Chapter 2

Relationship between Plasma Proteins and Boar Semen Freezability

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Abstract

Currently, there is great interest in using frozen boar semen to enhance pig-breeding processes. Yet semen freezability, as well as its limited lifespan in the uterus, limits the efficacy of such a procedure. Pig spermatozoa membrane is less stable and more sensitive to low temperatures as it contains lower levels of cholesterol. It is also highly susceptible to lipid peroxidation (LPO) during freezing, since it is rich in polyunsaturated fatty acids (PUFA). Seminal plasma (SP) has beneficial effects on post-thaw semen quality and its composition may have a genetic basis, specifically in protein content. To date, studies on boar semen freezability have focused on sperm cell proteins with very little attention having been paid to SP proteins. In boar SP, there are 82 identified proteins with spermadhesins (90%) and fibronectins (FN) the most abundant. The only plasma protein thus far identified as a freezability marker is FN1. Other plasmatic proteins of recognized importance in the freezing of porcine semen are: DQH, HSP90AA1, NPC2, L-PGDS, ß-HEX, SOD, and PON-1. The purpose of this chapter is to examine the most efficacious elements of the above plasma proteins with regard to their role as biological or potential biological markers of porcine semen freezability.

Keywords: pigs, reproduction, boar, semen freezability, plasma proteins

1. Introduction

Despite the excellent results in fertility and prolificacy achieved with fresh-cooled boar semen in commercial pork production operations [1], interest in the use of frozen boar semen persists. Applications of frozen boar semen include: the transfer of genes between population pyramids; availability of in-farm insemination plans; the export of germplasm; prevention of particular pathogenic transmission agents; and the establishment of germplasm banks [2].

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However, there are clear constraints on the use of frozen semen in commercial pig breeding. Most salient among these are the extremely high volumes and concentrations required per artificial insemination (AI) dose, as well as lower farrowing rates and litter size [1, 3–5]. Such issues are mainly caused by the variability in the capacity of boar spermatozoa to survive the freezing and thawing process (freezability) and the shortened lifespan of frozen–thawed boar sperm in the uterus [6–8]. Despite following the best-known cryopreservation protocols, seminal quality is affected to such an extent [3], that quality may vary as much as 70% [9]. Genetic factors [8] and proteomics variations [10–13] are the most commonly cited reasons for such variances.

Farrowing rates using frozen semen now approach the 85–90% success rate already seen in semen refrigeration techniques, thanks to innovative deep intrauterine insemination and sow management methods [9, 14]. Insemination using frozen–thawed boar sperm should not be performed using conventional AI but rather through post-cervical or deep intrauterine insemination [15–18].

Lower levels of cholesterol exhibited within its cell membrane renders pig sperm more sensitive to cold and less stable [1]. This is evidenced by a molar ratio cholesterol to phospholipids of 0.26 (the bull has a ratio of 0.45) [19]. This makes the sperm more prone to initiate the capacitation and acrosome reaction process [20, 21]. The freezing of porcine semen reduces the sperm survival rate by more than 50%, while causing most of the surviving cells to prematurely develop a phenomenon similar to capacitation—cryo-capacitation [22, 23]. Sperm capacitation is a biochemical process that the sperm must undergo during the passage through the reproductive tract of the female on their way to fertilizing an oocyte [24, 25].

Sperm membranes are rich in polyunsaturated fatty acids (PUFA), and therefore highly susceptible to lipid peroxidation (LPO) in conditions of oxidative stress by the increase of free oxygen radicals or reactive oxygen species (ROS), [26, 27]. Within these cells, the oxidative stress can be induced by different endogenous and exogenous factors that are activated during their passage through the male and female reproductive tracts [28]. As a counterbalance, there are multiple protection mechanisms that reduce oxidative injury [29].

It is also known that seminal plasma (SP) has beneficial effects on post-thaw semen quality [30]. SP is a mixture of secretions from the testicular network, the epididymis, and the accessory sex glands [25, 31] and there is evidence that its composition may have a genetic basis [8] and varies among boars [32, 33], specifically in its protein content [25, 30, 32]. All these suggest that SP is a factor closely related to freezability. SP of the pre-sperm fraction originates in the urethral and bulbourethral glands [34, 35]; SP of the fraction rich in sperm comes from the prostate, epididymis, and seminal vesicles; and SP of the sperm-poor fraction originates solely from the prostate and seminal vesicles [25]. SP is the sole vehicle in which sperm is immersed after ejaculation [25, 31] and regulates processes related to nutrition, protection, maturation, motility, and sperm capacitation [30, 36, 37].

Findings show that differences in freezability between males disappear when, (1) cryopreserving sperm obtained directly from the epididymis (which has had no contact with SP) [38] and, (2) are minimized when freezing the sperm peak fraction poor in SP and abundant in sperm (the first 10 ml of the rich fraction) [30]. Moreover, after mixing high and

low freezability SP and sperm from boars, the combination of high freezability sperm and plasma from boars recorded the highest value of acrosome integrity, while the addition of SP from high freezability boars to any category of sperm cell, yielded the highest values of membrane structural integrity [39].

The effect of SP on sperm function is extremely variable, and although several proteins and their effects have been identified, little is known about their wider effects and potential applications. It is therefore necessary to examine these molecules and how they interact and impact on cell function [40]. SP is composed of water, inorganic ions, citric acid, organic salts, prostaglandins, and proteins. The latter has been linked to having the greatest effect on sperm function and freezability [32]. The literature touches on SP's cryoprotective effect on capacitation and survival of spermatozoa [31], together with its positive correlation to membrane structural integrity, acrosome integrity, sperm motility, and mitochondrial membrane potential after manipulation of semen [35, 39].

The purpose of the following chapter is to review the most relevant aspects of plasma proteins recognized as biological or potential biological markers of porcine semen freezability.

2. Plasmatic proteins and your relationship with semen freezability

Current studies on the relationship between protein markers of boar semen freezability have been focused on sperm cell proteins with very little attention being paid to seminal plasma proteins [12]. Tandem mass spectrometry of two-dimensional liquid chromatography (2D–LC) derived samples identifying a total of 82 proteins in the seminal plasma of the boar [41]. Proteins with the greatest presence in the porcine SP are spermadhesins (90%) and fibronectins (FN) [32, 41]. Other plasmatic proteins of recognized importance in the freezing of porcine semen that are unconfirmed as freezability markers are: DQH protein [42, 43], Heat shock protein 90 alpha A1 (HSP90AA1), Niemann-pick disease type C2 protein (NPC2), Prostaglandin D synthase lipocalin type (L-PGDS) [44], ß subunit of N-acetyl-b-hexosaminidase (ß-HEX) [45], superoxide dismutase (SOD), and paraoxonase 1 (PON-1) [46]. The only plasma protein identified as a freezability marker is Fibronectin 1 (FN1) [12].

2.1. Spermadhesins

The spermadhesins are a family of sperm surface-associated glycoproteins of 12–16 kDa, possessing between 109 and 133 amino acids, and constituted by a single-domain CUB that serves as a structural support [32]. The spermadhesinas are multifunctional proteins that have the capacity to unite different ligands. These include: heparin, phospholipids, protease inhibitors, and carbohydrates. These change with glucosylation and aggregation, and can be divided into heparin-binding (AQN-1, AQN-2, AQN-3, AWN-1 and AWN-2) and non-heparin-binding (PSP-I and PSP-II) families [34, 47–50]. AWN, AQN-1, AQN-3, PSP-I, and PSP-II spermadhesins are transcribed and translated in seminal vesicles and the prostate. mRNA transcripts of these spermadhesins have been detected in caudal epididymis, and, in the case of PSP-I, have found mRNA in caput epididymis and rete testis [51]. The spermadhesins increase as the

sperm concentration decreases, resulting in a greater concentration of spermadhesins in the sperm-poor fraction [25, 52]. The PSP-I and PSP-II spermadhesins make up 50% of the SP proteins [47]. These bind to the sperm membrane in the acrosomal domain [53], and, in the case of PSP-II, are known to bind to phosphorylcholine [42], forming a cover that protects sperm from premature capacitation [32, 47]. When PSP spermadhesins are added to the semen in sperm sexing and freezing processes, they reduce the percentage of sperm with high concentrations of intracellular calcium, and maintain the survival, motility, and integrity of the mitochondrial membrane [47, 52]. In addition, it is known that exposure of frozen–thawed semen to low concentrations of PSP-I and PSP-II spermadhesins maintains viability and sperm motility but also has an inhibitory effect on the oocyte penetration rate [35]. In addition, it has been found that spermadhesins induce the migration of polymorphonuclear neutrophils within the uterine tract of the sow [54].

The spermadhesin AWN-1 makes up between 7 and 8% of the total protein of SP [55], binding to cholesterol and phosphorylcholine of the plasma membrane, during storage in the epididymis and during ejaculation [42]. This protein is considered a decapacitating factor, with 90% being released during the sperm capacitation [55]. In addition, the AWN-1 participates in the union between gametes through its ability to bind to ß-galactosides from the zona pellucida [32].

The spermadhesin AQN-1 is related to the union of the spermatozoon with the epithelium of the uterus in the reservoir located in the uterotubal junction [56]. This is due to its ability to bind to different ligands, including mannose [32]. This protein is united to the acrosome membrane, and 50–75% are released during the sperm capacitation, suggesting a decapacitating effect [55].

2.2. Fibronectins

Fibronectin (FN) is an abundant soluble constituent of plasma (300 μg/ml) and other body fluids and also part of the insoluble extracellular matrix; FN can be subdivided into two forms: soluble plasma FN (pFN) and less-soluble cellular (cFN) FN [57]. The fibronectins are products of expression of a single gene, the final protein may vary since alternative splicing of a single pre m-RNA is generated up to 20 variants in humans [58]. FN generally is a dimer with 2 similar ~250 kDa subunits linked covalently; each monomer is made up of 3 types of repeating units (type I, type II and type III) and approximately 90% of the FN sequence consists of 12 type I repeats, two type II repeats, and 15–17 type III repeats [57, 59]. All three types of FN repeat are also found in other molecules, suggesting that FN evolved through exon shuffling [59].

In boar seminal plasma, FN1 has also been identified and described as one of the most abundant proteins in the seminal plasma of this species [41, 60]. FN1 is considered a molecular marker for boar sperm freezability since it presents different concentrations of high and low freezability among males [12]. These differences were also confirmed by Rungruangsak et al. [61]. FN1 is related to defects of the intermediate tract and tail of the spermatozoon. Binding to the plasma membrane through integrins, and providing a protective protein of sperm; FN1 interacts with albumin, which reduces oxidative stress [60]. The binding of FN2 to the phosphorylcholine of the sperm membrane may prevent the movement of phospholipids and maintain membrane stability. Meanwhile, its binding to heparin may also enable cholesterol release during sperm capacitation [32].

2.3. Aspartic acid-glutamine-histidine protein or DQH protein

While DQH is a protein with a structure distinct from that of the spermadhesins, it forms a complex with spermadhesins AWN and AQN to perform similar functions [60]. This protein is produced in seminal vesicles and is not detected in spermatozoa of the epididymis [43]. It interacts with cholesterol, and also binds to phosphorylcholine of the spermatic membrane, to glycoproteins of the zona pellucida of the oocyte [42] and to epithelial cells of the oviduct. It is thus related to the formation of the spermatic reservoir and the sperm-oocyte junction [43].

2.4. Heat shock protein 90 alpha A1

In a study of the genes that code for heat shock proteins of 90 kDa (HSP90) or HSP90 (Heat shock protein 90 kDa), carried out by Chen et al. [62], 17 different genes were grouped into the following 4 classes: HSP90AA, HSP90AB, HSP90B, and TRAP. HSP90A indicates that the protein is cytosolic, HSP90B that the protein is from the reticulum endoplasmic and TRAP that the protein is mitochondrial. In addition, the HSP90A class was divided into two categories: (1) alpha (HSP90AA) to indicate that the gene is inducible and (2) beta (HSP90AB) to indicate that the gene is constitutive.

HSP90 proteins are highly conserved molecular companions that recognize hydrophobic regions exposed in denatured proteins and, in case of folding defects, to correct them in order to avoid irreversible protein aggregation [63]. These proteins intervene as protectors in oxidative stress, mediate cell repair, and increase resistance in case of persistent damage [64]; they have also been associated with cellular protection in thermal stress caused by high and low temperatures [63]. Another HSP90 function is the interruption of the apoptosis process via the interaction with and structural conservation of protein kinase B [65]. The thermal shock protein 90 alpha (inducible) A1 (cytosolic) (HSP90AA1) has been found in the sperm flagellum and has been associated with freezability in boars [13]; there are also reports of the existence of mRNA for this protein in sperm cells [66], which is synthesized from spermatogenesis and spermiogenesis [67]. The HSP90 interact with other proteins in different biochemical pathways to ensure a correct folding and proper functioning [65]. In the sperm capacitation process, the transduction signals that initiate phosphorylation are mediated by the cyclic AMP pathway and protein kinase A [24]. The phosphorylation of HSP90AA1 demonstrates the possibility of mediating this protein through threonine/serine kinases or by tyrosine kinase [68]. In addition, this protein is responsible for the signals of the activation of phosphorylation in tyrosine residues of flagellar proteins [69].

There is evidence of decreased intracellular concentration of HSP90AA1 during freezing, concomitant with reduced sperm motility, which is possibly associated with the protective function of this protein and its relationship with the activation of the enzyme nitric oxide synthase [70] and the beneficial role of nitric oxide in mobility [71]. In a study conducted by Hou et al. [72], it was found that the antibiotic geldanamycin, which is a specific inhibitor of HSP90, increases the production of nitric oxide and promotes sperm capacitation in the boar—a fact that indicates a different role of the HSP90 in these processes involving the porcine species.

HSP90AA1 is considered a molecular marker for boar semen freezability and it is found intracellularly in lower quantities in low freezability than in higher freezability spermatozoa [13].

This may be due to their exit from the spermatozoa into the extracellular space because of the loss in the integrity of the plasma membrane during the cooling [70]. The concentration of the HSP90AA1 protein increased in seminal plasma during the period from ejaculation up to 3 h of holding time, and this increase was greater among low freezability spermatozoa [44].

2.5. Niemann-pick disease type C2 protein

Niemann-pick C disease is a hereditary disease in which there is accumulation of cholesterol in the endosomes and lysosomes of the cell; it is caused by the mutation of two genes: NPC2 and NPC1 [73]. The mutation of the NPC2 gene causes a defect in the exit of cholesterol from the lysosomes, due to the absence of the Niemann-pick protein type C2 (NPC2) (also called HE1, which is expressed in lysosomes) [74]. The NPC2 protein is in different tissues and is most widely secreted in the epididymis of humans [75] and porcine [76]; it is also secreted in seminal vesicles, prostate, and vas deferens, and is therefore found in seminal plasma [77]. In the porcine species 2 isoforms of 19 and 16 kDa have been found, with differences in weight due to the greater amount of carbohydrates added by N-glycosylation in the 19 kDa. Both exhibit a micromolar affinity to cholesterol to the extent that for each mole of protein, one mole of cholesterol is attached [76]. The importance of the NPC2 protein is that it binds to the cholesterol of the sperm membrane with great efficiency [77], In contrast, NPC2 isoform 16 kDa does not have this capacity [76].

The exit of cholesterol from the membrane is a trigger of the sperm capacitation process [78] and the procedures performed on semen during freezing encourage this output. This leads to premature capacitation or cryo-capacitation [79]. NPC2 maintains the proportion of cholesterol in the sperm membrane and is a decapacitating factor [80]. In addition, this protein has a heparin-binding capacity, which suggests an action in sperm capacitation, due to the fact that heparin has a capacitating effect in bovine species [81].

Two isoforms of 16 and 19 kDa of the NPC2 protein have been found [44, 76]. The concentration of 19 kDa was higher in boars with seminal plasma of good freezability, with this concentration reducing in the period between ejaculation and the conclusion of 3 hours of interaction with spermatozoa. These results may be associated with the ability of NPC2 to bind to the cholesterol of the sperm membrane and suggest a better preventative mechanism against cryocapacitation [44]. These findings serve as a basis to confirm this protein as a new marker of boar freezability.

2.6. Prostaglandin D synthase lipocalin type

Prostaglandin D synthase type lipocalin (L-PGDS) is the only lipocalin that has enzymatic activity of prostaglandin H2 transformation, produced from arachidonic acid by cycloxygenase, in prostaglandin D2 [82]. L-PGDS is the most abundant protein in cerebrospinal fluid and is 75% similar to a homologous protein of 26 kDa. The latter is found in seminal plasma and is related to fertility in bulls [83] and boars [84]. The synthesis of this protein is mainly carried out in epididymal epithelial cells, with mRNA having also been found in Leydig cells in the testis and in prostate epithelial cells [85]. The main function of lipocalins is the transportation

of lipophilic substances, and in the case of L-PGDS, a high affinity has been recorded between it and retinoids, such as retinoic acid and retinol [86]. Stillwell and Wassall [87] reported that the presence of these two molecules in the plasma membrane affects their permeability by interacting with phospholipids, which results in greater ion entry from the outside. This could be related to capacitation, given that the beginning of the molecular cascade of this event is signaled by the change of membrane permeability to ions such as calcium, which results in acrosome reaction and hypermotility [88]. Gerena et al. [83] detected that L-PGDS is present in the acrosomal membrane in ejaculated spermatozoa, and then disappears in spermatozoa with acrosome reaction. In addition, in vitro incubation of spermatozoa with the L-PGDS protein increases the union of these with the zona pellucida [89], an action that is only possible after membrane changes that occur during capacitation [90]. L-PGDS also has an affinity for docosahexaenoic acid, of which it is also a transporter [91]. Docosahexaenoic acid interacts with other membrane lipids such as cholesterol and can play an important role in local structure and membrane function [92].

Another of the most important functions of the L-PGDS is its intervention in spermatogenesis and sperm maturation, through the thyroid hormone (triiodothyronine, T3) that regulates the growth, maturation, and functioning of Sertoli cells. This is made possible due to the ability of this protein to pass through membranes and to establish a connection between the blood fluid, the testicle, and the epididymis [83]. This protein is a potential marker of boar freezability because it has been found in different concentrations in the seminal plasma of semen with both high and low freezability [44].

2.7. ß subunit of N-acetyl-b-hexosaminidase

Among the most important enzymes related to the freezability of swine semen is the ß subunit of N-acetyl-b-hexosaminidase (ß-HEX). ß-HEX binds to the acrosome and is released during the acrosome reaction. A reliable indicator of sperm cryotolerance, this enzyme was found to be negatively correlated with motility, plasma membrane integrity, and post-thawing lipid peroxidation [45].

2.8. Antioxidants enzymes

In sperm cells, there are multiple protection mechanisms against oxidative stress [29], most saliently the antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) [93]. Spermatozoa may also depend upon the presence of extracellular free-radical scavenging systems, which interact with biochemical components of the seminal plasma [94].

2.8.1. Paraoxonase type 1

The paraoxonase (PON) enzyme family is functionally linked to cholesterol and is composed of three members: PON-1, PON-2, and PON-3 [95]. PON-1 and PON-3 are extracellular enzymes present in blood plasma, while PON-2 is an intracellular enzyme not found in blood plasma [95, 96].

PON-1 is an high-density lipoprotein associated enzyme that possess antioxidant and anti-inflammatory properties, which prevents low-density lipoprotein and high-density lipoprotein oxidation and, consequently, protects cells against oxidative stress [96].

Barranco et al. [97] revealed and characterized the presence of the antioxidant enzymes PON-1 and PON-2 in boar semen. PON-1 binds to membrane cholesterol, preventing its oxidation and thereby positively influencing both motility and the sperm membrane integrity [46]. Likewise, the presence of this protein is correlated with quality, functionality of liquid-stored semen samples and can also be related to fertility outcomes in boars. Its antioxidant properties, specifically the decrease of intracellular ROS generation, could contribute to the superior ability of the spermatozoa present in the sperm-peak portion of the ejaculate and to colonize the sperm reservoir in the oviduct [98]. The sperm-peak portion contains SP with better antioxidant capacities, greater cryotolerance, and lower ROS generation than the post sperm-rich fraction. This coincides with high SOD and PON-1 values in this portion [46].

Given the fact that PON-1 is positively related to total motility and viability of frozen–thawed boar semen, [46], that there is evidence that activity levels in SP differ among boars [98], and the possibility exists that PON-1 levels are genetically determined [96], it can be concluded that PON-1 is a potential molecular marker of boar semen freezability.

2.8.2. Superoxide dismutase

Superoxide dismutases are a family of metalloenzymes involved in intracellular and extracellular antioxidant defense system by catalyzing the dismutation of superoxide anions into hydrogen peroxide and oxygen. In mammals, three SOD isoenzymes have been described by their cellular localization, metal composition in the active site, and sensitivity to inhibitors [99, 100]. It is known that there are two subtypes of copper and zinc containing SOD (Cu/Zn-SOD): cytosolic and secretory extracellular SOD, occurring in the seminal plasma (EC-SOD). However, there are no research on its properties and functions. The EC-SOD is an important antioxidant enzyme of boar seminal plasma, which plays an important physiological role in counteracting oxidative stress in spermatozoa [94].

Conflict of interest

The author declares that he does not have any competing interest.

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