the cryopreservation process to optimize sperm quality. Int. J. Mol. Sci, 21(8), 2781.

Peris, S. I., Bilodeau, J. F., Dufour, M., Bailey, J. L., 2007. Impact of cryopreservation and reactive oxygen species on DNA integrity, lipid peroxidation, and functional parameters in ram sperm. Mol. Reprod. Dev., 74(7), 878-892.

Perumal, P., Srivastava, S., Ghosh, S., Baruah, K. K., 2014. Computer-Assisted Sperm Analysis of Freezable and Nonfreezable Mithun (*Bos frontalis*) Semen. J. Anim., 2014, 1-6.

Pini, T., Leahy, T., Soleilhavoup, C., Tsikis, G., Labas, V., Combes-Soia, L., Harichaux, G., Rickard, J. P., Druart, X., de Graaf, S. P., 2016. Proteomic investigation of ram spermatozoa and the proteins conferred by seminal plasma. J. Proteome Res., 15(10), 3700-3711.

Pool, K. R., Rickard, J. P., de Graaf, S. P., 2020. Global methylation and protamine deficiency in ram spermatozoa correlate with sperm production and quality but are not influenced by melatonin or season. Animals, 10(12), 2302.

Pradieé, J., Sánchez-Calabuig, M. J., Castaño, C., O'Brien, E., Esteso, M. C., Beltrán-Breña, P., Maillo, V., Santiago-Moreno, J., Rizos, D., 2018. Fertilizing capacity of vitrified epididymal sperm from Iberian ibex (*Capra pyrenaica*). Theriogenology, 108, 314-320.

Pukazhenthi, B. S., 2016. Saving wild ungulate diversity through enhanced management and sperm cryopreservation. Reprod. Fertil. Dev., 28(8), 1133-1144.

Rajoriya, D. J., Prasad, J., Ghosh, S., Ramteke, S., Barik, N. C., Das, G., Pande, M., 2014. Cholesterol loaded cyclodextrin increases freezability of buffalo bull (*Bubalus bubalis*) spermatozoa by increasing cholesterol to phospholipid ratio. Vet. World, 7, 702-706.

Rajoriya, J. S., Prasad, J. K., Ramteke, S. S., Perumal, P., De, A. K., Ghosh, S. K., Bag, S., Raje, A., Singh, M., Kumar, A., Kumaresan, A., 2020. Exogenous cholesterol prevents cryocapacitation like changes, membrane fluidity and enhances in-vitro fertility in bubaline spermatozoa. Reprod. Domest. Anim., 55(6), 726-736.

Rajoriya, J. S., Prasad, J. K., Ramteke, S. S., Perumal, P., Ghosh, S. K., Singh, M., Pande, M., Srivastava, N., 2016. Enriching membrane cholesterol improves stability and cryosurvival of buffalo spermatozoa. Anim. Reprod. Sci., 164, 72-81.

Ramón, M., Pérez-Guzmán, M. D., Jiménez-Rabadán, P., Esteso, M. C., García-Álvarez, O., Maroto-Morales, A., Anel-López, L., Soler, A. J., Fernández-Santos, M. R., Garde, J. J., 2013. Sperm cell population dynamics in ram semen during the cryopreservation process. Plos One, 8(3), e59189.

Rasul, Z., Ahmad, N., Anzar, M., 2001. Changes in motion characteristics, plasma membrane integrity, and acrosome morphology during cryopreservation of buffalo spermatozoa. J. Androl., 22(2), 278-283.

Rego, J. P., Martins, J. M., Wolf, C. A., van Tilburg, M., Moreno, F., Monteiro-Moreira, A. C., Moreira, R. A., Santos, D. O., Moura, A. A., 2016. Proteomic analysis of seminal plasma and sperm cells and their associations with semen freezability in Guzerat bulls. J. Anim. Sci., 94(12), 5308-5320.

Rickard, J. P., Leahy, T., Soleilhavoup, C., Tsikis, G., Labas, V., Harichaux, G., Lynch, G. W., Druart, X., de Graaf, S. P., 2015. The identification of proteomic markers of sperm freezing resilience in ram seminal plasma. J. Proteomics, 126, 303-311.

Riesco, M. F., Alvarez, M., Anel-Lopez, L., Neila-Montero, M., Palacin-Martinez, C., Montes-Garrido, R., Boixo, J. C., de Paz, P., Anel, L., 2021. Multiparametric study of antioxidant effect on ram sperm cryopreservation-from field trials to research bench. Animals, 11(2), 283.

Ros-Santaella, J. L., Domínguez-Rebolledo, A. E., Garde, J. J., 2014. Sperm flagellum volume determines freezability in red deer spermatozoa. Plos One, 9(11), e112382.

Ryu, D. Y., Song, W.-H., Pang, W., Yoon, S.-J., Rahman, M. S., Pang, M., 2019. Freezability biomarkers in bull epididymal spermatozoa. Sci. Rep., 9, 12797.

Sang, L., Du, Q. Z., Yang, W. C., Tang, K. Q., Yu, J. N., Hua, G. H., Zhang, X. X., Yang, L. G., 2011. Polymorphisms in follicle stimulation hormone receptor, inhibin alpha, inhibin beta A, and prolactin genes, and their association with sperm quality in Chinese Holstein bulls. Anim, Reprod. Sci., 126(3), 151-156.

Santiani, A., Evangelista, S., Sepúlveda, N., Risopatrón, J., Villegas, J., Sánchez, R., 2014. Addition of superoxide dismutase mimics during cooling process prevents oxidative stress and improves semen quality parameters in frozen/thawed ram spermatozoa. Theriogenology, 82(6), 884-889.

Sellappan, S., Sivashanmugam, P., Lakshminarayana, S., Kolte, A., B Krishnan, B., Arangasamy, A., Ravindra, J., 2017. Occurrence and functional significance of the transcriptome in bovine (Bos taurus) spermatozoa. Sci. Rep., 7, 42392.

Shah, N., Singh, V., Yadav, H. P., Verma, M., Chauhan, D. S., Saxena, A., Yadav, S., Swain, D. K., 2017. Effect of reduced glutathione supplementation in semen extender on tyrosine phosphorylation and apoptosis like changes in frozen thawed Hariana bull spermatozoa. Anim. Reprod. Sci., 182, 111-122.

Shangguan, A., Zhou, H., Sun, W., Ding, R., Li, X., Liu, J., Zhou, Y., Chen, X., Ding, F., Yang, L., Zhang, S., 2020. Cryopreservation induces alterations of miRNA and mRNA fragment profiles of bull sperm. front. Genet., 11, 419.

Shi, L., Ren, Y., Zhou, H., Hou, G., Xun, W., Yue, W., Zhang, C., Yang, R., 2014. Effect of rapid freezing–thawing techniques on the sperm parameters and ultrastructure of Chinese Taihang black goat spermatozoa. Micron, 57, 6-12.

Singh, M., Ghosh, S. K., Prasad, J. K., Kumar, A., Tripathi, R. P., Bhure, S. K., Srivastava, N., 2014. Seminal PDC-109 protein vis-à-vis cholesterol content and freezability of buffalo Spermatozoa. Anim. Reprod. Sci., 144(1), 22-29.

Sivakumar, A., Kumar, S., Yathish, H. M., Mishra, C., Modi, R. P., Chaudhary, R., Khan, S., Sivamani, B., Ghosh, S. K., Sarkar, M., 2018. Expression profiling and identification of novel SNPs in CatSper2 gene and their influence on sperm motility parameters in bovines. Anim. Biotechnol., 29(1), 34-40.

Soleilhavoup, C., Tsikis, G., Labas, V., Harichaux, G., Kohnke, P. L., Dacheux, J. L., Guérin, Y., Gatti, J. L., de Graaf, S. P., Druart, X., 2014. Ram seminal plasma proteome and its impact on liquid preservation of spermatozoa. J. Proteomics, 109, 245-260.

Song, W. H., Ryu, D. Y., Pang, W. K., Yoon, S. J., Rahman, M. S., Pang, M. G., 2020. NT5C1B and FH are closely associated with cryoprotectant tolerance in spermatozoa. Andrology, 8(1), 221-230.

Srivastava, N., Srivastava, S.K., Ghosh, S.K., Kumar, A., Perumal, P., Jerome, A., 2013. Acrosome membrane integrity and cryocapacitation are related to cholesterol content of bull spermatozoa. Asian Pac. J. Reprod., 2(2): 126-131.

Sukardi, S., Elliott, R. M., Withers, J. O., Fontaine, U., Millar, J. D., Curry, M. R., Watson, P. F., 2001. Calcium-binding proteins from the outer acrosomal membrane of ram spermatozoa: potential candidates for involvement in the acrosome reaction. Reproduction, 122(6), 939-946.

Talukdar, D., Ahmed, K., Deka, B. C., Sinha, S., Deori, S., Das, G., 2016. Cryo-capacitation changes during cryopreservation of swamp buffalo spermatozoa. Indian J. Anim. Sci., 86, 397-400.

Tiwari, A., Singh, D., Kumar, O. S., Sharma, M. K., 2008. Expression of cytochrome P450 aromatase transcripts in buffalo (*Bubalus bubalis*)-ejaculated spermatozoa and its relationship with sperm motility. Domest. Anim. Endocrinol., 34(3), 238-249.

Treulen, F., Arias, M. E., Aguila, L., Uribe, P., Felmer, R., 2018. Cryopreservation induces mitochondrial permeability transition in a bovine sperm model. Cryobiology, 83, 65-74.

Ugur, M. R., Saber Abdelrahman, A., Evans, H. C., Gilmore, A. A., Hitit, M., Arifiantini, R. I., Purwantara, B., Kaya, A., Memili, E., 2019. Advances in cryopreservation of bull sperm. Front. Vet. Sci., 6, 268.

Varela, E., Rojas, M., Restrepo, G., 2020. Membrane stability and mitochondrial activity of bovine sperm frozen with low-density lipoproteins and trehalose. Reprod. Domest. Anim., 55(2), 146-153.

Velho, A., Bezerra de Menezes, E., Dinh, T., Kaya, A., Topper, E., Moura, A., Memili, E., 2018. Metabolomic markers of fertility in bull seminal plasma. Plos One, 13, e0195279.

Verma, A., Rajput, S., De, S., Kumar, R., Chakravarty, A. K., Datta, T. K., 2014. Genome-wide profiling of sperm DNA methylation in relation to buffalo (*Bubalus bubalis*) bull fertility. Theriogenology, 82(5), 750-759.e751.

Wang, P., Wang, Y., Wang, H., Wang, C., Zan, L., Hu, J., Li, Q., Jia, Y., Ma, G., 2014. HSP90 expression correlation with the freezing resistance of bull sperm. Zygote, 22(2), 239-245.

Waterhouse, K. E., Haugan, T., Kommisrud, E., Tverdal, A., Flatberg, G., Farstad, W., Evenson, D. P., De Angelis, P. M., 2006. Sperm DNA damage is related to field fertility of semen from young Norwegian Red bulls. Reprod. Fertil. Dev., 18(7), 781-788.

Watson, P. F., 2000. The causes of reduced fertility with cryopreserved semen. Anim. Reprod. Sci., 60-61, 481-492.

Westfalewicz, B., Dietrich, M., Słowińska, M., Judycka, S., Ciereszko, A., 2019. Seasonal changes in the proteome of cryopreserved bull semen supernatant. Theriogenology, 126, 295-302.

Wojtusik, J., Wang, Y., Pukazhenthi, B. S., 2018. Pretreatment with cholesterol-loaded cyclodextrins prevents loss of motility associated proteins during cryopreservation of addra gazelle (Nanger dama ruficollis) spermatozoa. Cryobiology, 81, 74-80.

Yadav, H. P., Kumar, A., Shah, N., Chauhan, D. S., Saxena, A., Yadav, S., Swain, D. K., 2017. Effect of cholesterol loaded cyclodextrin supplementation on tyrosine phosphorylation and apoptosis like changes in frozen thawed Hariana bull spermatozoa. Theriogenology, 96, 164-171.

Yang, W.-C., Tang, K.-Q., Yu, J.-N., Zhang, C., Zhang, X.-X., Yang, L.-G., 2010. Effects of MboII and BspMI polymorphisms in the gonadotropin releasing hormone receptor (GNRHR) gene and sperm quality in Holstein bulls. Mol. Biol. Rep., 38, 3411-3415.

Yathish, H. M., Kumar, S., Chaudhary, R., Mishra, C., A, S., Kumar, A., Chauhan, A., Ghosh, S. K., Mitra, A., 2018. Nucleotide variability of protamine genes influencing bull sperm motility variables. Anim. Reprod. Sci., 193, 126-139.

Yoon, S. J., Rahman, M. S., Kwon, W. S., Park, Y. J., Pang, M. G., 2016a. Addition of cryoprotectant significantly alters the epididymal sperm proteome. Plos One, 11(3), e0152690.

Yoon, S. J., Rahman, M. S., Kwon, W. S., Ryu, D. Y., Park, Y. J., Pang, M. G., 2016b. Proteomic identification of cryostress in epididymal spermatozoa. J. Anim. Sci. Biotechnol., 7(1), 67.

Zhang, X. G., Hu, S., Han, C., Zhu, Q. C., Yan, G. J., Hu, J. H., 2015. Association of heat shock protein 90 with motility of post-thawed sperm in bulls. Cryobiology, 70(2), 164–169.

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