Seminal plasma proteins of adult boars and correlations with sperm parameters

Verónica González-Cadavid, Jorge A.M. Martins, Frederico B. Moreno, Tiago S. Andrade, Antonio C.L. Santos, Ana Cristina O. Monteiro-Moreira, Renato A. Moreira, Arlindo A. Moura*

*Department of Animal Science, Federal University of Ceará, Ceará, Brazil
bSchool of Pharmacy, University of Fortaleza, Fortaleza, Brazil
cXerez Comercial Swine Unit, Maranguape, Ceará, Brazil

ARTICLE INFO

Article history:
Received 23 January 2014
Received in revised form 28 May 2014
Accepted 28 May 2014

Keywords:
Proteomics
Seminal plasma
Swine
Semen parameter

ABSTRACT

The present study was conducted to identify the major seminal plasma protein profile of boars and its associations with semen criteria. Semen samples were collected from 12 adult boars and subjected to evaluation of sperm parameters (motility, morphology, vitality, and percent of cells with intact acrosome). Seminal plasma was obtained by centrifugation, analyzed by two-dimensional SDS-PAGE, and proteins identified by mass spectrometry (electrospray ionization quadrupole time-of-flight). We tested regression models using spot intensities related to the same proteins as independent variables and semen parameters as dependent variables (P < 0.05). One hundred twelve spots were identified in the boar seminal plasma gels, equivalent to 39 different proteins. Spermadhesin porcine seminal protein (PSP)-I and PSP-II, as well as spermadhesins AQN-1, AQN-3 and AWN-1 represented 45.2 ± 8% of the total intensity of all spots. Other proteins expressed in the boar seminal plasma included albumin, complement proteins (complement factor H precursor, complement C3 precursor and adipsin/complement factor D), immunoglobulins (IgG heavy chain precursor, IgG delta heavy chain membrane bound form, IgG gamma-chain, Ig lambda chain V-C region PLC3, and CH4 and secreted domains of swine IgM), IgG-binding proteins, epididymal-specific lipocalin 5, epididymal secretory protein E1 precursor, epididymal secretory glutathione peroxidase precursor, transferrin, lactotransferrin and fibronectin type 1 (FN1). On the basis of the regression analysis, the percentage of sperm with midpiece defects was related to the amount of CH4 and secreted domains of swine IgM and FN1 (r² = 0.58, P = 0.006), IgG-binding protein (r² = 0.41, P = 0.024), complement factor H precursor (r² = 0.61, P = 0.014) and lactadherin (r² = 0.45, P = 0.033). The percentage of sperm with tail defects was also related to CH4 and secreted domains of swine IgM and FN1 (r² = 0.40, P = 0.034), IgG-binding protein (r² = 0.35, P = 0.043) and lactadherin (r² = 0.74, P = 0.001). Sperm motility, in turn, had association with the intensities of spots identified as lactadherin (r² = 0.48, P = 0.027). In conclusion, we presently describe the major proteome of boar seminal plasma and significant associations between specific seminal plasma proteins and semen parameters. Such relationships will serve as the basis for determination of molecular markers of sperm function in the swine species.

© 2014 Elsevier Inc. All rights reserved.
1. Introduction

Artificial insemination (AI) is the most important reproductive biology tool for genetic improvement aiming high prolificacy in pig industry [1,2]. The most successfully used germplasm material in AI is the chilled semen, because boar spermatozoa cryopreservation procedures promote damages in sperm membranes and organelles, which in turn lead to lower fertility rates [1]. The knowledge of factors or substances that stabilize or protect boar spermatozoa for a successful fertilization process is crucial for animal production. This is the reason for the large number of researches in the area that aim not only to define good biological markers for the reproductive potential of boar, but also the development of products to be used as additive for the improvement of the present assisted reproduction methods.

In this context, seminal plasma is a promising source for the study of such potential biomarkers, because it is a complex mixture of secretions from testis, epididymis, and male accessory sex glands. In fact, the addition of 50% of boar seminal plasma to thawed sperm has been found to have beneficial effects on the physiology of that cell [3]. Seminal plasma proteins, among many other substances present in the seminal fluid, are numerous and possess the ability to bind to the sperm during ejaculation, influencing several sperm criteria such as motility, capacitation, sperm transport, survival and longevity, protection against damages, and the formation of the sperm reservoir inside the female reproductive tract [4–6]. Given these multifunctional attributes of the seminal plasma components, the present study was conducted to identify the major seminal plasma protein profile of adult boars and also to evaluate its associations with several semen criteria.

2. Materials and methods

2.1. Animals and materials

The experiment was conducted at a commercial swine farm in the Northeast of Brazil (Maranguape, CE; 03° 54′ 46″ S, 38° 39′ 19.8″ W; Xerez Avicola, Ltda). Twelve sexually mature boars, routinely used as semen donors for AI, were housed in individual stalls (3.1 × 2.5 m) equipped with cooling systems. Animals were fed 2 kg/day with a commercial diet according to the nutritional requirement guidelines for adult boars and received water ad libitum.

Chemicals and equipment used in this work were purchased from Sigma-Aldrich (St. Louis, MO, USA), GE Life sciences (Piscataway, NJ, USA), Bio-Rad (Rockville, MD, USA), Promega (Madison, WI, USA), Eppendorf (Hauppaugne, NY, USA), and Waters (Milford, MA, USA).

2.2. Semen evaluation

Semen samples were collected by the gloved hand technique [7]. Aliquots of 20 μL were placed over glass slides and covered with cover slips, warmed at 37 °C, and subjected to light microscopy (200×) to evaluate the percentage of motile sperm [8] and sperm wave motion in a scale of scores from 0 to 5 [9]. Sperm concentration was determined by the Neubauer chamber. Sperm acrosome status was evaluated by the Trypan blue and Giemsa staining method [10]. Sperm cells were then visualized by light microscopy (1000×) and grouped as cells with intact or damaged acrosome.

Sperm morphology was evaluated by fixing the cells in a 1% (wt/vol) formaldehyde in PBS solution and evaluated under light microscopy (1000×), according to a previously described method [11], counting 200 cells per ejaculate. The integrity of the sperm plasma membrane was evaluated by the hypo-osmotic swelling test. Briefly, we incubated 100 μL of semen mixed with 1 mL of 100 mOs/kg distilled water for 40 minutes at 37 °C. After incubation, cells were fixed in a 1% formaldehyde solution. One aliquot (20 μL) of the mixture was mounted on glass slide and covered with cover slip and subjected to light microscopy (1000×). In this case, we counted 200 cells per ejaculation and evaluated the percentage of sperm with functional or damaged membranes, characterized by straight or coiled tail, respectively [12].

2.3. Two-dimensional electrophoresis of seminal plasma proteins

After ejaculation and separation of aliquots for sperm evaluation, the remaining semen samples were immediately mixed with a protease inhibitor cocktail containing 4-(2-aminoethyl) benzenesulfonyl fluoride, pepstatin A, E-64, bestatin, leupeptin, and aprotinin. Ten microliters of the cocktail was used per 1 mL of semen [13]. Boar semen was then centrifuged at 800×g for 15 minutes at 4 °C to separate the sperm cell. The supernatant seminal fluid was placed into a new tube and centrifuged at 5000×g for 60 minutes at 4 °C. The resulting supernatant was aliquoted and stored at −20 °C until use.

An aliquot of each sample of seminal plasma was thawed at room temperature and quantified as the total protein concentration [14] using bovine serum albumin as a standard. The assay was performed in triplicates. Then, a volume from each sample of seminal plasma containing 750 μg of total protein was mixed with hydration buffer (8 M urea, 1 M thiourea, 2%–3%[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonate, 2% nonlinear ampholytes in the pH range 3–10, 25 mM dithiothreitol [DTT], and traces of bromophenol blue) sufficient to make 450 μL. These mixtures were then incubated with 24 cm immobilized pH gradient (IPG) strips (pH 3–10, nonlinear) in individual reswelling tray channels for approximately 20 hours. Isoelectric focusing was carried out in Ettan IPGphor 3 apparatus at 20 °C according to the following program: 200 V (60 minutes), 1000 V (60 minutes gradient), 7000 V (30 minutes gradient), 7000 V (60,000 Volt hours [Vh]) e 100 V (12 hours and 30 minutes), with a total of 16.300 Vh. After focusing, IPG strips were incubated (15 minutes) in equilibration buffer I (6 M urea, 50 mM Tris–HCl, pH 8.8, 29.3% (v:v) glycerol, 2% SDS, and 1% DTT) and re-equilibrated for additional 15 minutes in equilibration buffer II (similar to equilibration buffer I, but containing 2.5% iodoacetamide instead of DTT). After equilibration, strips were placed on the top of homogeneous SDS-PAGE gels (12.5%), sealed with agarose (5% in SDS-PAGE running buffer), and run at 500 V with 40 mA per gel.
Gels were stained in colloidal Coomassie blue based on a protocol first described by Candiano, et al. [15] and later adapted in our laboratory [16,17]. Briefly, the resulting gels were fixed overnight in a solution containing 2% (v:v) phosphoric acid and 30% (v:v) ethanol. Then, gels were washed three times (20 minutes each) in 2% (v:v) phosphoric acid, followed by incubation (20 minutes) in a solution with 2% (v:v) phosphoric acid, 18% (v:v) ethanol, and 15% ammonium sulfate. Finally, 8 mL (2% of final volume) of a Coomassie blue G-250 stock solution was added. The gels remained in this final solution for 72 hours.

2.4. Analysis of gel images

Two-dimensional (2D) gels were scanned at 300 dpi in a transparent mode, saved as tagged image file format (tiff) files, and analyzed using PDQuest software, version 7.3.0 (Bio-Rad Laboratories, Hercules, CA, USA). We followed a strategy previously described in detail [18]. For this analysis, we used a group of 12 gels. A master gel was constructed by the software, which included spots of a reference gel and spots consistently present in the other 2D maps. Spots present in key regions of the gels were used as landmarks so that spots were correctly matched in every member of the match set. Quantitation of protein spots in the gels was given as parts per million (ppm) of the total integrated optical density of spots according to PDQuest.

2.5. Digestion of protein spots from 2D gels

Proteins separated by 2D electrophoresis and marked as spots in the seminal plasma gels were destained and subjected to in-gel trypsin digestion [19,20]. Briefly, each spot was excised individually from three different gels, pooled, cut into 1 mm³ pieces, and transferred to clean tubes. Dye and SDS were removed from the spots after three washes in 400 μL of a solution containing 50% (v:v) acetonitrile and 25 mM ammonium bicarbonate (pH 8.0). Gel pieces were then dehydrated after two washes with 200 μL of absolute acetonitrile for 5 minutes and air-dried at room temperature. Gel pieces were incubated with trypsin (20 ng per spot) for 20 hours at 37 °C. After the final incubation, peptides were extracted by three washings with 5% (v:v) trifluoroacetic acid and 50% (v:v) acetonitrile in 50 mM ammonium bicarbonate. Supernatants were concentrated in microtubes using a speed vacuum concentrator. A piece of blank gel, without spots, and a piece of albumin from the molecular mass markers were submitted to the same procedure and used as negative and positive controls, respectively.

2.6. Protein identification by mass spectrometry (electrospray ionization quadrupole time-of-flight)

As previously described [13,17,21], the proteolytic digests (5 μL) were injected into solvent A (5% [v:v] acetonitrile and 0.1% [v:v] formic acid in water) supplied by the auxiliary pump of the capillary high performance liquid chromatography unit and trapped in a Waters Symmetry 300 column (C-18, 5 μm film; 0.3 mm × 5 mm) for online desalting and preconcentration. After washing for 3 minutes with solvent A at 5 μL/min, trapped peptides were then back flushed with the gradient solvent flow onto the analytical column, an High Strength Silica T3 fused silica capillary column (C-18 5 μm, 0.075 mm × 150 mm), using a 10-port switching valve. The analytical column was washed with a gradient solution (5%-80% solvent B; acetonitrile-water-formic acid, 95:5:0.2) for 40 minutes. The mass spectrometer was calibrated using Glu-Fib product ion fragments as needed to maintain mass accuracy within 10 ppm.

The electrospray ionization quadrupole time-of-flight mass spectrometer was operated to acquire tandem mass spectrometry of tryptic peptides in a data-dependent acquisition mode for precursor ion selection using charge-state recognition and intensity threshold as selection criteria using MassLynx 4.0 service pack 1 (Waters Corp., Milford, MA, USA). To carry out the tandem mass spectrometric data acquisition, a survey scan (1.5 seconds) over the m/z of 400 to 1500 was performed. From each survey scan, up to three most intense precursor ions based on the selection criteria were chosen for tandem mass spectrometry to obtain the production spectra resulting from collision-induced dissociation in the presence of argon.

The product ion spectra (6–8 seconds) collected were processed using Protein Lynx Global Server 2.1 (Waters Corp., Milford, CA, USA) and were converted to peak list text files for database searching. To identify the proteins, MS-MS ion searches were performed on the processed spectra against the NCBInr and Swissprot databases using MASCOT Daemon and search engine (Matrix Science Inc., Boston, MA, USA). The searches were made using the NCBInr database with the assumption that there was a maximum of one missed trypsin cleavage and peptides were monoisotopic and using partially oxidized methionine residues and completely carbamidomethylated cysteine residues. Peptide mass tolerance and fragment mass tolerance were initially set to 0.1 and 0.1 Da, respectively, for MS/MS ion searching. However, candidate peptide identifications were only accepted if the m/z values were observed within 0.1 Da of the theoretical mass of the candidate identification, as determined when manually reviewing MASCOT search results. The false-positive rate was determined by running searches using the same parameters against a decoy database.

2.7. Gene ontology analysis

Data from the seminal plasma proteins listed obtained after MASCOT search were analyzed using the software for researching annotations of proteins, an open-source application [17,22]. Gene ontology terms for biological process, cellular component, and molecular function were obtained from UniProtKB and EBI GOA databases.

2.8. Protein interaction analysis

Protein-protein interaction networks were retrieved from STRING (http://string-db.org/) version 9.1 database [23]. This database consists of known and predicted protein interactions collected from direct (physical) and indirect
(functional) associations. Network analysis was evaluated for spermadhesins (PSP-I and PSP-II complex, AQN, and AWN), fibronectin type1 (FN1), CH4 and secreted domains of swine IgM, IgG-binding protein, and lactadherin. Analyses were conducted with no more than 10 interactions, using Sus scrofa specie. No annotations were available in the STRING platform for the CH4 and secreted domains of swine IgM and IgG-binding protein. Thus, we used their amino acid sequences obtained from NCBI nr for search of protein networks.

2.9. Statistical analysis

We tested linear and quadratic regression models, using the sum of spot intensities related to the same proteins as independent variables and semen parameters as dependent variables, using the REG procedure of SAS software (9.0, 2002; SAS Institute Inc, Cary, NC, USA). All parameters in the models were tested for normality of distribution of the residues by Shapiro-Wilk test, asymmetry, and kurtosis, using the UNIVARIATE procedure of SAS with the NORMAL option. Only independent variables with  \( P \leq 0.05 \) and residues with normal distribution were included in the regression models.

3. Results

3.1. Sperm traits

Boars used in the present study had a total sperm number of 164 ± 118.9 \( \times 10^8 \) sperms per ejaculate (minimum 50 \( \times 10^8 \); maximum 434.3 \( \times 10^8 \)), sperm motility of 76.7 ± 9.6% (minimum 60%; maximum 90%), and wave motion of 3.6 ± 0.6 (minimum 2.5; maximum 4.0). The percentage of morphologically normal sperm cells was 68.8 ± 16.1% (minimum 37%; maximum 87%). Total sperm abnormalities (31.2 ± 16.3%) were grouped in head (1.7 ± 2.5%), midpiece (2.3 ± 2.1%), and tail (15.8 ± 8.4%) defects, as well as cytoplasmic droplets (11.4 ± 14.3%). Moreover, the percentage of sperm with functional plasma membrane (reactive cells in hypo-osmotic swelling test) was 62.4 ± 13.8% and the percentage of live sperm with intact acrosome reached 26.4 ± 16.9%.

3.2. Seminal plasma proteins

On average, 245.8 ± 69.5 spots were detected per gel of boar seminal plasma (Fig. 1). Thirty-six of these spots were consistently present in all 2D gels stained with colloidal Coomassie blue, and their intensities represented 35.6 ± 7.9% of the total intensities of all spots detected in the maps. One hundred twelve spots, including the 36 spots present in all gels, and equivalent to 45.6% of the intensities of all detected spots in the master gel were identified by mass spectrometry as 39 different proteins (Supplementary Fig. 1, Table 1, Supplementary Table 1). A cluster of low molecular weight and acidic spots (15.3–25.1 kDa; pI3–5.3, box in Supplementary Fig. 1A, B) represented 78 ± 13.1% of the total intensity of all spots depicted in the 2D gels. Spots identified as spermadhesins PSP-I, PSP-II (chain B, the crystal structures of two members of the spermadhesin family reveal the folding of the CUB - complement C1r/C1s, Uegf, Bmp1 - domain), AQN-1, AQN-3 and AWN-1 were together the most abundant group of proteins in the boar seminal plasma (45.2 ± 8% of total intensity) and mainly found in the cluster described previously.

Albumin was identified in nine high molecular weight spots that together represented 2.5 ± 2.5% of the total intensity of all spots shown in the gels. Other proteins identified as complement proteins (complement factor H precursor, complement C3 precursor and adipsin/complement factor D), immunoglobulins (IgG heavy chain precursor, immunoglobulin delta heavy chain membrane bound form, immunoglobulin gamma-chain, Ig lambda chain V-C region PLC3, and CH4 and secreted domains of swine IgM) and IgG-binding proteins represented, respectively, 3.7 ± 2.7, 1.9 ± 2.1 and 0.5 ± 0.3% of the total intensity of spots detected in the gels. We also identified proteins such as epididymal-specific lipocalin 5, epididyml secretory protein E1 precursor and epididymal secretory glutathione peroxidase precursor that together represented 2.7 ± 2%. Transferrin and lactotransferrin accounted with 0.7 ± 0.8%, whereas FN1 isoforms, X1, X2 and X6, composed by 15 basic spots participated with only 0.6 ± 0.9% of total intensity. Other 18 proteins were present in the seminal plasma with individual intensities representing less than 1%. Combined intensities of all these proteins reached 6.4 ± 3.5% of all spots described in the 2D gels (Supplementary Fig. 1).

3.3. Gene ontology of seminal plasma proteins

Proteins identified in the seminal plasma of boars mainly participate in biological processes associated with regulation (22%), metabolic process (18%), and reproduction (13%) among other stimuli events and interactions with cells and organisms (Table 1). The major molecular function of such proteins is binding (48%), followed by catalytic (35%), antioxidant (6%), and enzyme regulator (3%) activities (Table 1). Regarding the cellular component (data not shown), most identified proteins are extracellular (43%), followed by plasma membrane and other intracellular organelles represented 11% each.

3.4. Protein-protein interaction analysis

According to the in silico analysis of protein networks, the PSP-I/PSP-II spermadhesin complex (Supplementary Fig. 2A) interacts with ubiquitin carboxyl-terminal esterase L3, paraspeckle component 1, BTB (POZ) domain-containing 10, GPN-loop GTPase 2, among others. Spermadhesins AQN (Supplementary Fig. 2B) and AWN (Supplementary Fig. 2C) in turn interact, respectively, with zonadhesin and peroxiredoxin 5 and with 3-hydroxybutyrate dehydrogenase and lactadherin. Both AQN and AWN share common interactions with seminal plasma protein pB1 precursor and ADAM (A desintegrin and metalloproteinase)3b. Fibronectin type 1 (Supplementary Fig. 2D) networks with integrins, the von Willebrand factor, albumin, and CD44 among others. Complement factor H (Supplementary Fig. 2E) shares link with complement components, C-reactive protein, among others. Reported lactadherin networking (Supplementary Fig. 2F)
Fig. 1. Regression models showing the variations in boar sperm parameters as related to seminal plasma proteins. Dependent variables (y): percentages of motile sperm and sperm with midpiece and tail defects. Independent variables (x) refer to the sum of intensities of spots identified as each protein. Specifically, CH4 and secreted domains of swine IgM/FN1: sum of intensities of spots 22, 23 and 87 (A and B); IgG-binding protein: spots 55, 56, 57, 58, 59, 60 and 73 (C and D); complement factor H: spots 88, 89, 90, 123, 124, 125, 126 and 127 (E); lactadherin: spots 42 and 43 (F–H). Spot numbers refer to those shown in Supplementary Figure 1, Table 1 and Supplementary Table 1. FN1, fibronectin type 1; IgG, immunoglobulin G; IgM, immunoglobulin M.
<table>
<thead>
<tr>
<th>No.</th>
<th>Protein</th>
<th>NCBI accession number</th>
<th>Mean intensity ± standard error of all spots of this protein</th>
<th>GO Biological process</th>
<th>Molecular function</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>Spermadhesin PSP-I</td>
<td>108346; 47523176</td>
<td>243633.9 ± 20381.5</td>
<td>Reproduction</td>
<td>NA</td>
</tr>
<tr>
<td>02</td>
<td>Chain B, the crystal structures of two members of the spermadhesin family reveal the folding of the CUB domain</td>
<td>3318759</td>
<td>353318.0 ± 25132.0</td>
<td>Reproduction</td>
<td>NA</td>
</tr>
<tr>
<td>03</td>
<td>AQN-1</td>
<td>114082</td>
<td>12650.5 ± 2973.8</td>
<td>Reproduction</td>
<td>Binding</td>
</tr>
<tr>
<td>04</td>
<td>AQN-3</td>
<td>72535165</td>
<td>46610.3 ± 7239.5</td>
<td>Reproduction</td>
<td>Binding</td>
</tr>
<tr>
<td>05</td>
<td>AWN-1</td>
<td>248304</td>
<td>63219.3 ± 9515.2</td>
<td>Reproduction</td>
<td>NA</td>
</tr>
<tr>
<td>06</td>
<td>Complement C3 precursor (S. scrofa)</td>
<td>47522844</td>
<td>30584.8 ± 6239.9</td>
<td>Cellular process; regulation; response to stimulus</td>
<td>Binding; enzyme regulator activity</td>
</tr>
<tr>
<td>07</td>
<td>Complement factor H precursor (S. scrofa)</td>
<td>47523636</td>
<td>2721.4 ± 795.7</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>08</td>
<td>Adipsin/complement factor D (S. scrofa)</td>
<td>773265</td>
<td>772.5 ± 181.8</td>
<td>Metabolic process; regulation</td>
<td>Catalytic activity</td>
</tr>
<tr>
<td>09</td>
<td>Fc fragment of IgG-binding protein</td>
<td>545830918</td>
<td>977.6 ± 224.1</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>10</td>
<td>Predicted: IgGfC-binding protein-like</td>
<td>545830921</td>
<td>1223.5 ± 194.4</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>11</td>
<td>IgG heavy chain precursor (S. scrofa)</td>
<td>441477616</td>
<td>14479.1 ± 4383.3</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>12</td>
<td>Ig lambda chain V-C region PLC3—pig</td>
<td>89216</td>
<td>1286.2 ± 578.7</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>13</td>
<td>Ig gamma-chain (S. scrofa)</td>
<td>164503</td>
<td>1396.2 ± 442.0</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>14</td>
<td>Ig delta heavy chain membrane bound form, partial (S. scrofa)</td>
<td>22774002</td>
<td>428.9 ± 143.6</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>15</td>
<td>CH4 and secreted domains of swine IgM (S. scrofa)</td>
<td>1236646</td>
<td>1227.1 ± 721.7</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>16</td>
<td>Lactotransferrin (S. scrofa)</td>
<td>41688298</td>
<td>3888.4 ± 838.9</td>
<td>Immune system process; interaction with cells and organisms; localization; metabolic process; regulation</td>
<td>Binding; catalytic activity</td>
</tr>
<tr>
<td>17</td>
<td>Transferrin (S. scrofa)</td>
<td>833800</td>
<td>3053.2 ± 1629.5</td>
<td>Localization; regulation</td>
<td>Binding</td>
</tr>
<tr>
<td>18</td>
<td>Albumin</td>
<td>833798</td>
<td>21006.5 ± 6040.0</td>
<td>Cellular process; localization; regulation</td>
<td>Binding</td>
</tr>
<tr>
<td>19</td>
<td>beta-Microseminoprotein precursor (S. scrofa)</td>
<td>62738431</td>
<td>15335.7 ± 5394.9</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>20</td>
<td>Epididymal secretory protein E1 precursor (S. scrofa)</td>
<td>47523496</td>
<td>20050.6 ± 4688.3</td>
<td>Cellular process; interaction with cells and organisms; localization; metabolic process; regulation</td>
<td>Binding</td>
</tr>
<tr>
<td>Spot</td>
<td>Protein Name</td>
<td>24-cm Gel</td>
<td>pI Range</td>
<td>GO Terms</td>
<td>Protein Functions</td>
</tr>
<tr>
<td>------</td>
<td>--------------</td>
<td>-----------</td>
<td>----------</td>
<td>---------</td>
<td>------------------</td>
</tr>
<tr>
<td>21</td>
<td>Epididymal secretory glutathione peroxidase precursor (S scrofa)</td>
<td>47523090</td>
<td>3 to 10</td>
<td>Response to stimulus</td>
<td>Antioxidant activity</td>
</tr>
<tr>
<td>22</td>
<td>Predicted: epididymal-specific lipocalin 5 (S scrofa)</td>
<td>31124684</td>
<td>3 to 10</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>23</td>
<td>Predicted: fibronectin isoform (S scrofa)</td>
<td>311273023, 545875530</td>
<td>3 to 10</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>24</td>
<td>Lactadherin precursor</td>
<td>172072653</td>
<td>3 to 10</td>
<td>Developmental process; interaction with cells and organisms; reproduction</td>
<td>NA</td>
</tr>
<tr>
<td>25</td>
<td>Inhibitor of carbonic anhydrase precursor (S scrofa)</td>
<td>47523160</td>
<td>3 to 10</td>
<td>Localization; regulation</td>
<td>Binding</td>
</tr>
<tr>
<td>26</td>
<td>Angiotensin-converting enzyme isoform 2 precursor</td>
<td>145279215, 77627998</td>
<td>3 to 10</td>
<td>Metabolic process</td>
<td>Catalytic activity</td>
</tr>
<tr>
<td>27</td>
<td>Haptoglobin precursor</td>
<td>47522826</td>
<td>3 to 10</td>
<td>Interaction with cells and organisms; metabolic process; response to stimulus</td>
<td>Antioxidant activity; catalytic activity</td>
</tr>
<tr>
<td>28</td>
<td>Ceruloplasmin</td>
<td>388890649</td>
<td>3 to 10</td>
<td>Localization; metabolic process; regulation</td>
<td>Binding; catalytic activity</td>
</tr>
<tr>
<td>29</td>
<td>Zinc-alpha-2-glycoprotein (S scrofa)</td>
<td>335284102</td>
<td>3 to 10</td>
<td>Immune system process; regulation</td>
<td>Binding</td>
</tr>
<tr>
<td>30</td>
<td>Cathepsin B precursor</td>
<td>147906534</td>
<td>3 to 10</td>
<td>Cellular process; metabolic process; regulation</td>
<td>Catalytic activity</td>
</tr>
<tr>
<td>31</td>
<td>Arylsulfatase A precursor (S scrofa)</td>
<td>47522624</td>
<td>3 to 10</td>
<td>NA</td>
<td>Catalytic activity</td>
</tr>
<tr>
<td>32</td>
<td>Peroxisiredoxin 5, mitochondrial (S scrofa)</td>
<td>47523086</td>
<td>3 to 10</td>
<td>Metabolic process</td>
<td>Catalytic activity</td>
</tr>
<tr>
<td>33</td>
<td>Predicted: vitamin D-binding protein (S scrofa)</td>
<td>335293644</td>
<td>3 to 10</td>
<td>NA</td>
<td>Binding</td>
</tr>
<tr>
<td>34</td>
<td>Phosphatidylethanolamine-binding protein 4 precursor (S scrofa)</td>
<td>242253862</td>
<td>3 to 10</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>35</td>
<td>Hemoglobin subunit beta (S scrofa)</td>
<td>261245058</td>
<td>3 to 10</td>
<td>NA</td>
<td>Binding</td>
</tr>
<tr>
<td>36</td>
<td>Predicted: ras-related protein Rab-22A isoform X2 (S scrofa)</td>
<td>545881384</td>
<td>3 to 10</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>37</td>
<td>Predicted: CUE domain-containing protein 1-like (S scrofa)</td>
<td>35090532</td>
<td>3 to 10</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>38</td>
<td>Hexosaminidase B (beta polypeptide) (S scrofa)</td>
<td>262072808</td>
<td>3 to 10</td>
<td>Metabolic process</td>
<td>Catalytic activity</td>
</tr>
<tr>
<td>39</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase (S scrofa)</td>
<td>2407184</td>
<td>3 to 10</td>
<td>Cellular process; regulation; other</td>
<td>Binding; catalytic activity</td>
</tr>
</tbody>
</table>

Abbreviations: GO, gene ontology; IgG, Immunoglobulin G; NA, not annotated; NCBI, National Center for Biotechnology Information; PSP, porcine seminal protein; STRAP, software for researching annotations of protein.

This table includes spots detected in 24-cm gels, within the 3 to 10 pH range. Gene ontology and definition of biological processes and molecular function categories were searched using the STRAP tool. Numbers and protein names refer to those shown in Supplementary Figure 1.
includes those with integral membrane protein 2B, apolipoproteins A-I, and A-IV among others. For the case of CH4 and secreted domains of swine IgM, theoretical interactions (Supplementary Fig. 2G) are with signal-regulatory protein alpha and its similar and the von Willebrand factor. Immunoglobulin G-binding protein (Supplementary Fig. 2H) links were detected with bone morphogenetic proteins, tubulin, gamma complex–associated protein, and potassium channel.

3.5. Associations between seminal plasma proteins and sperm traits

Regression models were estimated using identified proteins as independent variables and sperm traits as dependent ones (Fig. 1). The sum of the intensities of spots that contain IgM and another protein FN1 (spots 22, 25, and 87; Supplementary Fig. 1, Table 1) were inversely associated with the variations in sperm midpiece (\( \text{r}^2 = 0.58 \)) and tail (\( \text{r}^2 = 0.40 \)) defects. According to the calculated regression equation, for each part per million increased in the sum of IgM and FN1 spot intensities, the percentage of such defects decreased 0.0025% and 0.0083%, respectively (Fig. 1A, B). Immunoglobulin G–binding protein was also inversely associated with both sperm midpiece (\( \text{r}^2 = 0.41 \)) and tail (\( \text{r}^2 = 0.34 \)) defects. In this case, for each part per million increased in the sum of IgG-binding protein spot (numbers 55–60 and 73, Supplementary Fig. 1, Table 1) intensities, we estimate that sperm midpiece and tail defects decrease 0.00049% and 0.0017%, respectively (Fig. 1C, D). The intensity of spots identified as complement factor H also showed a quadratic relation with sperm midpiece defects (\( \text{r}^2 = 0.61; \) Fig. 1E). Lactadherin (spot numbers 42 and 43), in turn, had a positive association with sperm motility (\( \text{r}^2 = 0.48; \) Fig. 1F) and an inverse relation with the percentage of sperm with midpiece (\( \text{r}^2 = 0.45 \)) and tail (\( \text{r}^2 = 0.74 \)) defects (Fig. 1G, H).

4. Discussion

In the present work, we describe the major seminal plasma proteome of adult boars and report that significant statistical associations exist between several of these proteins (CH4 and secreted domains of swine IgM, FN1, IgG-binding protein, lactadherin, and complement factor H precursor) and sperm traits.

As well known, the boar seminal plasma is a complex mixture of testis, epididymis, and accessory sex glands fluids rich in organic and inorganic elements. Seminal plasma proteins are high-molecular-weight molecules that exert many functions related to sperm development, maturation, transport, and survival in the female reproductive tract, as well as capacitation and acrosome reaction, sperm-egg recognition, and protection against microbial and oxidative damages [5,23–25]. On the basis of the present study, the most abundant proteins of the boar seminal fluid belong to the spermadhesin family, which are glycoproteins expressed in several mammalian species, such as bulls [26,27], rams [17,28], goats [29], peccary [30], and rabbits [31]. In all these species, spermadhesins are usually low abundance components contrasting to what we described for the domestic swine, in which these proteins represented 45.2% of all protein spots detected in the 2D gels constructed with 24-cm strips and stained with colloidal Coomassie. Other studies also reported a large contribution of spermadhesins for the protein content of the seminal plasma of boars and, depending on the method used to evaluate such contribution (chromatography, Western blots), spermadhesins in fact make from 75% to 90% of all proteins expressed in that secretion [25,32,33]. We have not identified all spots in the low-molecular-weight acidic cluster described in the 2D gels (Fig. 1). However, given the outline of these spots, we believe that most of these spots are spermadhesins. Because the total intensity of that cluster represents 78% of all spots, it is plausible thus that the relative participation of spermadhesins in the boar seminal plasma is actually close to this value.

Five spermadhesins were identified in the boar semen: PSP-I, PSP-II, AQN-1, AQN-3, and AWN-1. Spermadhesins PSP-I and PSP-II, the most abundant proteins in the seminal fluid, form a heterodimer and exhibit diverse functional attributes. The PSP-I/PSP-II complex and AQN-1 bind to the acrosome [33] and act to preserve sperm membrane integrity, motility, and mitochondrial activity [34,35]. Porcine seminal proteins control leukocyte invasion into the female reproductive tract once sperm is deposited there [25,36–38]. In fact, our in silico analysis of protein networks indicates that PSP-I/PSP-II interacts with MS4A8B, a transmembrane protein homolog to the CD20 expressed in lymphocytes B and T. Proteins MS4A inhibit propagation of T lymphocytes and signal transduction of B cells [39,40]. Thus, this interaction involving PSPs and MS4A8B probably relate to the protection of sperm against the female immune system.

Spermadhesins AWN-1 and AQN-3, in turn, display phospholipid–binding properties and adhere to the sperm during ejaculation, promoting sperm capacitation and stabilization of the acrosome [41]. In the S. scrofa, such proteins also mediate sperm-zona pellucida binding [42]. As indicated by our STRING-based analysis, both AQN and AWN proteins interact with seminal plasma protein pB1 (Protein DQH) and ADAM3b. Protein DQH forms a spermadhesin complex and exerts phosphorylcholine-binding activity [24]. Protein ADAM3b, in turn, is a type of ADAM component and has the capacity to bind to the zona pellucida [43]. Moreover, AQN appears to network with zonadhesin, another molecule that binds to the zona pellucida [44].

Albumin is present in boar seminal plasma as a series of high-molecular-weight isoforms (76.1–78.0 kDa). Albumin is a common component of seminal plasma and plays a role on protection of sperm against oxidative stress caused by lipid peroxidation. Albumin also seems to act in conjunction with superoxide dismutase and glutathione peroxidase, the latter identified in the present study as the epididymal secretory form, scavenging the reactive oxygen species derived from the cellular metabolism [45]. Protein network analysis also shows (data not shown) that epididymal secretory glutathione peroxidase interacts with transferrin, which is, along with lactotransferrin, an iron-binding protein secreted by Sertoli cells, the epididymal epithelium and the accessory sex glands [46]. Both transferrin and lactotransferrin, also identified in our study, are
iron carriers that prevent sperm lipid peroxidation. Because iron is an important ion to pathogenic microorganisms, transferrin and lactotransferrin may be also defined as antimicrobial proteins [47].

Immunoglobulins were previously associated with autoimmune subfertile men [48]. They bind to antigens present in sperm surface and promote agglutination and immobilization, leading to a lack of cervical mucus penetration and fails in acrosome reaction and in gamete interaction [49,50]. Immunoglobulins (IgG, IgA, IgG, and IgM) have been detected in the seminal plasma of healthy and nonvaccinated adult boars [51]. In the present work, IgG and IgM were also identified in 2D gels of boar seminal plasma, the latter in the form of CH4 and secreted domains of swine IgM. Interestingly, the sum of the intensities of spots that contain IgM and another protein FN1 were inversely associated with the variations in sperm midpiece and tail defects. Our in silico analysis of protein network shows that FN1 interacts with several integrins and the CD44, which are present on the sperm surface and bind to osteopontin, an important fertility-associated protein expressed in the seminal plasma of bulls [52–54]. Fibronectin type 1 also interacts with albumin, as mentioned previously, that has a protective role on sperm. On the basis of such information, we suggest that FN binds to sperm via membrane integrins and by its interaction with albumin may reduce the oxidative stress that occurs in sperm midpiece and tail, the main sector of energy yield for sperm function and major reactive oxygen species generators.

Boar seminal plasma IgG-binding protein was also inversely associated with both midpiece and tail defects in our study. Human seminal plasma IgG-binding proteins reduce the interactions between antisperm antibodies and phagocytic cells [55], and thus it is possible that such proteins decrease the damaging effects of Igs on sperm function and structure. Moreover, on the basis of our in silico network analysis, IgG-binding protein interacts with tubulin gamma complex–associated protein, which has been related to low sperm motility in men [56]. Overall, the presented attributes of IgG-binding proteins would explain the empirical associations with the number of sperms with midpiece and tail defects found in the boar ejaculates. However, as for the case of IgM, the possible mechanism by which these proteins act to reduce specific sperm defects must be further investigated.

The quadratic association between sperm midpiece defects and the intensity of spots identified as complement factor H suggests that an increase in such protein initially reduces the percentage of abnormal cells, but its continuous expression may become deleterious for semen quality. Functions of complement factor H relate to maintenance of the homeostasis and protection of human host cells and tissues from damages caused by complement activation [57]. Boar seminal plasma factor H is mainly secreted by the seminal vesicles and this protein has also been detected on the surface of acrosome. Both seminal plasma and acrosome factor H display the same complement regulatory activity, protecting sperm cells from complement damages in the male and female genital tract [58]. In fact, as evidenced with the STIRNG web-based search, factor H interacts with complement proteins such as C3, factors B and I, and the C-reactive protein, confirming its potential for regulation of the immune system.

Lactadherin, also known as P47 and milk fat globule-epidermal growth factor factor 8 protein (MFGE8), was the only protein showing positive association with sperm motility. Also, in agreement with this association, MFGE8 presented an inverse relation with the percentage of sperm with midpiece and tail defects, as detected for the previously mentioned proteins. Lactadherin has been identified by immunocytochemistry in boar testis and all parts of the epididymis [59]. It seems that newly formed spermatozoa are released in the seminiferous lumen and transported to the epididymis already with lactadherin bound to the acrosome. It is suggested that binding of this protein to sperm may also be regulated when epididymal maturation occurs and during capacitation and acrosome reaction [59]. Because most sperm defects are originated during spermiogenesis and sperm motility is defined during epididymal transit, we hypothesize that lactadherin has an important participation in such events, and its present associations with sperm parameters is strong evidence of it. Also, covering rat sperm with epididymal lactadherin facilitates sperm-egg binding [60], and in boars, lactadherin was isolated from sperm plasma membrane by its affinity to zona pellucida glycoproteins [61]. In fact, in our protein network analysis, lactadherin interacts with integral membrane protein 2B, which is able to bind to sperm. Protein MFGE8 also links with apolipoproteins A1 and A4, which participate in cholesterol metabolism, are expressed in the oviduct fluid, and contribute to sperm capacitation [59]. Another study still reports that lactadherin prevents Escherichia coli binding to intestinal epithelial cells of piglets, acting as a protection promoter [62]. Thus, we believe that lactadherin actions go beyond sperm maturation and capacitation, affecting both the survival of sperm when in contact with bacteria and sperm-fertilizing capacity.

4.1. Conclusions

We presently describe the major proteome of boar seminal plasma, which comprises mainly several classes of spermadhesins. It is also reported that significant associations exist between semen parameters and specific seminal plasma proteins (CH4 and secreted domains of swine IgM, FN1, IgG-binding protein, lactadherin, and complement factor H precursor). Most of these proteins empirically associated with some boar sperm traits are related with immune system, downregulating deleterious factors to the sperm, with the exception of lactadherin, which seems to be involved in modifications of post spermatogenesis, preparing sperm to functional capabilities. Empirical relationships between seminal plasma components and semen traits will serve as the foundation for determination of molecular markers of sperm function and fertility in the swine species.

Acknowledgments

The authors would like to thank “Xerez Avicola Ltda” (Maranguape, CE, Brazil) for allowing the use of animals and facilities. We also appreciate the support from Formil
Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.theriogenology.2014.05.024.

References


