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REVIEW

Sperm cryopreservation: A review on current molecular cryobiology and advanced approaches

BIOGRAPHY

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KEY MESSAGE

Understanding the new aspects of sperm cryobiology, such as epigenetic and proteomic modulation, as well as novel techniques, is essential for clinical applications and the improvement of ART protocols. Long-term follow-up studies on the resultant offspring obtained from cryopreserved spermatozoa is recommended for future studies.

ABSTRACT

The cryopreservation of spermatozoa was introduced in the 1960s as a route to fertility preservation. Despite the extensive progress that has been made in this field, the biological and biochemical mechanisms involved in cryopreservation have not been thoroughly elucidated to date. Various factors during the freezing process, including sudden temperature changes, ice formation and osmotic stress, have been proposed as reasons for poor sperm quality post-thaw. Little is known regarding the new aspects of sperm cryobiology, such as epigenetic and proteomic modulation of sperm and trans-generational effects of sperm freezing. This article reviews recent reports on molecular and cellular modifications of spermatozoa during cryopreservation in order to collate the existing understanding in this field. The aim is to discuss current freezing techniques and novel strategies that have been developed for sperm protection against cryo-damage, as well as evaluating the probable effects of sperm freezing on offspring health.

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INTRODUCTION

The first record of semen cryopreservation dates back approximately 200 years, when Lazaro Spallanzani (1776) attempted to preserve spermatozoa by cooling it in snow (*Royere et al., 1996*). Further scientific progress was made considerably later with Polge's discovery of glycerol's cryoprotectant properties (*Polge et al., 1949*). This advance marked a turning point in the field of fertility preservation. Since that advance, there have been considerable improvements in techniques for cryopreservation of semen of different species. The earliest offspring produced from cryopreserved spermatozoa were reported in 1951 (cow), 1953 (human), 1957 (pig, horse) and 1967 (sheep) (*Curry, 2000*). Sperm cryobanks were developed in the 1960s for cattle and in the 1970s for humans (*Sanger et al., 1992*). Today, artificial insemination of animals and human assisted reproductive technology routinely use cryopreserved semenatozoa (*Kopeika et al., 2015; Yeste, 2016*). However, despite numerous achievements in sperm cryobiology, the search continues for methods that can optimally recover viable spermatozoa after cryopreservation.

Sperm cryopreservation is an effective route to the management and preservation of male fertility in humans and domestic animals (*Sharma, 2011*). Cytotoxic treatments, such as chemotherapy and radiotherapy, as well as surgical treatments, may lead to testicular failure or ejaculatory dysfunction (*Agarwal et al., 2014b; Rousset-Jablonski et al., 2016*). In such situations, freezing of spermatozoa can be a suitable solution to preserve fertility; the frozen-thawed semen can be used for intrauterine insemination (IUI), IVF or intracytoplasmic sperm injection (ICSI) (*Dohle, 2010*). Cryopreservation is widely used to preserve spermatozoa obtained from azoospermic patients who have undergone testicular sperm extraction (*Di Santo et al., 2012*) and can also be routinely used in men who want to begin assisted reproduction treatment and have a back-up sperm source. Furthermore, cryopreservation facilitates the storage of donor semen, while infectious disease screening can be completed and confirmed negative (*Anger et al., 2003*). In animals, artificial insemination is an extensively employed technique that uses frozen-thawed spermatozoa to manage or accelerate the rate of genetic improvement (*Flores et al., 2011; Masoudi et al., 2016*) by inseminating select or multiple females, respectively, with the semen obtained from a male of desired genetic quality (*Comizzoli, 2015*).

BIOLOGY OF SPERM CRYOPRESERVATION

A complete understanding of sperm physiology during cryopreservation is mandatory to ensure maximum success. A key factor in sperm cryobiology is that they are small cells with a large surface area (*John Morris et al., 2012; Morris, 2006*). These characteristics affect the viscosity and glass transition temperature of the intracellular cytosol in sperm cells, which makes them less susceptible to potential damage (*Isachenko et al., 2003*). In the absence of cryoprotective agents, cold shock and the induction of ice crystal formation can lead to the destruction of organelles in the sperm cells (*AbdelHafez et al., 2009*). This event may manifest in the oxidation of cellular compounds, as well as disruption and damage of cellular structures, such as the DNA, acrosome and plasma membrane, which ultimately reduces fertility (*O'Connell et al., 2002*). Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anions (O_2_) and hydroxyl radicals (OH^-) may induce apoptosis, membrane lipid peroxidation, disruption of mitochondria and DNA damage (Bollwein *et al.*, 2008). Conventionally, the concentration of cryoprotective agents, as well as membrane-stabilizing additives, is correlated with the rate and sensitivity of spermatozoa to sub-zero temperatures. The lipid composition of the sperm plasma membrane is a major factor that can influence the cryotolerance and cold sensitivity of spermatozoa. Differences in the fatty acid profile and omega-3/omega-6 ratio in spermatozoa of different species results in different levels of cryotolerance (*Esmaeili et al., 2015*). Moreover, spermatozoa obtained from different species may be different in size, shape and lipid composition, potentially affecting their resistance to cryo-injuries (*Esmaeili et al., 2015; Fattah et al., 2017; Maldjian et al., 2005*). Pre-freezing semen quality parameters, such as sperm motility and the abstinence period of sperm donors, can also affect the cryosurvival rate of post-thaw sperm (*Zhou et al., 2014*). Spermatozoa with abnormal motility traits (e.g. asthenozoospermic, oligoasthenozoospermic) are particularly susceptible to cryo-damage, possibly reducing their fertilizing ability (*Borges et al., 2007*). The following sections discuss the effects of freezing on the structures and macromolecules (proteins, transcriptome and epigenome) of spermatozoa, as well as the effects of cryopreservation on post-thaw sperm parameters.

ULTRASTRUCTURAL CHANGES OF SPERMATOZOA DURING FREEZING

Several studies have examined cryo-damage in spermatozoa of different species (*Ozkavukcu et al., 2008; Yeste, 2016*). Acrosome disintegration and partial removal of the outer acrosomal membrane with depletion of acrosomal content are common alterations that are attributed to physical freezing events (*Barthelemy et al., 1990*). These defects are probably attributable to ice crystal formation during the freezing of extracellular fluids, which results in expansion of the sub-acrosomal region. Alternatively, osmotic changes may cause damage to the lipid membrane structure, leading to tension changes in water canal proteins and ionic leakage in plasma membranes and resulting in morphological changes (*Sa-Ardrit et al., 2006*). Interestingly, it has been shown that rapid freezing markedly reduced the ultrastructural changes and preserved the integrity of sperm heads compared with slow freezing (*Serafini et al., 1986*). Studies have shown that glycerol is preferable to dimethyl sulfoxide (DMSO) as a cryoprotectant to protect sperm structures (*Oettle and Soley, 1986; Serafini et al., 1986*).

Woolley and Richardson have observed that extenders containing glycerol and egg yolk improved the apical segment of the acrosome and circular mitochondria after thawing (*Woolley and Richardson, 1978*). Cytoskeleton proteins, such as vimentin and actin, are other sperm structures that may incur damage during freezing. Transmission electron microscopy (*Harvey et al., 2013*) of post-thaw spermatozoa showed an incremental increase in wrinkling of the plasmalemma and sub-acrosomal swelling, as well as loss of acrosomal content and the appearance of vesiculations (*Ozkavukcu et al., 2008*).

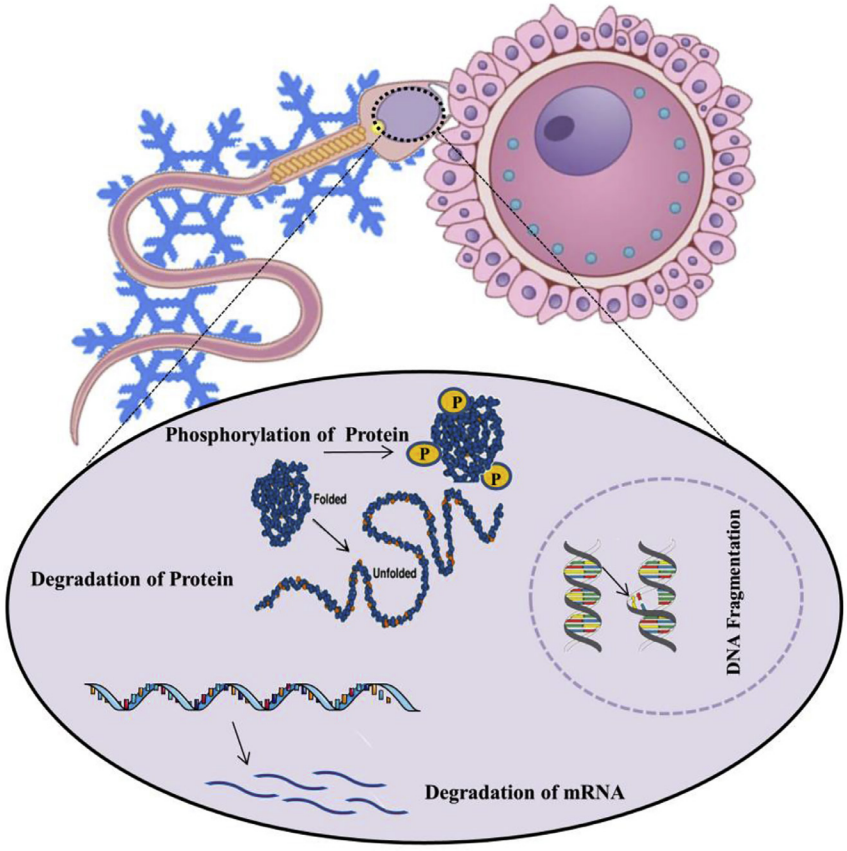

FIGURE 1 A general overview of modification in protein, mRNA and genome of cryopreserved spermatozoa that may affect the paternal contribution and early embryo development.

Post-thaw scanning electron microscopy has revealed an increase in the number of loose sperm head and tail defects. New nanoscopic techniques, such as scanning near-field optical microscopy (*Andolfi et al., 2015*), may help to elucidate the topographic images and subcellular structural details of spermatozoa without special staining or sample preparation, including the assessment of different cytoplasmic organs and the structure of the mitochondria (*Di Santo et al., 2012*).

PROTEOME, TRANSCRIPTOME AND EPIGENOME MODIFICATIONS OF POST-THAWED SPERMATOZOA

The underlying mechanisms behind the effect of cryopreservation on sperm parameters are not completely understood. Genes and protein expression, mRNA stability and epigenetic content of spermatozoa are thought to be modulated during the freeze-thaw process. Cryopreservation can affect the expression of key genes (e.g. *SNORD116/PWSAS* and *UBE3A*) related to fertility potential (*Valcarce et al., 2013b*). Alterations in protein expressions in post-thawed boar spermatozoa have been verified by differences in the expression of 41 proteins (*Chen et al., 2014*). Expression of AKAP3, superoxide dismutase 1 (SOD1), TPI1 and ODF2 proteins have been shown to be increased in frozen-thawed spermatozoa. It has also been reported that levels of heat shock protein 90 (HSP90), which plays a direct role in the motion characteristics of spermatozoa, significantly decreased after freezing-thawing (*Huang et al., 2009*). Proteomic analysis of normozoospermic spermatozoa showed significant changes in proteins such as mitochondrial aconitase hydratase (ACO2), OXCT1, tektin1 (TEKT1), alpha-enolase (ENO1), vimentin, and the level of tyrosine phosphorylation in frozen spermatozoa compared with the fresh state (*Wang et al., 2014*). These proteins are related to motility, viability and acrosomal integrity of spermatozoa. In another recent study, it was shown that levels of several quantifiable proteins, such as superoxide dismutase 1 (SOD1), peroxiredoxin 6 (PRDX6), isoform 2 of thioredoxin domain-containing protein 2 (TXNDC2), glutathione S-transferase Mu 3 (GSTM3), NADH-cytochrome b5 reductase 2 (CYB5R2), zona pellucida-binding proteins 1 and 2 (ZPBP1 and ZPBP2), acrosin-binding protein (ACRBP), acrosome membrane-associated protein 3 (SPACA3) and sperm equatorial segment protein 1 (SPESP1), decreased after cryopreservation, while those of other proteins, such as those of the annexin family (ANX1, ANX3 and ANX4), clusterin (CLU), importin-1b (KPNB1), histone H4 (HIST1H4A), tubulin-a1 A chain (TUBA1A) and sperm-associated antigen 17 (SPAG17), increased (*Bogle et al., 2017*). A recent study on chicken spermatozoa showed increases in 36 proteins and reductions in 19 proteins after thawing compared with pre-freezing. These proteins were related to sperm metabolism and the flagellum structure of spermatozoa (*Cheng et al., 2015*). Proteins such as ACRBP, FN1, HSP90AA1 and VDAC2 are biomarkers that predict boar sperm resistance to cryopreservation. ENO1 and glucose-6-phosphate isomerase (GPI) are

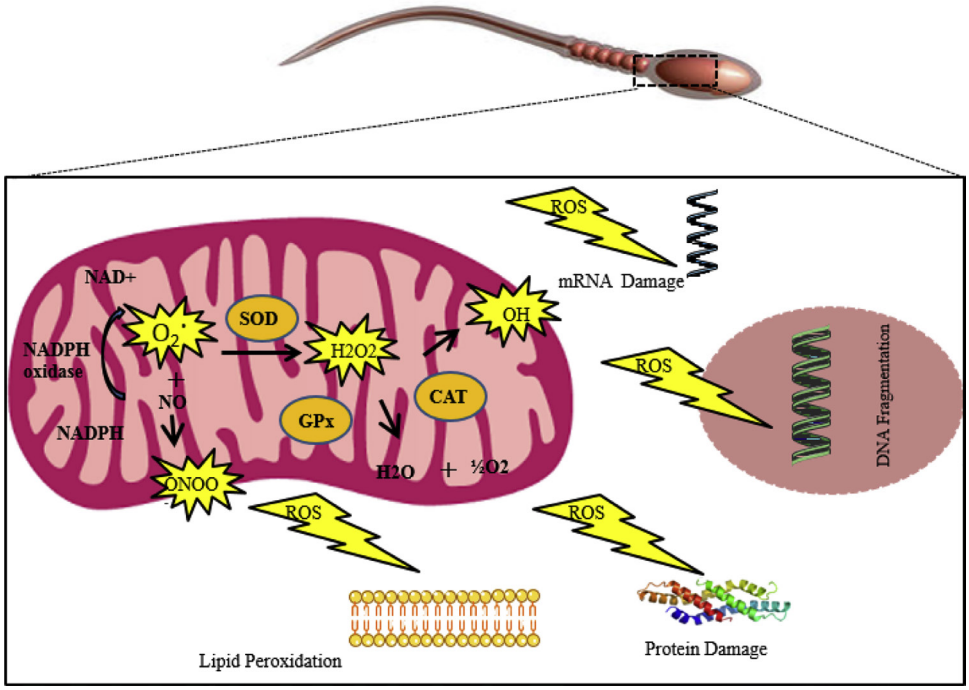

FIGURE 2 A summary of production of reactive oxygen species (ROS) in cryopreserved sperm mitochondria that leads to reduced sperm quality. NADPH = dihydronicotinamide-adenine dinucleotide phosphate; NAD = nicotinamide adenine dinucleotide; O_2° = superoxide; H_2O_2 = hydrogen peroxide; SOD = superoxide dismutase; ONOO = peroxynitrite; GPx = glutathione peroxidase; CAT = catalase.

markers related to the quality of human spermatozoa before freezing (*Jiang et al., 2015*).

Spermatozoa deliver paternal mRNA to the oocyte during fertilization (*Harvey et al., 2013; Lalancette et al., 2008; Stoeckius et al., 2014*) and thus play an important role in early embryo development (*Jodar et al., 2013*). Furthermore, during the freezing process, transcripts and mRNA–protein interactions in spermatozoa can be lost, which may directly influence embryo development (*Valcarce et al., 2013a*). Correlations between sperm mRNA and early development have been reported in humans and several animals such as pig and murine research models (*Avendano et al., 2009; Depa-Martynow et al., 2007; Fang et al., 2014; Hwang et al., 2013*). *Valcarce et al. (2013a)* showed that cryopreservation decreased the expression of several key transcripts (*PRM1*, *PRM2*, *PEG1/MEST* and *ADD1*) related to human sperm fertility.

In a study by *Riesco and Robles (2013)*, cryopreservation was observed to alter several transcripts (i.e. *eif2s1* and *lhcgr)*, while several others (i.e. human *HOXB1* and *ACTB*) remained stable in spermatozoa. Transcript analysis of frozen boar spermatozoa showed that the transcripts *B2M*, *BLM*, *HPRT1*, *PGK1*, *S18*, *SDHA*, *YWHAZ*, *PPIA*, *RPL4*, *DNMT3A*, *DNMT3B*, *JHDM2A*, *KAT8* and *PRM1* differed after cryopreservation (*Zeng et al., 2014a*). In the latter study, several micro-RNA (i.e. *let-7c*, *ssc-miR-26a* and *ssc-miR-186*) also underwent changes.

Recently, other studies have begun to explain certain of the epigenetic modifications that may occur in sperm cells during the freezing process. For instance, histone H1-DNA binding proteins and protein–DNA disulphide bonds are altered in boar spermatozoa after cryopreservation (*Flores et al., 2011*). *Zeng et al. (2014b)* stated that epigenetic-related expression of genes such as *DNMT3A*, *DNMT3B*, *JHDM2A*, *KAT8*, *PRM1*, *PRM2* and *IGF2* may change after freezing. Higher methylation in the *vasa* and *cxcr4b* promoters have been observed in zebra fish spermatozoa, but there were no differences in DNA methylation after cryopreservation (*Riesco and Robles, 2013*). Another study reported that cryopreservation did not affect the DNA methylation pattern of human sperm genes, such as maternally imprinted *LIT1, SNRPN* and *MEST*, as well as paternally imprinted *MEG3* and *H19* genes (*Klaver et al., 2012*).

FIGURE 1 shows a hypothetical overview of potential modifications that may be seen in proteins, mRNA and the genome of cryopreserved spermatozoa that could affect the paternal contribution to early embryo development.

SPERM PARAMETERS AFFECTED BY FREEZING

Motility, plasma membrane functionality, acrosome integrity and overall viability of spermatozoa post-thaw typically decreases in contrast to the pre-freeze state (*Ozkavukcu et al., 2008*). *Nijs et al. (2009)* reported that the percentage of motile spermatozoa decreased from 50.6% to 30.3% after cryopreservation. However, the mechanism through which motility decreases has not been thoroughly elucidated to date. A strong correlation exists between the percentage of immotile spermatozoa and mitochondrial defects after thawing (*Ozkavukcu et al., 2008*). It has also been suggested that rapid changes in osmolarity and intracellular ice crystal formation in the cryopreservation process may lead to changes in membrane proteins and carbohydrate composition, which can disrupt membrane structures (*Pedersen and Lebech, 1971*) and reduce sperm viability.

TABLE 1 THE EFFECTS OF VARIOUS ANTIOXIDANTS ON FROZEN-THAWED SPERM QUALITY

Additives	Species	Improvement effects	References
Vitamin E analogous	Ram, bovine, human, boar	Viability, LPO, motility, DNA integrity	*Azawi and Hussein (2013)*
			Hu et al. (2011)
			Kalthur et al. (2011)
			Satorre et al. (2012)
			Jeong et al. (2009)
			Minaei et al. (2012)
			Nekoonam et al. (2016)
Glutathione	Human, boar, deer	LPO and DNA integrity	*Ghorbani et al. (2016)*
			Giaretta et al. (2015)
			Varo-Ghiuru et al. (2015)
			Wang and Dong (2017)
L-cysteine	Boar, ram, human, buffalo	Motility, acrosome integrity, viability, LPO	*Chanapiwat et al. (2009)*
			Kaeoket et al. (2010)
			Topraggaleh et al. (2014)
			Iqbal et al. (2016)
			Noguchi et al. (2015)
			Sharafi et al., (2015a)
Melatonin	Ram, human	Viability, motility, intracellular ATP concentrations, DNA integrity and fertilizing ability	*Succu et al. (2011)*
			Karimfar et al. (2015)
L-carnitine	Human, rooster, cat	Motility, vitality, LPO, DNA integrity	*Banihani et al. (2014)*
			Zhang et al. (2016)
			Fattah et al. (2017)
			Manee-In et al. (2014)
Taurine, hypotaurine	Human, buffalo	Motility, viability and membrane integrity	*Brugnon et al. (2013)*
			Shiva Shankar Reddy et al. (2010)
Catalase	Human, rooster, goat, bull	Motility, vitality and DNA integrity	*Moubasher et al. (2013)*
			Moghbeli et al. (2016)
			Shafiei et al. (2015)
			Paudel et al. (2010)
Superoxide dismutase	Human, ram, bull, goat	Motility, vitality, LPO and ROS levels	*Bateni et al. (2014)*
			Santiani et al. (2014)
			Olfati Karaji et al. (2014)
			Shafiei et al. (2015)
Cholesterol-loaded cyclodextrins (CLC)	Goat, boar, rabbit	Fertilizing ability, acrosome integrity and viability	*Konyali et al. (2013)*
			Tomas et al. (2014)
			Nishijima et al. (2014b)

LPO = lipid peroxidation; ROS = reactive oxygen species.

ROS production and lower antioxidant enzyme activity in spermatozoa induce apoptotic pathways that can lead to a reduction in sperm viability (*Di Santo et al., 2012*). DNA integrity is a concern during cell freezing because cryopreservation easily changes mitochondrial membrane properties and increases the production of ROS, which may subsequently result in the oxidation of DNA, producing high frequencies of single and double-strand DNA breaks (*Said et al., 2010*). Furthermore, defects in DNA repair enzymes have been reported as another reason for DNA damage after freezing (*Bogle et al., 2017*).

Another parameter affected by freezing is morphology (*Donnelly et al., 2001*), as an uncontrolled liquid influx into spermatozoa may change cellular osmolality and deform the membrane structure, consequently altering sperm morphology (*O'Connell et al., 2002; Ozkavukcu et al., 2008*). Loose head and

tail defects, such as coiled and looped tails, are frequently observed following the freezing of spermatozoa.

It has been postulated that DNA changes in spermatozoa during cryopreservation may be caused by oxidative stress and apoptosis-inducing factors, which lead to disruption of nucleoprotein structure, disulphide bonds and the DNA–protamine complex (*Johnston et al., 2012; Yeste et al., 2013*). It has also been shown that spermatozoa with abnormal morphology are more sensitive to DNA damage during cryopreservation compared with those with normal morphology (*Di Santo et al., 2012*). Furthermore, the percentage of single-strand breaks in cryopreserved spermatozoa obtained from infertile men is higher than those obtained from fertile donors (*Hammadeh et al., 1999*). Single-stranded DNA may be more susceptible to the denaturing stress of oxidation, as samples of *in vitro* cultured testicular spermatozoa with higher motility possess more double-stranded DNA (*Emiliani et al., 2001*).

Conversely, other studies have indicated that cryopreservation does affect the stability of sperm DNA (*Duru et al., 2001; Isachenko et al., 2004b; Paasch et al., 2004; Schuffner et al., 2001*). This discrepancy may be related to factors such as the freezing method or the method used for DNA integrity evaluation (TUNEL, SCSA, SCD, Comet neutral or Comet alkaline) (*de Paula et al., 2006; Spano et al., 1999*). For example, a significant increase in DNA fragmentation has been reported following solid surface vitrification (SSV) and rapid freezing techniques (*Satirapod et al., 2012*), whereas *Isachenko et al. (2004a)* observed no significant differences in DNA integrity following vitrification or rapid freeze techniques. **Figure 2** displays possible intercellular events in cryopreserved spermatozoa that may lead to a reduction of antioxidant capacity and sperm quality.

STRATEGIES AGAINST CRYO-INJURIES

For nearly 70 years, scientists have attempted to reduce the detrimental effects of cryopreservation on spermatozoa. In this regard, defensive and controllable offensive strategies have been proposed and are discussed in the following sections.

Defensive strategies

Defensive strategies are methods in which different supplements are added to freezing media to protect sperm cells against damage. These additives could be cryoprotectants, antioxidants, antifreeze proteins (AFP), fatty acids, animal serum, nanoparticles or plant essential oils.

Cryoprotectants

Cryopreservation-induced changes in carbohydrate composition may reduce the integrity of the sperm plasma membrane, subsequently affecting their fertilization potential (*Di Santo et al., 2012; Parks and Graham, 1992*). Alterations in the content and location of proteins, such as ion channels, are another possible cause of impairment of membrane function after cryopreservation. Consequently, cryoprotectants are added to sperm freezing media to protect spermatozoa against the latter damage. The cryoprotective effect of cryodiluents against freezing damage acts through several mechanisms, such as decreasing the freezing point of intracellular and extracellular water (*Royere et al., 1996*), penetrating and interacting with cytoplasmic components, as well as forming a protective layer around the membranes of spermatozoa. Generally, cryoprotectants are classified into two groups, permeable and non-permeable. Permeable cryoprotectants including glycerol, dimethyl sulfoxide (DMSO), dimethyl acetaldehyde, propylene glycol and ethylene glycol (*Di Santo et al., 2012*) pass through the plasma membrane and replace water in the sperm cell. Such cryoprotectants are toxic at higher concentrations and numerous reports have shown that sperm fertility potential is dramatically decreased in freezing medium supplemented with high concentrations of these permeable agents (*Gilmore et al., 1997*). However, non-permeable agents, such as raffinose, sucrose, egg yolk citrate, albumin, polyethylene glycol and polyvinyl pyrrolidone, are common additives that cannot pass through the plasma membrane but do provide protective characteristics (*Di Santo et al., 2012*). In recent years, novel cryoprotective supplements, such as soybean lecithin and low-density lipoprotein, have been evaluated in human and animal sperm freezing (*Emamverdi et al., 2015*). These new cryoadditives have lipid properties that can directly combat ROS (*Chaudhari et al., 2015; Shahverdi et al., 2015*).

Antioxidants

ROS generation and oxidative stress during the freezing process may lead to serious sperm damage (*Anger et al., 2003*). Numerous studies have recently shown that the addition of antioxidants to freezing extenders can neutralize ROS and improve post-thaw sperm function (*Agarwal et al., 2014a; Zhang et al., 2012*). While some antioxidants can improve post-thaw sperm quality, others lack these beneficial effects (*Zhandi and Sharafi, 2015*). Based on their chemical structure, antioxidants are divided into two categories, namely enzymatic and non-enzymatic. Enzymatic antioxidants include glutathione peroxidase (GPx), SOD and catalase. Non-enzymatic antioxidants consist of radical scavengers such as vitamin E (α-tocopherol), vitamin C (ascorbic acid), glutathione (GSH), taurine and cofactors such as selenium or zinc that are necessary for the function of antioxidant enzymes. These represent defence systems that can combat free radicals (*Amidi et al., 2016; Moghbeli et al., 2016; Sharafi et al., 2015a*).

Table 1. characterizes the effects of various antioxidants used for the cryopreservation of spermatozoa of different species. Notable antioxidants include vitamin E, vitamin C, catalase, quercetin, pentoxifylline, genistein, biotin, butylated hydroxytoluene, resveratrol, honey, l-carnitine and nerve growth factor (*Najafi et al., 2014; Saeednia et al., 2016; Shabani Nashtaei et al., 2017*). Significant improvements in sperm parameters have been shown after supplementation of freezing media with these components. Further investigations are required to evaluate the clinical applications of antioxidants and the effect of combinations of antioxidant compounds in order to establish optimal conditions for semen cryopreservation technology.

Antifreeze proteins

Antifreeze proteins (AFP) and antifreeze glycoproteins (AFGP) are biological antifreeze agents found in numerous species that have adapted to extreme temperature conditions, such as polar fish, as well as some insects and plants (*Cheung et al., 2017; Kim et al., 2017*). These components act through decreasing the freezing point, inhibiting ice crystal formation, and stabilizing phospholipids and unsaturated fatty acids of plasma membranes. It is believed that AFP and AFGP can help preserve the

TABLE 2 EFFECTS OF VARIOUS SUB-LETHAL MILD STRESSORS FOR PRECONDITIONING OF SPERMATOZOA BEFORE CRYOPRESERVATION

Sub-lethal stress	Species	Effects	References
HHP	Bull, boar	Motility, viability and fertility potential	*Pribenszky et al. (2010*, 2011)
			Huang et al. (2009)
			Horvath et al. (2016)
H_2O_2	Bull	Fertilization and blastocyst rates	*Rahman et al. (2012)*
NO	Bull, human	Motility, mitochondria potential and apoptotic rates	*Sharafi et al. (2015b)*
			Hezavehei *et al*. (unpublished observations)
Osmotic	Rhesus macaque	Motility, viability, heat shock protein phosphorylation	*Cole and Meyers (2011)*
Ethanol	Bull	Motility, viability and apoptotic rates	*Dodaran et al. (2015)*

HHP = high hydrostatic pressure; NO = nitric oxide.

integrity of cellular membranes during sperm freezing (*Inglis et al., 2006*). The possible beneficial effects of AFP for freezing human spermatozoa have not been elucidated in detail; however, these compounds have been found to significantly increase the motility and viability of ram, chimpanzee, mouse, fish and sea bream spermatozoa after freeze-thawing (*Karanova et al., 2002; Koshimoto and Mazur, 2002; Payne et al., 1994; Younis et al., 1998; Zilli et al., 2014*). Both AFP and AFGP improved the osmotic resistance of bull spermatozoa during cryopreservation (Prathalingam *et al*., 2006), while AFPIII has increased the post-thaw motility and plasma membrane integrity of rabbit (*Nishijima et al., 2014a*) and buffalo spermatozoa (*Qadeer et al., 2014, 2015*) during freezing. Nonetheless, use of AFP as a cryopreservation additive for preserving spermatozoa from different species still needs to be further investigated.

Other defensive strategies

The use of magnetized water as a base solution of freezing media is an interesting suggestion in recent years. By passing water through a specially manufactured permanent magnet, water molecules are activated and ionized, changing them into a hexagonal structure with numerous hydrogen bonds and a high electron donor capacity (*Cai et al., 2009; Toledo et al., 2008*). It has been suggested that freezing media composed of magnetized water may form smaller ice crystals during freezing (*Cai et al., 2009; Xu and Yu, 2008*). A recent report stated that a magnetized extender could protect plasma, acrosomal and mitochondrial membranes of boar spermatozoa against cryopreservation-induced damage (*Lee and Park, 2015*).

Low-level laser irradiation (LLLI) is also a novel biophysics-based approach to improving the quality of post-thaw spermatozoa. It has been suggested that spermatozoa can absorb low-power laser energy via respiratory chain components, such as cytochrome C oxidase (complex IV of the mitochondrial electron chain), that can modify various biological processes occurring in spermatozoa. Several studies have revealed that this method can improve the viability, acrosome integrity and ATP production of spermatozoa after thawing (*Iaffaldano et al., 2010*). LLLI affects the spermatozoa's mitochondrial respiratory chain, resulting in increased ATP production and decreased ROS. Hence, sperm cell survival can be enhanced during freezing (*Fernandes et al., 2015*).

Offensive controllable strategies

Stress preconditioning of spermatozoa before cryopreservation is a novel strategy for sperm cryopreservation (*Horvath et al., 2016*). Application of sub-lethal stress in spermatozoa may induce a general adaptation and increased resistance to various future stresses (*Pribenszky et al., 2010, 2011*). It has been reported that the key factors that respond to sub-lethal stress include biosynthesis of stress-related proteins such as heat shock proteins and intracellular antioxidants because these proteins reduce the activation of the apoptotic cascade, and thus protect spermatozoa against cryo-injury (*Pribenszky et al., 2011*). Sperm preconditioning to high hydrostatic pressure (HHP), osmotic pressure, heat or oxidative agents before cryopreservation has been evaluated with human and animal spermatozoa (TABLE 2) (*Pribenszky and Vajta, 2011*). Pribenszky and colleagues reported that the mild stress induced by hydrostatic pressure (HP; 30 MPa for 90 min) in bull and boar spermatozoa resulted in higher post-thaw motility, membrane integrity and viability after cryopreservation (*Pribenszky et al., 2010*). Furthermore, HHP increased the life-span of stored semen and litter size compared with fresh semen (*Pribenszky et al., 2011*). *Huang et al. (2009)* reported that HHP treatment increased levels of sperm proteins that play a key role in fertilization (*Huang et al., 2009*). This research group also reported that the levels of proteins, such as ubiquinol-cytochrome C reductase complex core protein 1, perilipin and carbohydrate-binding protein AWN precursor, increased following the exposure of boar spermatozoa to sub-lethal amounts of HHP (*Pribenszky et al., 2010*).

Another sub-lethal stressor used for spermatozoa is an osmotic challenge. Increased HSP70 expression and post-translational modification of phosphoproteins such as tyrosine have been observed in macaque sperm treated with sub-lethal osmotic stress (*Cole and Meyers, 2011*). Mild oxidative stress induced by 200 μmol/l H_2O_2 increased fertilization and penetration rates of post-thaw bull spermatozoa (*Rahman et al., 2012*). In other studies, the quality of frozen-thawed spermatozoa significantly improved when very small doses of stressors (e.g. ethanol and nitric oxide) were added to cryopreservation medium before freezing (*Dodaran et al., 2015*; Sharafi *et al*., *2015*c). Further investigations in this area would facilitate understanding of the cellular and subcellular mechanisms involved in the application of sub-lethal doses of stress before and after freezing.

UPDATED TECHNIQUES FOR SPERM CRYOPRESERVATION

Many techniques are available for the cryopreservation of human and animal spermatozoa. In recent years, various procedures have been added to the technology of sperm freezing (Sharma *et al*., 2015).

Conventional method of cryopreservation

Slow freezing, rapid freezing and ultra-rapid freezing (i.e. kinetic vitrification) are conventional cryopreservation methods. Slow freezing is a method in which sperm cells are cooled progressively over a period of 2–4 h in two or three steps, either manually or automatically, using a programmable machine. It has been suggested that in slow freezing, ice crystal formation results in high electrolyte concentrations inside cells and possible chemical-physical damage to the spermatozoa (*Di Santo et al., 2012*). Cooling rates must be controlled at a certain value to reduce osmotic injury (*Said et al., 2010*). In the rapid freezing technique, spermatozoa are mixed with the cryoprotectant and the suspension is loaded into a cryo-straw or cryovial and subsequently exposed to a liquid nitrogen vapour phase for at least 10 min before being plunged into liquid nitrogen (*Di Santo et al., 2012*). Several studies have compared the slow and rapid freezing methods (*Tongdee et al., 2015; Vutyavanich et al., 2010*), consistently reporting a significantly greater rate of chromatin deterioration with rapid cooling compared with slow freezing (*Hammadeh et al., 2001*).

Vitrification is an alternative method used for the storage of human spermatozoa without the use of permeable cryoprotectants (*Isachenko et al., 2003*). Using this method, the sperm suspension is plunged directly into liquid nitrogen and the sperm cells are cooled in an ultra-rapid manner, known as kinetic vitrification (*Isachenko et al., 2003*). Recently, *Isachenko et al. (2012b)* have reported successful ICSI with vitrified spermatozoa and the birth of two healthy babies. A few other studies have shown a reduction in sperm quality after vitrification compared with slow freezing (*Agha-Rahimi et al., 2014; Vutyavanich et al., 2012*). Some researchers believe that kinetic vitrification is more suitable for clinical trials, because spermatozoa are separated from seminal plasma before cryopreservation and warmed spermatozoa do not need an additional centrifugation step for plasma removal (*Isachenko et al., 2011, 2012a*).

Advanced methods of cryopreservation

Freezing small numbers of sperm

Conventional freezing methods are not suitable for freezing small numbers of spermatozoa because in these methods, sperm samples are diluted in a large volume of cryoprotectant and recovery for ICSI is difficult. For this purpose, biological and non-biological carriers have been used to prevent the loss of small numbers of spermatozoa during the freezing of micro-volume aliquots of spermatozoa (*AbdelHafez et al., 2009*).

Empty zona pellucida (ZP) as a bio-carrier for sperm freezing (*Cohen and Garrisi, 1997*) efficiently sustained recovery rates, post-thaw motility, DNA integrity and the subsequent fertilization rate of small numbers of spermatozoa after thawing (*Borini et al., 2000; Levi-Setti et al., 2003; Ye et al., 2009*). The first pregnancy using micro-encapsulated cryopreserved spermatozoa from this method was obtained in 1998 (*Walmsley et al., 1998*). This technique is not simple because micromanipulation is required for sperm insertion into the ZP and spare homogeneous empty human ZP is not necessarily accessible (*AbdelHafez et al., 2009*). Other instruments that were also studied for storing small numbers of spermatozoa include cryo-loops, microdroplets, cut straws, mini-straws, open pulled straws, alginate beads, agarose gel microspheres, cryotop, plastic capillaries and high-security straws (HSV) (*Desai et al., 2004; Isachenko et al., 2005*). It was reported that open pulled straws appeared to be better because they had the lowest risk of contamination (*Isachenko et al., 2005*). Cryopreservation of small numbers of spermatozoa in a cryoloop is an approach derived from embryo vitrification. The spermatozoa obtained from this method sustain their viability and can fertilize oocytes (*Desai et al., 2004*). In the micro-droplet method, the sperm suspension is mixed with a vitrification solution and pelleted directly onto a super-cooled stainless steel surface, with the vitrified droplets then being placed into cryovials and stored in liquid nitrogen. The latter methods are considered open systems with a possible risk of contamination during freezing and storage (*Di Santo et al., 2012*).

ICSI pipettes have also been used to freeze small numbers of spermatozoa. However, their fine glass tips are fragile and unsealed, which may increase the risk of contamination in liquid nitrogen (*AbdelHafez et al., 2009*). Recently, *Endo et al. (2012)* have proposed the use of vitrification devices, such as the cryotop and cell sleeper, for the cryopreservation of small numbers of spermatozoa. In these two carriers, small numbers of sperm contained in a micro-droplet of freezing medium are placed on the cryotop strip or tray of the cell sleeper. Next, the spermatozoa are suspended above a liquid nitrogen surface for 2 min and subsequently plunged into sterilized liquid nitrogen. A desirable recovery rate (83–96%) was achieved after freezing in cell sleeper and cryotop devices, respectively. Following ICSI using vitrified spermatozoa, the fertilization rate was good (71%) and a single live birth was achieved (*Endo et al., 2012*).

Another ultra-rapid cooling approach for cryopreserving individual spermatozoa without cryoprotectant uses polydimethylsiloxane (PDMS) chips in microfluids that can cryopreserve a small number of spermatozoa in micro-vessels that remain stable during freeze-thawing. The rate of sperm recovery was found to be acceptable with this method, but it is not considered technically simple (*Zou et al., 2013*).

Solid surface vitrification (SSV)

This method is a combination of several methods in which sperm suspension is mixed with cryoprotectant and directly exposed to a freezing metal surface at a rapid cooling rate. Initially, this method was developed for use with bovine oocytes (*Dinnyes et al., 2000*). In this work, sperm cells were loaded into thin capillaries. Then, the capillaries were allowed to come in contact with the cold (−180°C) surface of a cryo-chamber. The vitrified droplets were transferred into a cryovial for storage in liquid nitrogen. Decreased DNA damage and reduced damage to the tails of the sperm are the advantages of this method (*Satirapod et al., 2012*).

Freeze-drying method (lyophilization)

Freeze-drying of spermatozoa is a preservation method in which liquid

nitrogen is not required. Lyophilized freeze-dried sperm can be kept at 4°C and transported at room temperature (*Keskintepe et al., 2002*). This method has been used for humans (*Kusakabe et al., 2008*) and several animals (*Keskintepe and Eroglu, 2015*). Although the spermatozoa recovered following this method are immotile, DNA damage was decreased compared with methods that use liquid nitrogen (*Gianaroli et al., 2012*). Several studies have reported successful fertilization of oocytes using lyophilized spermatozoa from mice (*Ward et al., 2003*), rats (*Kaneko and Serikawa, 2012*), pigs and rabbits (*Kwon et al., 2004*), as well as humans (*Keskintepe and Eroglu, 2015*). This inexpensive method is safe for bio-banking but requires ICSI for successful fertilization because sperm cells obtained by this procedure are not motile.

Effect of freezing on reproductive outcome and offspring health

Currently, sperm cryopreservation is a necessary tool in assisted reproductive techniques (ART) (*Kopeika et al., 2015; Tournaye et al., 1999*). The fertility potential of cryopreserved spermatozoa may depend on the method of fertilization (IUI, IVF and ICSI). Using cryopreserved spermatozoa, pregnancy outcomes after IVF or ICSI are not significantly different and are similar compared with the use of fresh spermatozoa (*Kalsi et al., 2011; Kopeika et al., 2015*) and as expected, pregnancy rates are lower after IUI (*Sun et al., 2013*). Poor-quality cryopreserved spermatozoa do not significantly affect pregnancy rates after ICSI (*Kuczynski et al., 2001*). Although motility is not required for fresh testicular spermatozoa to achieve fertilization, it is a prerequisite to ensure the selection of viable spermatozoa for ICSI to maintain optimal fertilization and pregnancy rates (*Schiewe et al., 2016*). The latter investigator has shown that the promotion of pre-freeze testicular sperm motility improves the ease and selection of motile spermatozoa post-thaw.

In a recent study, embryo quality and blastocyst formation were negatively affected by cryopreserved spermatozoa injected into oocytes (*Braga et al., 2015*). Perez-Cerezales *et al*. (2011) reported that post-thaw spermatozoa with fragmented DNA may exhibit altered gene expression in surviving embryos. However, in other studies, morphokinetic parameters of early embryo development were not affected by sperm freezing (*Eastick et al., 2017; Vicdan et al., 2016*). Furthermore, there was no evidence of chromosomal abnormality in offspring obtained from IVF using frozen-thawed spermatozoa (*Kopeika et al., 2015*). On a cautionary note, it has been reported that mouse zygotes fertilized by cryopreserved spermatozoa had higher methylation levels and reduced rates of cleavage and formation of blastocysts (*Jia et al., 2015*), and the offspring may experience improper growth and mesenchymal tumours (*Kopeika et al., 2015*). However, there are only few follow-up studies reporting on the correlation between sperm cryopreservation and child health after birth.

CONCLUSIONS AND FUTURE DIRECTIONS

Sperm cryopreservation is an important technique of fertility management in ART, but cryo-damage to cellular components may have detrimental effects on sperm function. Understanding the cellular and molecular modifications involved in the cryopreservation process can be useful for the optimization of sperm freezing. Whole genome sequencing and proteomics are powerful technological advances that could yield insightful supplemental information on the mechanism of cryo-injury, which may improve the efficacy of cryopreserved spermatozoa and related ART procedures. Because the post-thaw production of proteins and RNA may be altered after cryopreservation, evaluating them may be useful in the future as biomarkers indicating cryostress. Although cryopreserved spermatozoa have been effectively used to assist reproduction for decades, it is recommended that long-term follow-up studies be performed on offspring obtained from cryopreserved spermatozoa in future generations to fully assess their biological safety.

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