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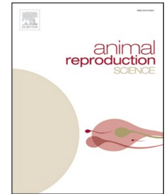

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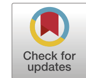

Advances in sperm cryopreservation in farm animals: Cattle, horse, pig and sheep

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ABSTRACT

Sperm cryopreservation is one of the most important procedures in the development of biotechnologies for assisted reproduction. In some farm animals, the use of cryopreserved sperm has so many benefits for which relevance has become more evident in recent decades. Values for post-thaw sperm quality, however, are variable among species and within individuals of the same species. There is no standardized methodology for each of the stages of the cryopreservation procedure (andrological examination, semen collection, dilution, centrifugation, resuspension of the pellet with the freezing medium, packaging, freezing and post-thaw sperm evaluation), which also contributes to differences among studies. Cryotolerance markers of sperm and seminal plasma (SP) have been evaluated for prediction of ejaculate freezability. In addition, in previous research, there has been a focus on supplementing cryopreservation media with different substances, such as enzymatic and non-enzymatic antioxidants. In most studies, inclusion of these substances have led to improved post-thaw sperm quality and fertilizing capacity as a result of minimizing the adverse effects on sperm structure and function. Another approach is the use of different cryoprotectants. The aim with this review article is to provide an update on sperm cryopreservation in farm animals. The main detrimental effects of cryopreservation are described, including the negative repercussion on reproductive performance. Furthermore, the potential use of molecular biomarkers to predict sperm cryotolerance is discussed, as well as the addition of substances that can mitigate the harmful impact of freezing and thawing on sperm.

1. Introduction

Worldwide, the current growing demand for access to food in general and animal protein in particular leads farmers to be as efficient as possible in breeding and management of livestock. Animal reproduction, therefore, is fundamental for addressing these needs. Understanding the reproductive dynamics of the different livestock species as well as the development of new technologies are

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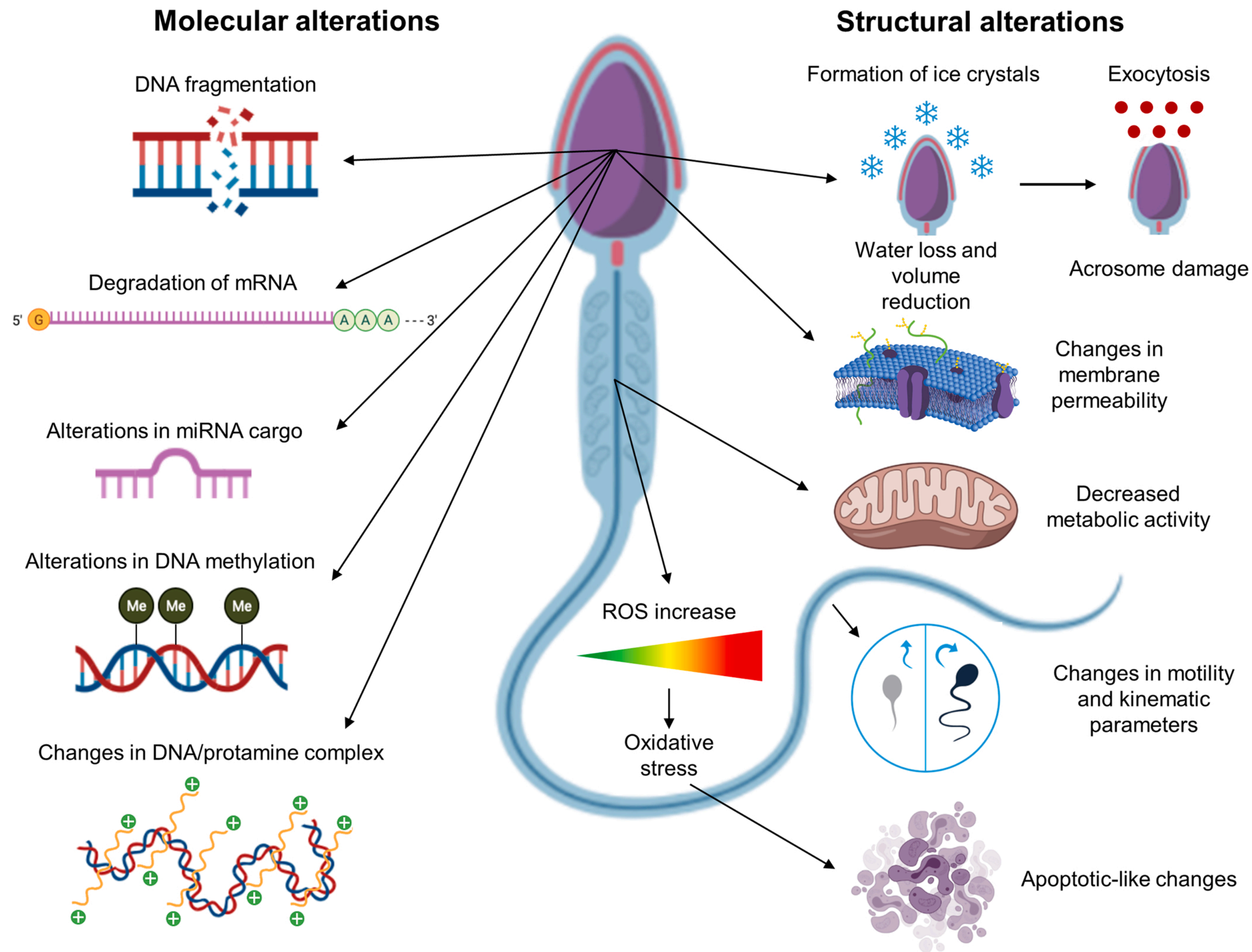

Fig. 1. Structural and molecular alterations in mammalian sperm following cryopreservation. Freeze-thawing decreases plasma membrane and acrosome integrity, motility, metabolic and mitochondrial activity, and increases ROS production. In addition, cryopreservation augments DNA fragmentation, may affect the DNA methylation signature, leads to degradation of mRNAs and miRNAs, and induces alterations in the integrity of the nucleoprotein structure (DNA/protamine complexes and translocation of H1).

thus required to improve animal product production efficiency (Davis and White, 2020).

Since the first attempts in the 1600 s (Sherman, 1964) and the discovery, in the mid-1950 s, of various cellular cryoprotective agents such as egg yolk (Phillips and Lardy, 1940) and glycerol (Polge et al., 1949) to protect sperm from cold shock during cooling, cryopreservation of genetic material has advanced markedly in mammalian reproduction. In effect, sperm cryopreservation allows for long-term storage, gene dispersal of genetically superior animals from generation to generation, and transport of semen for long distances regardless of the location (Veerkamp and Beerda, 2007). Another advantage of using frozen-thawed sperm is that farmers can easily inseminate females at the optimal time of reproduction, instead of depending on the presence of the breeding male at the farm. Furthermore, cryopreservation is an essential procedure for the management of germplasm banks, thus supporting biodiversity conservation and protection of endangered species (Fickel et al., 2007).

Mounting evidence indicates that, in most species, there is a group of animals that produce sperm with a relatively greater freezing capacity ("good freezers") than some other males; another group that has an acceptable freezing capacity; and a group of animals that, even though they have superior genetic value and acceptable reproductive performance when there is natural mating or use of fresh semen for artificial insemination, show little to no sperm cryotolerance ("bad freezers") (Loomis and Graham, 2008; Yeste, 2015). Nevertheless, in several studies there has been a focus on improving freeze-thawing regimens in species of agricultural interest. This includes optimizing the concentration of cryoprotectants and the freezing curve, with the aim to maintain sperm structure and function during freeze-thawing processes (Salamon and Maxwell, 1995), as well as to identify proteins that are related to the sperm resilience to cryopreservation, which are known as freezability markers (Yeste, 2015). However, there are several barriers associated to cryopreservation because sperm are very sensitive to temperature changes, and their viability is compromised after thawing (Nijs et al., 2009). This, therefore, has led to an exhaustive series of studies on post-thaw sperm quality (i.e., integrity of plasma membrane, acrosome and DNA, and production of reactive oxygen species (ROS), among others). In this context, it is worth mentioning that other attempts envisaged the addition of substances that improve the low temperature environment to which spermatozoa is exposed, as well as the modification of freezing curves, thus leading to better post-thaw sperm quality. In this review, there is an attempt to address all these aspects focusing on livestock (cattle, pigs, sheep and horses).

2. Structural alterations following sperm cryopreservation

Despite the efforts made to improve freezing media and protocols, mainly considering the addition of cryoprotectants, elucidating how low temperatures cause lesions on sperm, bearing in mind that the quality of frozen-thawed sperm basically relies on their capacity to withstand temperature changes without losing their main functions (Sieme et al., 2008), is still needed. The damage that occurs during cryopreservation results from the exposure of sperm to temperature variations (thermal stress), which leads to the formation of ice crystals inside the cell and in the surrounding environment (Morris et al., 2012). In addition, there are changes in osmolality (osmotic stress) that include: a) the formation of a hyperosmotic extracellular medium during freezing, to which the cell responds by losing water and as a result there is a lesser cell volume so as to balance the extracellular and intracellular solute contents (Yeste, 2016); and b) the submersion of sperm cells in a hypotonic extracellular medium during thawing, thereby allowing water to enter the cell by passive diffusion with the consequent increase in sperm volume (Pommer et al., 2002). With all these processes, the sperm plasma membrane is the primary irreversibly affected structure (Loomis and Graham, 2008), due to alterations in lipid-protein complexes during freezing and thawing (Mazur et al., 1972) (Fig. 1). With the decrease in temperature, the configuration of phospholipids is modified as these compounds move laterally in the membrane, which allows for the adhesion of proteins. This causes the sperm plasmalemma to become more rigid and fragile, due to conversion from a liquid to gel state (De Leeuw et al., 1990), resulting in an increase in plasmalemma permeability and a decrease in sperm metabolism (Hammerstedt and Graham, 1992).

3. Molecular alterations due to sperm cryopreservation

Even with the injuries to the sperm membrane during freezing and thawing processes, the damage to the molecular components induced by cryopreservation may be greater than that resulting from this procedure (Fig. 1). There needs to be determination of the extent of chromatin damage ensuing from freeze-thawing that results in DNA fragmentation (Fraser and Strzezek, 2007). It has been suggested that the nucleoprotein structure, which is composed of protamine 1 (P1) and protamine 2 (P2), and histones (5%−15%), could be responsible for the cryodamage to the DNA because freezing and thawing have been reported to disrupt the disulfide bridges between cysteine residues (Flores et al., 2011). The greater or lesser extent of this disruption relies on the type of protamines (P1 and P2) in sperm chromatin (Ribas-Maynou et al., 2021). While P1 but not P2 is present in all species, there are differences in the proportions (P1:P2) and relative content of protamines, as well as in the amount of retained histones (Gosálvez et al., 2011). In addition, other mechanisms, such as the decrease in temperature, oxidative stress induced by the production of large quantities of ROS (McCarthy et al., 2010), and the mechanical stress caused by the cellular contraction that compacts chromatin in some regions of the genome (Kopeika et al., 2015) could also disrupt the integrity of the double helix of DNA. The effects of the damage to molecules during cryopreservation is likely to be reflected on the fertilizing capacity of frozen-thawed sperm.

3.1. *Effects on fertilizing capacity*

An important aspect arising from cryopreservation is related to the processes occurring during fertilization. During fertilization, the spermatozoon releases messenger RNAs (mRNA) within the oocyte. Cryopreservation, however, can affect the sperm mRNA content (Stoeckius et al., 2014) and thus impair the function of these mRNAs, which are known to have a function during the early stages of

embryonic development when there are translation processes that result in the synthesis of proteins by the oocyte (Wang et al., 2014). It, therefore, is noteworthy that sperm are transcriptionally "silent" cells and lack the capacity to replace the mRNAs that are lost during cryopreservation (Ortiz-Rodriguez et al., 2019; Fig. 1). In addition, Urrego et al. (2014) and Zeng et al. (2014) described that epigenetic factors involved in gene expression, such as non-coding RNA (ncRNA), DNA methylation, chromatin remodeling, and post-translational histone modifications, could also be affected by freezing and thawing procedures. In cattle, cryopreservation leads to differences in the ncRNA content as determined when there were comparisons between frozen-thawed and fresh sperm (R1A10, R1C4, R4A1 and R4D2) (Chen et al., 2015). Furthermore, results from another study, also conducted in cattle, indicated there was a differential abundance of 86 microRNAs (miRNA, a type of small ncRNA) in frozen-thawed sperm; 40 of these miRNAs were related to sperm function (motility, viability), apoptotic-like changes and metabolic pathways (Capra et al., 2017). Likewise, Dai et al. (2019) reported that 135 miRNAs were in differential abundance between fresh and frozen-thawed pig sperm, with 34 being involved in apoptotic-like changes and metabolic pathways. It is thus thought that cryopreservation alters the miRNAs involved in the expression of genes related to apoptotic-like changes, which include alterations in mitochondrial membrane potential, phosphatidylserine externalization, DNA fragmentation, and caspase activation (Said et al., 2010; Shangguan et al., 2020). As previously described in this review article, sperm are transcriptionally "silent" cells; additional studies in this area, therefore, are warranted to determine the effects on specific molecules that modulate sperm function.

3.2. Effects on the embryo

Another important facet that can be altered by freeze-thawing is sperm DNA methylation, which is known to be essential for embryo development before implantation (Benchaib et al., 2003). Basically, this process involves the covalent addition of a methyl group to cytosines of CpG (5'-cytosine-phosphate-guanine-3') regions (Urrego et al., 2014; Ugur et al., 2019). The extent of DNA methylation in horse sperm is markedly greater following cryopreservation (5.4% in frozen-thawed sperm compared with 0.6% in fresh semen) (Aurich et al., 2016). The failure of fertilization to occur when frozen-thawed sperm are used for artificial insemination could be explained by there being an aberrant methylation of the DNA during cryopreservation. Similarly, cryopreservation procedures could have effects on early embryonic development because there is epigenetic inheritance by the oocyte from the few sperm nucleosomes and methylated DNA, and paternal chromatin also contributes to the embryonic epigenome (van der Heijden et al., 2008; Ortiz-Rodriguez et al., 2019b).

Transcription factors present in the embryo have essential functions in embryonic development (Jia et al., 2015). There are 0.78% of the transcripts that have been identified in horse embryos that are downregulated (with the presence of 84 transcription factors, for example: *NF-1, KLF13, CPBP, BTEB3, TCF7L1,* and *KLF3*) when frozen-thawed sperm are used for artificial insemination (Ortiz-Rodríguez et al., 2021). The downregulation of these transcription factors could be associated with the delay in embryonic development and the greater mortality, which are attributed to inseminations with cryopreserved sperm (Jia et al., 2015). In cattle, cryopreservation alters mRNA and miRNA profiles because 526 mRNAs and 55 miRNAs are in differential abundance when there are evaluations of fresh and frozen-thawed sperm (Shangguan et al., 2020). Sperm cryodamage occurring during freezing and thawing could explain the alteration of these mRNA and miRNA profiles due to the degradation of mRNAs and miRNAs (Fig. 1). Furthermore, sperm-borne miRNAs could regulate the maternal function of the mRNAs involved in cleavage, epigenetic reprogramming, and embryonic apoptosis (Wang et al., 2017). Sperm cryopreservation, therefore, could further alter the mRNA profile in embryos because some relevant functions are affected by miRNA modulation of genes, which would induce negative effects on embryo development (Braga et al., 2015). Changes in the miRNA profile have also been observed in frozen-thawed pig sperm because when there is cryopreservation with and without glycerol there are 23 and 14 differentially abundant miRNAs, respectively, but only two are significantly downregulated with and without the inclusion of glycerol as cryoprotectant (Zhang et al., 2017). Because these miRNAs are mainly associated to metabolic and cellular processes, these previously described variations could also affect embryonic development.

4. Redox balance and mitochondrial function

As previously described in this review article, the imbalance between the cellular antioxidant defense system and ROS production during cryopreservation leads to oxidative stress. The ROS include oxygen free radicals, such as hydroxyl radical (OH^-), superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) (Martínez-Cayuela, 1995; Peña et al., 2019; Zhang et al., 2019). Although ROS are necessary for sperm to have homeostatic physiological functioning, freezing results in an increase in lethal consequences of greater than optimal ROS concentrations due to the activation of apoptotic-like pathways (Dutta et al., 2019). Furthermore, sperm can also undergo marked structural damage, primarily at the DNA, which has detrimental consequences on fertility of some individuals (Januskauskas et al., 2003; Waterhouse et al., 2010; Estrada et al., 2014).

Among the effects resulting from the production of ROS during cryopreservation are the changes that occur in the mitochondrial membrane potential of sperm (Said et al., 2010). In addition to humans, there have been the most studies of the effects on sperm of greater than optimal ROS concentration in horses. The research focused on determination of the redox balance has led to measuring the static oxidative-reducing potential (sORP) in cryopreserved sperm (Ortiz-Rodriguez et al., 2019a). Rosiglitazone inclusion in cryopreservation media can improve the mitochondrial function of frozen-thawed sperm because of reduction in the activity of caspase-3, consequently delaying the activation of apoptotic-like pathways. Specifically, addition of rosiglitazone to cryopreservation media leads to maintenance of redox homeostasis, which results in AKT protein continuing to be phosphorylated. This protein is involved in the balance between survival and apoptotic pathways (Ortiz-Rodriguez et al., 2019a).

Compared to horses, the relevance of the ROS produced in other species during sperm cryopreservation is less clear. In pigs, Flores

et al. (2008b) reported that the capacity for ROS production at the mitochondria is little before freezing (Flores et al., 2008b). Likewise, Gómez-Fernández et al. (2013) and Yeste et al. (2013) observed intracellular concentrations of ROS and membrane lipid peroxidation, both in viable and non-viable frozen-thawed sperm, do not differ between ejaculates with relatively greater and lesser freezability. In buffalo and cattle, the effects of ROS on lipid peroxidation, mitochondrial membrane potential and DNA integrity in frozen-thawed sperm is not as apparent as in the fresh sample of the same ejaculate (Kadirvel et al., 2009).

5. Sperm motility after cryopreservation and motile subpopulations

Cryopreservation affects the movement and modifies the size and shape of the sperm head. The evaluation of the effects of freezing and thawing on sperm kinematics and morphometry is usually conducted utilizing computer-assisted sperm analysis (CASA), which provides a more objective evaluation (Ugur et al., 2019). This system allows for assessment of sperm movement trajectory at a specific time by evaluating a sequence of continuous images (frames/s, Hz) obtained utilizing a phase-contrast microscope, a video camera, and viewed on a computer screen (Holt et al., 2007, 2018). By using a CASA system, not only are total and progressive sperm motility values recorded, but also the velocity [curvilinear (VCL, μm/s), rectilinear (VCL, μm/s) and mean (VAP, μm/s)] of movement of each in a unit of time. From these variables, other indices such as linearity (LIN, %), straightness (STR, %) and oscillation (WOB, %) of sperm movements can be determined. The system also allows for determining the lateral displacement (ALH, μm) and the beat frequency of the head (BCF, Hz) (Yániz et al., 2018).

Obtaining all the values for these variables with utilization of the CASA system, together with the application of statistical models, has allowed sperm to be grouped into subpopulations according to motility patterns (Martínez-Pastor et al., 2011). The assessments of sperm subpopulations has greatly allowed researchers and staff at reproductive centers to have the capacity for interpretation and understanding of various aspects of sperm morphology and motility. One of these facets is the possibility of analyzing in much greater detail the effects of varying temperatures on sperm movement. As reported for bulls (Muiño et al., 2009), boars (Estrada et al., 2017), rams (Ledesma et al., 2017) and stallions (Ortega-Ferrusola et al., 2009), freezing and thawing modify the sperm structure in motile subpopulations in a semen sample.

There has been a focus in some studies on the association of motile subpopulation type with sperm viability and fertilizing capacity. The proportions of the subpopulation that included sperm with rapid and non-linear movements have been reported to be greater in ejaculates with greater sperm viability post-thawing (Ibanescu et al., 2020). Furthermore, the damage to the plasma membrane as a result of cryopreservation can affect motility patterns, causing sperm to have different patterns of movement, which can affect the population categorization of sperm cells. With the aim of understanding how sperm move, there have been several studies focused on improving the software of analysis, mainly for the implementation of use of high-resolution cameras that can be utilized to obtain as many as 500 frames/second (fps) for evaluations (Bompart et al., 2018), the usage of various types of counting chambers with different design and depth (Gacem et al., 2020), and the implementation of 3D technology (Soler et al., 2018).

6. Variations in sperm cryopreservation outcomes between species

As indicated previously in this review article, sperm cryopreservation causes structural and molecular changes, reduces the fertilizing capacity and may also impair subsequent embryo development. In addition to the inherent factors related to the freezing regimens and techniques used to improve post-thaw sperm quality, there is an important variability in cryopreservation outcomes between species because the resilience of the sperm of different species to freezing-thawing processes differs. In cattle, as an outcome of cryopreservation there is a gradual reduction by as much as 50% in sperm motility and viability post-thawing (Khalil et al., 2019), whereas in pigs, although the reduction in motility is similar to that of cattle, the decrease of sperm viability is greater (~ 60%) (Roca et al., 2006). In sheep, there is a lesser post-thaw sperm motility and viability compared to cattle and pigs (around 40% and 30%, respectively) (García et al., 2017). Furthermore, in horses, there are least desirable cryopreservation outcomes compared to the other farm animals (approximately, 30% motility and viability post-thawing) (Catalán et al., 2020).

Regarding other post-thaw sperm function variables in cattle, 40% of frozen-thawed sperm have a swollen plasma membrane (Khalil et al., 2019). In pigs, plasma membrane fluidity is compromised in as many as 50% of sperm post-thawing (Martinez-Alborcia et al., 2012). These values are even greater in sheep and horses because as many as 80% (Salmon et al., 2017) and 70% (Catalán et al., 2020) of the cryopreserved sperm have disrupted plasma membrane integrity. For acrosome integrity, 10% and 19% of frozen-thawed cattle sperm have typical and atypical acrosomal exocytosis, respectively, and there is loss of the acrosome in about 6% of sperm (Khalil et al., 2019). In horses, 12% of frozen-thawed sperm have acrosome damage (Ferrer et al., 2020), whereas in pigs and sheep this percentage is about 30% (Pezo et al., 2021) and 50% (García et al., 2017), respectively.

Cryopreservation also results in a lesser mitochondrial function of frozen-thawed sperm in cattle (15%; Khalil et al., 2019), sheep (30%; García et al., 2017), pigs (30%; Delgado-Bermúdez et al., 2019) and horses (35%; Catalán et al., 2020). There are slightly greater percentages of viable sperm with greater than optimal ROS concentrations (peroxides and superoxides) after cryopreservation in horses (1%; Catalán et al., 2020), sheep (1.5% Falchi et al., 2018) and pigs (2% peroxides and 4% superoxides; Delgado-Bermúdez et al., 2019). There is a marked increase in intracellular superoxide concentrations in frozen-thawed cattle sperm (up to 48%), even though the percentage of viable sperm with the relatively greater peroxide concentrations is similar to that in the other species (1%) (Hitit et al., 2020).

Chromatin and DNA integrity is about 9% in frozen-thawed cattle sperm with there being nuclear damage (Khalil et al., 2019). In pigs, 1% of cryopreserved sperm have DNA fragmentation and approximately 10% show chromatin decondensation (Caamaño et al., 2021). In sheep, 14% of frozen-thawed sperm have DNA fragmentation (Öztürk et al., 2020), a value that is similar to that in horses

(12%; Al-Essawe et al., 2018).

7. Freezability markers

Sperm quality data from frozen-thawed samples indicate the individual component has an important effect on sperm cryopreservation because there is a large variability among sires, ejaculates and even between fractions of the same ejaculate (reviewed in Yeste, 2016). Differences in the individual response are related to the sperm resilience to thermal and osmotic stressors, which are prevalent during freezing and thawing. The SP proteins appear to have effects on sperm to enhance the capacity of these gametes to withstand cryopreservation. The relative content of these proteins differs among individuals and has been related to sperm cryotolerance (Moura and Memili, 2016). The mechanism through which these proteins can exert a positive effect on sperm viability after thawing is directly related to: a) the resistance to oxidative stress and especially to apoptotic-like changes, because proteins undergo carbonylation, which favors oxidation and leads to the loss of biological functions of these proteins (Mostek et al., 2017); and b) to the changes in protamine-DNA complexes that occur during cryopreservation (Flores et al., 2008a).

Sperm proteins, such as AKAP4 and its precursor (proAKAP4), can be used as marker factors for frozen-thawed sperm quality that are associated with cryodamage, such as oxidative stress in pigs (Perez-Patino et al., 2019a), horses (Blommaert et al., 2019) and sheep (Riesco et al., 2020; Table 1). Likewise, two heat shock proteins, HSP90AA1 and HSPA8, are associated with sperm cryotolerance because when there are greater relative concentrations of these proteins, there is greater resilience of sperm to freeze-thawing (Zhang et al., 2015; Holt et al., 2015). In pigs, Casas et al. (2010) found that when sperm have greater concentrations of HSP90AA1, these gametes have higher resilience to cryopreservation as indicated by greater sperm motility and viability post-thawing. Likewise, aquaporins (AQP) modulate the permeability of plasma membrane to water and permeable cryoprotectants during cryopreservation (Agre et al., 2002; Yeste et al., 2017). Prieto-Martínez et al. (2017a) and Prieto-Martínez et al. (2017b) reported that AQP3 and AQP7 are associated with the cryotolerance of pig and cattle sperm. Another possible marker for sperm freezability in pigs is the voltage-dependent anion channel 2 (VDAC2), a pore-forming protein in the mitochondrial membrane that is facilitative for the transport of ions; sperm having greater abundances of this protein have greater cryotolerance (Vilagran et al., 2014; Table 1). Furthermore, the relative abundance of Glutathione S-transferase Mu 3 (GSTM3) in pig sperm is greater in sperm with lesser compared to those with greater (GFE) freezability both before and after cryopreservation (Llavanera et al., 2019).

Furthermore, abundances of SP proteins are also associated with sperm cryotolerance. In effect, pig sperm from ejaculates with greater quantities of fibronectin-1 (FN1) have a greater resilience to withstand freezing and thawing processes (Vilagran et al., 2015; Table 1). Two protein complexes (the 26 S proteasome and TCP-1, which contain chaperonin, CCT) confer greater resilience to sperm cryopreservation in sheep (Rickard et al., 2015). Also, in cattle, acid seminal fluid proteins (aSFP) have functions in scavenging the oxidative stress factors induced by cryogenic damage, reducing lipid peroxidation, which could explain why abundances of these proteins are associated with sperm cryotolerance (Einspanier et al., 1994).

8. Functions of cryoprotectants

The basic principle for preserving cells, tissues or organs for an indeterminate time at cold temperatures lies in the suppression of cellular metabolism. A key factor for cryopreserved sperm to maintain their functional integrity is the surrounding milieu. This must provide the required conditions of energy, pH, osmolality, and ionic strength for sperm to withstand the decrease in temperature and thus minimize the physical variations that lead to structural damage. This includes the inhibition of formation of ice crystals and water

Table 1
Freezability markers identified in cattle, pig, sheep and horse sperm.

Marker	Effects	Species	Reference
Sperm proteins			
proAKAP4	Could help predict factors underlying cryogenic damage, such as oxidative stress	Horse	(Blommaert et al., 2019)
		Pig	(Perez-Patino et al., 2019a)
		Sheep	(Riesco et al., 2020)
Heat shock protein 90AA1 (HSP90AA1)	Maintain metabolic and structural integrity of cells under stress conditions	Cattle	(Zhang et al., 2015)
		Pig	(Casas et al., 2010)
Heat shock protein 8 (HSPA8)		Cattle	(Holt et al., 2015)
Aquaporin 7 (AQP7)	Involved in the permeability of the plasma membrane to water and permeable cryoprotectants	Cattle	(Prieto-Martínez et al., 2017b)
Aquaporin 3 (AQP3)		Pig	(Prieto-Martínez et al., 2017a)
Voltage-dependent anion channel 2 (VDAC2)	Allows the transport of ions	Pig	(Vilagran et al., 2014)
Glutathione S-transferase Mu 3 (GSTM3)	Involved in cellular protection against oxidative stress	Pig	(Llavanera et al., 2019)
SP proteins			
Fibronectin-1 (FN1)	Could help predict factors underlying cryogenic damage, such as oxidative stress	Pig	(Vilagran et al., 2015)
26 S proteasome and CCT complex		Sheep	(Rickard et al., 2015)
Acid seminal fluid proteins (aSFP)		Cattle	(Einspanier et al., 1994)

Abbreviations: SP, seminal plasma.

loss from inside the cell (Morrell and Mayer, 2017), and the oxidation of cellular compounds, such as DNA, acrosome, and plasma membrane, which ultimately lead to a lesser fertilizing capacity of spermatozoa (O'Connell et al., 2002). In addition, the media components induce modifications of the sperm surface due to the interaction with the plasma membrane, which leads to changes in the lateral organization of proteins and lipids that ultimately result in its destabilization and the occurrence of capacitation-like changes (Leahy and Gadella, 2011; Pini et al., 2018). In most species, sperm cryopreservation media are composed of a buffer (Tris), non-permeable cryoprotectant (egg yolk), permeable cryoprotectant (glycerol), energy source (glucose) and other additives (antibiotics, vitamins and antioxidants). The cryoprotective agents prevent the formation of ice crystals with the decrease in temperature and minimize the effect of cold shock that culminates in the alterations of organellar structures and functions in sperm (Abdelhafez et al., 2009).

There have been studies conducted where there was a focus on modification of components in the medium and/or inclusion of other additives, taking into account the variability between species and also between individuals. Most freezing media contain 20% egg yolk and 3–5% glycerol as non-permeable and permeable cryoprotectants, respectively, to protect sperm from damage during freeze-thawing (Table 2). The beneficial effect of egg yolk has been attributed to the presence of low-density lipoproteins (LDL), which have the capacity to bind to the plasma membrane and form a protective film that stabilizes the lipid bilayer (Bergeron and Manjunath, 2006). Replacing the whole egg-yolk with LDL results in a greater motility and viability post-thawing of cattle (Amirat et al., 2004), pig (Jiang et al., 2007), sheep (Tonieto et al., 2010) and horse sperm (Pillet et al., 2011). In the case of frozen-thawed pig sperm, using LDL at an optimal concentration of 9% rather than the whole egg yolk leads to less sperm DNA damage induced by cryopreservation (Jiang et al., 2007b). The current trend in reducing substances of animal origin, however, has fostered the search for alternatives to replace egg yolk as a cryoprotectant due to its potential contamination, mainly bacterial. One of these alternatives is the evaluation of substances of plant origin as potential substitutes for egg yolk and non-permeable cryoprotectants in freezing media (Murphy et al., 2018; Table 2). Soy lecithin is one of the alternatives to this compound having the capacity to facilitate a mechanism of action that is similar to that of LDL (Vidal et al., 2013) while there is greater biosafety and lesser cytotoxicity (Bousseau et al., 1998). The results of replacing egg yolk with soy lecithin to freeze bull sperm, however, are inconsistent because results from some studies focused in this area indicate that there is greater sperm motility post-thawing (up to 19%) than with the use of egg yolk (Aires et al., 2003), whereas results from others indicate there is not an impact on motility or there is a negative effect (Crespilho et al., 2012). Because of the variability in the cryopreservation outcomes when there was use of substances of vegetable origin and that of egg yolk, with most freezing regimens there continues to be the use of egg yolk as the primary non-permeable cryoprotectant in freezing media. Another alternative approach for reducing the potential of contamination with use of egg yolk is a previous treatment consisting of dehydration or pasteurization; the variability observed in sperm quality post-thawing, however, does not allow for ascertaining the efficacy of this approach (Alcay et al., 2015; García et al., 2017). Furthermore, chemically composed liposomes, which do not contain infectious agents (Kumar et al., 2015), have been used as an alternative to egg yolk in freezing media for cryopreserving bull, boar, ram and stallion sperm (Luna-Orozco et al., 2019; Medina-León et al., 2019; Miguel-Jimenez et al., 2020). The protective effect of liposomes is due to their capacity to modify the

Table 2
Non-permeable and permeable cryoprotectants included in freezing media for cattle, pig, sheep and horse sperm.

Component	Effects	Species	Concentration	Reference
Non-permeable cryoprotectants				
Egg yolk	Able to bind plasma membrane and form a protective film that stabilizes the lipid bilayer	Cattle	20% (v:v)	(Amirat et al., 2004)
		Pig	9% (v:v)	(Jiang et al., 2007a)
		Sheep	20% (v:v)	(Tonieto et al., 2010)
		Horse	2% (v:v)	(Pillet et al., 2011)
Soy lecithin	Prevents ice crystal formation, minimizes plasmalogen substitution and reduces mechanical membrane damage	Cattle	1% (v:v)	(Aires et al., 2003)
		Sheep	2% (v:v)	(Forouzanfar et al., 2010)
		Horse	45 g/L	(Papa et al., 2011)
Liposomes	Modification of the conformation and permeability of the plasma membrane to water and permeable cryoprotectants	Cattle	40 mg/mL	(Miguel-Jimenez et al., 2020)
		Pig	0.3119 µmol/mL	(He et al., 2001)
		Sheep	40 mg/mL	(Luna-Orozco et al., 2019)
		Horse	50 µL	(Medina-León et al., 2019)
Permeable cryoprotectants				
Glycerol	Decreases the concentration of electrolytes so that sperm undergo osmotic contraction and withstand low temperatures	Cattle	7% (v:v)	(De Leeuw et al., 1993)
		Horse	3.5% (v:v)	(Alvarenga et al., 2005)
		Pig	4% (v:v)	(Buhr et al., 2001)
		Sheep	5% (v:v)	(Silva et al., 2012)
Trehalose	Maintains post-thaw motility and acrosome integrity better	Cattle	25 mM	(Büyükleblebici et al., 2014)
		Pig	250 mM	(Gutiérrez-Pérez et al., 2009)
		Sheep	60 mM	(Öztürk et al., 2020)
Amides	Induce less osmotic damage because of their lower molecular weight	Cattle	3% (v:v)	(Forero-Gonzalez et al., 2012)
		Horse	1.5% (v:v)	(Wu et al., 2015)

conformation and permeability of the plasma membrane to water and permeable cryoprotective agents (Röpke et al., 2011; Table 2).

In the case of permeable cryoprotectants used to freeze sperm, there are alcohols, typically glycerol and to a lesser extent ethylene glycol. Glycerol inserts into the lipid bilayer and changes membrane diffusion rates, causing the electrolyte concentration to decrease and for there to be an osmotic contraction of sperm volume that allows for these gametes to withstand cold temperatures (Holt, 2000). Some problems can be encountered upon thawing, however, because glycerol is not completely removed from the plasma membrane. The sensitivity of sperm to the harmful effects of glycerol is related to the concentration and this varies among species (Alvarenga et al., 2005; Table 2). There has been a marked detrimental effect in frozen-thawed pig sperm because when there are relatively greater concentrations of glycerol, there is a reduction in the fluidity of specific plasma membrane domains stained by *trans*- (tPNA) and *cis*-parinaric acid (cPNA) (Buhr et al., 2001). In cryopreserved horse sperm, concentrations of greater than 3.5% glycerol cause a rapid depolymerization of F-actin, which is essential for the integrity of the sperm cytoskeleton (Macías García et al., 2012).

While there have been many evaluations for alternative compounds, there is none as efficacious as glycerol as a permeable cryoprotectant. Alternatively, there have been several attempts at blending relatively lesser concentrations of glycerol with non-permeable cryoprotectants such as L-glutamine or trehalose (de Mercado et al., 2009). In effect, the combined use of 250 mM trehalose and 1% glycerol leads to maintenance of motility and acrosome integrity post-thawing of pig sperm to a greater extent than with the use of 4% glycerol alone (Gutiérrez-Pérez et al., 2009). Similarly, the mixing of trehalose (60–100 mM) with glycerol in a small concentration (1.5%−3%) results in a greater cryoprotective effect on DNA of frozen-thawed sheep sperm than with inclusion of 5% glycerol (Öztürk et al., 2020). This, however, differs from what has been reported for frozen-thawed cattle sperm, where there was no such effect on sperm motility and viability when these two components were combined (Büyükleblebici et al., 2014).

Another group of permeable cryoprotectants are amides, especially methylformamide and dimethylformamide (Table 2). The main advantage of amides compared with glycerol is the lesser osmotic damage because of the lesser molecular weight of the amides (Alvarenga et al., 2005). Yet, the effects of including amides in the freezing medium differ among species because of sperm resilience cryopreservation. When there is replacement of glycerol with methylformamide and dimethylformamide, there is a greater maintenance of the horse sperm motility, viability, mitochondrial membrane potential and acrosomal integrity subsequent to freezing and

Table 3
Supplementation of freezing media for cattle, pig, sheep and horse sperm with seminal plasma components and additives.

Supplement	Effects	Species	Concentration	Reference
Bulk seminal plasma (SP)	Contain proteins and antioxidants that may increase sperm cryotolerance	Pig	5% (v:v)	(Hernández et al., 2007)
		Sheep	20% (v:v)	(Ramírez-Vasquez et al., 2019)
SP proteins				
Bovine seminal plasma proteins (BSP)	Stabilize the plasma membrane and delay sperm capacitation	Pig	3 mg/mL	(Vadnais and Roberts, 2010)
Spermadhesins	Stabilize the plasma membrane and prevents premature acrosome exocytosis	Addition not reported		
Low molecular weight proteins (14–16 kDa)	Maintain post-thaw sperm viability better	Cattle	1–1.5 mg/10 × 10^6 sperm/mL	(Rueda et al., 2013)
		Sheep	2.1 mg/10^6 sperm/mL	(Pérez-Pé et al., 2001)
Bovine serum albumin (BSA)	Maintain post-thaw sperm viability better	Sheep	2.5 mg/mL	(Susilowati et al., 2020)
		Goat	4 mg/mL	(Kaewkesa et al., 2016)
Antioxidants				
Enzymatic antioxidants (mainly SOD)	Maintain the redox balance and scavenge the ROS produced	Cattle	100 IU/mL	(Olfati Karaji et al., 2014)
		Pig	200–400 IU/mL	(Roca et al., 2005)
		Sheep	100 IU/mL	(Silva et al., 2011)
Non-enzymatic antioxidants (mainly GSH)		Cattle	0.5 mM	(Gangwar et al., 2018)
		Horse	2.5 mM	(Oliveira et al., 2013)
		Pig	1 mM	Gadea et al. (2005)
		Sheep	2–5 mM	(Silva et al., 2011)
Other additives				
Manganese (III) porphyrin tetrakis (benzoic acid 4–69) (MnTBAP)	Counteract ROS production and lipid peroxidation	Horse	150 μM	(Treulen et al., 2019)
Taurine		Sheep	40 mM	(Banday et al., 2017)
Astaxanthin		Pig	15 μM	(Basioura et al., 2020)
L-carnitine		Sheep	5 mM	(Souza et al., 2019)
Moringa oleifera seed extract		Sheep	0.5 mg/mL	(Carrera-Chávez et al., 2020)
Rosmarinus officinalis aqueous extract		Sheep	4–6% (v:v)	(Motlagh et al., 2014)
Syzygium aromaticum extract		Sheep	75 μg/mL	(Baghshahi et al., 2014)
Resveratrol		Horse	10 μM	(Nouri et al., 2018)
		Pig	50 μM	(Zhu et al., 2019)
Plasma rich of platelets (PRP)	Protects from ROS damage and maintains sperm function at post-thaw	Sheep	5%	(Hernández-Corredor et al., 2020)
Antifreeze proteins (AFP)	Able to prevent the formation of crystals through hydrogen bonding to ice	Cattle	0.1–10 μg/mL	(Prathalingam et al., 2006)
		Pig	0.1–1 μg/mL	(Kim, 2016)
		Sheep	10 μg/mL	(Payne et al., 1994)

Abbreviations: SP, seminal plasma; GSH, reduced glutathione; SOD, superoxide dismutase.

thawing procedures (Wu et al., 2015). There is also the greatest yield with use of glycerol (2.3%) and methylformamide (4.7%) combined, substituting alcohols (glycerol and ethylene glycol), because with dimethylformamide there is a decrease in the motility and integrity of plasma and mitochondrial membranes of frozen-thawed cattle sperm (Forero-Gonzalez et al., 2012).

9. Supplementing freezing media with seminal plasma (SP) components and other additives

Sperm cryopreservation protocols generally include the elimination of SP and replacement with freezing media. While SP is considered to be detrimental for in vitro sperm survival, the total or partial removal of SP results in sperm having less motility and metabolic activity and, thus, fertilizing capacity (Ashworth et al., 1994; Maxwell and Johnson, 1999; Caballero et al., 2004). It, therefore, is reasonable to advise that the addition of SP or components of SP, mainly proteins and antioxidants, may increase sperm cryotolerance.

For samples that contain SP, compared to the control, the addition of 5% SP before freezing results in improved motility (6.4%−9.2%) and plasma membrane integrity (7.7%−10.5%) of frozen-thawed pig spermatozoa (Hernández et al., 2007; Table 3). Similarly, the addition of SP to sperm that have been subjected to selection techniques such as density gradient washing before freezing can have a reversal effect from the cryodamage induced by a temperature decrease (Maxwell and Johnson, 1999). Indeed, results from previous studies indicate the addition of 20% SP to frozen-thawed sheep sperm that are previously washed with Percoll® leads to greater total motility (14.7%), progressive motility (17.3%), integrity of plasma membrane (10.4%), percentage of sperm with an intact acrosome (8.5%) and the resilience of chromatin to decondensation (13.9%), compared with when there is not inclusion of SP in the storage medium (Ramírez-Vasquez et al., 2019). In the following subsection, the addition of separate SP components to freezing media will be described.

9.1. Proteins

Seminal plasma proteins have important functions from the time of ejaculation to when there are interactions in the female reproductive tract and oocyte fertilization (Rodríguez-Martínez et al., 2011). Spermadhesins and fibronectin-2 type (FN2) proteins, BSP-A1/2, -A3 and − 30k in cattle, SP-1/2 in horses and pB1 in pigs (Töpfer-Petersen et al., 1995; Fan et al., 2006) are the main proteins present in the SP of various animal species (Caballero et al., 2012).

Spermadhesins have been associated with the capacity of sperm to bind different ligands, including glycoproteins of the oviductal cell membrane, and have also been reported to stabilize the sperm membrane and prevent premature acrosome exocytosis (Dostàlovàl et al., 1995; Töpfer-Petersen et al., 2005). Nevertheless, the function of these proteins when added to liquid-stored and cryopreserved sperm is not clear. In effect, while the addition of SP proteins of the heparin-binding fraction (HBP) belonging to the spermadhesin family, AQN-1, AQN-3 and AWN, prevents capacitation-like changes induced by cooling to 5 ºC in pig sperm, there is not the same effect when there are additions of these proteins to the freezing medium (Vadnais and Roberts, 2010).

Within the group of FN2 proteins, however, BSPs have been evaluated to the greatest extent because these proteins stabilize the plasma membrane and delay capacitation in the presence of high-density lipoproteins (HDL) and glycosaminoglycans (GAG) (Manjunath et al., 2007). The addition of two SP proteins of the BSP family, RSVP14 and RSVP20, to the freezing medium leads to enhanced motility, viability, acrosome integrity and mitochondrial activity of frozen-thawed sheep sperm (Muiño-Blanco et al., 2008; Table 3). This finding is indicative of the capacity of these proteins to protect sperm from cold shock, preserving the integrity of the plasma membrane (Barrios et al., 2005; Bernardini et al., 2011).

The addition of the low molecular weight proteins of SP to freezing media, especially those between 14 and 16 kDa, leads to maintenance of the viability of bull sperm, regardless of the composition of the medium (Rueda et al., 2013; Table 3). Sperm viability increases by 20% when the 1–1.5 mg of protein fraction (14–16 kDa) per 10^6 sperm is added to a freezing medium composed of citrate, fructose and egg yolk. There are similar results when the commercial medium BioXcell® (made from soy lecithin) is used, as the addition of 0.5 mg of protein (14–16 kDa) per 10^6 sperm results in an increased sperm viability post-thawing by 25% (Rueda et al., 2013).

Considering these beneficial effects, interspecies studies, consisting of adding SP proteins from one species to the medium used for cryopreserving the sperm of another, have also been conducted. This research has mainly been carried out in sheep (Susilowati et al., 2020) and goats (Uysal and Bucak, 2007), the freezing media being supplemented with SP proteins of cattle. Specifically, replacing egg yolk with 10–15% bovine serum albumin (BSA) ameliorates the quality and fertilizing capacity of frozen-thawed sheep sperm (Matsuoka et al., 2006; Fukui et al., 2007). Similarly, the addition of 4 mg/mL BSA to an egg yolk-based freezing medium improves buck sperm quality post-thawing (Kaewkesa et al., 2016; Table 3).

9.2. Enzymatic antioxidants

The capacity of sperm to synthesize antioxidants is limited (Aitken, 1995; Ortega-Ferrusola et al., 2019). The antioxidant defense system basically relies on the enzymes present in SP. Among the main enzymes are glutathione peroxidase (GPX), glutathione reductase (GSR), superoxide dismutase (SOD) and catalase (CAT). The activity of SOD in SP is related to sperm cryotolerance in equids (Papas et al., 2019b). Furthermore, the potential effects of these antioxidant enzymes depends on whether species breed seasonally. In effect, the activities of the antioxidant enzymes present in the SP of sheep (Marti et al., 2007) and horses (Papas et al., 2019a) are affected by reproductive seasonality with there being a large amount of variability in antioxidant concentration and, therefore, potential protective effects throughout the year.

Most freezing protocols for mammalian sperm involve removal of SP using centrifugation before cryopreservation (Oliveira et al., 2013), which limits the protective action of the SP antioxidant enzymes against oxidative stress (Peris-Frau et al., 2020). Supplementing freezing media with antioxidants has been proposed to have a beneficial effect on maintenance of sperm function post-thawing. In cattle, the addition of 100 IU/mL SOD to the freezing medium leads to an increased sperm motility and viability post-thawing and reduces the amount of malondialdehyde (MDA) produced by sperm (Olfati Karaji et al., 2014; Table 3). There have been similar results reported in sheep, where the addition of 100 IU/mL SOD to the freezing medium results in greater acrosome integrity and mitochondrial membrane potential of sperm post-thawing, without changes in values for motility and kinematic variables compared to the control sample (Silva et al., 2011).

9.3. Non-enzymatic antioxidants

In addition to enzymatic antioxidants, the non-enzymatic ones are also important in maintaining redox balance during the period when there are temperature decreases. Among these, the most important antioxidant is reduced glutathione (GSH) which interacts with GPX and GSR enzymes. The addition of 2.5 mM GSH to the freezing medium leads to improvement in the proportions of progressively motile, viable and acrosome-intact sperm post-thawing in horses (Oliveira et al., 2013). Likewise, there is a similar effect in cattle using a lesser concentration of GSH (0.5 mM) (Gangwar et al., 2018). In pigs, Gadea et al. (2005) observed an increase in total and progressive motility after the addition of 1 mM GSH to the freezing medium. In sheep, supplementing the freezing medium with 2–5 mM GSH results in greater maintenance of the acrosome integrity of frozen-thawed sperm, without affecting values for sperm motility variables (Silva et al., 2011; Table 3).

9.4. Other additives with an antioxidant effect

Another approach is the addition of other substances with an antioxidant effect to freezing media so as to counteract ROS production and lipid peroxidation (Al-Mutary, 2021). As previously described in this review article, adequate concentrations of ROS are important for sperm capacitation, acrosome reaction and oocyte-sperm fusion, but when these concentrations exceed the cellular antioxidant defense capacity, there are disruptions of sperm function processes (Guthrie and Welch, 2012). In horses, sperm supplemented with 50–150 μM Manganese (III) porphyrin tetrakis (benzoic acid 4–69) (MnTBAP), a synthetic metalloporphyrin that easily penetrates cell membranes, have greater motility and viability and less lipid peroxidation and DNA damage post-thawing, due to the activity of SOD and CAT (Treulen et al., 2019; Table 3). The addition of 40 mM taurine to a Tris-based freezing medium leads to a reduction in the concentrations of MDA and is beneficial for the quality of frozen-thawed sheep sperm (Banday et al., 2017). The use of other substances does not appear to result in any antioxidant protective effects. For example, in pigs, the addition of 0.5–5 μM astaxanthin to the freezing medium has no effect on membrane lipid peroxidation, and that of 15 μM astaxanthin results in increases in the aforementioned peroxidation (Basioura et al., 2020). Also, in sheep, the addition of 5–10 mM L-carnitine to the commercial freezing extender OptiXcell® drives to inhibition of oxidative stress (Souza et al., 2019).

As indicated previously in this review article, alternatives have been evaluated to reduce the use of substances of animal origin, such as fresh egg yolk, in sperm freezing extenders, to avoid possible contamination. In different studies, therefore, there has been addition of different extracts from plants (fruits, vegetables, oil seeds and herbs) that contain phytochemical substances with antioxidant properties, such as carotenoids, polyphenols and flavonoids (Del Valle et al., 2013). It is noticeable that the vast majority of these studies were conducted with sheep semen and it was reported that there was a positive outcome. The addition of 0.5 mg/mL of *Moringa oleifera* seed extract to freezing media counteracts the disruptive effects on plasma membrane integrity and progressive motility in frozen-thawed sperm (Carrera-Chávez et al., 2020; Table 3). Similarly, Motlagh et al. (2014) reported that the addition of 4–6% aqueous extract of rosemary (*Rosmarinus officinalis*) to the freezing medium has a beneficial effect on sperm motility and viability post-thawing leading to a decrease in MDA concentration and maintaining the integrity of the acrosome. Another substance that was examined is the clove extract (*Syzygium aromaticum*), an herbal antioxidant containing eugenol, a phenolic compound. When added to an egg yolk-based freezing medium at 35–75 μg/mL, there was improvement in the values for sperm motility variables of frozen-thawed sheep sperm (Baghshahi et al., 2014).

Another substance with an antioxidant capacity is resveratrol, a non-flavonoid polyphenol constituent in red wine (Kyselova et al., 2003; Table 3). Horse sperm cryopreserved in the presence of 10 μM resveratrol have greater total and progressive motility, plasma membrane integrity and mitochondrial membrane potential, and less DNA damage post-thawing (Nouri et al., 2018). Likewise, the addition of 50 μM resveratrol to pig sperm freezing medium ameliorates total sperm motility, results in enhanced values for kinematic variables, plasma membrane and acrosome integrity and decreases lipid peroxidation and oxidative damage to DNA post-thawing. The inclusion of resveratrol at the 50 μM concentration also leads to an increase in the activities of GPX, SOD and CAT subsequent to sperm thawing (Zhu et al., 2019).

9.5. Addition of antifreeze proteins

One of the major effects on sperm of thermal and osmotic stresses during cryopreservation is DNA damage. In an attempt to solve this problem, there has been evaluation of the effects of supplementing cryopreservation media with antifreeze proteins (AFP). The AFPs have the capacity to prevent the formation of crystals as a result of hydrogen binding to ice (Davies et al., 2002), which inhibits recrystallization and thus protects cell membranes from cryodamage (Robles et al., 2019). These proteins are in large concentrations in the body fluids of certain Antarctic organisms resulting in their tolerance to very cold temperatures (Bayer-Giraldi et al., 2011). In

livestock, the first study was conducted with sheep sperm, with the supplementation of the freezing medium with 10 μg/mL of AFP leading to an increase in sperm viability and integrity of the acrosome (Payne et al., 1994; Table 3). The addition of 0.1–10 μg/mL of one of the AFP proteins, AFP Type I (AFPI), increases the capacity of cattle sperm to withstand the osmotic stress as a consequence of cryopreservation (Prathalingam et al., 2006). In pigs, the addition of AFPs (0.1–1 μg/mL) to the freezing medium leads to an improved sperm viability and acrosome integrity post-thawing, without affecting DNA integrity (Kim, 2016). These antifreeze proteins are not currently produced commercially because the isolation from marine species is difficult and expensive; thus, there has been little use in sperm cryopreservation regimens in farm animals (Zandiyeh et al., 2020).

9.6. Addition of plasma rich in platelets

An interesting method that has emerged in recent years to mitigate the adverse effects of cryopreservation is the addition of platelet-rich plasma (PRP) to freezing media. The PRP consists of a unique autologous group of platelets with the concentration being three to seven times greater than the physiological concentration. This is one of the components of PRP that result in the biological activity and therapeutic effects of SOD (Marx, 2004; Irmak et al., 2020), which makes the compound a cryoprotective alternative for inhibiting the damage induced by ROS as a consequence of the maintenance of sperm function post-thawing. In humans, the inclusion

Table 4
Procedures and methodological differences in the cryopreservation of cattle, pig, sheep and horse sperm.

Process	Method	Species	Reference
Semen collection	Artificial vagina	Cattle	(Miguel-Jimenez et al., 2020)
		Sheep	(Carrera-Chávez et al., 2020)
		Horse	(Papas et al., 2019a)
	Electroejaculation	Cattle	(Fernandez-Novo et al., 2021)
		Sheep	(Özmen et al., 2020)
	Gloved-hand technique	Pig	(Basioura et al., 2020)
	Epididymal recovery	Cattle	(Losano et al., 2018)
		Sheep	(Bergstein-Galan et al., 2017)
		Pig	(Perez-Patino et al., 2019b)
		Horse	(Neuhauser et al., 2018)
Dilution of sperm in the freezing medium	1:1 (v:v)	Cattle	(Miguel-Jimenez et al., 2020)
		Sheep	(Banday et al., 2017)
		Pig	(Basioura et al., 2020)
		Horse	(Treulen et al., 2019)
	1:2 (v:v)	Cattle	(Fernandez-Novo et al., 2021)
	1:3 (v:v)	Sheep	(Pini et al., 2018)
	1:4 (v:v)	Horse	(Papas et al., 2019b)
	1:5 (v:v)	Horse	(Gacem et al., 2020)
Seminal plasma removal	Centrifugation	Cattle	(Zoca et al., 2021)
		Pig	(Delgado-Bermúdez et al., 2019)
		Sheep	(Ramírez-Vasquez et al., 2019)
		Horse	(Catalán et al., 2020)
Sperm concentration	8×10^6 sperm/mL	Cattle	(Wang et al., 2021)
	15×10^6 sperm/mL	Cattle	(Murphy et al., 2018)
	23×10^6 sperm/mL	Cattle	(Miguel-Jimenez et al., 2020)
	50×10^6 sperm/mL	Horse	(Ferrer et al., 2021)
	100×10^6 sperm/mL	Cattle	(Zoca et al., 2021)
	100×10^6 sperm/mL	Sheep	(Riesco et al., 2020)
	200×10^6 sperm/mL	Horse	(Catalán et al., 2020)
	300×10^6 sperm/mL	Pig	(Estrada et al., 2017)
	400×10^6 sperm/mL	Sheep	(Öztürk et al., 2020)
	600×10^6 sperm/mL	Sheep	(Banday et al., 2017)
Straw volume	0.25 mL	Cattle	(Murphy et al., 2018)
		Sheep	(Souza et al., 2019)
	0.50 mL	Cattle	(Zoca et al., 2021)
		Pig	(Zhu et al., 2019)
		Horse	(Catalán et al., 2020)
Freezing	Liquid nitrogen vapor	Cattle	(Miguel-Jimenez et al., 2020)
		Sheep	(Ramírez-Vasquez et al., 2019)
		Pig	(Zhu et al., 2019)
		Horse	(Macedo et al., 2018)
	Automatic (controlled-rate freezer)	Cattle	(Wang et al., 2021)
		Sheep	(Banday et al., 2017)
		Pig	(Schäfer et al., 2017)
		Horse	(Catalán et al., 2020)
	Vitrification	Cattle	(Baiee et al., 2020)
		Sheep	(Arando et al., 2017)
		Pig	(Arraztoa et al., 2017)
		Horse	(Hidalgo et al., 2018)

of 5% PRP in the freezing medium leads to greater sperm progressive motility and viability without having any effect on ROS production, mitochondrial membrane potential or DNA integrity (Yan et al., 2021). In livestock, the addition of PRP to cryopreservation media has only been evaluated in sheep. Supplementing the freezing medium for sheep semen with a 1:1 (v:v) ratio of PRP:sperm brings to a greater sperm total and progressive motility post-thawing (Hernández-Corredor et al., 2020; Table 3).

10. Cryopreservation protocols

There has to be consideration of several factors (freezing curve, composition of sperm plasma membrane, cell volume…) when developing protocols used to cryopreserve sperm from different mammalian species. Furthermore, differences in results among published reports may also be related to the availability of equipment in laboratories, as well as to specific procedures for semen collection. For example, in horses, semen is usually collected utilizing an artificial vagina, with electroejaculation and recovery of epididymal sperm only occurring when necessary, such as when there are bone fractures or colic (Cary et al., 2004). Furthermore, elimination of seminal plasma before cryopreservation is, in most species, an essential process for obtaining viable sperm post-thawing (Brinsko et al., 2000; Table 4).

Most of the results do not explain how long the cool down phase takes nor whether it is a slow or rapid procedure. Similarly, while in many reports there is a description of freezing conditions (i.e., liquid nitrogen vapor; automatic, controlled-rate freezing or vitrification), the distance in placement above the liquid nitrogen when using the conventional method is not often provided, nor is the time applied to freeze sperm. In Table 4, there is a summary of the main procedures for cryopreservation of cattle, pig, sheep and horse sperm. Remarkably, there is no standardized procedure for each of these species, which leads to a large variation among studies and in conducting routine field procedures.

11. Alternative sperm cryopreservation methods

11.1. Automatic freezing

Undoubtedly, the technological advances in the different areas of biotechnology have resulted in improved protocols, methods and equipment used in the laboratory, which has a positive effect on the reliability, precision and robustness. There has been substantial progress in the use of controlled-rate cooling/freezing systems, which were introduced in the 1970 s (Landa and Almquist, 1979). Subsequently, there has been the preferred use in research centers of automatic freezing curves rather than that of the conventional method (i.e., liquid nitrogen vapor), due to the greater sperm quality post-thawing (Macedo et al., 2018). In some studies, there were no differences in outcomes when using traditional and automatic, controlled-rate freezing (Forero-Gonzalez et al., 2012) (Table 4).

11.2. Ultra-fast cryopreservation

Slow freezing, rapid freezing, and ultra-rapid freezing have been described as sperm cryopreservation methods (Hezavehei et al., 2018). To avoid the sperm cryodamage resulting from the physical effects ensuing from the decrease in temperature and the subsequent crystallization of intracellular water, the use of an ultra-rapid cryopreservation method, termed vitrification, was proposed. Basically, this technique, unlike traditional freezing procedures, is based on sperm being placed in an aqueous solution that involves incorporation of permeable cryoprotectants at very large concentrations. When temperature decreases to less than 0 °C, the aqueous solution becomes viscous and goes from liquid to glassy state without undergoing ice crystal formation (Isachenko et al., 2004). Throughout the development period for this technique as an alternative to slow-freezing, the medium used has been modified, mainly with the incorporation of non-permeable cryoprotectants to reduce cell damage at the time of cryopreservation (Isachenko et al., 2008). Sperm vitrification has been performed in the absence of cryoprotectants with promising results after vitrification (Sánchez et al., 2015).

Although there are advantages of this technique in modulating osmotic stress resilience and there are encouraging results in humans (Aizpurua et al., 2017), the use of this procedure in farm animals is not yet encouraged. In cattle, vitrification has been reported to result in a large percentage of non-viable and non-motile sperm (Baiee et al., 2020; Table 4). In pigs, whereas vitrification markedly reduces the motility, viability and acrosome integrity of vitrified-warmed sperm, there are not effects on the integrity and condensation of their chromatin (Arraztoa et al., 2017). In sheep, there is markedly lesser quality of vitrified-warmed sperm compared to that of fresh sperm (Arando et al., 2017). In horses, vitrification results in a lesser sperm motility, viability and plasma membrane functionality (Baca-Castex and Miragaya, 2015).

12. Conclusions

Sperm cryopreservation is an essential Assisted Reproductive Technique in practically all mammalian species, including humans. Cryogenic damage to sperm components and function, however, are very apparent when there is cryopreservation of sperm. In previous studies, there has been a focus on developing alternatives and strategies aimed at enhancing sperm resilience to freezing and thawing. In future studies, there should be an aim on the description, determination, and establishment of specific components, such as proteins and microRNAs, that may serve as potential indicators of sperm cryodamage, without neglecting to evaluate additives that may have a positive effect on sperm cells. Furthermore, the application of proteomic and metabolomic procedures may lead to a greater understanding of the differences in sperm cryotolerance among species.

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Conflicts of interest

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