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Functional aspects of seminal plasma and sperm proteins and their potential as molecular markers of fertility

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Abstract

Molecular components of sperm and in the media surrounding them influence bull fertility. Given this concept, proteins of the seminal plasma modulate crucial functions and events of reproduction, such as sperm motility and capacitation, cell protection, acrosome reaction, fertilization and embryonic development. Sperm proteins are also important for successful fertilization, egg activation and embryo development. Empirical associations between seminal and sperm proteins and fertility in the bovine indicate that these proteins are potential molecular markers of the male reproductive status.

Keywords: bull, fertility, proteins, seminal plasma, sperm.

Introduction

Pregnancy per artificial insemination (AI) is the best indicator of the reproductive potential of sires. However, this information becomes available only after animals are mature and have been selected for commercial use in the AI industry. Moreover, criteria such as sperm motility and morphology have limited associations with the actual fertility of sires, particularly in groups of animals that have been extensively selected by the AI industry (Killian *et al.*, 1993; Moura, 2005; Moura *et al*., 2006; De Oliveira *et al*., 2013; Dogan *et al*., 2015; Kaya and Memili, 2016). Significant differences in fertility exist among bulls with "normal" semen parameters and bulls with non-compensable sperm defects may never achieve adequate fecundity (De Oliveira *et al*., 2013; Dogan *et al*., 2015; Kaya and Memili, 2016). This information suggests that mechanisms by which sperm molecular profiles influence bull fertility are not fully understood. In this context, research effort has been made to identify molecular markers of gamete function and fertility in nearly all farm animals and humans. Candidates for such markers include sperm RNA, sperm proteins and molecules of the reproductive fluids, among others. All these studies are based on the hypothesis that molecular components of sperm and/or from the surrounding media influence the fertilizing capacity of sires. Considering this concept, analysis of both sperm and seminal plasma proteome will provide meaningful information about the mechanisms regulating sperm fertilizing potential and reproductive performance of sires. Thus, the present review discusses the roles of selected seminal plasma proteins and how their expression relates to fertility. Sperm proteins and their potential use as fertility markers are also discussed mainly focused on the bovine species.

Seminal plasma proteins

Proteins involved in sperm protection

Seminal plasma contains proteins that protect sperm in the epididymis (Kraus *et al*., 2005; Hinton *et al*., 1995), after ejaculation and in the female reproductive tract. Production of reactive oxygen species (ROS) is part of sperm physiology (MacLeod, 1943) but, when in excess, ROS disturb sperm homeostasis through formation of lipid peroxidation, and reduction of enzymes that regulate calcium influx and loss of ATP (Ohta *et al*., 1989; Aitken *et al*., 1993). To control the deleterious effects of ROS, epididymis secretes antioxidant enzymes (Hinton *et al*., 1996), such as glutathione S-transferase, tioredoxin peroxidase, superoxide dismutase, glutathione peroxidase (GSHPx) and catalase (Alvarez and Storey, 1983; Jeulin *et al*., 1989; Fouchécourt *et al*., 2000; Dacheux *et al*., 2006). GSHPx is one the main enzymes that protects sperm (Perry *et al*., 1992; Dacheux *et al*., 2005) and it catalyzes the reduction of hydrogen peroxide (Halliwell and Gutteridge, 1990). Increased GSPHx activity in ram semen is linked to antioxidant effects and maintenance of sperm viability (Casao *et al*., 2010). Another seminal plasma molecule known as acidic seminal fluid protein (aSFP) also has actions on the control of oxidative stress in the bovine reproductive tract (Einspanier *et al*., 1993; Schöneck *et al*., 1996). aSFP shares identity with molecules of spermadhesin family (Romão *et al*., 1997) and, in the bull, it is secreted by both the epididymides and accessory sex glands (Moura *et al*., 2007a, 2010). Binding of aSFP to ejaculated sperm occurs but it is lost after capacitation (Dòstolovà *et al*., 1994), suggesting that, unlike porcine spermadhesins (Caballero *et al*., 2004, 2005), bovine aSFP does not participate in sperm-oocyte interaction. However, aSFP has been associated with survival of bull sperm to cryopreservation (Jobim *et al*., 2004), suggesting that its anti-oxidant activities may be beneficial to reproductive technology.

Additionally, ion chelators of the seminal plasma, such as lactoferrin, protect sperm against effects of lipid peroxidation (Ochsendorf, 1999). Lactoferrin sequesters ionic iron (Nozaki *et al.*, 2003) and adsorbs sperm during epididymal transit (Jin *et al*., 1997) and ejaculation (Thaler *et al*., 1990), when it also has

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antimicrobial effect. Seminal albumin, in turn, binds to lipid peroxides, contributing to sperm protection (Alvarez and Storey, 1983), and presents a positive correlation with the percentage of normal sperm in bovine semen (Elzanaty *et al*., 2007). Clusterin is another seminal plasma molecule with protecting roles, acting as a chaperone (Humphreys *et al*., 1999) and inhibiting cell lysis by complement-mediated mechanisms present in female secretions (Ibrahim *et al*., 1999; Meri and Jarva, 2001). In the epididymis, some clusterin functions relate to maturation, lipid transport (Tenniswood *et al.*, 1992) and sperm membrane remodeling (Humphreys *et al.*, 1999). Clusterin chaperone activity is consistent with its ability to interact with different types of proteins *in vivo* (Carver *et al.*; 2003). *In silico* analysis of clusterin networking indicates its potential interactions with proteases and protease inhibitors, such as plasminogen, alpha-2-macroglobulin, TIMP-1, alpha-2-antiplasmin precursor and plasminogen activator inhibitor 1. Clusterin also has putative links to fibronectins, which participate in cell adhesion, healing of wounds and maintenance of cell structure; GTP protein-coupled receptors and modulators of cell growth, such as TGFB3. Seminal plasma clusterin is inversely related to the percentage of sperm with intact membrane in peccaries (*Peccari tajacu* L.; Santos *et al*., 2014) and with the percentage of normal sperm in semen samples of Brahman bulls (Boe-Hansen *et al*., 2015). In contrast, bull and ram sperm with morphologic defects exhibit extensive clusterin binding (Ibrahim *et al.,* 2001a, b). This association probably occurs as a result of clusterin´s ability to bind to the damaged portions of hydrophobic regions of sperm membrane (Bailey and Griswold, 1999).

Proteins associated with sperm motility

Seminal plasma contains proteins associated with processes of sperm motility (Baas *et al*., 1983), such as the kallikrein-cinins. In this case, kininogen present in seminal plasma acts as a specific substrate for kalikrein (Fink *et al.*, 1989), producing the main effectors for stimulation of sperm motility after ejaculation (Schill *et al.*, 1989). Studies have demonstrated positive correlation between seminal plasma kallikrein activity and sperm motility, and addition of exogenous kallikrein benefits bovine sperm motility as well (Somlev *et al*., 1996). Angiontensin converting enzyme (ACE) is another seminal plasma component related to the kalikrein system (Hohlbrugger *et al*., 1984). ACE catalyzes the formation of angiontensin II and binds to receptors on the sperm, intensifying events related to motility (Vinson *et al*., 1996). ACE activity in the ram seminal plasma positively correlates with sperm concentration and fertility (Métayer *et al*., 2001; Gatti *et al.*, 2004). Inhibition of ACE activity in the bovine seminal plasma decreases the number of sperm with progressive motility and inhibits acrosome reaction after *in vitro* capacitation (Costa and Thundathil, 2012).

Proteins involved in sperm capacitation

Phospholipid binding proteins belonging to the family of BSPs (Binder of Sperm Proteins) are present in the seminal plasma of several species, including bulls, bucks (male goats and rabbits), rams, men, male rodents, and stallions (Moura *et al*., 2007a; Manjunath *et al*., 2009; Souza *et al*., 2012; Plante *et al*., 2016). BSPs comprise approximately 60% of all proteins of the accessory sex gland fluid (Moura *et al*., 2007a) and seminal plasma (Manjunath and Sairam, 1987) from *Bos taurus* bulls and nearly the same amount in *Bos indicus* seminal plasma (Rego *et al*., 2014). In the bovine, BSP proteins are secreted as several isoforms with 14-15 kDa (BSP1 and BSP3) and 30 kDa (BSP5). Both BSP1 and BSP5 possess two fibronectin type II domains arranged in tandem and amino terminal extensions that are O-glycosylated at threonine residues. Such biochemical attributes allow BSP1 and BSP5 to interact with sperm and to modulate ligand-binding activities by similar mechanisms (Calvete *et al*., 1996; Manjunath *et al*., 2009), sharing functional similarities as well (Manjunath and Therien, 2002). Bovine BSPs are typical accessory sex gland proteins and there are no reports of their expression in the epididymis (Manjunath and Therien, 2002; Moura *et al*., 2007a, 2010). BSPs bind to bull sperm at ejaculation (Manjunath and Therien, 2002) and remain as such after sperm come in contact with oviductal secretions *in vitro* (Souza *et al*., 2008); after sperm become acrosome reacted and after sperm are subjected to freezing and thawing (Rodriguez-Villamil *et al*., 2016). BSPs also mediate the interaction between sperm and the oviduct epithelium (Gwathmey *et al*., 2006; Suarez, 2016). The most studied role of BSPs is their effects to bind and remove phospholipids and cholesterol from the sperm membrane, one of the initial events of capacitation (Thérien *et al*., 1999). Capacitating effects of BSPs seem to hold true in other species as well, such as mice (Plante and Manjunath, 2015) and humans (Plante *et al*., 2014). However, while ruminant BSPs originate mainly from the accessory sex glands (Manjunath *et al*., 2009; Souza *et al*., 2012; van Tilburg *et al*., 2014), its expression in mice and humans occurs in the epididimis.

Roles played by BSPs seem to go beyond sperm capacitation as experimental evidence indicates that BSP1 contributes to *in vitro* fertilization and embryonic development when added to the fertilization media. The study conducted by Rodriguez-Villamil *et al*. (2016) evaluated cumulus-oocyte complexes (COCs) incubated with frozen-thawed ejaculated sperm (18 h) in Fert-TALP medium containing: heparin and different concentrations of purified BSP1 (10, 20 or 40 µg/ml). With ejaculated sperm, cleavage rates were similar when Fert-TALP medium was incubated with heparin (74.1 ± 2.7%), 10 µg/ml BSP1 (77.8 ± 3.1%) or 20 µg/ml BSP1 (74 ± 2.0%). Day-7 blastocyst rates were equivalent after incubations with heparin (40.8 ± 5.0%) and 10 µg/ml BSP1 (34.1 ± 4.4%), but there were marked reductions in blastocyst formation after IVF

media were supplemented with 20 µg/ml BSP1 (22.4 ± 2.9%) and 40 µg/ml BPS1 (19.3 ± 4.1%; $P < 0.05$). Such results confirm that BSP1 is as efficient as heparin for induction of capacitation and the fertilizing capacity of frozen-thawed ejaculated sperm. However, the study also reports a damaging effect of BSP1 on *in vitro* embryo development. This outcome may be the result of BSP1-induced damage on sperm, given the exposure period during fertilization (18 h). In agreement with such results, studies show that excess of BSP proteins and time exposure are harmful to cryopreserved sperm because of membrane destabilization and excessive phospholipid and cholesterol efflux (Therien *et al*., 1995; 1998; Manjunath and Therien, 2002). Also, an earlier investigation claimed that the content of BSP5 in accessory sex gland fluid has a quadratic association with bull fertility (Moura *et al*., 2006), suggesting that too much BSP is detrimental to sperm physiology and/or embryo development. Finally, inclusion of BSP1 in the Percoll solution with frozen-thawed ejaculated sperm for a short period of time (30 min), prior to *in vitro* fertilization, was detrimental to embryo development (our unpublished results). In this case, 40 µg/ml BPS1 was as efficient as heparin to enhance the fertilizing capacity of sperm. Result obtained with the Percoll incubation substantiates the concept that damaging effect of BSP1 relates to exposure time of sperm to this molecule.

In other experiments, COCs were incubated with frozen-thawed cauda epididymal sperm (18 h) in Fert-TALP medium containing: no heparin, heparin, 10, 20 or 40 µg/ml BPS1. In this case, cleavage and blastocyst rates were similar after treatments with heparin (68.5 ± 1.3% and 24.7 ± 3.2%, respectively) or without heparin (65.5 ± 1.8% and 27.3 ± 1.6%, respectively). Cleavage was higher after treatment with any BSP1 concentrations (74.2 ± 2.7% to 79.0 ± 1.1%) than without heparin ($P < 0.05$). Also, cleavage was better with 40 µg/ml BSP1 (79.0 ± 1.1%) than with heparin (68.5 ± 1.3%; $P < 0.05$). Embryo development was higher ($P < 0.05$) after treatment with 20µg/ml BSP1 (35.6 ± 2.5%) and 40 µg/ml (41.1 ± 2%) than after incubations with heparin (24.7 ± 3.2%) or without heparin (27.3 ± 1.6%). It has been established that BSPs bind to membrane cholesterol and phospholipids but how this occurs is still a matter of debate. Interestingly, supplementing IVF media with BSP1 + heparin (for 18 h) did not improve the effects of BSP1 when fertilization occurred with frozen-thawed epididymal sperm (our unpublished results). Thus, we may conclude that: 1. heparin has limited effect on cauda epididymal sperm *in vitro*, as evaluated by fertilization and blastocyst formation; 2. BSP1 had better effect on embryo growth than heparin; 3. combination of BSP1 with heparin does not enhance cleavage rates and embryo development beyond those obtained with BSP1 only. When conducting studies with IVF systems, we also verified that BSP1 did not cause reductions in blastocyst rates after fertilization with epididymal sperm, in contract to what we had observed with ejaculated sperm. This obviously emphasizes that previous exposure or not of spermatozoa to seminal plasma determines how sperm will respond to BSP *in vitro*.

Despite all important roles played by BSPs, these molecules can also damage sperm during cryostorage as they extract phospholipids and cholesterol from the membrane in a concentration- and time-dependent manner (Manjunath *et al*., 2002; Plante *et al*., 2016). Such deleterious effects occur when sperm are exposed for a long time and/or to excess of BSP proteins, as it happens during cooling and freezing. In this regard, extenders used for sperm preservation, such as egg-yolk (EY) and milk, contain components that associate with BSPs (Manjunath *et al*., 2002). In the case of EY, its low-density lipoproteins interact with bovine BSPs (Bergeron and Manjunath, 2006) and milk proteins also interact with BSPs. This interaction between milk components and BSPs prevent excessive BSP binding to sperm and excessive phospholipid removal from the membrane, being thus the fundamental mechanism by which milk components protect sperm during cryopreservation (Plante *et al*., 2015). In fact, experiments conducted with goats (Menezes *et al*., 2016) show that not only did milk proteins (casein and β-lactoglobulin) bind to BSPs, but they also reduced BSP interactions with sperm membranes. Currently, BSP proteins are one of the most studied seminal plasma proteins in mammalian species. In addition to being abundant in ruminants, studies conducted in the bull confirm that BSPs have broad effects on ejaculated sperm, including capacitation, interaction with the oviduct epithelium and fertilization. The fact that BSPs interact with components of semen extenders suggest that these proteins are potential targets for the development of new fertility markers and biomolecules that can improve assisted reproductive technologies.

Proteins involved in acrosome reaction and fertilization

Seminal plasma phospholipase A2 (PLA2) participates in capacitation, acrosome reaction and sperm-oocyte membrane fusion (Soubeyrand *et al*., 1997; Pietrobon *et al*., 2005; Roldan and Shi, 2007). PLA2 also has antimicrobial effects and its expression in seminal plasma is associated with fertility in bulls (Moura *et al*., 2006). The analysis of seminal plasma proteome showed that osteopontin (OPN) was related to *in vivo* fertility of Holstein bulls (Killian *et al*., 1993; Moura *et al*., 2006) and to the fertilizing capacity of cauda epididymal sperm treated with accessory sex gland fluid during IVF trials (Henault *et al*., 1995; Moura *et al*., 2007b). In fact, the amount of OPN in the seminal plasma of high fertility bulls is 4-fold higher in comparison to that in low fertility sires (Moura *et al*., 2006). The importance of OPN in reproduction is also demonstrated by experiments using IVF. In this regard, the percentage of fertilized bovine oocytes was significantly reduced by addition of OPN antibodies to fertilization media and exposure of sperm or oocytes to antibodies against alphaV and alpha5 integrins before fertilization (Gonçalves *et al*., 2007). Also, pre-treatment of sperm and oocytes with OPN enhances

both *in vitro* fertilization and early embryo development in the bovine (Gonçalves *et al*., 2008a, b). The RGD amino acid sequence of osteopontin mediates its link with α5 and αv integrins (Denhardt, 2002; Wai and Kuo, 2004) and the ability of osteopontin to support cell adhesion is prevented when the RGD sequence is mutated (Liaw *et al*., 1995; Xuan *et al*., 1995). Treatment of sperm or oocytes with an RGD peptide, but not with an RGE sequence, reduced both the number of sperm bound to the zona pellucida and fertilization rates, similar to what was found using antibodies against osteopontin. Results involving RGD and anti-integrin antibodies suggest that OPN interacts with sperm through integrins (Gonçalves *et al*., 2007). Incubation of oocytes with osteopontin purified from bovine milk increased cleavage rates on day 4 (from 78.1 ± 1.3 to 85.8 ± 1.4 %), blastocyst development on day 8 (from 24.2 ± 1.2 to 33.8 ± 1.4 %) and hatched blastocysts on day 11 (from 10.6 ± 1.6 to 18.5 ± 1.4 %; Gonçalves *et al*., 2007). Following this concept, purified milk OPN improved sperm capacitation and, when added to the IVF media, OPN enhanced bovine blastocyst formation (Monaco *et al*., 2009). Moreover, freezing bull semen with different concentrations of OPN elicited better *in vitro* fertilization rates (85 to 78 ± 4 % *vs*. 75 to 69 ± 4 %) and blastocyst development on day 8 (45 ± 2.9 to 37 ± 1.6 % *vs*. 33 ± 2.3 to 29 ± 2.8 %) in comparison with untreated semen (Gonçalves *et al*., 2008a). In the swine, supplementation of fertilization media with recombinant rat OPN enhanced fertilization rates by 41% and reduced polyspermy (Hao *et al*., 2006). Exogenous OPN added to IVF media improved cleavage rates and swine embryo development, and inhibited apoptosis and DNA fragmentation (Hao *et al*., 2008). Moreover, a recent study described that anti-OPN antibodies efficiently decreased the rates of *in vitro* fertilization and blastocyst growth in mice (Liu *et al*., 2015). Such pieces of evidence gathered after experiments in bulls, porcine and mice support OPN's effects on fertilization and post-fertilization events.

Osteopontin is typically involved in cell adhesion, tissue and extracellular remodeling, inflammation and immune-mediated events (Denhardt, 2002; Wai and Kuo, 2004; Rittling and Singh, 2015; Bouleftour *et al*., 2016). Despite substantial information about the actions of osteopontin in several tissues, an understanding of its function in male reproduction is far from complete. Based on the current literature and general attributes of osteopontin, a plausible hypothesis has been suggested by several authors about OPN and fertilization. In this case, OPN secreted by the accessory sex glands binds to sperm during ejaculation through integrins and that the integrin-OPN complex interacts with the zona pellucida (D'Cruz, 1996). This model is supported by the fact that bovine oviductal fluid contains OPN (Gabler *et al*., 2003). Additionally, OPN binds to CD44 receptor, which usually participates in cell adhesion (Cichy and Puré, 2003), and has been expressed both in sperm (Bains *et al*., 2002) and oocyte membrane (Schoenfelder and Einspanier, 2003). In the bull, osteopontin binds to sperm acrosome cap at ejaculation (Cancel *et al*., 1999) and this sperm-OPN link still exists after sperm comes in contact with secretions of the oviductal fluid and undergoes acrosome reaction *in vitro* (Souza *et al*., 2008). In addition to sperm binding, OPN interacts with the zona pellucida and oolemma of bovine oocytes (Souza *et al*., 2008). In light of these findings, we propose that OPN adheres to sperm and this complex connects to the zona pellucida or to OPN-zona pellucida, given that osteopontin is capable of forming bonds with other OPN molecules with high affinity (Kaartinen *et al*., 1999; Goldsmith *et al*., 2002). When entering the periviteline space, OPN attached to the post-equatorial segment would mediate the interaction of sperm with the oolema, also through integrins and/or CD44. Integrins (αv and α5) have been identified in the bovine (Erikson *et al*., 2008) and human spermatozoa (Fusi *et al*., 1996; Reddy *et al*., 2003), as well as on human oolema (D'Cruz, 1996). The CD44 transmembrane glycoproteins are present in bovine sperm and oocyte. Interface of sperm osteopontin with oocyte integrins and CD44 receptors could trigger intracellular signaling, as reported for other cell types (Wai and Kuo, 2004; Rangaswami *et al*., 2006), and affect fertilization and early embryo development.

Sperm proteins and fertility

Molecular and cellular health of sperm are important for mammalian reproduction and proteins of spermatozoa are vital for fertility, ability of the sperm to fertilize and activate the egg and to support embryo development. Sperm proteins play important roles in sperm integrity, morphology and functions, including motility, capacitation, fertilization, egg activation and embryo development (Parisi *et al*., 2014). For example, Outer dense fiber protein 2 (ODF2) is a component of sperm tail structure and is involved in regulation of motility in mouse (Tarnasky *et al*., 2010). In addition, tubulins, such as tubulin beta-2C chain located in sperm tail, are down regulated in asthenozoospermia (Siva *et al*., 2010). Also, mutations in gene encoding for sperm cation channel protein (CatSper) in mouse result in less directed sperm movements in the mouse (Ren *et al*., 2001).

Sperm capacitation is important for sperm's ability to fertilize the egg and many proteins and pathways that have been shown to be involved with sperm capacitation. In this regard, extracellular signal-regulated kinase (ERK) pathway is involved in tyrosine phosphorylation and capacitation of boar sperm (Awda and Buhr, 2010), and casein kinase 2 (CKII) participates in EGF signaling in sperm from Holstein bulls with different fertility. Following fertilization, sperm proteins appear to play roles during egg activation and are also markers of fertility. For example, sperm phospholipase C Zeta 1 (PLCZ 1) is involved in intracellular Ca2+ oscillation (Cooney *et al*., 2010) and postacrosomal sheath WW domain-binding protein (PAWP) participates in porcine egg activation and pronuclear formation, with potential effects on embryo development as well (Wu *et al*., 2007). Moreover, using methods in proteomics, Peddinti *et al*. (2008) identified

125 differentially expressed proteins in sperm from high *vs.* low fertility bulls and Grant *et al*. (2015) demonstrated that superoxide dismutase (SOD) is expressed at higher levels in sperm from high fertility sires.

Sperm nuclear structure is also crucial important for fertility. Experiments have demonstrated that bull sperm contain a repertoire of histones fertility (De Oliveira *et al.,* 2013) and that amounts of Protamine 1 (PRM1) in sperm are directly correlated with bull reproductive performance (Dogan *et al.,* 2015). These studies indicate that different amounts of histones, as well as the ratios between protamines and histones, determine sperm viability and thus bull fertility.

Conclusions

In recent decades, new methods in proteomics have allowed the detection of unprecedented number of proteins in the seminal plasma and spermatozoa of humans, domestic and wild species. This obviously broadens our knowledge about the roles of molecules present in seminal fluid and sperm, and how they define male fertility. Empirical associations exist between some of those molecules proteins and fertility indexes and sperm parameters. Experiments also confirm cause and effect relations between male factors (such as osteopontin and BSPs), and *in vitro* fertilization and early embryo development. Therefore, seminal and sperm proteins are potential markers of fertility and they may be used in the animal biotechnology industry.

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