Sperm Cryopreservation in Ruminant Species

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Sperm cryopreservation is a powerful tool to preserve threatened animal species or for livestock breeding. However, this process is not free of disadvantages. Thus, during the cryopreservation process a significant amount of sperm suffers considerable cryodamage, which may affect sperm quality and fertility. Recently, the use of different “omics” technologies in sperm cryobiology, especially proteomics studies, has led to a better understanding of the molecular modifications induced by sperm cryopreservation, facilitating the identification of different freezability biomarkers and certain proteins that can be added before cryopreservation to enhance sperm cryosurvival. This entry provides an updated overview of the molecular mechanism involved in sperm cryodamage, as well as the molecular aspects of those novel strategies that have been developed to reduce sperm cryodamage, including including new cryoprotectants, antioxidants, proteins, nanoparticles and vitrification.

Keywords: cryopreservation ; sperm ; proteomics ; molecular ; ruminants

1. Molecular Damaged Caused by the Freezing-Thawing Process

Sperm cryopreservation has been reported to induce an increase in plasma membrane fluidity–permeability, overproduction of reactive oxygen species (ROS), reduction of acrosome integrity, impairment of mitochondrial membrane potential and lower sperm motility in bull[1][2][3][4][5], buffalo[6][7][8], buck[9][10][11], ram[12][13][14][15][16] and red deer[17]. Molecular studies during sperm cryopreservation offer the possibility of recognizing those specific elements (proteins, lipids, ions, carbohydrates, etc.) altered by the freezing–thawing process that are in part responsible for the structural and functional changes observed in cryopreserved sperm (Figure 1).

In this sense, understanding the molecular modifications inflicted by the freezing–thawing process is essential to diminish or prevent cryodamage. Owing to the reduced[18], if not seemingly absent,[19][20] transcriptional and translational activity in mature sperm, proteomics studies represent the best option for investigating the molecular mechanisms regulating sperm functionality[21]. Moreover, it is also important to study the impact of cryopreservation on sperm RNAs transcripts since some of them are delivered to the oocyte participating in fertilization and embryo development, while others are involved in capacitation, motility, metabolism and other relevant sperm functions[22].

Figure 1. Main consequences of sperm cryodamage in ruminants. During the cryopreservation process, ruminant sperm suffer several structural and functional damages, which are probably the result of different molecular changes. This figure summarizes those structural, functional and molecular changes produced during the freezing–thawing procedure.

One of the first structures affected by the cryopreservation is the sperm plasma membrane[23]. During the freezing some sperm surface proteins as well as membrane proteins are lost or translocated with the consequent loss of their function. For example, proteins involved in capacitation, sperm–oocyte interaction and gamete fusion, such as TCP1, LOC101123268, RPN1, P25b, HEXB, CSNK1G2, ICA, LOC101123216, ADAM2 and TIMP-2, decreased in abundance in ram, gazelle and bull sperm after cryopreservation[24][25][26][27], while another protein associated with fertilization, HSP70, was lost in buffalo sperm[28]. Other proteins involved in transport, membrane stabilization and protection against lipid peroxidation or cold-shock, such as GLUT, CLU, BSP5, BSP1, aSFP, HSPA4L, TRAP1, GPX4 and GPX5 also decreased in abundance in these species along with antiapoptotic and decapacitating proteins (CSNK2A2 and Spermadhesin Z13) [24][26][29][30].

Cryopreservation also induces significant changes in the distribution or abundance of those proteins that act as ROS scavengers. Relevant antioxidant enzymes such as glutathione peroxidase (GPx), glutathione reductase (GR) and superoxide dismutase (SOD) were redistributed on ram sperm surface following cryopreservation[31]. These findings, together with the reduced antioxidant activity of SOD and reduced glutathione (GSH) observed in bull and ram sperm after cryopreservation, could explain in part the increased susceptibility of frozen–thawed sperm to suffer lipid peroxidation and oxidative damage[30][31].

Disturbances in the sperm antioxidant system during cryopreservation and the activation of L-Amino acid oxidase in dead or defective cryopreserved sperm significantly contribute to the increased ROS production detected in ruminant sperm after freezing–thawing, the sperm plasma membrane being the primary site where ROS-induced damage is manifested (Figure 2)[3][14][32][33]. Excessive generation of ROS during cryopreservation leads to major protein, lipid and carbohydrate changes in the sperm membrane due to the reduction of disulfide bonds between membrane proteins[34], peroxidation of membrane phospholipids and modifications of the sperm glycocalyx[35].

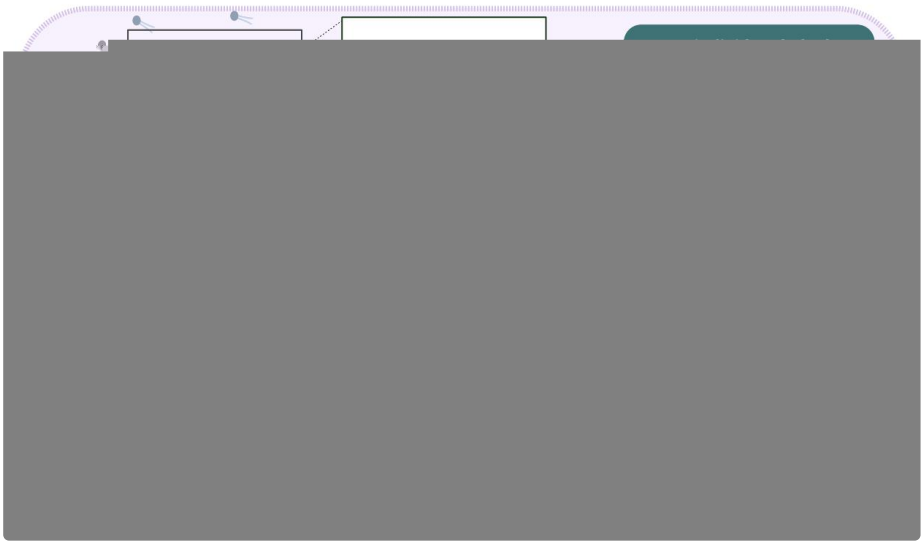

Figure 2. Plasma membrane damage during sperm cryopreservation and its relationship with oxidative stress. A reorganization of sperm membrane phospholipids takes places during freezing–thawing, altering lipid–protein, lipid–carbohydrate and protein–carbohydrate interactions which are necessary for proper membrane activity. Excessive production of reactive oxygen species (ROS) leads to major protein, lipid and carbohydrate changes in the sperm membrane due to the reduction of disulfide bonds between membrane proteins, peroxidation of membrane phospholipids and modifications of the sperm glycocalyx. As a result, the sperm membrane becomes fragile and its semipermeable property is lost. Overproduction of ROS during sperm cryopreservation may also cause DNA damage and impair several axonemal and mitochondrial proteins, which negatively affect mitochondrial activity and axonemal integrity, resulting in the loss of sperm motility.

Besides sperm membrane damage, oxidative stress disrupts mitochondrial activity with the consequent loss of sperm motility[36]. In ruminants, two main metabolic pathways, oxidative phosphorylation and glycolysis, produce the energy required to maintain sperm motility in the form of ATP[37]. Comparative proteomics studies between fresh and cryopreserved sperm revealed that freezing–thawing procedures alter the abundance of several enzymes implicated in oxidative phosphorylation and glycolysis in ram, bull and gazelle sperm[24][29][38][39]. Among them, different ATP synthases, COX5B, AK1, NDUFV2, ODPB2, ACO2 and NDPK7 were some of those proteins related to oxidative phosphorylation, while different hexokinases, GPI, ALDOA, GAPDH5, PGK2, PGAM2, PKM2 and TPI were some of those proteins related to glycolysis.

Sperm with damaged DNA can complete the fertilization process; however, embryo development can be seriously interrupted or altered once the embryo genome is activated at the 4- or 8-cell stage due to the transcription of damaged paternal genes[40][41]. Moreover, several coding and non-coding RNAs, nuclear proteins and other epigenetics marks from sperm are delivered to the offspring together with the paternal genome[42]. In consequence, aside from DNA damage, changes in the relative abundance of RNAs, aberrant DNA methylation, abnormal histone modifications or improper chromatin compaction in sperm due to alterations in the nucleoprotein structure could have a severe impact on fertilization or embryogenesis[40][43][44]. While the effect of freezing–thawing on sperm DNA stability has been widely investigated, few studies in ruminants explored the influence of freezing–thawing on sperm epigenome. Messenger RNA (mRNA) carries the genetic code to translate proteins, but there are other RNAs termed non-coding RNAs (ncRNAs) that do not code for proteins. Both types of RNAs (mRNA and ncRNAs) have been found to modulate a variety of biological functions in sperm[22]. In addition, some ncRNAs are also involved in epigenetic regulation[45]. In consequence, variations in RNA transcripts during cryopreservation could adversely affect sperm integrity, functionality and its fertilizing potential or make the sperm vulnerable to epigenetic errors. Chen et al.[46] reported that cryopreservation modified in bull sperm the relative abundance of four ncRNAs involved in embryo development. Moreover, in horses, sperm cryopreservation increased global DNA methylation[47], whereas in boar sperm, freezing–thawing decreased the relative abundance of mRNAs as well as the protein levels of some genes associated with DNA methylation (DNMT3A, DNMT3B), histone modifications (JHDM2A, KAT8) and genomic imprinting (IGF2)[48].

2. Molecular Aspects of those Novel Strategies to Reduce Sperm Cryodamage

Currently, there is a wide variety of extenders that can be used during sperm cryopreservation in different ruminant species (reviewed by[49][50][51][52][53][54]); however, not all of them offer the same protection against sperm cryodamage. Extenders usually contain various components (buffers, antibiotics, sugars, fatty acids, cryoprotectants, antioxidants and other substances) to efficiently protect sperm viability and fertility during cryopreservation[53].

Cryoprotectants protect sperm from ice crystal formation, osmotic and chemical stress. Such components can be classified into permeating and non-permeating, and both types of cryoprotectants are usually included in the extenders. Glycerol is the permeating cryoprotectant most commonly used in ruminants during sperm cryopreservation, while egg yolk is the non-permeating cryoprotectant. The former is cytotoxic beyond certain concentration and has been shown to alter in bull sperm some proteins associated with sperm–oocyte binding (IZUMO4), energy metabolism (PDB1, NUDFV2, NDPK7), cytoskeleton organization (CAPZB, ODF2) and ROS metabolism (SOD2), which may negatively affect sperm function[55]. Recently, a novel cryoprotective agent, carboxylated poly-L-lysine, has been used to reduce glycerol concentration in the freezing medium, enhancing in vivo fertility of cryopreserved buffalo and bull sperm[56][57]. Regarding non-permeating cryoprotectants, it has been reported that egg yolk also alters the proteome of ram sperm before cryopreservation[26]. Therefore, special attention should be payed to sperm-cryoprotectant interactions since these interactions may affect sperm cryopreservation outcomes. Additional studies should be conducted to elucidate whether glycerol and egg yolk exert the same impact on the sperm proteome of other ruminant species.

Another strategy for protecting sperm against cryodamage is the increment of the cholesterol membrane content prior to cryopreservation by adding cholesterol-loaded cyclodextrins (CLC) to the freezing medium. This treatment improves sperm membrane stability after incorporating exogenous cholesterol to the plasma membrane, which in turn enhances sperm cryosurvival, motility, mitochondrial activity and the number of sperm attached to zona pellucida, reducing at the same time cryo-capacitation and premature tyrosine phosphorylation[58][59][60]. The beneficial effects of CLC seem to be greater in those ejaculates with low freezability, at least in ram sperm[61]. Moreover, the addition of CLC to the extender attenuated in gazelle sperm the degradation of three proteins related to energy metabolism and cytoskeletal organization (CAPZB, HSP90A, PAGM2) during the freezing–thawing process compared to untreated sperm, which may explain the increased motility observed in CLC treated sperm[24].

Supplementation of the freezing medium with antioxidants reduces the negative effects generated by the excessive ROS production during cryopreservation, which improves sperm cryosurvival. Antioxidants can be classified into enzymatic and non-enzymatic, and both types can be added to the freezing medium, yielding different results[62][63]. The former includes superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx) and catalase (CAT), while the latter includes reduced glutathione (GSH), vitamins, plant extracts (e.g., cinnamtannin B-1), minerals, amino acids, proteins and other exogenous compounds (e.g., resveratrol or quercetin) with antioxidant properties[53][64].

Recent studies investigated the addition of different nanoparticles to the freezing medium to overcome the main drawbacks that conventional antioxidants could present, like the low durability to harsh conditions[65][66][67][68]. Nanotechnology advances have contributed to the design of novel nano-compounds that possess antioxidant properties,

such as selenium, zinc oxide and apoferritin containing gold-silver nanoparticles. Addition of selenium nanoparticles to semen extender enhanced viability, motility and chromatin integrity of cryopreserved bull sperm, obtaining greater in vivo fertility results[65]. Similar results were reported in cryopreserved ram sperm when selenium particles were added to the freezing medium[66].

Melatonin is another potential candidate to include in the freezing medium due to its protective effect against oxidative stress, which is dose-dependent[69]. The beneficial effects of melatonin on sperm cryopreservation rely on its powerful antioxidant property and its ability to stimulate the enzymatic activity of SOD, GPx and CAT[70]. Moreover, addition of melatonin to the freezing medium prevents a prolonged opening of MPTP during cryopreservation, which in turn increases ATP production, improving post-thaw sperm motility[71].

Proteomics studies on seminal plasma have greatly contributed to identifying those proteins with beneficial effects on sperm cryopreservation, facilitating the generation of recombinant proteins as a promising strategy for sperm cryopreservation. Recently, supplementation of the extender with recombinant seminal plasma proteins such as regucalcin (RGN), a recombinant peptide containing four FNII domains (TrxA-FNIIx4-His_6) and serine protease inhibitor kazal-type 3 (SPINK3) have been shown to exert a cryoprotective effect on sperm[72][73][74].

Antifreeze proteins and glycoproteins are other cryoprotective elements that deserve special attention. These proteins, which are produced by some insects, Antarctic fishes, crustaceans, bacteria, fungi and microalgae, have the capacity to protect sperm membrane from cryodamage by preventing ice crystal formation[75]. Addition of antifreeze protein and glycoprotein type I to semen extender significantly increased post-thaw motility in ram sperm[76], whereas in bull, supplementation with antifreeze protein type I only improved the osmotic resistance of sperm during cryopreservation[77].

3. Future Directions

Cryopreservation alters a variety of proteins and ARNs transcripts involved in relevant sperm functions, such as sperm motility, capacitation, fertilization and embryo development. Understanding the molecular damages caused by the freezing–thawing process is fundamental to protect these molecular elements and prevent or reduce those changes in sperm structure or function that negatively affect the reproductive performance. Moreover, supplementation of the freezing medium with novel cryoprotectants, antioxidants and other new components such as proteins or nanoparticles requires a further optimization to be an effective alternative to the commercial extenders currently used for cryopreservation of ruminant sperm.

Abbreviations

TCP1	T-complex protein 1 subunit alpha
LOC101123268	Dolichyl-diphosphooligosaccharide-protein glycosyltransferase
RPN1	Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 1
HEXB	Beta-hexosaminidase subunit beta-like isoform X1
CSNK1G2	Casein kinase I isoform gamma-2 isoform X2
ICA	Inhibitor of carbonic anhydrase-like isoform X3
LOC101123216	Disintegrin and metalloproteinase domain-containing protein 20
ADAM2	Fertilin beta
TIMP-2	Tissue inhibitor of metalloproteinases 2
HSP70	Heat shock 70 kDa protein

GLUT	Glucose transporter
CLU	Clusterin
BSP5	Binder of sperm protein 5
BSP1	Binder of sperm protein 1
aSFP	Acidic seminal fluid protein
HSP4AL	Heat shock 70 kDa protein 4 L isoform C1
TRAP1	Heat shock protein 75 kDa, mitochondrial isoform X3
GPX4	Phospholipid hydroperoxide glutathione peroxidase
GPX5	Epididymal secretory glutathione peroxidase
CSNK2A2	Casein kinase II subunit alpha
SOD2	Superoxide dismutase 2
COX5B	Cytochrome c oxidase subunit 5B, mitochondrial
AK1	Adenylate kinase isoenzyme 1
NUDFV2	NADH dehydrogenase flavoprotein 2
ODPB2	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial isoform 2
ACO2	Aconitate hydratase, mitochondrial
NDPK7	Nucleoside diphosphate kinase 7
GPI	Glucose-6-phosphate isomerase
ALDOA	Fructose-bisphosphate aldolase
GAPDH5	Glyceraldehyde-3-phosphate dehydrogenase, testis-specific
PGK2	Phosphoglycerate kinase 2
PGAM2	Phosphoglycerate mutase 2
PKM2	Pyruvate kinase M2
TPI	Triosephosphate isomerase

HSP90	Heat shock 90 kDa protein
DNMT3A	DNA (cytosine-5-)-methyltransferase 3 alpha
DNMT3B	DNA (cytosine-5-)-methyltransferase 3 beta
JHDM2A	JmjC domain-containing histone demethylation protein 2A
KAT8	K(lysine) acetyltransferase 8
IGF2	Insulin-like growth factor 2
IZUMO4	Izumo sperm–egg fusion protein 4
PDB1	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial precursor
HSP90A	Heat shock 90 kDa protein alpha

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