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Review article

Insights into crucial molecules and protein channels involved in pig sperm cryopreservation

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ABSTRACT

Cryopreservation is the most efficient procedure for long-term preservation of mammalian sperm; however, its use is not currently dominant for boar sperm before its use for artificial insemination. In fact, freezing and thawing have an extensive detrimental effect on sperm function and lead to impaired fertility. The present work summarises the basis of the structural and functional impact of cryopreservation on pig sperm that have been extensively studied in recent decades, as well as the molecular alterations in sperm that are related to this damage. The wide variety of mechanisms underlying the consequences of alterations in expression levels and structural modifications of sperm proteins with diverse functions is detailed. Moreover, the use of cryotolerance biomarkers as predictors of the potential resilience of a sperm sample to the cryopreservation process is also discussed. Regarding the proteins that have been identified to be relevant during the cryopreservation process, they are classified according to the functions they carry out in sperm, including antioxidant function, plasma membrane protection, sperm motility regulation, chromatin structure, metabolism and mitochondrial function, heat-shock response, premature capacitation and sperm-oocyte binding and fusion. Special reference is made to the relevance of sperm membrane channels, as their function is crucial for boar sperm to withstand osmotic shock during cryopreservation. Finally, potential aims for future research on cryodamage and cryotolerance are proposed, which might be crucial to minimise the side-effects of cryopreservation and to make it a more advantageous strategy for boar sperm preservation.

1. Introduction

Cryopreservation is the most efficient procedure for long-term preservation of mammalian sperm and is useful for the establishment of genetic banks for both endangered and domestic species (Pickard and Holt, 2004). In spite of that, sperm cryopreservation is not the preferred strategy for preservation prior to carrying out artificial insemination (AI) procedures, as liquid preservation is still the most used strategy in this context (reviewed by Waberski et al., 2019). Nevertheless, cryopreservation must be considered as a major challenge for sperm, as it is associated to temperature, osmotic and oxidative shocks. As a consequence of this process, both sperm integrity and function are compromised, and fertilising ability is decreased (reviewed by Yeste, 2016, 2015). In fact, whereas fertility rates after using liquid-preserved pig semen commonly reach about 80–90 % and are similar to or even better than in natural mating; when frozen-thawed pig sperm is used farrowing rates only reach about 70 %, even under the best breeding management conditions.

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This is the main reason explaining why cryopreserved sperm is only used in about 1 % of AI procedures (Roca et al., 2006b).

The aim of the present article is to summarise the basis of pig sperm cryodamage from a molecular point of view. First, an overview of the cryopreservation process is exposed, and differences on the ability of ejaculates to withstand cryopreservation and the strategies that have been adopted to minimise cryoinjury are discussed. Then, the basis of sperm cryoinjury from a structural and physiological point of view is described. After that, the impact of cryopreservation on different sperm molecules is outlined with a special focus on proteins. The sperm proteins that have a key role for cryotolerance and those that undergo alterations because of cryopreservation are summarised in relation to their functions with special attention being paid to membrane channels, a key group of proteins that allow sperm adaptation to the extracellular medium changes that occur during freezing and thawing.

2. Cryopreservation: a tough challenge for pig sperm

Sperm from different mammalian species present disparate resilience to cryopreservation mainly due to the composition of their sperm membranes, which is strictly related to their ability to withstand freezing and thawing procedures (reviewed by Holt, 2000a). Considering that, cryopreservation protocols have been optimised for each species and thus, these protocols differ in terms of freezing method, cryopreservation medium composition, type and concentration of cryoprotective agents, dilution, cooling rate, equilibration, thawing method, and thawing rate (reviewed by Kumar et al., 2019).

With regard to the freezing method, protocols with slow cooling rates until reaching 5°C and exposition of sperm to cryopreservation medium for long equilibration times have been determined to be more suitable to pig sperm (reviewed by Johnson et al., 2000). Beyond this temperature, sperm membranes are more susceptible to cryodamage and, for this reason, optimal cooling rates below 5°C are specific for each cell type and must be highly controlled. Remarkably, the use of controlled-rate freezers is the most reliable strategy to ensure optimal cooling rates (Medrano et al., 2009). The optimal cooling rate takes two different aspects into consideration: 1) it must be low enough to allow intracellular water to flow out of the cell and avoid cytoplasmic ice formation, which is highly likely to cause cryoinjury; and 2) it must be fast enough to avoid excessive cell dehydration and organelle and membrane shrinkage, as well as exposure to highly concentrated solutes (reviewed by Yeste, 2016). In relation to the thawing method, shorter protocols at high thawing rates have proven to yield the best success rates in terms of sperm function and survival (Tomás et al., 2014). The use of cryopreservation media supplemented with cryoprotecting agents, both permeating and non-permeating, is crucial to optimise cryopreservation protocols (reviewed by Pezo et al., 2019).

Cryoprotecting agents are added to cryopreservation media to reduce the risk of ice crystal formation inside the cells and to protect intracellular structures from the different forms of stress associated to freeze-thawing. Nevertheless, cryoprotectants are not completely harmless for sperm, as high concentrations might have toxic effects. Identifying species-specific optimal combinations of cryoprotectants and their concentrations is, therefore, crucial for cryopreservation success (reviewed by Yánez-Ortiz et al., 2021). Two main types of cryoprotecting agents can be used in cryopreservation media: non-permeating and permeating. On the one hand, non-permeating cryoprotectants are molecules that remain in the extracellular medium and contribute to both optimal cell dehydration through the generation of an osmotic gradient and stabilisation of membranes and proteins. On the other hand, not only can permeating cryoprotective agents penetrate the plasma membrane and be partially incorporated into that membrane, but they also have the ability to alter cytoplasm viscosity and molecular diffusion rates (Elliott et al., 2017). Pig semen freezing extenders mainly contain, as non-permeating cryoprotectants, lactose (reviewed by Yeste, 2016) and proteins from hen egg yolk combined with Orvus ES Paste (Equex), a surfactant that enhances the interaction between egg yolk proteins and the plasma membrane. In terms of permeating cryoprotectants, the most efficient molecule for pig sperm is glycerol (reviewed by Johnson et al., 2000).

It must be considered that individual differences exist in terms of tolerance to the cryopreservation process, which have even been identified between ejaculates from the same individual (reviewed by Holt, 2000b). For this reason, individuals and ejaculates can be classified into good and poor freezers, which differ in terms of post-thaw sperm viability and motility (reviewed by Watson, 1995). Based on that, research efforts have been directed to identifying cryotolerance biomarkers, which would predict the ability of an ejaculate to maintain its function after freezing and thawing. In fact, in good freezability ejaculates, using an optimal insemination protocol for cryopreserved semen, which includes a precise control of insemination timing and deep uterine insemination (Roca et al., 2006a), yields fertilisation rates and litter sizes almost equivalent to those of preserved semen (Almiñana et al., 2010).

3. Cryodamage: the negative impact of cryopreservation on sperm structure and its relationship to sperm functional alterations

While cryopreservation protocols, the composition of freezing media and the use of cryoprotecting agents have been optimised over the last decades, cryopreservation is still an extremely challenging process for sperm as it detrimentally impacts cell structures, which inevitably ends up impairing their function.

3.1. Sperm membranes

The consequences of cryopreservation on the sperm plasma membrane are strictly related to its biophysical properties and, therefore, to its composition. In pig sperm, plasma membrane has a high proportion of unsaturated phospholipids (Leßig et al., 2004), a low cholesterol:phospholipid ratio (Labbé et al., 2001) and an asymmetrical cholesterol distribution between inner and outer monolayers. These characteristics are strictly related to the high sensitivity of pig sperm membrane to cold shock (reviewed by Gautier and Aurich, 2022; Holt, 2000a). Briefly, at low temperatures the lateral movement of membrane phospholipids is restricted, which

results in transition from a fluid to a gel phase. Different phospholipids have different transition temperatures, therefore different phases might coexist and cause membrane protein clustering, lipid reorganisation and cholesterol release (reviewed by Yánez-Ortiz et al., 2021; Yeste, 2016). As a consequence of this membrane remodelling, proteins might lose their function because of the alteration of protein-lipid interactions (reviewed by Johnson et al., 2000). This, together with greater membrane permeability, leads to an impaired regulation of the exchange of ions and other molecules with the extracellular medium (reviewed by Watson, 2000).

This negative impact on plasma membrane integrity also affects other sperm membranes, such as that of the acrosome. After freezing and thawing, the percentage of sperm that present damaged acrosomes increases (Maxwell and Johnson, 1997) and, in fact, the ability of frozen-thawed sperm to respond to the induction of capacitation and acrosome reaction is impaired (Guthrie and Welch, 2005).

Considering this high sensitivity of sperm membranes to low temperatures, other insults that this cell withstands might also impair membrane integrity. This includes the exposure to hypertonic media during freezing, which leads to cell shrinkage, and to hypotonic media upon thawing, which increases cell volume. As the effects of osmolality changes may be more drastic when they occur at low temperatures (Gilmore et al., 1996), they must be particularly considered when designing sperm cryopreservation protocols.

3.2. Cytoskeleton elements

The perinuclear theca is a cytoskeletal envelope that surrounds the sperm nucleus. It apically resides between the inner acrosomal membrane and the nuclear envelope, and caudally it is located between the plasma membrane and the nuclear envelope to form the postacrosomal sheath. It is a reservoir of signalling molecules that are involved in different functions, such as oocyte activation or early embryo development (Sutovsky et al., 2003). Moreover, it stabilises the sperm nucleus until fertilisation occurs, when it is disrupted to allow for chromatin restructuration and the formation of the male pronucleus (Oko and Sutovsky, 2009). Structural alterations of the perinuclear theca induced by cryopreservation have been described in different studies (Gutiérrez-Pérez et al., 2011; Orozco Benítez et al., 2008). It must also be considered that glycerol, a broadly used cryoprotecting agent in freezing media for pig sperm, also has a detrimental impact on perinuclear theca integrity (Arenas Núñez et al., 2013), which evidences the need to find an equilibrium between the beneficial effects of cryoprotecting agents at low concentrations and temperatures and their toxic effects at higher concentrations and temperatures, as it has been previously discussed in Section 2.

Another structural element with a relevant role in sperm function is the actin cytoskeleton. The actin filament network is a dynamic structure that undergoes polymerisation changes during capacitation, and must depolymerise prior to the acrosome reaction to allow the fusion between the plasma membrane and the outer acrosome membrane (Breitbart et al., 2005; Brener et al., 2003). In addition, the actin network contributes to maintaining the sperm structure and volume, which is of special relevance for the adaptive response of sperm to osmotic shock (Petrunkina et al., 2004). As a consequence of freezing and thawing, the ratio between polymerised and depolymerised actin (F-actin:G-actin) is impaired in sperm from different species, and increases in the case of pigs. The hyperosmotic shock that sperm undergo during cryopreservation seems to induce actin polymerisation, which might be the basis of the sublethal damage that leads to other alterations in frozen-thawed sperm, such as alterations of the surrounding protein environment (Flores et al., 2010). In fact, polymerised actin is lost from the equatorial region, where it contributes to the stabilisation of the perinuclear theca (Gutiérrez-Pérez et al., 2011). Another region where actin filaments content might be relevant is the midpiece, where mitochondrial activity is controlled by surrounding proteins and where volume regulation is crucial as it may directly have a negative impact on sperm motility (Flores et al., 2010).

Finally, the basic units of the axoneme microtubule are α-tubulin and β-tubulin, and outer dense fibres (ODF) surround the axoneme and offer strong support to the sperm tail (Linck et al., 2016). As a consequence of cryopreservation, the ODFs have a blurrier appearance under the microscope, which suggests a reorganisation of its protein constituents (Courtens and Paquignon, 1985). Despite the passive role of ODFs with regard to sperm motility, their restructuration during freezing and thawing is likely to affect their elastic properties, which may hamper sperm motility (Christensen et al., 1995).

3.3. DNA integrity and alterations in chromatin architecture

Concerning DNA integrity, it is a latent side-effect of cryopreservation, as a relevant increase in DNA fragmentation cannot be immediately detected after thawing (Flores et al., 2011), but after the incubation of sperm at 38 °C for at least 2 hours DNA damage becomes evident (Alkmin et al., 2013; Yeste et al., 2013a, 2013b). This DNA damage might be the consequence of the overall structural remodelling of sperm chromatin that occurs during freeze-thawing. This structural remodelling is related to the sperm resilience to cryopreservation, as it is more evident in samples with low cryotolerance (Hernández et al., 2006). This remodelling might be related to the destabilisation of the chromatin architecture leading to a higher susceptibility to DNA fragmentation (Fraser and Strzezek, 2007; Yeste et al., 2014a). DNA damage has been described to have a detrimental effect on sperm fertility potential, as it leads to a lower embryo development potential (Mateo-Otero et al., 2022). As a consequence, further efforts on optimising cryopreservation protocols should seek a more optimal protection of sperm chromatin.

4. Molecular insights into pig sperm cryoinjury

4.1. Changes in reactive oxygen species (ROS) levels

Reactive oxygen species are naturally generated by sperm, and they are crucial at low levels for the regulation of different aspects of

sperm physiology, such as capacitation, motility hyperactivation and the acrosome reaction (de Lamirande et al., 1998; O'Flaherty et al., 1999). In pig sperm, the overall levels of ROS are very low, both in fresh and cryopreserved samples, which suggests that these cells either present low levels of ROS formation or their mechanisms to enzymatically neutralise these ROS are highly efficient (Guthrie et al., 2008). The impact of cryopreservation on ROS levels, however, is not clear. A slight increase in intracellular levels of hydrogen peroxide has been described as a collateral effect of freezing and thawing (Kim et al., 2011), which might be related to the impairment in sperm motility that occurs in these samples. Strikingly, the negative impact of ROS on sperm motility does not seem to be mediated by an alteration in mitochondrial function, as mitochondrial membrane potential and sperm ATP content are not impaired in the presence of high levels of hydrogen peroxide. The mechanism that has been proposed as a mediator of this negative impact on sperm motility is through an alteration of cytoskeletal elements such as the axoneme (Guthrie et al., 2008). In fact, dynein, an axonemal protein, is involved in the formation of adducts with byproducts of lipooxidation when sperm are exposed to high ROS concentrations, and which has been related to lower sperm motility rates in human sperm (Baker et al., 2015).

Nevertheless, Flores et al. (2010) described a decreased ability of cryopreserved sperm mitochondria to produce ROS, and Awda et al. (2009) found that after freezing and thawing sperm presented lower levels of superoxide, but equivalent levels of peroxide. Similarly, (Guthrie and Welch, 2006) did not find differences in ROS levels between fresh and frozen-thawed sperm, and Gómez-Fernández et al. (2013) and Yeste et al. (2013a) described an absence of relationship between intracellular levels of peroxides and sperm freezability. Hence, further studies exploring the impact of cryopreservation on ROS production and ROS scavenging mechanisms are needed to provide a more insightful perspective on this aspect.

4.2. Lipid peroxidation and lipid relocation

The higher content of polyunsaturated fatty acids in the plasma membrane of pig sperm leads to a high susceptibility to lipid peroxidation induced by ROS. In the presence of high levels of ROS a peroxidation cascade may start, which leads to the depletion of unsaturated fatty acids from the plasma membrane. This decreases membrane stability and, thus, both sperm function and survival are compromised (Aitken, 1995; Chatterjee and Gagnon, 2001). Seminal plasma provides antioxidant protection to the sperm plasma membrane through the presence of ROS scavengers (Li et al., 2018). During sperm processing for cryopreservation, seminal plasma is removed and sperm are diluted in freezing media, so that this natural protection to lipid peroxidation is almost completely eliminated. Nevertheless, greater levels of antioxidants in seminal plasma prior to its elimination appear to underlie sperm cryotolerance (Li et al., 2018). Moreover, freezing and thawing are related to an increase in the lipid peroxidation of pig sperm plasma membrane (Hu et al., 2014). In spite of this, good and poor freezability ejaculates do not differ in plasma membrane lipid peroxidation, which could be related to the low levels of ROS present in pig sperm (Gómez-Fernández et al., 2013).

Special attention should be paid to the relocation of a specific phospholipid, phosphatidyl inositol bisphosphate (PIP_2), which occurs as a consequence of the cryopreservation process (Duma-Pauta et al., 2023). This phospholipid has a crucial role as a signalling molecule, as it binds gelsolin and triggers its phosphorylation by c-SRC tyrosine kinases, which allows actin polymerisation. In effect, during capacitation, PIP_2 levels increase in the sperm head and decrease in the tail, which causes gelsolin translocation to the sperm head. The lower levels of gelsolin in the sperm tail then allow actin polymerisation, which is essential for motility hyperactivation (Finkelstein et al., 2013).

4.3. Differences in the metabolomic profile

The ability of metabolite levels in seminal plasma to predict sperm cryotolerance has been explored in different studies. Zhang et al. (2021) identified 50 metabolites in pig seminal plasma that presented significant differences between good and poor freezability samples, amongst which they identified D-aspartic acid, N-acetyl-L-glutamate and inosine, which are involved in amino acid metabolic pathways. Sui et al. (2023) described 185 metabolites that were differentially expressed in association to cryotolerance in seminal plasma from Chinese native pigs, which were mainly associated to AMP kinase and cAMP signalling pathways.

The potential as predictive biomarkers of cryotolerance of metabolite levels in sperm has also been studied. Sui et al. (2023) identified 21 metabolites that were differentially expressed in good and poor freezability ejaculates from Chinese native pigs, which were mainly related to the metabolism of amino acids and caffeine. Torres et al. (2022) described differences in the metabolomic profile between samples with good and poor cryotolerance. These profiles essentially differed in the components of metabolic pathways associated to sperm energetics. Furthermore, in this study, a different evolution of the sperm metabolomic profile between samples with good and poor freezability during holding time at 17 C prior to cryopreservation suggested that these two types of samples use different sources of energy. This preconditioning step that is an essential part of the cryopreservation protocol to optimise sperm quality after freezing and thawing has, therefore, a different impact on good and poor freezability samples. These results evidence the high relevance of choosing the timing to analyse metabolites as potential cryotolerance biomarkers.

4.4. Alterations of the transcriptomic profile

Zeng et al. (2014a) proposed *GAPDH*, *RPL4* and *PPIA* as reference genes to normalise mRNA levels between different samples in cryopreservation-related studies, as these were the most stable genes in frozen-thawed sperm amongst a panel of 11 genes that were screened. In this context, different studies have evaluated the impact of cryopreservation on the levels of separate transcripts. On the one hand, a decrease in the mRNA levels of epigenetic-related genes as a consequence of cryopreservation has been described, including *DNMT3, DNMT3B, JHDM2A, KAT8, PRM1, PRM2* and *IGF2* (Zeng et al., 2014b). It is relevant to highlight that in this study it

was described that different cryopreservation protocols have a disparate impact on mRNA levels (Zeng et al., 2014b). This evidences the relevance of considering how cryopreservation protocols may affect mRNA integrity when optimising them. Relative expression levels of some mRNAs related to sperm apoptotic-like changes and DNA repair also decreased as a consequence of freeze-thawing, including *FAS, BCL-2, API5, H2AFX* and *TP53* mRNAs (Zhang et al., 2017). Other research unveiled the differential expression of 567 mRNAs involved in processing of environmental information, metabolism and cellular processes. The functions of these mRNAs included cell adhesion and cytokine-receptor interaction, as well as signalling pathways involving PI3K-Akt, AMPK, MAPK, chemokines and calcium, among others (Dai et al., 2019). This dramatic repercussion on epigenetic-related mRNA levels must be considered as highly relevant because of its direct impact on successful fertilisation and further embryo development (reviewed by Jodar et al., 2013).

Regarding microRNAs, changes of intracellular levels in relation to the cryopreservation process have been described in previous research. In this type of studies, it is very appropriate to identify valid and cell-specific reference miRNAs, as Zhang et al. (2015) did by proposing a list of 15 miRNAs that fulfilled these requisites. Moreover, Dai et al. (2019) identified 135 differentially expressed miRNAs in frozen-thawed sperm compared to fresh counterparts, these miRNAs being involved in different cell processes including protein binding and response to stimuli. Similarly, Zhang et al. (2017) evaluated a panel of 46 miRNAs as potential cryoMiRs or associated to sperm cryotolerance. The target mRNAs of these miRNAs were involved in apoptosis regulation (both its activation and suppression) and DNA damage response and therefore, they were predicted to be involved in sperm quality. From this panel, 16 miRNAs were differentially up-regulated or down-regulated in frozen-thawed sperm.

4.4.1. Could mRNAs or miRNAs be useful as cryotolerance biomarkers in pig sperm?

The potential of mRNAs as predictors of sperm resilience to cryopreservation has also been explored in different works. Fraser et al. (2020) compared sperm from poor and good freezability samples, and identified differently expressed mRNAs related to inflammation and apoptosis (*FOS, NFATC3, ITGAL, EAF2* and *ZDHHC14*), spermatogenesis (*FGF-14* and *BAMBI*), autophagy (*RAB33B*), protein phosphorylation (*PTPRU* and *PTPN2*) and energy metabolism (*ND6* and *ACADM*). For this reason, some of these transcripts, namely *FOS*, *NFATC3*, *EAF2*, *BAMBI*, *PTPRU*, *PTPN2*, *ND6* and *ACADM*, were proposed as potential pig sperm freezability biomarkers. Similarly, *ATP1B1* mRNA levels in fresh sperm showing good freezability were greater than in those exhibiting poor freezability, which makes it a good candidate as cryotolerance biomarker (Mańkowska et al., 2022). While the relationship between mRNA abundance and the resilience of samples to cryotolerance seems to be evident, the mechanisms underlying this relationship are still to be unveiled. In this context, a differential impact of cryopreservation on mRNA content on samples with poor freezability compared to samples with good freezability was described by Mańkowska et al. (2022). According to this work, relative levels of *TXNRD1* and *HSPA4L* significantly increased after cryopreservation in sperm from poor freezability samples, whereas this did not happen in good freezability samples. Nevertheless, the fact that sperm are transcriptionally silent must be kept in mind when the reasons for these variations in mRNA levels are being considered. In this context, a decrease in mRNA abundance might be explained because of their degradation or loss through the highly permeable membranes because of cryodamage; however, an increase in mRNA levels is difficult to explain and further research is much warranted to unveil the potential mechanisms underlying this event.

Regarding miRNAs, (Pedrosa et al., 2021) proposed ssc-miR-503 measured in sperm, as well as ssc-miR-130a and ssc-miR-9 measured in seminal plasma extracellular vesicles as cryotolerance biomarkers, as they were present at higher levels in samples with poor freezability.

4.5. Alterations of the proteomic profile

Different studies have described changes in the proteomic profile of pig sperm during freezing and thawing. Chen et al. (2014) identified variations in the abundance of 41 proteins between fresh and frozen-thawed pig sperm, amongst which 35 showed higher levels after the cryopreservation process. Pérez-Patiño et al. (2019) identified 13 proteins that were less abundant in frozen-thawed sperm compared to fresh samples, and 10 proteins that were more abundant after cryopreservation and thawing. Finally, Kim et al. (2023) identified nine proteins that were present at higher levels after cryopreservation and 67 proteins that presented lower levels in frozen-thawed samples. These proteins were found to be involved in oxidative phosphorylation, mitochondrial function, pyruvate metabolic pathways and sperm tail structure.

The elucidation of the mechanism underlying such proteome modifications occurring during cryopreservation is challenging. Since transcription and translation are repressed in mature sperm, changes in sperm proteome mainly rely either on the exchange of proteins with the extracellular environment or on post-translational modifications (Aitken and Baker, 2008; Druart and de Graaf, 2018). The most plausible explanation for the decrease in the abundance of cytoplasmic proteins after cryopreservation might be their efflux due to the impaired integrity of the plasma membrane that occurs during this process (Bogle et al., 2017), or their degradation due to alterations in the activity of different proteases and protease regulators (Gurupriya et al., 2014). Regarding the increase in the abundance observed for some proteins, in the study of Perez-Patiño et al. (2019), these modifications were determined through proteomic analysis and the causes proposed for this change were related to secondary or tertiary structural changes that might occur during cryopreservation.

Previous research has unveiled that sperm cryopreservation also leads to post-translational modifications, including glycosylation, acetylation, SUMOylation, methylation and ubiquitination, among others (reviewed by Maciel Jr et al., 2019). Protein acetylation allows for the regulation of the activation status in different molecules and compounds, and is involved in the regulation of different sperm functional variables in pigs, including motility and acrosome exocytosis (Chen et al., 2021). The impact of cryopreservation on protein post-translational modifications was evidenced by a recent study that evaluated changes in the acetylation status of the pig

sperm proteome after freeze-thawing (Ali et al., 2023). A total of 1440 proteins had a modified acetylation status, accounting for 4705 modified acetylated sites. The functions of these proteins were mostly related to metabolic processes, as well as catalytic and antioxidant activities. These changes in protein post-translational modification pattern might result from the alteration of the molecular context that occurs as a result of sperm cryopreservation, including changes in metabolite availability (Shi and Tu, 2015). Another study described an increase in protein tyrosine phosphorylation levels as a consequence of cryopreservation (Kumaresan et al., 2012). Despite previous studies had described both intracellular calcium levels and tyrosine phosphorylation increase in pig sperm during cryopreservation (Green and Watson, 2001), these two signalling events do not seem to be correlated (Kumaresan et al., 2012). In fact, it has been suggested that, despite increased levels of tyrosine phosphorylation occur as a consequence of higher intracellular levels of calcium during capacitation, the mechanisms that trigger this post-translational modification during cryopreservation seem to be different (Kumaresan et al., 2012). It is in fact probable that membrane destabilisation associated to cryopreservation leads to a higher permeability to different ions (reviewed by Watson, 2000) that in turn, may trigger a variety of kinases that are responsible for these changes in the phosphorylation pattern.

Considering the relevance of the proteomic profile for sperm physiology, the proteins whose amounts change due to freeze-thawing and that are identified in the studies summarised in this sub-section, as well as other proteins that have been recognised as important for sperm resilience to cryopreservation are detailed in Section 5.

5. Sperm proteins are crucial for cryotolerance but are susceptible to cryodamage

Freezing and thawing lead to extensive sperm proteome remodelling. Alterations in different groups of proteins lead to characteristic consequences on sperm physiology in relation to the role played by these proteins. In the following subsections, the impact of cryopreservation on proteins with different functions is detailed.

5.1. Antioxidants

Redox balance in sperm is crucial for their physiology. Even though mild levels of reactive oxygen species (ROS) are needed to promote sperm capacitation (reviewed in Aitken, 1997), excessive exposure to these molecules is detrimental for sperm function. In fact, sperm present different ROS scavenger systems to prevent lipid peroxidation-derived damage (reviewed in Lenzi et al., 2002). After cryopreservation, which is a process that involves a thorough manipulation of sperm and exposes them to challenging conditions, the levels of intracellular ROS and free radicals might increase (Kim et al., 2011). In this context, a rise in the intracellular levels of different proteins that are involved in ROS detoxification, such as superoxide dismutase 1 (SOD1) and phospholipid hydroperoxide glutathione peroxidase (PHGPx), has been described in frozen-thawed pig sperm (Chen et al., 2014). This might reflect the activation of a protective response of sperm to oxidative stress with the aim to prevent the damage derived from cold exposure and toxicity from certain components of cryopreservation media. Nevertheless, considering that sperm are transcriptionally and translationally silent, increases in protein levels are difficult to explain, and might be mediated, as previously proposed, by the release of these proteins from membranes, by post-translational modifications or by changes in secondary or tertiary structure (Bogle et al., 2017). Furthermore, another study assessing the impact of cryopreservation on antioxidants including SOD1 in stallion sperm identified a decrease rather than an increase in antioxidant levels of this protein(Gaitskell-Phillips et al., 2021). Further research might help clarify the actual impact of cryopreservation on these proteins as well as the mechanisms mediating these changes.

Glutathione S-transferases (GSTs) are a family of proteins involved in the cellular protection against oxidative stress through a number of reduced glutathione-dependent reactions (Hayes et al., 2005). In sperm, Mu members of the GSTs family are membrane-bound proteins, which are not only involved in ROS scavenging, but also in oocyte-sperm interaction (Gopalakrishnan et al., 1998; Hemachand et al., 2002; Hemachand and Shaha, 2003; Petit et al., 2013). Glutathione S-transferase Mu 3 (GSTM3) was proposed as a potential biomarker of poor sperm cryotolerance, as ejaculates with lower resilience to cryopreservation present higher levels of this protein (Llavanera et al., 2019).

5.2. Structural proteins involved in sperm motility

The relevance of the equilibrium between actin polymerisation and depolymerisation for sperm motility has already been discussed in Section 3.2. Regulation of this balance is crucial for the maintenance of sperm motility after cryopreservation, which is one of the main indicators of cryotolerance. One of the mechanisms that allows the stabilisation of F-actin is F-actin capping protein (CAPZ), a protein that binds to actin filaments and inhibits both the addition and removal of actin monomers (Zigmond, 2004). This protein is a polymer formed by different subunits amongst which subunit α has been described as a potential cryotolerance biomarker, as it is present at higher levels in good freezability pig sperm samples (Guimarães et al., 2017), which is in direct relation with the better preservation of post-thaw motility of these ejaculates.

It must also be mentioned that, as a consequence of cryopreservation, relative levels of ODF2, a protein that constitutes ODFs (Petersen 1999), as well as those of β-tubulin, one of the basic constituents of the axoneme, increase (Chen et al., 2014). This might contribute to the reorganisation of the characteristic axonemal structure, which is associated to freezing and thawing and impairs motility in cryopreserved sperm.

5.3. Nucleoproteins

The localisation and distribution of histone H1 and protamine-1 also change throughout the cryopreservation process (Flores et al., 2011, 2008). In fact, disulphide bridges formed between cysteine radicals to allow protamine-protamine interactions are impaired as a consequence of freezing and thawing (Flores et al., 2011). This disruption of nuclear protein interactions is not only relevant for freezability (Yeste et al., 2013a), but also for the fertilising ability of cryopreserved sperm samples (Estrada et al., 2014).

It must be highlighted that, compared to other mammalian species, pig sperm seem to be more resilient to DNA fragmentation, which has been related to the absence of protamine-2 in this species. The higher number of cysteine residues that are present in protamine-1 seem to allow the formation of a higher number of disulphide bonds between protamines, which confers a more protective architecture to pig sperm chromatin (Gosálvez et al., 2011). Nevertheless, a recent study unveiled that the classical protocols that have been used for the evaluation of DNA fragmentation in mammalian sperm need to be adapted for its use in species that only present protamine-1, such as pig sperm (Ribas-Maynou et al., 2021). In this context, it seems that such a highly condensed chromatin needs an additional decondensation step to fully unveil DNA damage in these species. Considering that, previous works aiming at assessing DNA fragmentation in pig sperm using the standard protocols must be considered with caution, as they may have underestimated DNA damage in species that do not express protamine-2.

5.4. Proteins involved in sperm metabolism and mitochondrial function

5.4.1. Glycolysis

Glycogen Synthase Kinase 3 (GSK3α), an enzyme that is involved in glycolysis regulation, is involved in sperm motility regulation in fresh sperm, and in fact its inhibition leads to an enhanced motility pattern (Aparicio et al., 2007). Nevertheless, its activation state is not modified during cryopreservation, as phospho-GSK3α levels do not vary during this procedure. In addition, its inhibition does not mitigate the harmful impact of cold shock on pig sperm motility either during freezing or upon thawing (Martín-Hidalgo et al., 2018). This protein, therefore, does not seem to be involved in cryopreservation-associated alterations of the motility pattern.

The case is different for triosephosphate isomerase 1 (TPI1), glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and phosphoglycerate kinase 2 (PGK2), which are glycolytic enzymes (Kurganov et al., 1985) whose intracellular levels in frozen-thawed sperm are higher than in their fresh counterparts (Chen et al., 2014). This suggests that the glycolysis pathway might be enhanced in sperm. Hence, it is important that, after thawing, the timing of insemination is highly controlled to avoid sperm exhaustion and thus lower fertilisation efficiency.

5.4.2. Tricarboxylic acid (TCA) cycle

The relative levels of oxoglutarate dehydrogenase (OGDH) (Perez-Patiño et al., 2019), isocitrate dehydrogenase (IDH2) and malate dehydrogenase (MDH2) (Ali et al., 2023), three enzymes involved in the tricarboxylic acid (TCA) cycle, were found to decrease in pig sperm as a consequence of the cryopreservation process. Not only were the relative levels of IDH2 and MDH2 modified as a consequence of cryopreservation, but these proteins were also differentially acetylated when compared to fresh samples (Ali et al., 2023). Whereas IDH2 acetylation leads to an activity decrease (Smolková et al., 2020), MDH2 lysine acetylation has been described to result in increased activity (Zhao et al., 2010). In fact, Ali et al. (2023) identified a differential acetylation status for many other proteins involved in the TCA cycle (pyruvate carboxylase, PC; and sirtuin 5, SIRT5), as well as in other metabolic pathways (enolase 1, ENO1; phosphoglycerate mutase 2, PGAM2; spermatogenic glyceraldehyde-3-phosphate dehydrogenase, GAPDHS; lactate dehydrogenase C, LDHC; glutathione peroxidase 4, GPX4; and glycerol-3-phosphate dehydrogenase 2, GPD2). As acetylation modulates energy metabolism because it modifies enzymatic activity, its changes during cryopreservation are essential to understanding sperm adaptation to the surrounding environment, but they might also be the reason for alterations in sperm physiology occurring because of freezing and thawing.

The results of these studies seem to evidence the existence of a cold stress response in sperm during cryopreservation, or an adaptation to a greater energetic demand due to sperm hyperactivation after thawing, which cause an enhancement of both glycolysis and TCA cycle in sperm.

5.4.3. Oxidative phosphorylation

Regarding the electron transport chain, the relative levels of NDUFS2, UQCR10 and COX2, which are components of complexes I, III and IV of the mitochondrial electron transport chain (Rich and Maréchal, 2010), were reported to decrease during cryopreservation (Perez-Patiño et al., 2019). On the other hand, DJ-1, a protein deglycase involved in complex I regulation that participates in oxidative stress control (Wang et al., 2018; Yasuda et al., 2013), was found to be present at higher levels after freeze-thawing. Higher levels of cardiac muscle ATP synthase H^+-transporting mitochondrial F1 complex α-subunit 1 (ATP5A1) and β-subunit (ATP5B), two subunits of the mitochondrial ATP synthase (Chen et al., 2014), which is involved in oxidative phosphorylation (reviewed by Okuno et al., 2011), were also detected in frozen-thawed sperm. Functional approaches to unveil the potential impact of these variations in protein levels on cryotolerance might help unveiling the actual relevance of these proteins during cryopreservation.

Mitofusin 2 (MFN-2) is a mitochondrial outer membrane protein with GTPase activity that is involved in the maintenance of the mitochondrial network architecture, and in the regulation of mitochondrial function, including oxidative phosphorylation and intracellular signalling pathways (Bach et al., 2003; Pich et al., 2005). During pig sperm cryopreservation, MFN-2 expands from the apical region of the midpiece to the whole midpiece which may be related to the decrease of mitochondrial function (Flores et al., 2010), and which might explain the alterations in the sperm motility pattern that present frozen-thawed sperm samples (Cremades

et al., 2005).

Differences in ATP and ROS levels, as well as mitochondrial function impairment in frozen-thawed sperm (reviewed by Gualtieri et al., 2021) might be, at least partially, due to variations in the relative levels of these proteins.

5.5. Heat-shock proteins

The impact of sperm cryopreservation on the intracellular levels of the 90 kDa heat shock protein (HSP90AA1 or HSP90), which is related to a wide variety of proteins involved in cell growth, differentiation, and survival, seems to be controversial. Even though higher levels have been detected by Chen et al. (2014) in frozen-thawed sperm, other studies observed that cryopreservation caused a decrease in the intracellular levels of this protein (Casas et al., 2010; Huang et al., 1999). Remarkably, this protein has been identified as a potential cryotolerance biomarker, as ejaculates with good freezability present higher levels of HSP90 than poor freezability ejaculates (Casas et al., 2010).

Another heat shock protein that has been related to cryotolerance in pig sperm is HSP70. Yeste et al. (2014b) described that during preservation at 17°C and before cryopreservation, phosphorylation levels of serine residues (pSer) in HSP70 increased, these higher levels of pSer-HSP70 prior to cryopreservation being related to higher sperm cryotolerance. Holding time seems to prepare them better for cold shock through the post-translational modification of HSP70 (Yeste et al., 2014b), which has been suggested to mediate protein folding and translocation across the sperm plasma membrane (Bohring et al., 2001).

Finally, HSPB10 has also been explored as a potential cryotolerance biomarker, but total levels of this protein have failed to predict pig ejaculate ability to maintain quality and function after freezing and thawing (Vilagran et al., 2014).

5.6. Proteins involved in capacitation

During pig sperm cryopreservation, different proteins and molecules involved in capacitation may undergo modifications or changes. In brief, membrane destabilisation occurring as a consequence of cryopreservation leads to a higher permeability of sperm membranes, which might lead to certain events that are characteristic of the capacitation process, such as an increase in intracellular calcium levels (Green and Watson, 2001). Nevertheless, cryopreservation does not trigger a physiological capacitation, as the components of the extracellular medium that initiate the signalling pathways which prepare sperm for the acrosome reaction are absent from cryopreservation media, and some hallmark intracellular signalling events that occur during capacitation are not present in frozen-thawed sperm (Pini et al., 2018). In spite of that, frozen-thawed sperm show a faster response to capacitating stimuli compared to fresh sperm (Kumaresan et al., 2014), which might be related to alterations in molecules and proteins playing a relevant role in capacitation.

As a consequence of its low cholesterol:phospholipid ratio and the higher proportion of cholesterol in the outer monolayer of the plasma membrane compared to the inner monolayer, the sensitivity of pig sperm to cold shock is greater than in other mammalian species (reviewed in Johnson et al., 2000). Interestingly, freezing and thawing leads to lower levels of a 16 kDa protein (Chen et al., 2014), which is secreted by the epididymis and regulates cholesterol content in plasma membranes (Okamura et al., 1999). As a consequence, it is possible that this contributes to lower membrane cholesterol levels (Chen et al., 2014), leading to the compromised stability of sperm plasma membranes. This might contribute to the faster response of cryopreserved sperm to capacitation stimuli (Kumaresan et al., 2014).

In addition, angiotensin I converting enzyme (ACE) levels increase as a consequence of freeze-thawing (Chen et al., 2014). The role of this protein in cryopreservation-associated alterations was unveiled because the use of ACE inhibitors reduced the incidence of this side-effect (Foresta et al., 1991). In fact, a negative correlation between relative levels of ACE on the sperm surface and fertilisation ability has also been described in human sperm (Kohn et al., 1998; Shibahara et al., 2001); the higher levels of ACE in frozen-thawed sperm might, therefore, contribute to the low fertility rate of these samples.

The relative levels of P47, a plasma membrane protein involved in membrane remodelling and zona pellucida binding, increase during pig sperm capacitation (Ensslin et al., 1998), and the fact that its levels also increase in response to cryopreservation (Chen et al., 2014) suggest that this protein might also be involved in the poor response of frozen-thawed sperm to the induction of capacitation associated to this preservation strategy.

Finally, polycystic kidney disease and receptor for egg jelly-related protein-like (PKDREJ), a protein that, in mouse sperm, has been found to be involved in acrosomal exocytosis as a culminating event after capacitation (Sutton et al., 2008), is also increased in frozen-thawed pig sperm (Chen et al., 2014).

5.6.1. Alterations of the PKA signalling pathway

As previously mentioned, membrane destabilisation due to cold shock leads to higher permeability to different molecules, including ions that can activate different intracellular signalling pathways. In this context, soluble adenylyl cyclase (sAC) and protein kinase A (PKA) present greater activity levels in frozen-thawed sperm, leading to downstream phosphorylation of different proteins that result in motility hyperactivation and capacitation-like changes (reviewed in de Lamirande and O'Flaherty, 2008). In fact, Chen et al. (2014) described an increase in intracellular levels of three different proteins involved in the PKA signalling pathway: A kinase anchoring protein 3 (AKAP3), AKAP4 and ROPN1. Regarding kinases AKAP3 (Vijayaraghavan et al., 1999) and AKAP4 (Turner et al., 1998), they can be activated by PKA through phosphorylation, which confers them the ability to regulate other target proteins involved in the regulation of different cellular processes. The interaction between the phosphorylated form of AKAP3 and PKA, for example, leads to PKA recruitment to the sperm tail in humans, which is crucial for sperm motility (Luconi et al., 2004). In addition,

phosphorylation of AKAP3 by PKA also regulates its binding to rhophilin-associated protein 1 (ROPN1) (Fiedler et al., 2008).

5.7. Sperm-oocyte binding and fusion proteins

Spermadhesins, which are mainly secreted by seminal vesicles and are present in seminal plasma, bind to the acrosomal region of the sperm head and have a crucial role in capacitation and in sperm-oocyte recognition and binding (Haase et al., 2005). Two members of this family, AWN1 and PSPC1, are reduced in frozen-thawed sperm compared to fresh samples (Chen et al., 2014). Whereas AWN1 is involved in the interaction with the zona pellucida (Sanz et al., 1992), PSPC1 is a glycosylated protein involved in sperm-oocyte binding (Töpfer-Petersen, 1999). Decreased levels of these proteins may therefore contribute to lower fertility in cryopreserved samples.

Acrosin is a serine proteinase with trypsin-like specificity that is stored in the acrosomal matrix as proacrosin. It is activated upon the acrosome reaction, and although it participates in zona pellucida digestion (Klemm et al., 1991), it is not essential for fertilisation in some species (Honda et al., 2002). Although acrosin levels have been described to increase in response to the cryopreservation process (Chen et al., 2014), a dramatic decrease in acrosin activity has been described right after the cooling step prior to freezing (Pinart et al., 2015), which might contribute to the lower ability of frozen-thawed sperm to fertilise. Strikingly, as acrosin activity in fresh semen is also correlated to sperm cryotolerance, this parameter has been suggested as a potential cryotolerance biomarker for pig sperm (Pinart et al., 2015).

Acrosin binding protein (ACRBP) is a calcium-dependent phosphoprotein that cleaves and becomes SP32 upon activation. During capacitation, SP32 interacts with proacrosin forming a protein complex (Zhang et al., 2023) that has been suggested to protect

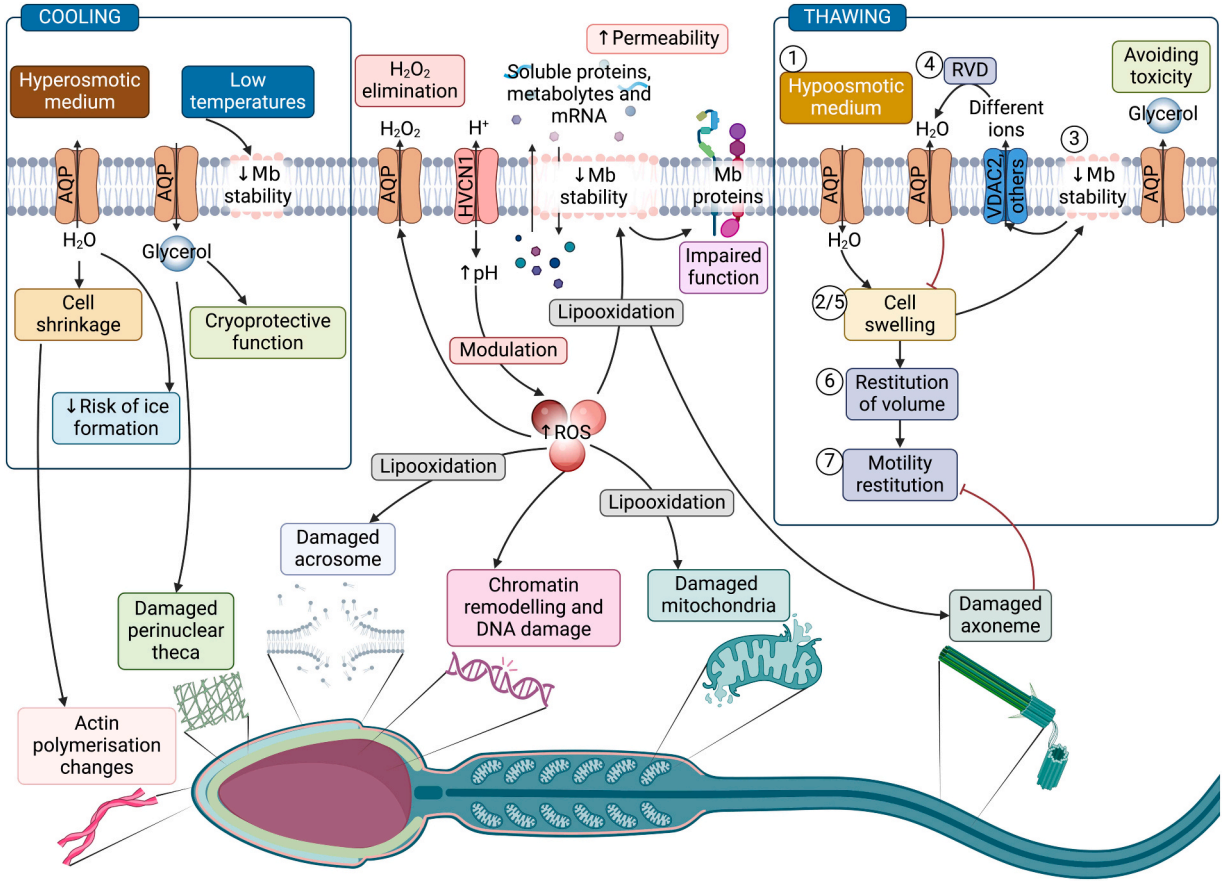

Fig. 1. The central role of membrane channels during cryopreservation. During the cooling phase (left, blue box), sperm are exposed to hyperosmotic cryopreservation media. Water outflux through aquaporins (AQPs) lowers the risk of ice formation, but cell shrinks and it compromises actin structure. AQPs also allow the entrance of the cryoprotectant glycerol, but it can also damage the perinuclear theca. Low temperatures impair membrane (mb) stability. Upon thawing (right, blue box), sperm are exposed to a hypoosmotic medium (1), causing cell swelling (2) due to water entrance through AQPs. Consequently, membrane instability (3) leads to ion leakage, which activates regulatory volume decrease (RVD) mechanisms (4). Then, water flows out (5) and cell volume is reestablished (6), which is crucial for motility (7). During the whole process, reactive oxygen species (ROS) impair chromatin structure and membrane stability (plasma, mitochondrial and acrosome membranes) through lipooxidation. Hence, membrane protein function is impaired, and soluble molecules leak from sperm compartments. Byproducts of lipooxidation also cause axonemal alterations. AQPs contribute to ROS detoxification, and proton transport modulates ROS levels through pH regulation. Created with BioRender.com.

proacrosin from degradation (Baba et al., 1994). As a consequence of capacitation process, SP32 is activated through phosphorylation (Dubé et al., 2005, 2003), and triggers proacrosin conversion to acrosin (Baba et al., 1994). Moreover, SP32 is part of a protein complex that is crucial for sperm-zona pellucida binding (Kato et al., 2021; Kongmanas et al., 2015). Vilagran et al. (2013) identified ACRBP as a potential cryotolerance biomarker, as ejaculates with good cryotolerance presented greater amounts of this protein. Another protein that is localised in the acrosomal matrix is the zona pellucida-binding protein 2 transcript variant 1 (ZPBP2), which is involved in sperm-zona pellucida binding in human sperm (Redgrove et al., 2011; Torabi et al., 2017). Higher levels of ZPBP2 were identified in frozen-thawed sperm compared to their fresh counterparts (Chen et al., 2014), and the potential implications of this variation should be further investigated.

Two different proteins that are expressed in the acrosome membrane and are involved in sperm-oocyte binding and fusion (Fujihara et al., 2010; Hao et al., 2002), sperm equatorial segment protein 1 (SPESP1) and sperm acrosome associated 1 (SPACA1), also present increased levels in frozen-thawed sperm (Chen et al., 2014). Three additional proteins that are involved in sperm-oocyte binding and fusion, apolipoprotein B-100-like (Huang et al., 1996), chaperonin-containing TCP1, subunit 7 (η) (CCT7) and ZPBP precursor, also increase as a consequence of cryopreservation in pig sperm (Chen et al., 2014).

IZUMO1 is an immunoglobulin that interacts with the oocyte receptor JUNO (Folate receptor 4, FOLR4), and it is therefore essential to allow sperm-oocyte binding after the acrosome reaction (Bianchi et al., 2014). As a consequence of the cryopreservation process, this protein relocates from the principal and end pieces of the sperm tail in fresh sperm (Llavanera et al., 2019) and the equatorial segment and the inner acrosomal membrane in fresh and capacitated sperm (Kim et al., 2013; Llavanera et al., 2019) to the equatorial segment of sperm after cryopreservation.

It is also relevant to note that GSTM3, which has been previously mentioned in Section 5.1 because of its function as an oxidative stress regulator, is also involved in sperm-oocyte binding and relocates from the equatorial subdomain and the sperm tail -except for the terminal piece- to the mid-piece region as a consequence of freezing and thawing, which might contribute to impairing the fertilising ability of cryopreserved sperm (Llavanera et al., 2019).

6. Membrane channels: the challenge of adapting to a changing medium

The relevance of membrane channels in relation to sperm cryopreservation must be considered from two different angles. On the one hand, while they are relevant for a wide variety of sperm functions, the fact that they are located in sperm membranes, which are highly susceptible to cryodamage, is associated to a high risk of function loss as a consequence of cryopreservation. On the other hand, the exchange of molecules between the extracellular medium and sperm cytoplasm is crucial during both freezing and thawing procedures. Special attention must thus be paid to this group of proteins, which are analysed in the following sub-sections and their central role during cryopreservation is summarised in Fig. 1.

6.1. *Glucose transporters*

Glucose transporters (SLC2A) are involved in hexose transport across sperm membranes. One member of this family, SLC2A3, which specifically transports glucose across the sperm plasma membrane, is located in the acrosomal region in fresh sperm, and disappears from the plasma membrane after freezing and thawing, which might compromise the sperm ability to obtain this energetic source from the medium. Yet, the distribution of SLC2A5, which is specifically involved in fructose transport, remains unaltered during the cryopreservation process, and does not relocate from the post-acrosomal region and along the midpiece and the principal piece of the tail, where it is present (Sancho et al., 2007). This different impact of cryopreservation on the SLC2A channels of the sperm plasma membrane might be in relation to the interactions of these channels with other structures. It is probable that SLC2A3 is exclusively embedded in the plasma membrane, whereas SLC2A5 could be attached to other structures, such as cytoskeletal proteins; this would minimise its loss when plasma membrane integrity is compromised during freezing and thawing (Sancho et al., 2007).

6.2. *Aquaporins*

The relevance of the different members of the family of aquaporins (AQP0–12) have also been studied in relation to their relevance during pig sperm cryopreservation. Aquaporins are water channels present in mammalian sperm in a species-specific distribution (reviewed in Delgado-Bermúdez et al., 2022). In pig sperm, different studies have described that different members of this family relocate during the cryopreservation process, such as AQP7 (Prieto-Martínez et al., 2017; Vicente-Carrillo et al., 2016). Moreover, relative levels of AQPs in fresh sperm samples can predict the freezability of an ejaculate. In pig sperm, both AQP3 and AQP7, but not AQP11, have been identified as cryotolerance biomarkers, since samples with higher levels of these proteins exhibit a greater ability to withstand cryodamage (Prieto-Martínez et al., 2017).

Different studies have evaluated the functional relevance of the different members of the family of AQPs, and have unveiled that the members of the aquaglyceroporins subfamily, which do not only allow water to penetrate through the plasma membrane, but also glycerol (Abrami et al., 1995) and other molecules such as H_2O_2 (Bienert et al., 2007), are more relevant for cryotolerance than orthodox AQPs, which mainly facilitate the transport of water (Delgado-Bermúdez et al., 2019a). Another study unveiled that GLPs function was more relevant for the freezability of GFEs than for that of PFEs (Delgado-Bermúdez et al., 2019b). The importance of water flow through the plasma membrane is evidently crucial since, as previously described, the outflow of water avoids the intracellular formation of crystals, which might cause physical alterations of sperm structures including plasma membrane and the membranes of intracellular organelles. Moreover, allowing glycerol to freely flow across the plasma membrane is relevant for both its

influx before cryopreservation, to let it exert its cryoprotective effect inside sperm; and also for its efflux after thawing, to minimise the time of exposure at temperatures at which sperm are metabolically active and at which its toxic effects could be manifested. Finally, the efflux of ROS, which are formed during cryopreservation as a consequence of oxidative stress (Gualtieri et al., 2021), through certain AQPs is crucial after thawing to avoid its intracellular accumulation, which might contribute to increase membrane damage through peroxidation.

6.3. Ion channels

Intracellular pH in pig sperm can be regulated through different strategies, including proton efflux across the plasma membrane voltage-gated proton channel HVCN1 (Yeste et al., 2020), which is relevant during capacitation and for motility regulation. During cryopreservation, pH regulation through this channel seems to be relevant for cryotolerance, as membrane integrity -including plasma, mitochondrial and acrosome membrane- relies on its function. Alterations of both pH and membrane integrity are highly relevant for the maintenance of sperm homeostasis, since intracellular levels of calcium and ROS, as well as mitochondrial membrane potential might also be collaterally impaired (Delgado-Bermúdez et al., 2021).

Another crucial role of ion channels is related to the regulatory volume decrease mechanism associated to the adaptation to hypotonic stress, such as the hypotonic shock that sperm undergo upon thawing. The exposure to a hypotonic environment causes sperm swelling because of an excess of water intake. In response to that, a series of intracellular mechanisms are activated to facilitate osmolyte efflux, which creates a driving force that flows water out of the cell, leading to volume restoring. In sperm, potassium and chloride transporters have been described as the main ion channels involved in regulatory volume decrease (reviwed in Cooper and Yeung, 2007). Strikingly, the general inhibition of the family of 6TM potassium channels and the specific inhibition of potassium channel SLO1 during cryopreservation had no impact on sperm function after thawing (Delgado-Bermúdez et al., 2021). These results might indicate the functional compensation exerted from other members of the superfamily of potassium channels during this regulatory volume decrease; however, further studies are needed to explore the precise role of potassium channels in sperm cryotolerance.

Regarding voltage-dependent anion channel 2 (VDAC2), Vilagran et al. (2014) proposed that this protein was a good candidate cryotolerance biomarker, as high VDAC levels in fresh samples were able to identify ejaculates with better freezability. This channel participates in the transport of a wide variety of ions and small molecules across membranes, including sodium, calcium, chloride, hydrogen carbonate, ATP and glutamate, among others (Shoshan-Barmatz et al., 2010); therefore, its relevance for sperm cryotolerance is with high probability also related to ion flow through sperm membranes as a mechanism to withstand osmotic shock during the cryopreservation process.

7. Conclusions

Both the structural and functional impact of cryopreservation on pig sperm have been explored over the last decades, and cryoinjuries involve membrane, cytoskeletal and chromatin damage, which are related to alterations of ROS levels and modifications of lipids, transcripts, and proteins. Considering the wide variety of functions that proteins carry on, alterations in their levels, localisation and structure induced by cryopreservation may lead to redox imbalance, impaired membrane and nuclear integrity, variations in metabolism and mitochondrial function, vulnerability to cold shock, deficient motility, dysregulation of capacitation signalling pathways, acrosome degeneration and deficient binding and fusion with the oocyte. The most recent research is focused on unveiling the underlying mechanisms, as well as on addressing how the changes of the transcriptome and proteome induced by cryopreservation affect sperm cryotolerance. The search for cryotolerance biomarkers that help predict the potential resilience of a given sperm sample to cryopreservation has also provided promising results in both transcriptomics and proteomics. A special reference to membrane channels should be made, as their function is crucial for pig sperm to endure the most challenging drawback associated to cryopreservation in this species: osmotic shock. Further research interrogating the mechanisms leading to certain insults derived from cryodamage is warranted, and it might contribute to the optimisation of cryopreservation media and protocols. This might encourage a more extended use of this technique in contexts in which it is currently relegated to a residual percentage of samples, such as its use prior to artificial insemination.

Competing interests statement

Author declares no competing interests.

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Declaration of Competing Interest

None.

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