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Biological Signals of Sperm Membrane Resistance to Cryoinjury in Boars

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Abstract

Despite the great progress achieved in the cryopreservation of boar semen, it has not been possible to effectively counteract the negative effects on fertility and prolificacy. The boar sperm membrane (SpM) has a particular composition of phospholipids, cholesterol, and proteins that make it highly sensitive to freezing. Just at the beginning and during the freezing protocol, the sperm are exposed to factors that destabilize the membrane and increase the sensitivity to cholesterol efflux and lipid peroxidation. This is a series of events similar to physiological capacitation; they are commonly called cryocapacitation. All the molecules reported as freezability marker and those considered potential markers are directly or indirectly related to the physiology of the SpM. The above gives rise to intensify studies tending to assess their importance as facilitators of the boar semen freezing.

Keywords: boars, reproduction, boar, semen freezability, biological signals

1. Introduction

The cryopreservation of boar semen is unquestionably an indispensable technology in the development of actions for the conformation of germplasm banks and commercial genetic improvement plans. Despite the great progress achieved in different topics related to this problem, it has not been possible to effectively counteract the damage caused by the freezing protocols to the sperm cell with the usual negative effects on fertility and prolificacy. The current extensive knowledge about the physiological, morphological, and molecular characteristics has been pointed out about many of the peculiarities of the (SpM) of the boar. Each day there is greater clarity about the lipid composition and its dynamics in the fluid mosaic and about the protein

fraction and its participation in crucial events that guarantee the integrity of the sperm and the complete fulfillment of its reproductive function. The present chapter is oriented to confront from the basic morphology and physiology of the cell and especially the membrane, the damages caused by the cryopreservation technique with the evidences registered around possible resistance phenomena characterized by abundance, and lack or absence of certain molecules from both the spermatozoa and the seminal plasma (SP). The aforementioned confrontation has focused on studies that classify boars according to the freezability of their semen, by virtue of the behavior of molecules, especially proteins, currently nominated as freezability markers.

2. Sperm membrane characteristics of boar

2.1. Lipids

The cell membrane is a highly fluid and dynamic lipid bilayer, composed mainly of phospholipids, cholesterol, and proteins. Thanks to the amphipathic character of phospholipids and cholesterol, the cellular membrane functions as a barrier between intracellular and extracellular environments [1]. The phospholipids have a hydrophilic head or polar group and two hydrophobic hydrocarbon acyl chains [2]. The polar group is constituted for a phosphate esterified with glycerol (short-chain alcohol) in phosphoglycerolipids (PG) or sphingosine (long-chain alcohol) in sphingomyelin (SM) [3, 4]. Likewise, cholesterol structure consists of a hydrophilic hydroxyl group linked to a hydrophobic steroid ring structure of cyclopentanoperhydrophenantrene with a hydrocarbon tail [3]. In the phosphate group of the PG, a second alcohol or an amino alcohol can also be attached and different phospholipids are produced: phosphatidylinositol (PI) (alcohol inositol), phosphatidylserine (PS) (aminoalcohol serine), phosphatidylethanolamine (PE) (amino alcohol ethanolamine), and phosphatidylcholine (PC) (amino alcohol choline) [3, 5]. PG are in greater abundance than SM [2], and in boar SpM, PC and PE are found in greater quantities than PS and PI [6]. In boar SpM, the outer leaflet is mainly composed of PC and SM and the inner leaflet by PS and PE, possibly by the action of an aminophospholipid translocase [4, 7]. This translocase is ATP dependent and causes a rapid movement of PS and PE toward the inner leaflet of the membrane [7, 8].

In human, ram, rabbit, bull, and boar spermatozoa, the SpM has higher amount of long-chain polyunsaturated fatty acids (PUFAs) with cis configuration than the membranes of the somatic cells [9–11]. In addition, differences have been found in the proportion of unsaturated to saturated [12].

The most abundant fatty acids in the phospholipids of the boar SpM are palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1, n-9), and the PUFAs: docosapentaenoic acid (DPA; 22:5, n-6) and docosahexaenoic acid (DHA; 22:6, n-3) [10].

The sterols are the second most abundant class of lipids in the SpM, mainly cholesterol (24% from total lipids) and in a lesser proportion, desmosterol [6, 13–17]. The ratio of cholesterol to phospholipid is 0.26 compared with 0.45 in the bull, 0.3 in the rooster, and 0.36 in the stallion [6]. In addition, cholesterol is distributed asymmetrically in the lipid bilayer, with greater amount

in the outer leaflet [15], by its affinity with PC and SM [18]. The cholesterol molecules are inserted between the phospholipids and interact with the fatty acids, and their rigid steroid structure provides stability and organization to the membrane [2, 19].

2.2. Fluidity and selective permeability of the boar SpM

The fluidity of the boar SpM depends on the temperature [20], the hydrocarbon acyl chains of the phospholipids [17, 21], the sterol content [19], and the charge of the polar groups of the phospholipids [22]. High amount of PUFAs with cis configuration in the boar SpM affects the degree of compaction of the phospholipids and increases the membrane fluidity [2, 9, 11, 22].

Depending on the temperature, the lipids of the membrane can be in liquid crystalline phase or in gel phase [22, 23]. The temperature, at which the lipids pass from one phase to another, is the phase transition temperature (Tm). Above Tm, the membrane is liquid, and the cholesterol limits the lateral diffusion of phospholipids and proteins, so that it maintains the membrane stability and moderates the fluidity. Below Tm, the membrane is in a gel phase, the cholesterol increasing the membrane fluidity [2, 25] and maintaining its stabilizing function [19, 23].

As the boar SpM is composed of different types of lipids (saturated fatty acids, PUFA, cholesterol, desmosterol, and others) [9, 16], the phase transition occurs in a temperature range between 30 and 5°C [23], which leads to lipid phase separations and irreversible alterations, when it is exposed to low temperatures [15, 23, 24].

The property of selective permeability of the membrane is determined by the presence of protein ionic channels and specific protein transporters [2]. The boar SpM has a ratio of phospholipids to proteins of approx. 0.68 [14], and it has different membrane proteins involved in capacitation, acrosome reaction, motility, and cell volume regulation, such as: HCO_3^-/Cl^- exchanger [4], voltage-dependent anion channel 2 (VDAC2) [26, 27], calmodulin-sensitive Ca^{2+}-ATPase [28], ATP-binding cassette transporters, class B scavenger receptor [19], K^+ and Cl^- channels [29] and aquaporins (AQP) [30].

2.3. Domains and microdomains in boar SpM

The boar spermatozoon has a morphology according to its physiology [31]. Each of its parts is highly differentiated by polarized membrane domains [32]. Protein structures separate these domains, the posterior ring separates the membrane head from the midpiece membrane, and the annular ring separates the midpiece membrane from the flagellum membrane [4]. The membrane of the boar sperm head can be subdivided into four regions: apical, acrosomal, equatorial segment, and post-acrosomal [31, 33, 34]. These regions are highly heterogeneous [32], in which it is evidenced by a series of specific sperm glycolipids, called seminolipids, in the outer leaflet of lipid bilayer, in the apical region of recently ejaculated spermatozoa from the boar [35, 36].

The boar SpM in liquid crystalline phase is in lipid-disordered membrane phase, with high lateral diffusion of lipids and proteins due to low amount of cholesterol [19, 37]. The outer leaflet of somatic cells membrane has lipid-ordered microdomains known as lipid rafts that are rich in SM, glycosphingolipids, saturated phospholipids, and proteins, ordered by the

presence of high amount of cholesterol [2, 38, 39]; they function as platforms for molecular signaling, cell adhesion, and cell-to-cell interaction [38, 39]. Lipid rafts have been identified in both boar spermatozoa and sow oocytes [40], and are associated with maturation, capacitation, acrosome reaction, and gamete interaction [37, 40, 41].

3. Physiological events in the SpM between ejaculation and fecundation

3.1. Characteristic events occurred during ejaculation

During boar ejaculation, mature spermatozoa from the epididymis tail are mixed with the secretions of the accessory sex glands [33, 42]; in this moment occur different events: first, the change of osmolarity from 331 mOsm/L in the epididymis tail to 300 mOsm/L of the SP [43–46] produces swelling of the sperm and the activation of a regulatory volume (RV) process (regulatory volume decrease (RVD) in this case) [46, 47]; second, the elimination of cytoplasmic droplet by increasing the levels of fructose [48] with possible participation of RVD [45, 49]; and third, the union of spermadhesins: AQN-1, AQN-2, AQN-3, AWN-1, AWN-2, DQH, PSP I, and PSP II (among other proteins) from the SP to the SpM, as decapacitating factors [50–52].

3.2. Regulation of cellular volume (CV)

The maintenance of CV and the adequate concentration of ions and molecules are vital for normal sperm physiology [53]. CV is determinate by the relationship between the intracellular content and the osmolarity of the extracellular medium [54]. Drevius [55] states that spermatozoa behave like perfect osmometers. The sperm exposure to anisotonic conditions causes a cellular swelling or shrinkage, phenomena counteracted by the activation of a regulatory volume process [46]. This process can be RVD or regulatory volume increase (RVI) depending on the osmotic change [29, 46] which lead to influx or efflux of water and osmolytes and ions [56]. During the epididymal maturation, the sperm acquires the ability to regulate cell volume [56], in the case of the boar, the sperm undergo an osmotic change that goes from approximately 296 mOsm/L in the rete testis until reaching around 331 mOsm/L in the tail of the epididymis [43]. Then, at ejaculation, sperm are subjected to an osmotic gradient, from a hyperosmotic environment in the tail of the epididymis to the isoosmotic conditions of the PS (around 300 mOsm/L) [43, 44]. Because of this osmotic change, there is influx of water into the sperm to reestablish the osmotic equilibrium through the dilution of the intracellular osmotic content and cell swelling occurs [57]. Sperm swelling induced by hypoosmosis produces the opening of K^+ ion channels, allowing the exit of intracellular K^+ ($[K^+]i$) under a concentration gradient [29, 46, 56]. In parallel, Cl^- ion channels are opened to promote the exit of Cl^- intracellular ($[Cl^-]i$) in order to achieve an electrical balance [29, 46]. The exit of the $[K^+]i$ and $[Cl^-]i$ reduces intracellular osmolarity, water loss, and reduction of cell volume [29, 53].

RVD's pathways are mediated by the enzymes protein kinase C (PKC) and protein phosphatase 1 (PP1), which change the balance of phosphorylation-dephosphorylation of threonine and serine residues [53, 58–60]. Using the boar spermatozoa as a model, Petrunkina [58] found that the

phosphorylation activity of PKC seems to be related with deactivation of RVD through closing and keeping closed the ion channels, especially Cl$^-$ ion channel, while the dephosphorylation activity of PP1 produces the opposite effect. These authors found that by activating phosphodiesterase that reduces cAMP levels, it results in inactivation of RVD, and by stimulating adenylate cyclase (AC) in order to increase cAMP levels, under hypoosmotic conditions, it is activated RVD (opening of ionic channels). However, in isosmotic conditions, high levels of cAMP increase the cell volume by premature activation of RVD-related channels, which consequently produces the entry of Cl$^-$ and Na$^+$ under a concentration gradient, increasing intracellular osmolarity. This increase in cell volume under isoosmotic conditions also occurred with the inhibition of PKC.

3.3. Main events occurred between sperm deposition in the cervix and its arrival to the spermatic reservoir (SR)

Between the cervix and the SR, sperm must overcome barrier represented by the cervical mucus and the polymorphonuclear neutrophils and T cells; the presence of semen in the uterine lumen causes endometrial inflammation and recruitment of polymorphonuclear neutrophils and T cells that attack the spermatozoa [61] and an adequate volume regulation have relation with migration capacity through the cervical mucus [62]. In the SR spermatozoa are protected from polymorphonuclear neutrophils, thanks to the bind of spermadhesin AQN-1 of the SpM with lysosome-associated membrane protein (LAMP) receptors 1 and 2 of the membrane oviductal epithelium [42, 63–65]. There is an association of good sperm RV capacity with high farrowing rates in pigs [54]. Boar sperm with problems in RV capacity [41], as well as with morphological alterations or cytoplasmic drops, and epididymal sperm have a negative relationship with binding index to pig oviductal epithelium [66]. Likewise, spermatozoa with chromatin instability, which have a high relationship with the retention of cytoplasmic droplets and with immatures, have low binding capacity to the oviduct epithelium [67]. A study developed by [68] showed that proteins from the plasma membrane of the oviduct epithelium suppress the activation of bicarbonate-linked motility selectively.

3.4. Relationship between boar sperm capacitation and CV regulation

During sperm capacitation there are changes in membrane permeability and ion entry and exit [69], and sperm undergoes osmotic changes in the sow's reproductive tract [29, 47, 62]; thus, it is necessary to establish a molecular model of capacitation that involves the cellular RV.

At the beginning of the capacitation, most of the decapacitation factors are removed (50–75% of the spermadhesins AQN-1, AQN-2, and AQN-3 and 90% of the spermadhesin AWN) [50]. The SpM is destabilized and the cholesterol becomes more accessible to the lipid-binding components of the sow's reproductive tract [42] or to the fatty acid-free bovine serum albumin (FAF-BSA) in the in vitro capacitation systems [13, 33].

In the oviduct sperm suffer a hypoosmotic shock and are exposed to high concentrations of bicarbonate and calcium [70, 71]. This osmotic change produces sperm swelling, and RVD is activated, with the subsequent exit of [K$^+$]i and [Cl$^-$]i and activation of the Cl$^-$/HCO$_3^-$ exchanger, involved in both RVD [46, 53] and sperm capacitation [72, 73]. The output of [K$^+$] i and [Cl$^-$]i in RVD, generates water loss and cell volume reduction [29, 53]. These changes in cell volume have been evidenced in *in vitro* capacitation [74].

The activation of Cl^-/HCO_3^- exchanger during the RVD allows the entry of HCO_3^- into the spermatozoa [4, 75] that activates the AC and increases the levels of cAMP [60, 70, 76]. The HCO_3^- in consortium with the FAF-BSA effect the efflux of cholesterol from the SpM, the FAF-BSA functioning as an external acceptor of this lipid [75, 77]. Much of this cholesterol presents a lateral translocation from the equatorial region to the apical region of the sperm head by the activation of the cAMP-dependent PKA [19, 75]. Activation of PKA produces a partial scrambling of PS and PE toward the outer leaflet of the lipid bilayer, in the apical region of sperm head [75]. Parallel, there is a lateral translocation of seminolipids from the apical region to the equatorial region of the sperm head [35, 36]. High levels of cAMP activate the premature opening of Cl^- and K^+ channels, with reduction of $[Cl^-]i$ [58]. This event leads to the entrance of Cl^- to the cell and increase of the CV by the entrance of water [58]. The output of $[K+]i$ and the increase of $[Cl^-]i$ produce a hyperpolarization of the membrane causing the opening of voltage-dependent Ca^{2+} channels [69]. Both Cl^- and HCO_3^- were determinants for sperm capacitation, and reduction of Cl^- concentration even in the presence of HCO_3^- suspended the capacitation process [73]. The exchanger channel Cl^-/HCO_3^- works in association with the cystic fibrosis transmembrane conductance regulator (CFTR), which recycles Cl^- to allow the entry of HCO_3^- [73], and the inhibition of CFTR blocks completely the membrane hyperpolarization during sperm capacitation [74].

The entrance of Ca^{2+} activates the tyrosine phosphorylation of proteins [70] necessary for motility hyperactivation and lipid rafts aggregation in the apical region of sperm head, in the presence of FAF-BSA, and HCO_3^- is also activated [37, 75]. Aggregation of lipid rafts is determinant for the binding to the ZP and the acrosomal reaction [13, 40]. These function as molecular signaling platforms where different proteins such as fertilin beta, sp32 precursor, spermadhesin AQN-3, preproacrosin, caveolin-1, and flotillin-1 are involved in the binding to the ZP [37].

4. Cryoinjury in boar SpM

The SpM is the main cellular structure where cryopreservation causes damage; therefore, this must have a special focus [13]. Just at the beginning and during the freezing protocol, the sperm are exposed to mechanical forces such as centrifugation and dilution that favor the depletion of decapacitated factors [78] and the formation of reactive oxygen species (ROS) [79]. The detachment of decapacitated factors destabilizes the membrane and increases the sensitivity to cholesterol efflux [13, 42], and ROS formation produces lipid peroxidation of the highly sensitive membrane by the high proportion of PUFAs and DNA fragmentation [80]. In this respect, there is evidence of cholesterol and PUFA exit from the membrane during freezing, causing loss of membrane integrity and greater peroxidation [10, 16, 17]. During the freezing, it has been observed that protamine 1 and histone 1 suffer determinant structural changes [24].

The cold induces changes in the lipids and proteins of the membrane that determine its functioning [23]. As the temperature goes from 30 to 5°C [23], restriction of the lateral movement of the phospholipids increases, and the membrane passes from a liquid crystalline phase to a gel phase [15, 24]. Because the boar SpM contains a high variety of lipids (PUFAs, saturated fatty acids, cholesterol, and others) with different Tm [9, 16], some lipids tend to jellified earlier than others

[23, 24]. Lipids that are jellified earlier (usually saturated fatty acids) exclude lipids that still maintain a liquid phase [23]. The different groups of fatty acids (saturated and unsaturated), in their different phases (liquid and gel), form order-disorder transitions phases, and the packaging of phospholipids especially in the edges between liquid phase and gel phase is disturbed, forming lipid phase separations [18]. It is worth noting that in human sperm cells that have high amount of cholesterol in the membrane, there is a minimum of lipid phase separation, while these separations occur in an exaggerated manner in the boar SpM that has low amount of cholesterol [23].

The formation of order-disorder transition phases excludes membrane proteins from the phospholipid groups in gel phase toward phospholipid groups that maintain in the liquid phase [18], resulting in loss of membrane-selective permeability by irreversible proteins clustering, disruption of lipid-protein interactions, and translocation or loss of function of ion channels [18, 24, 81]. As a consequence of loss of selective permeability occur: (i) exit of enzymes and cations such as K^+ [15, 18]; (ii) alteration of the water transport and the entry of cryoprotectants such as glycerol [13]; and (iii) influx of Ca^{2+} and HCO_3^- from the extracellular environment [15, 82].

This series of events similar to physiological capacitation, commonly called cryocapacitation, triggering biochemical pathways that result in protein phosphorylation and hypermotility [82, 83]. The main differences between capacitation and cryocapacitation are: (i) cholesterol efflux and the reorganization of membrane lipids during freezing lead to irreversible protein aggregation with loss of function [18]; (ii) the loss of selective permeability of the membrane generates the entry and exit of ions in an uncontrolled manner with differences in the concentration of determining ions [23]; (iii) there are differences in the patterns of phosphorylated proteins [83]; (iv) as the separation of lipid phase is not a reversible process, this possibly affects the aggregation of lipid rafts in the apical region of the spermatic head and therefore the binding to the ZP and the acrosome reaction [13, 18].

5. Biological signals of SpM resistance to cryoinjury in boars

All the molecules reported as freezability marker and those considered potential markers are directly or indirectly related to the physiology of the SpM. For this reason, the following synthesis is plotted according to the location of the molecule in both the extracellular (SP) and intracellular medium (spermatozoa).

5.1. Boar freezability markers

5.1.1. Freezability markers in seminal plasma

Fibronectin 1 is one of the most abundant proteins in the boar SP [52]. This protein possibly interacts with integrins, CD44, and albumin, which suggests its binding to the sperm and a protective action by reducing the effects of oxidative stress [85]. Integrins are proteins expressed in the membrane that connect the extracellular matrix with the interior of the cell and fibronectins, among other proteins, are their ligands [85]. Currently, fibronectin 1 is the only protein recognized as freezability marker [84, 85].

5.1.2. Freezability markers in spermatozoa

Heat shock protein 90 alpha A1 (*HSP90AA1*) has been identified in the spermatozoa flagellum, where it activates the phosphorylation of flagellar proteins in tyrosine residues [86, 87]. In addition, this protein is associated with thermal stress protection [86] and sperm capacitation [88] and is considered a freezability marker in boar [89].

Both *acrosin binding protein* (*ACRBP*) *and acrosin* have been proven as predictors of freezability in boar semen [90, 91]. Acrosin is a proteinase present in the acrosomal domain of sperm, related with the binding and proteolysis of ZP [92, 93]. This enzyme is stored in the acrosome of epididymal and ejaculated spermatozoa in its inactive zymogen or proacrosin [92]. Proacrosin is converted into its mature form, the acrosin [93], during the capacitation, and there is an increase in acrosin activity [94]. In this respect, ACRBP is tyrosine phosphorylated during boar sperm capacitation [95] and intervenes in the conversion of proacrosin [96].

In the capacitation there is a lateral translocation of proacrosin and acrosin, involved in the penetration of the ZP, toward the apical region of the sperm head [94]. This event may coincide with the redistribution of the proteins flotillin-1 and caveolin-1 and the aggregation of lipid rafts in apical region necessary for recognition with ZP and the subsequent acrosomal reaction [37], a phenomenon that may not occur due to alterations in the distribution of lipids in the freezing [13].

High levels of *triosephosphate isomerase* (*TPI*) in refrigerated boar semen correspond with poor freezability [90]. In human semen higher amount of this enzyme in asthenozoospermic samples has been found than in cases of normospermia [97]. In the case of frozen-thawed boar semen, TPI promotes premature capacitation of sperm reducing the freezability [90].

Recently, a relationship of *VDAC2* protein with capacitation in boar spermatozoa has been tested [98]. This association seems to be due to its role in the transport of Ca^{2+} to the mitochondria, which is a determining factor in the capacitation process [99]. In addition, this protein mediates the transport of ions as Ca^{2+}, HCO_3^-, Cl^-, and Na^+ [100] determinants in the processes of capacitation [70, 75] and cell volume regulation in the face of osmotic stress [101]. The condition of freezability marker of this protein [26] can be explained by the occurrence of phenomena similar to capacitation and osmotic stress during freezing [87].

AQP3 and *AQP7* belong to one family of hydrophobic integral proteins of the cell membrane that participate in the transport of water and glycerol [102], essential in the cryopreservation of cells [24]. These proteins are associated with cell volume regulation [45], which is a fundamental process to counteract the osmotic stress caused by freezing [101]. Indeed, AQP 3 and AQP 7 have been previously validated as freezability markers [30].

5.2. Potential boar freezability markers

There is abundant evidence on the leading role of a large group of proteins located both in SP (spermadhesins, Niemann-pick disease type C2 protein (NPC2), lipocalin-type prostaglandin D synthase (L-PGDS); heat shock protein 90 alpha A1 (HSP90AA1); paraoxonase type 1(PON-1), extracellular superoxide dismutase (EC-SOD); and spermatozoon (Cl^- /HCO_3^- exchanger,

Cl^- channel, K^+ channel, AQP, Ca^{2+}-ATPase, ATP-binding cassette transporters, and scavenger receptors), in crucial events of the sperm physiology. This gives rise to intensify studies tending to assess their importance as freezability markers.

5.2.1. Potential boar freezability markers in seminal plasma

Due to the multifunctionality of the *spermadhesins* conferred by their capacity to unite different ligands (heparin, phospholipids, cholesterol, protease inhibitors, and carbohydrates), they participate in several physiological events associated with premature capacitation [51, 103, 104] and with reduction of concentrations of intracellular calcium, sperm survival, motility, and integrity of the mitochondrial membrane [103, 105]. For all the above, it is feasible to think about the possibility of incorporating in the future some of these proteins to the group of freezability markers located in the SP.

The importance of the *NPC2* protein lies in the great affinity of its isoform 19 kDa for the SpM cholesterol [106]. NPC2 is very important in capacitation because it maintains the proportion of cholesterol in the SpM [107] and because it has heparin binding capacity [108]. It is known that during freezing, cholesterol efflux from the membrane leads to cryocapacitation or premature capacitation [10, 16, 17, 109] and that the concentration of 19 kDa protein is higher in semen of high freezability boars and it reduces in 3 h after the ejaculation [110]. These properties and findings suggest a better preventative mechanism against capacitation and serve as a basis to evaluate this protein as a new marker of boar freezability [110].

L-PGDS has high affinity with retinoic acid and retinol [111], two molecules that affect plasma membrane permeability by interacting with phospholipids [112]. Then, L-PGDS could be related to capacitation (acrosome reaction and hypermotility) [113]. L-PGDS is present in the acrosomal membrane in ejaculated spermatozoa and disappears with acrosome reaction [114], and it increases the union in vitro of spermatozoa with the ZP [115], after capacitation [4]. For its affinity for DHA [116] can play an important role in membrane structure and function [117]. This protein is a potential marker of boar freezability because its concentration varies in the seminal plasma of semen with both high and low freezability [110].

HSP90AA1 is a protein considered an intracellular molecular marker for boar semen freezability; it is found in lower quantities in low freezability than in higher freezability spermatozoa [89]. The concentration of this protein increased in seminal plasma of low freezability boars up to 3 h after ejaculation [110], possibly, because of the alteration the plasma membrane integrity during the cooling [118].

The presence in boar semen of the antioxidant enzyme *PON-1* has been reported [119]. The PON-1 influences motility and the SpM integrity because it binds to membrane cholesterol and prevents its oxidation [80]. It is possible that high PON-1 concentration found in the SP of the sperm-peak portion is related with better antioxidant capacities, greater cryotolerance, and lower ROS generation than the post sperm-rich fraction [80]. The above added to the differences detected among boars [120], possibly of genetic origin [121], allows to assume that PON-1 has a potential value as a molecular marker of boar semen freezability. For the case of the *EC-SOD*, it is known that it is in the boar seminal plasma playing an important role as

an antioxidant enzyme in spermatozoa [122]; however, there is a lack of more determinant studies on this protein, which allows establishing its value as a freezability marker.

5.2.2. Potential freezability markers in spermatozoa

The Cl^-/HCO_3^- exchanger is part of the solute carrier family 26, number 3 (SLC26A39) [73]. This exchanger has been related to the regulation of CV [46, 53] and has been postulated as one of the possible mediators of the entry of HCO_3^- into spermatozoa and intracellular alkalization during sperm capacitation [37, 75]. The role for HCO_3^- in cholesterol efflux, in the scrambling of phospholipids [75] and the aggregation of lipid rafts during capacitation is clear [37, 75]. Taking into account that in the cryocapacitation membrane reorganization occurs [24, 81], and the need for an adequate regulation of the sperm volume is to counteract the osmotic stress [101], the Cl^-/HCO_3^- exchanger is a molecule that can be tested as a freezability marker.

Individual Cl^- and K^+ channels are the main regulators of the volume of the sperm cell under hypoosmotic conditions [29]. In view of the fact that freezing affects the functionality of the channels by aggregation or translocation and that the spermatozoon suffers a hypoosmotic shock during thawing with consequences on seminal quality [18, 24, 81], the Cl^- channel and the K^+ channel have great importance as possible markers of freezability.

Considering that AQP 3 and AQP 7 have already been tested as freezability markers [98] and that there is an extensive family of *AQP* involved in the transport of water and glycerol [123], aquaporins continue to have great potential as predictors of freezability in boars. This can be supported, also, in the results obtained in bull sperm where differences among individuals have been found on the basis of volume regulation and glycerol permeability [123].

Ca^{2+}-ATPase is an intracellular Ca^{2+} extractor protein located in the head of the sperm that helps regulate the concentrations of this ion [28]. In the boar sperm, when inhibiting this protein, there is reduction of head-to-head agglutination, capacitation characteristic [124]. In knowledge of the entry of Ca^{2+} into the spermatic cell due to loss of selective permeability and membrane lipid phase separations [18, 24, 81], to test whether Ca^{2+}-ATPase levels allow to reduce the cryocapacitation and the differences between individuals in freezing is of relevant importance.

There is little information about cholesterol transporters such as *ATP-binding cassette transporters* and *scavenger receptors*, as well as about the dynamics of reverse cholesterol transport in boar sperm [19]. The low amount of cholesterol in the boar SpM [14, 15] and the efflux of this molecule in the freezing process [16, 17], with the consequences of lipid phase separation in the membrane of this specie [23], can be key events in the study of freezability.

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