Chapter 7

Biological Signals of Sperm Membrane Resistance to Cryoinjury in Boars

Julian Valencia and Francisco Javier Henao

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.80824

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) (aminoal cohol 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.

Author details

Julian Valencia and Francisco Javier Henao*

*Address all correspondence to: fhenao@ucaldas.edu.co

Doctoral Program in Agrarian Sciences, Faculty of Agricultural Sciences, University of Caldas, Manizales, Caldas, Colombia

References

[1] Singer SJ, Nicolson GL. The fluid mosaic model of the structure of cell membranes. Science. 1972;**175**:720-731

[2] Murray RK, Weil PA. Membranes: Structure and function. In: Harper's Illustrated Biochemistry 30th. United States: McGraw Hill; 2015. pp. 477-535

[3] Stillwell W. An Introduction to Biological Membranes. 2nd ed. Amnsterdam: Elsevier; 2016. 579 p

[4] Flesch FM, Gadella BM. Dynamics of the mammalian sperm plasma membrane in the process of fertilization. Biochimica et Biophysica Acta. 2000;**1469**:197-235

[5] Bothan KM, Mayes PA. Lipids of physiologic significance. In: Harper's Illustrated Biochemistry 30th. United States: McGraw Hill; 2015. pp. 477-535

[6] Parks JE, Lynch DV. Lipid composition and thermotropic phase behavior of boar, bull, stallion, and rooster sperm membranes. Cryobiology. 1992;**29**:255-266

[7] Gadella BM, Miller NG, Colenbrander B, van Golde LM, Harrison RA. Flow cytometric detection of transbilayer movement of fluorescent phospholipid analogues across the boar sperm plasma membrane: Elimination of labeling artifacts. Molecular Reproduction and Development. 1999;**53**:108-125. DOI: 10.1002/(SICI)1098-2795(199905)53:1<108::AID-MRD13>3.0.CO;2-K

[8] Kurz A, Viertel D, Herrmann A, Muller K. Localization of phosphatidylserine in boar sperm cell membranes during capacitation and acrosome reaction. Reproduction. 2005;**130**:615-626. DOI: 10.1530/rep.1.00561

[9] Johnson LA, Pursel VG, Gerrits RJ. Total phospholipid and phospholipid fatty acids of ejaculated and epididymal semen and seminal vesicle fluids of boars. Journal of Animal Science. 1972;**35**:398-403

[10] Waterhouse KE, Hofmo PO, Tverdal A, Miller RR. Within and between breed differences in freezing tolerance and plasma membrane fatty acid composition of boar sperm. Reproduction. 2006;**131**:887-894. DOI: 10.1530/rep.1.01049

[11] Poulos A, Darin-Bennett A, White IG. The phospholipid-bound fatty acids and aldehydes of mammalian spermatozoa. Comparative Biochemistry and Physiology. B, Comparative Biochemistry. 1973;**46**:541-549

[12] Bailey JL, Bilodeau JF, Cormier N. Semen cryopreservation in domestic animals: A damaging and capacitating phenomenon. Journal of Andrology. 2000;**21**:1-7

[13] Leahy T, Gadella BM. Sperm surface changes and physiological consequences induced by sperm handling and storage. Reproduction. 2011;**142**:759-778. DOI: 10.1530/rep-11-0310

[14] Nikolopoulou M, Soucek DA, Vary JC. Changes in the lipid content of boar sperm plasma membranes during epididymal maturation. Biochimica et Biophysica Acta (BBA)—Biomembranes. 1985;**815**:486-498. DOI: 10.1016/0005-2736(85)90377-3

[15] Johnson LA, Weitze KF, Fiser P, Maxwell WM. Storage of boar semen. Animal Reproduction Science. 2000;**62**:143-172. DOI: 10.1016/s0378-4320(00)00157-3

[16] Cerolini S, Maldjian A, Pizzi F, Gliozzi TM. Changes in sperm quality and lipid composition during cryopreservation of boar semen. Reproduction. 2001;**121**:395-401

[17] Maldjian A, Pizzi F, Gliozzi T, Cerolini S, Penny P, Noble R. Changes in sperm quality and lipid composition during cryopreservation of boar semen. Theriogenology. 2005;**63**:411-421. DOI: 10.1016/j.theriogenology.2004.09.021

[18] De Leeuw FE, Chen HC, Colenbrander B, Verkleij AJ. Cold-induced ultrastructural changes in bull and boar sperm plasma membranes. Cryobiology. 1990;**27**:171-183

[19] Leahy T, Gadella BM. New insights into the regulation of cholesterol efflux from the sperm membrane. Asian Journal of Andrology. 2015;**17**:561-567. DOI: 10.4103/1008-682X.153309

[20] Canvin AT, Buhr MM. Effect of temperature on the fluidity of boar sperm membranes. Journal of Reproduction and Fertility. 1989;**85**:533-540

[21] Am-in N, Kirkwood RN, Techakumphu M, Tantasuparuk W. Lipid profiles of sperm and seminal plasma from boars having normal or low sperm motility. Theriogenology. 2011;**75**:897-903. DOI: 10.1016/j.theriogenology.2010.10.032

[22] Mantsch HH, RN ME. Phospholipid phase transitions in model and biological membranes as studied by infrared spectroscopy. Chemistry and Physics of Lipids. 1991;**57**: 213-226. DOI: 10.1016/0009-3084(91)90077-O

[23] Drobnis Erma Z, Crowe Lois M, Berger T, Anchordoguy Thomas J, Overstreet James W, Crowe John H. Cold shock damage is due to lipid phase transitions in cell membranes: A demonstration using sperm as a model. Journal of Experimental Zoology. 1993;**265**: 432-437. DOI: 10.1002/jez.1402650413

[24] Yeste M. Sperm cryopreservation update: Cryodamage, markers, and factors affecting the sperm freezability in pigs. Theriogenology. 2016;**85**:47-64. DOI: 10.1016/j.theriogenology.2015.09.047

[25] Sieme H, Oldenhof H, Wolkers WF. Sperm membrane behaviour during cooling and cryopreservation. Reproduction in Domestic Animals = Zuchthygiene. 2015;**50**(Suppl 3): 20-26. DOI: 10.1111/rda.12594

[26] Vilagran I, Yeste M, Sancho S, Casas I, Alamo MMR, Bonet S. Relationship of sperm small heat-shock protein 10 and voltage-dependent anion channel 2 with semen freezability in boars. Theriogenology. 2014;**82**:418-426. DOI: 10.1016/j.theriogenology.2014.04.023

[27] Martinez-Abad S, Castillo-Martin M, Gadani B, Rodriguez-Gil JE, Bonet S, Yeste M. Voltage-dependent anion channel 2 is involved in in vitro capacitation of boar sperm. Reproduction in Domestic Animals = Zuchthygiene. 2017;**52**(Suppl 4):65-68. DOI: 10.1111/rda.13060

[28] Fraser LR, Abeydeera LR, Niwa K. Ca2+-regulating mechanisms that modulate bull sperm capacitation and acrosomal exocytosis as determined by chlortetracycline analysis. Molecular Reproduction and Development. 1995;**40**:233-241. DOI: 10.1002/mrd.1080400213

[29] Petrunkina AM, Waberski D, Gunzel-Apel AR, Topfer-Petersen E. Determinants of sperm quality and fertility in domestic species. Reproduction. 2007;**134**:3-17. DOI: 10.1530/REP-07-0046

[30] Prieto-Martinez N, Vilagran I, Morato R, Rivera Del Alamo MM, Rodriguez-Gil JE, Bonet S, et al. Relationship of aquaporins 3 (AQP3), 7 (AQP7), and 11 (AQP11) with boar sperm resilience to withstand freeze-thawing procedures. Andrology. 2017;**5**:1153-1164. DOI: 10.1111/andr.12410

[31] Hancock JL. The morphology of boar spermatozoa. Journal Royal Microscopical Society. 1957;**76**:84-97

[32] Gadella BM, Tsai PS, Boerke A, Brewis IA. Sperm head membrane reorganisation during capacitation. The International Journal of Developmental Biology. 2008;**52**:473-480. DOI: 10.1387/ijdb.082583bg

[33] Gadella BM. Reproductive tract modifications of the boar sperm surface. Molecular Reproduction and Development. 2017;**84**:822-831. DOI: 10.1002/mrd.22821

[34] Flechon JE, Kraemer DC, Hafez ES. Scanning electron microscopy of baboon spermatozoa. Folia Primatologica; International Journal of Primatology. 1976;**26**:24-35. DOI: 10.1159/000155727

[35] Gadella BM, Lopes-Cardozo M, van Golde LM, Colenbrander B, Gadella TW Jr. Glycolipid migration from the apical to the equatorial subdomains of the sperm head plasma membrane precedes the acrosome reaction. Evidence for a primary capacitation event in boar spermatozoa. Journal of Cell Science. 1995;**108**(Pt 3):935-946

[36] Gadella BM, Gadella TW Jr, Colenbrander B, van Golde LM, Lopes-Cardozo M. Visualization and quantification of glycolipid polarity dynamics in the plasma membrane of the mammalian spermatozoon. Journal of Cell Science. 1994;**107**(Pt 8):2151-2163

[37] van Gestel RA, Brewis IA, Ashton PR, Helms JB, Brouwers JF, Gadella BM. Capacitation-dependent concentration of lipid rafts in the apical ridge head area of porcine sperm cells. Molecular Human Reproduction. 2005;**11**:583-590. DOI: 10.1093/molehr/gah200

[38] Brown DA, London E. Structure and function of sphingolipid- and cholesterol-rich membrane rafts. The Journal of Biological Chemistry. 2000;**275**:17221-17224. DOI: 10.1074/jbc.R000005200

[39] Simons K, Vaz WL. Model systems, lipid rafts, and cell membranes. Annual Review of Biophysics and Biomolecular Structure. 2004;**33**:269-295. DOI: 10.1146/annurev.biophys.32.110601.141803

[40] Kawano N, Yoshida K, Miyado K, Yoshida M. Lipid rafts: Keys to sperm maturation, fertilization, and early embryogenesis. Journal of Lipids. 2011;**2011**:264706. DOI: 10.1155/2011/264706

[41] Bou Khalil M, Chakrabandhu K, Xu H, Weerachatyanukul W, Buhr M, Berger T, et al. Sperm capacitation induces an increase in lipid rafts having zona pellucida binding ability and containing sulfogalactosylglycerolipid. Developmental Biology. 2006;**290**:220-235. DOI: 10.1016/j.ydbio.2005.11.030

[42] Rodriguez-Martinez H, Saravia F, Wallgren M, Tienthai P, Johannisson A, Vazquez JM, et al. Boar spermatozoa in the oviduct. Theriogenology. 2005;**63**:514-535. DOI: 10.1016/j.theriogenology.2004.09.028

[43] Einarsson S, Gustafsson B. A case of epididymal dysfunction in boar. Andrologie. 1973;**5**:273-279

[44] Strzežek J, Torska J, Borkowski K, Glogowski J, Wysocki P, Holody D. The biochemical characteristics of boar seminal plasma during high ejaculation frequency. Reproduction in Domestic Animals. 1995;**30**:77-84. DOI: 10.1111/j.1439-0531.1995.tb00608.x

[45] Cooper TG, Yeung CH. Acquisition of volume regulatory response of sperm upon maturation in the epididymis and the role of the cytoplasmic droplet. Microscopy Research and Technique. 2003;**61**:28-38. DOI: 10.1002/jemt.10314

[46] Yeung CH, Barfield JP, Cooper TG. Physiological volume regulation by spermatozoa. Molecular and Cellular Endocrinology. 2006;**250**:98-105. DOI: 10.1016/j.mce.2005.12.030

[47] Druart X, Gatti JL, Huet S, Dacheux JL, Humblot P. Hypotonic resistance of boar spermatozoa: Sperm subpopulations and relationship with epididymal maturation and fertility. Reproduction. 2009;**137**:205-213. DOI: 10.1530/REP-08-0225

[48] Harayama H, Shibukawa T, Miyake M, Kannan Y, Kato S. Fructose stimulates shedding of cytoplasmic droplets from epididymal boar spermatozoa. Reproduction, Fertility, and Development. 1996;**8**:1039-1043

[49] Cooper TG. The epididymis, cytoplasmic droplets and male fertility. Asian Journal of Andrology. 2011;**13**:130-138. DOI: 10.1038/aja.2010.97

[50] Dostalova Z, Calvete JJ, Sanz L, Topfer-Petersen E. Quantitation of boar spermadhesins in accessory sex gland fluids and on the surface of epididymal, ejaculated and capacitated spermatozoa. Biochimica et Biophysica Acta. 1994;**1200**:48-54

[51] Jonakova V, Manaskova P, Kraus M, Liberda J, Ticha M. Sperm surface proteins in mammalian fertilization. Molecular Reproduction and Development. 2000;**56**:275-277. DOI: 10.1002/(SICI)1098-2795(200006)56:2+<275::AID-MRD13>3.0.CO;2-G

[52] Caballero I, Caballero I, Parrilla I, Almiñana C, del Olmo D, Roca J, et al. Seminal plasma proteins as modulators of the sperm function and their application in sperm biotechnologies. Reproduction in Domestic Animals. 2012;**47**:12-21. DOI: 10.1111/j.1439-0531.2012.02028.x

[53] Pereira R, Sa R, Barros A, Sousa M. Major regulatory mechanisms involved in sperm motility. Asian Journal of Andrology. 2017;**19**:5-14. DOI: 10.4103/1008-682X.167716

[54] Petrunkina AM, Harrison RA, Ekhlasi-Hundrieser M, Topfer-Petersen E. Role of volume-stimulated osmolyte and anion channels in volume regulation by mammalian sperm. Molecular Human Reproduction. 2004;**10**:815-823. DOI: 10.1093/molehr/gah106

[55] Drevius L-O. Bull spermatozoa as osmometers. Journal of Reproduction and Fertility. 1972;**28**:29-39. DOI: 10.1530/jrf.0.0280029

[56] Yeung CH, Barfield JP, Anapolski M, Cooper TG. Volume regulation of mature and immature spermatozoa in a primate model, and possible ion channels involved. Human Reproduction. 2004;**19**:2587-2593. DOI: 10.1093/humrep/deh466

[57] Jeyendran RS, Van der Ven HH, Perez-Pelaez M, Crabo BG, Zaneveld LJ. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. Journal of Reproduction and Fertility. 1984;**70**:219-228

[58] Petrunkina AM, Harrison RA, Tsolova M, Jebe E, Topfer-Petersen E. Signalling pathways involved in the control of sperm cell volume. Reproduction. 2007;**133**:61-73. DOI: 10.1530/rep.1.01137

[59] Kennelly PJ, Rodwell VW. Enzymes: Regulation of activities. In: Harper's Illustrated Biochemistry 30th. United States: McGraw Hill; 2015. pp. 87-96

[60] Tardif S, Dubé C, Chevalier S, Bailey JL. Capacitation is associated with tyrosine phosphorylation and tyrosine kinase-like activity of pig sperm proteins. Biology of Reproduction. 2001;**65**:784-792. DOI: 10.1095/biolreprod65.3.784

[61] Rodriguez-Martinez H, Saravia F, Wallgren M, Martinez EA, Sanz L, Roca J, et al. Spermadhesin PSP-I/PSP-II heterodimer induces migration of polymorphonuclear neutrophils into the uterine cavity of the sow. Journal of Reproductive Immunology. 2010;**84**:57-65. DOI: 10.1016/j.jri.2009.10.007

[62] Yeung CH, Cooper TG. Effects of the ion-channel blocker quinine on human sperm volume, kinematics and mucus penetration, and the involvement of potassium channels. Molecular Human Reproduction. 2001;**7**:819-828

[63] Hunter RH. Sperm transport and reservoirs in the pig oviduct in relation to the time of ovulation. Journal of Reproduction and Fertility. 1981;**63**:109-117

[64] Ekhlasi-Hundrieser M, Gohr K, Wagner A, Tsolova M, Petrunkina A, Topfer-Petersen E. Spermadhesin AQN1 is a candidate receptor molecule involved in the formation of the oviductal sperm reservoir in the pig. Biology of Reproduction. 2005;**73**:536-545. DOI: 10.1095/biolreprod.105.040824

[65] Topfer-Petersen E, Ekhlasi-Hundrieser M, Tsolova M. Glycobiology of fertilization in the pig. The International Journal of Developmental Biology. 2008;**52**:717-736. DOI: 10.1387/ijdb.072536et

[66] Petrunkina AM, Gehlhaar R, Drommer W, Waberski D, Topfer-Petersen E. Selective sperm binding to pig oviductal epithelium in vitro. Reproduction. 2001;**121**:889-896

[67] Ardon F, Helms D, Sahin E, Bollwein H, Topfer-Petersen E, Waberski D. Chromatin-unstable boar spermatozoa have little chance of reaching oocytes in vivo. Reproduction. 2008;**135**:461-470. DOI: 10.1530/REP-07-0333

[68] Satake N, Elliott RM, Watson PF, Holt WV. Sperm selection and competition in pigs may be mediated by the differential motility activation and suppression of sperm

subpopulations within the oviduct. The Journal of Experimental Biology. 2006;**209**: 1560-1572. DOI: 10.1242/jeb.02136

[69] Santi CM, Orta G, Salkoff L, Visconti PE, Darszon A, Trevino CL. K+ and Cl– channels and transporters in sperm function. Current Topics in Developmental Biology. 2013;**102**:385-421. DOI: 10.1016/B978-0-12-416024-8.00014-3

[70] Tardif S, Dube C, Bailey JL. Porcine sperm capacitation and tyrosine kinase activity are dependent on bicarbonate and calcium but protein tyrosine phosphorylation is only associated with calcium. Biology of Reproduction. 2003;**68**:207-213

[71] Visconti PE, Kopf GS. Regulation of protein phosphorylation during sperm capacitation. Biology of Reproduction. 1998;**59**:1-6

[72] Chávez JC, Hernández-González EO, Wertheimer E, Visconti PE, Darszon A, Treviño CL. Participation of the Cl^-/HCO_3^- exchangers SLC26A3 and SLC26A6, the Cl^- channel CFTR, and the regulatory factor SLC9A3R1 in mouse sperm capacitation. Biology of Reproduction. 2011;**86**:11-14. DOI: 10.1095/biolreprod.111.094037

[73] Chen WY, Xu WM, Chen ZH, Ni Y, Yuan YY, Zhou SC, et al. Cl^- is required for HCO_3^- entry necessary for sperm capacitation in Guinea pig: Involvement of a Cl^-/HCO_3^- exchanger (SLC26A3) and CFTR1. Biology of Reproduction. 2009;**80**:115-123. DOI: 10.1095/biolreprod.108.068528

[74] Petrounkina AM, Harrison RA, Petzoldt R, Weitze KF, Topfer-Petersen E. Cyclical changes in sperm volume during in vitro incubation under capacitating conditions: A novel boar semen characteristic. Journal of Reproduction and Fertility. 2000;**118**:283-293

[75] Flesch FM, Brouwers JF, Nievelstein PF, Verkleij AJ, van Golde LM, Colenbrander B, et al. Bicarbonate stimulated phospholipid scrambling induces cholesterol redistribution and enables cholesterol depletion in the sperm plasma membrane. Journal of Cell Science. 2001;**114**:3543-3555

[76] Boerke A, Tsai PS, Garcia-Gil N, Brewis IA, Gadella BM. Capacitation-dependent reorganization of microdomains in the apical sperm head plasma membrane: Functional relationship with zona binding and the zona-induced acrosome reaction. Theriogenology. 2008;**70**:1188-1196. DOI: 10.1016/j.theriogenology.2008.06.021

[77] Visconti PE, Ning X, Fornes MW, Alvarez JG, Stein P, Connors SA, et al. Cholesterol efflux-mediated signal transduction in mammalian sperm: Cholesterol release signals an increase in protein tyrosine phosphorylation during mouse sperm capacitation. Developmental Biology. 1999;**214**:429-443. DOI: 10.1006/dbio.1999.9428

[78] Nixon B, MacIntyre DA, Mitchell LA, Gibbs GM, O'Bryan M, Aitken RJ. The identification of mouse sperm-surface-associated proteins and characterization of their ability to act as decapacitation factors. Biology of Reproduction. 2006;**74**:275-287. DOI: 10.1095/biolreprod.105.044644

[79] Urrego R, Rios A, Olivera-Ángel M, Camargo O. Efecto de la centrifugación sobre la membrana plasmática y el ADN de espermatozoides bovinos. Revista Colombiana de Ciencias Pecuarias. 2008;**21**:19-26

[80] Li J, Barranco I, Tvarijonaviciute A, Molina MF, Martinez EA, Rodriguez-Martinez H, et al. Seminal plasma antioxidants are directly involved in boar sperm cryotolerance. Theriogenology. 2018;**107**:27-35. DOI: 10.1016/j.theriogenology.2017.10.035

[81] Watson PF. The causes of reduced fertility with cryopreserved semen. Animal Reproduction Science. 2000;**60-61**:481-492

[82] Bailey JL, Lessard C, Jacques J, Breque C, Dobrinski I, Zeng W, et al. Cryopreservation of boar semen and its future importance to the industry. Theriogenology. 2008;**70**: 1251-1259. DOI: 10.1016/j.theriogenology.2008.06.014

[83] Green CE, Watson PF. Comparison of the capacitation-like state of cooled boar spermatozoa with true capacitation. Reproduction. 2001;**122**:889-898. DOI: 10.1530/rep.0.1220889

[84] Vilagran I, Yeste M, Sancho S, Castillo J, Oliva R, Bonet S. Comparative analysis of boar seminal plasma proteome from different freezability ejaculates and identification of Fibronectin 1 as sperm freezability marker. Andrology. 2015;**3**:345-356. DOI: 10.1111/andr.12009

[85] Gonzalez-Cadavid V, Martins JA, Moreno FB, Andrade TS, Santos AC, Monteiro-Moreira AC, et al. Seminal plasma proteins of adult boars and correlations with sperm parameters. Theriogenology. 2014;**82**:697-707. DOI: 10.1016/j.theriogenology.2014.05.024

[86] Wang P, Shu Z, He L, Cui X, Wang Y, Gao D. The pertinence of expression of heat shock proteins (HSPs) to the efficacy of cryopreservation in HELAs. Cryo Letters. 2005;**26**:7-16

[87] Bris MD, Fábrega A. The boar spermatozoon. In: Bonet S, Casas I, Holt WV, Yeste M, editors. Boar Reproduction: Fundamentals and New Biotechnological Trends. New York Dordrecht London: Springer-Verlag Berlin Heidelberg; 2013. pp. 3-47

[88] Hou ML, Huang SY, Lai YK, Lee WC. Geldanamycin augments nitric oxide production and promotes capacitation in boar spermatozoa. Animal Reproduction Science. 2008;**104**:56-68. DOI: 10.1016/j.anireprosci.2007.01.006

[89] Casas I, Sancho S, Ballester J, Briz M, Pinart E, Bussalleu E. The HSP90AA1 sperm content and the prediction of the boar ejaculate freezability. Theriogenology. 2010;**74**: 940-950. DOI: 10.1016/j.theriogenology.2010.04.021

[90] Vilagran I, Castillo J, Bonet S, Sancho S, Yeste M, Estanyol JM. Acrosin-binding protein (ACRBP) and triosephosphate isomerase (TPI) are good markers to predict boar sperm freezing capacity. Theriogenology. 2013;**80**:443-450. DOI: 10.1016/j.theriogenology. 2013.05.006

[91] Pinart E, Yeste M, Bonet S. Acrosin activity is a good predictor of boar sperm freezability. Theriogenology. 2015;**83**:1525-1533. DOI: 10.1016/j.theriogenology.2015.02.005

[92] Polakoski KL, Parrish RF. Boar proacrosin. Purification and preliminary activation studies of proacrosin isolated from ejaculated boar sperm. Journal of Biological Chemistry. 1977;**252**:1888-1894

[93] Zahn A, Furlong LI, Biancotti JC, Ghiringhelli PD, Marijn-Briggiler CI, Vazquez-Levin MH. Evaluation of the proacrosin/acrosin system and its mechanism of activation in human sperm extracts. Journal of Reproductive Immunology. 2002;**54**:43-63

[94] Puigmule M, Fabrega A, Yeste M, Bonet S, Pinart E. Study of the proacrosin-acrosin system in epididymal, ejaculated and in vitro capacitated boar spermatozoa. Reproduction, Fertility, and Development. 2011;**23**:837-845. DOI: 10.1071/RD10345

[95] Dube C, Leclerc P, Baba T, Reyes-Moreno C, Bailey JL. The proacrosin binding protein, sp32, is tyrosine phosphorylated during capacitation of pig sperm. Journal of Andrology. 2005;**26**:519-528. DOI: 10.2164/jandrol.04163

[96] Baba T, Niida Y, Michikawa Y, Kashiwabara S, Kodaira K, Takenaka M, et al. An acrosomal protein, sp32, in mammalian sperm is a binding protein specific for two proacrosins and an acrosin intermediate. The Journal of Biological Chemistry. 1994;**269**:10133-10140

[97] Siva AB, Kameshwari DB, Singh V, Pavani K, Sundaram CS, Rangaraj N, et al. Proteomics-based study on asthenozoospermia: Differential expression of proteasome alpha complex. Molecular Human Reproduction. 2010;**16**:452-462. DOI: 10.1093/molehr/gaq009

[98] Martínez-Abad S, Martínez-Abad S, Castillo-Martín M, Gadani B, Rodríguez-Gil JE, Bonet S, et al. Voltage-dependent anion channel 2 is involved in in vitro capacitation of boar sperm. Reproduction in Domestic Animals. 2017;**52**:65-68. DOI: 10.1111/rda.13060

[99] Yeste M, Fernandez-Novell JM, Ramio-Lluch L, Estrada E, Rocha LG, Cebrian-Perez JA, et al. Intracellular calcium movements of boar spermatozoa during 'in vitro' capacitation and subsequent acrosome exocytosis follow a multiple-storage place, extracellular calcium-dependent model. Andrology. 2015;**3**:729-747. DOI: 10.1111/andr.12054

[100] Shoshan-Barmatz V, De Pinto V, Zweckstetter M, Raviv Z, Keinan N, Arbel N. VDAC, a multi-functional mitochondrial protein regulating cell life and death. Molecular Aspects of Medicine. 2010;**31**:227-285. DOI: 10.1016/j.mam.2010.03.002

[101] Petrunkina AM, Gropper B, Topfer-Petersen E, Gunzel-Apel AR. Volume regulatory function and sperm membrane dynamics as parameters for evaluating cryoprotective efficiency of a freezing extender. Theriogenology. 2005;**63**:1390-1406. DOI: 10.1016/j.theriogenology.2004.07.006

[102] Edashige K, Tanaka M, Ichimaru N, Ota S, Yazawa K, Higashino Y, et al. Channel-dependent permeation of water and glycerol in mouse morulae. Biology of Reproduction. 2006;**74**:625-632. DOI: 10.1095/biolreprod.105.045823

[103] Caballero I, Vazquez JM, Garcia EM, Parrilla I, Roca J, Calvete JJ, et al. Major proteins of boar seminal plasma as a tool for biotechnological preservation of spermatozoa. Theriogenology. 2008;**70**:1352-1355. DOI: 10.1016/j.theriogenology.2008.07.013

[104] Caballero I, Vazquez JM, Gil MA, Calvete JJ, Roca J, Sanz L, et al. Does seminal plasma PSP-I/PSP-II spermadhesin modulate the ability of boar spermatozoa to penetrate homologous oocytes in vitro? Journal of Andrology. 2004;**25**:1004-1012

[105] Garcia EM, Calvete JJ, Sanz L, Roca J, Martinez EA, Vazquez JM. Distinct effects of boar seminal plasma fractions exhibiting different protein profiles on the functionality of

highly diluted boar spermatozoa. Reproduction in Domestic Animals = Zuchthygiene. 2009;**44**:200-205. DOI: 10.1111/j.1439-0531.2007.01028.x

[106] Okamura N, Kiuchi S, Tamba M, Kashima T, Hiramoto S, Baba T. A porcine homolog of the major secretory protein of human epididymis, HE1, specifically binds cholesterol. Biochimica et Biophysica Acta. 1999;**1438**:377-387. DOI: 10.1016/s1388-1981(99)00070-0

[107] Lasserre A, Barrozo R, Tezon JG, Miranda PV, Vazquez-Levin MH. Human epididymal proteins and sperm function during fertilization: An update. Biological Research. 2001;**34**:165-178

[108] Manaskova-Postlerova P, Davidova N, Jonakova V. Biochemical and binding characteristics of boar epididymal fluid proteins. Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences. 2011;**879**:100-106. DOI: 10.1016/j.jchromb.2010.11.023

[109] Leahy T, Gadella BM. Capacitation and capacitation-like sperm surface changes induced by handling boar semen. Reproduction in Domestic Animals = Zuchthygiene. 2011;**46**:7-13. DOI: 10.1111/j.1439-0531.2011.01799.x

[110] Valencia J, Gómez G, López W, Mesa H, Henao FJ. Relationship between HSP90a, NPC2 and L-PGDS proteins to boar semen freezability. Journal of Animal Science and Biotechnology. 2017;**8**:21. DOI: 10.1186/s40104-017-0151-y

[111] Tanaka T, Urade Y, Kimura H, Eguchi N, Nishikawa A, Hayaishi O. Lipocalin-type prostaglandin D synthase (beta-trace) is a newly recognized type of retinoid transporter. The Journal of Biological Chemistry. 1997;**272**:15789-15795

[112] Stillwell W, Wassall SR. Interactions of retinoids with phospholipid membranes: Optical spectroscopy. Methods in Enzymology. 1990;**189**:373-382

[113] Breitbart H. Intracellular calcium regulation in sperm capacitation and acrosomal reaction. Molecular and Cellular Endocrinology. 2002;**187**:139-144

[114] Gerena RL, Irikura D, Eguchi N, Urade Y, Killian GJ. Immunocytochemical localization of lipocalin-type prostaglandin D synthase in the bull testis and epididymis and on ejaculated sperm. Biology of Reproduction. 2000;**62**:547-556

[115] Goncalves RF, Barnabe VH, Killian GJ. Pre-treatment of cattle sperm and/or oocyte with antibody to lipocalin type prostaglandin D synthase inhibits in vitro fertilization and increases sperm-oocyte binding. Animal Reproduction Science. 2008;**106**:188-193. DOI: 10.1016/j.anireprosci.2007.12.019

[116] Beuckmann CT, Gordon WC, Kanaoka Y, Eguchi N, Marcheselli VL, Gerashchenko DY, et al. Lipocalin-type prostaglandin D synthase (beta-trace) is located in pigment epithelial cells of rat retina and accumulates within interphotoreceptor matrix. The Journal of Neuroscience: The Official Journal of the Society for Neuroscience. 1996;**16**:6119-6124

[117] Stillwell W, Wassall SR. Docosahexaenoic acid: Membrane properties of a unique fatty acid. Chemistry and Physics of Lipids. 2003;**126**:1-27

[118] Huang SY, Kuo YH, Lee WC, Tsou HL, Lee YP, Chang HL. Substantial decrease of heat-shock protein 90 precedes the decline of sperm motility during cooling of boar spermatozoa. Theriogenology. 1999;**51**:1007-1016. DOI: 10.1016/s0093-691x(99)00046-1

[119] Barranco I, Roca J, Tvarijonaviciute A, Ruber M, Vicente-Carrillo A, Atikuzzaman M, et al. Measurement of activity and concentration of paraoxonase 1 (PON-1) in seminal plasma and identification of PON-2 in the sperm of boar ejaculates. Molecular Reproduction and Development. 2015;**82**:58-65. DOI: 10.1002/mrd.22444

[120] Barranco I, Tvarijonaviciute A, Perez-Patino C, Alkmin DV, Ceron JJ, Martinez EA, et al. The activity of paraoxonase type 1 (PON-1) in boar seminal plasma and its relationship with sperm quality, functionality, and in vivo fertility. Andrology. 2015;**3**:315-320. DOI: 10.1111/andr.309

[121] Camps J, Marsillach J, Joven J. The paraoxonases: Role in human diseases and methodological difficulties in measurement. Critical Reviews in Clinical Laboratory Sciences. 2009;**46**:83-106. DOI: 10.1080/10408360802610878

[122] Kowalowka M, Wysocki P, Fraser L, Strzezek J. Extracellular superoxide dismutase of boar seminal plasma. Reproduction in Domestic Animals = Zuchthygiene. 2008;**43**: 490-496. DOI: 10.1111/j.1439-0531.2007.00943.x

[123] Chen Q, Duan EK. Aquaporins in sperm osmoadaptation: An emerging role for volume regulation. Acta Pharmacologica Sinica. 2011;**32**:721-724. DOI: 10.1038/aps.2011.35

[124] Harayama H, Okada K, Miyake M. Involvement of cytoplasmic free calcium in boar sperm: Head-to-head agglutination induced by a cell-permeable cyclic adenosine monophosphate analog. Journal of Andrology. 2003;**24**:91-99