Seminal plasma proteins as potential biomarkers for sperm motility and velocities

Gemma Gaitskell-Phillips, Francisco E. Martín-Cano, José M Ortiz-Rodríguez, Eva da Silva-Álvarez, Javier Massot, Eloy Redondo, Maria C. Gil, Cristina Ortega-Ferrusola, * Fernando J. Peña

Laboratory of Equine Reproduction and Equine Spermatology, Veterinary Teaching Hospital, University of Extremadura, Cáceres, Spain.

*Correspondence to Dr. FJ Peña, Veterinary Teaching Hospital, Laboratory of Equine Spermatology and Reproduction, Faculty of Veterinary Medicine University of Extremadura Avd. de la Universidad s/n 10003 Cáceres Spain. E-mail fjuanpvega@unex.es

phone + 34 927-257167

fax +34 927257102

Acknowledgements

The authors received financial support for this study from the Ministry of Science-FEDER, Madrid, Spain, grants, AGL2017-83149-R Junta de Extremadura-FEDER (GR 18008 and IB 20008). JMOR holds a PhD grant from the Junta de Extremadura- FEDER (PD 18005), Mérida, Spain. GG-P holds a PhD grant from the Ministry of Science, Madrid, Spain (PRE2018-083354).

Abstract

Seminal plasma proteins have important roles in sperm functionality, and different mechanisms including micro-vesicle transport of proteins are involved in the regulation of sperm biology. Due to the role of seminal plasma, we hypothesized that specific proteins present in seminal plasma may be used as discriminant variables with potential to identify stallions producing different quality ejaculates; 10 fertile stallions, with different motility and velocity values (although within normal ranges) were used in this study. Motilities and velocities were studied using computer assisted sperm analysis (CASA), while protein composition of the seminal plasma was studied using UHPLC-MS/MS. Specific proteins were more abundant in samples with poorer percentages of total motility, average path velocity and circular velocity, and were: *Secreted phosphoprotein 1*, *Fructose-bisphosphate aldolase* (p=1,95E-09; q=0.0005) and *Malate dehydrogenase 1* (p=1,41E-11; q= 0.002), to the contrary samples with better straight-line velocity values were enriched in *Glutathione peroxidase (p=0.00013; q=0.04) and Triosephosphate isomerase (p=0.00015; q=0.04).*

Key words: stallion, seminal plasma, proteomics, CASA

1.- Introduction

The stallion ejaculate is composed of a mixture of spermatozoa and the secretions from the accessory sex glands [1]. Numerous proteins are vehiculated in the seminal plasma, mainly in micro vesicles such as prostasomes; microRNAs are also vehiculated in this way [2, 3]. Seminal plasma influences sperm functionality in different ways, for example the presence of high amounts of Annexin A2 may impair the ability of the ejaculates to sustain prolonged conservation periods under refrigeration [4]. However seminal plasma also contains proteins that support sperm metabolism, probably through vesicle mediated transport [4]; this group of proteins support sperm metabolism and also help in the redox regulation of these cells. [2, 3, 5, 6]. Seminal plasma proteins may interact with the spermatozoa vehiculated in micro-vesicles; thus, these proteins may influence and regulate sperm functionality [6]. Nowadays, the use of mass spectrometry allows for the identification of numerous proteins in the spermatozoa and the seminal plasma, in addition to the increased availability of bioinformatic software and on line platforms which allow curation of the information gathered from large data sets [7-10]. We hypothesized that bioinformatic analysis of seminal plasma proteins may provide

candidates for biomarkers of sperm quality in stallions, with potential to be used as discriminant variables to forecast the quality of a particular ejaculate.

2.- Material and methods

2.1.- Reagents and media

All chemicals were purchased from Sigma-Aldrich (Madrid, Spain), unless otherwise stated.

2.2.- Semen collection and processing

Semen was collected from 10 stallions (three ejaculates per stallion) of various breeds maintained as indicated in institutional and European regulations for animal care (Law 6/2913 June 11th and European Directive 2010/63/EU). Data on the reproductive record of the stallions used are presented in supplementary table 1. All stallions were of proven fertility, with a median age of 10.8 years old. Semen was collected from all stallions on a regular basis following the standard protocol in our center in which collections are performed in the morning between 10 and 12 h. Ejaculates used in this study were collected after depletion of the extragonadal sperm reserves, during the 2019 breeding season. The University ethics committee approved this study. Ejaculates were collected using a warmed, lubricated Missouri model artificial vagina, and the gel was removed with an inline filter. Semen was transported immediately to the laboratory after collection for evaluation and processing. Upon arrival at the laboratory, the seminal plasma (SP) was removed by serial centrifugation (2 x 1500g 10') and stored at -80º C until proteomic analysis. An aliquot of the ejaculate was processed through colloidal centrifugation [11, 12] to remove dead spermatozoa, and contaminating cells, and then was re-suspended in Tyrode's medium (20mM HEPES, 5mM Glucose, 96mM NaCl, 15mM NaHCO3, 1mM Na-Pyruvate, 21.6 mM Na-Lactate, 2mM CaCl2*2H2O, 3.1mM KCl, 0.4mM MgSO4*7H2O, 0.3mM NaH2PO4*H2O, 0.3% BSA) 315 mOsm/kg and pH 7.4 [13], for assessment of motility and sperm velocities.

2.3.- Sample preparation

Samples were processed immediately after collection. Aliquots of isolated SP were kept frozen at -80ºC until further analysis. Phase contrast microscopy was used to control the absence of spermatozoa, moreover SP was filtered (0.22μM) before snap freezing and further processing.

2.4.- Protein solubilization and quantification

Aliquots of SP were solubilized in lysis buffer and incubated under constant rotation at 4ºC for 1 hour as described in previous studies [2, 14]. The amount of protein was then normalized to obtain a final concentration of 100 μg of protein per sample.

2.5.- In-solution trypsin digestion.

Trypsin digestion was performed as described in preceding studies [2, 14, 15]. In brief the proteins were mixed with a bicarbonate buffer, reduced with DTT and lastly alkylated. They were then digested by trypsin overnight.

2.6.- UHPLC-MS/MS analysis.

The separation and analysis of the samples were performed as previously described [2, 14, 16] using an Agilent 1290 Infinity II Series UHPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with an automated multisampler module and a high-speed binary pump coupled to an Agilent 6550 Q-TOF Mass Spectrometer (Agilent Technologies, Santa Clara, CA, USA) using an Agilent Jet Stream Dual electrospray (AJS-Dual ESI) interface. Control of the UHPLC and Q-TOF was using Mass Hunter Workstation Data Acquisition software (Agilent Technologies, Rev. B.06.01).

2.7.- Data processing

Data processing and analysis was performed using the Spectrum Mill MS Proteomics Workbench (Rev B.04.01, Agilent Technologies, Santa Clara, CA, USA) as previously described [14].

2.8.- Bioinformatic Analysis

2.8.1.- Variance filtering and PCA

Data were normalized and $\log_2$ transformed using Qlucore Omics Explorer (https://qlucore.com) as described in previous publications [14]. Principal Component Analysis (PCA) was used to visualize the data set in a three-dimensional space, after filtering out variables with low overall variance to reduce the impact of noise and centering and scaling the remaining variables to zero mean and unit variance. The projection score [17] was used to determine the optimal filtering threshold.

2.8.2.- Identifying discriminating variables

Qlucore Omics Explorer Ver. 3.7 (https://qlucore.com) was used to identify the discriminating variables able to find differences in motility, and velocities (VCL, VAP and VSL) among the stallions. This software works by fitting a linear model for each variable with condition proteins of the seminal plasma from stallions showing different values of the above parameters as predictors, including the stallion, breed, and age as nuisance covariates. P-values were adjusted for multiple testing using the Benjamini-Hochberg method [18, 19] and variables with adjusted p-values (q values, equivalent to false discovery rate FDR) below 0.1 were considered significant. Then, to further validate the potential biomarkers previously identified (seminal plasma proteins) we also used the Biomarker Workbench in Qlucore version 3.7.21; this functionality allows the simultaneous analysis of multiple variables. A model including, age, breed, individual stallion, and the computer assisted sperm analysis (CASA) derived parameters: percentage of total motile spermatozoa, percentage of linear spermatozoa, circular velocity (VCL), average path velocity (VAP) and straight-line velocity (VSL) was used. In this analysis variables were considered potential biomarkers only if q values were below 0.05.

2.9.- Computer-assisted sperm analysis (CASA)

Sperm motility and velocities were assessed with a computer-assisted sperm analysis (CASA) system (ISAS Proiser, Valencia, Spain) [12, 20]. Samples were loaded into

Leja® chambers with a depth of 20 µm (Leja, Amsterdam, The Netherlands) and placed on a warmed stage at 38 °C. Sixty consecutive digitized images obtained using a 10x negative phase-contrast objective (Olympus CX 41), and 500 spermatozoa per sample were analyzed in random fields. Spermatozoa with an average path velocity (VAP) > 35 µm/s were considered motile. Spermatozoa deviating < 45% from a straight line were classified as linearly motile.

2.10.- Statistical analysis

The following parameters were measured: percentages of total and linear motile spermatozoa, circular (VCL), straight line (VSL) and average (VAP) velocities in µm/s. The normality of the data was assessed using the Kolmogorov-Smirnoff test. Paired t-tests and one-way ANOVA followed by Dunnett's multiple comparisons test were performed using GraphPad Prism version 8.00 for Mac, La Jolla California USA, (www.graphpad.com).

3.- Results

3.1.- Identification of stallions with different motility and velocities

The percentage of motile sperm ranged from 74.3 ± 6.7 to 92.0 ± 1.1 % ($P< 0.05$; Figure 1). Stallions showing ejaculates with median total motility over 90% were classified as "good", those with total motility over 80% were classified as "average" and those with percentages below 80% were classified as "poor" (Figure 1A). The values for good, average, and poor percentages of linearly motile spermatozoa were 70% 60% and 40% respectively (Fig 1B). Sperm velocities also showed significant variation, in terms of circular velocity, good stallions had VCL values of 212.3±6.7 µm/s, for stallions classified as average VCL was 181.1 ± 6.17 µm/s and for stallions classified as poor VCL was 141.7 ± 10.8 µm/s (Fig 1C). Average path velocity in the good, average, and poor groups was 117.3±1.8, 99.9± 3.0 and 70.3 ± 7.6 respectively (Fig 1D). Finally, VSL was 81.5 ±3.7 µm/s in good stallions, 65.5±2.6 µm/s in average and 51.3± 2.4 µm/s in the poor group (Fig 1 E)

3.2.- Seminal plasma proteins differ in stallions with good and poor motility and velocities

Raw data were uploaded to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD026375 [21] and the **Username:** reviewer_pxd026375@ebi.ac.uk **Password:** CobS4ffZ. Volcano plots were constructed to obtain an overview of seminal plasma proteins differentially expressed in stallions showing poorer motility (Figure 2A), lower VCL and VAP (Figure 2 B and C) and better (faster) VSL (Figure 2D). Next, discriminant variables (p= 9.6e-04; q=0.069) were identified for % total motility for VCL and VAP (p= 8.7x e-4, q=0.0069) and for VSL (p= 0.001; q=0.08). We identified 6 proteins in seminal plasma with discriminant power for stallions with lower percentages of total motility in their ejaculates, then these proteins were further curated (q<0.05) using the biomarker workbench (Table 1) to find the proteins with the highest potential as biomarkers, and *fructose-bisphosphate aldolase* (p=2.56e-5; q=0.0070), *secreted phosphoprotein 1* (p=2.58e-5; q=0.0070) and *malate dehydrogenase 1* (p=0.00015; q= 0.028) were identified as strong biomarkers of ejaculates with poor motility (Figure 3). Discriminant variables were also identified for VCL and VAP (Figures 4 and 5) and in both cases were found to be the same proteins as in the case of the percentages of total motile spermatozoa. However, in the case of straight-line velocity two proteins were identified as being discriminant variables for stallions with higher VSL (Table 1). Higher VSL values were better explained by two variables, *glutathione peroxidase (p=0.00013; q=0.04) and triosephosphate isomerase (p=0.00015; q=0.04).*

4.- DISCUSSION

Stallion ejaculates presenting percentages of total motility and velocities which were different, but within normal ranges, differed in the amounts of specific seminal plasma proteins, present in ejaculates. These findings should be considered in the context of in vitro situation. Many proteins present in seminal plasma are vehiculated in exosomes (epididysomes, prostasomes and vesiculosomes), in fact it has already been discovered that many seminal plasma proteins attach to stallions sperm membranes [22]. Although further research is needed, presence of these proteins in the seminal plasma may reflect the secretory activity of the male genital tract and thus its functionality [3, 6]. Specific

proteins were associated with samples showing lower percentages of total motility, VCL and VAP. To the contrary, two specific seminal plasma proteins were present in higher amounts in samples with higher VSL. Interestingly, the same discriminant proteins were able to predict samples which had poor motility, VCL and VAP. Fructose bisphosphate aldolase catalyzes the reversible reaction that splits fructose 1, 6 bisphosphate into dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3 -phosphate (GP3). These are intermediate metabolites in glycolysis, and in a subsequent step phosphate groups are eliminated. Irreversible elimination of the phosphate groups from DAHP and 3GP forms methylglyoxal (MG) as a by product; this is a 2-oxoaldehyde that is a strong electrophile due to adjacent carbonyl groups that rapidly and spontaneously react with nucleophiles from proteins, lipids and DNA forming advanced glycation end products (AGEs) [23]. These compounds are potentially cytotoxic and mutagenic, although they may also be involved in regulatory functions. Besides, MG can form adducts with superoxide dismutase 1 (SOD1) impairing the antioxidant action of this enzyme and promoting oxidative stress [24]. In relation with this fact recent research from our laboratory identifies SOD1 as one of the most important antioxidant systems in the spermatozoa [14, 25]. Furthermore we have recently described the toxic nature of MG for the stallion spermatozoa [26]. Extenders containing high amounts of glucose, produce high amounts of MG causing sperm malfunction including drops in motility and sperm velocities [26]. This provides an explanation to our findings linking high amounts of this enzyme in seminal plasma and poor motility, VCL and VAP. While relevant potential biomarkers were found for poor motility, average and circular velocity, to the contrary biomarkers for good straight-line velocity were evidenced. *Glutathione peroxidase* and *triosephosphatase isomerase* were biomarkers of good VSL. A BLAST analysis showed that the glutathione peroxidase found in our study had an 89% homology to the *epididymal secretory glutathione peroxidase* from *Sus scrofa*, 85.5% to the same protein in *Canis lupus familiaris* and 79.3% to the same protein in *Homo sapiens*. This enzyme catalyzes the reduction of hydrogen peroxide, lipid peroxides and organic hydroperoxide by glutathione. This protein constitutes a glutathione peroxidase-like protective system against peroxide damage in sperm membrane lipids [27]. It is not surprising that a protein with roles in lipid peroxide detoxification relates to better characteristics of sperm kinematics. Spermatozoa are cells that need a tight redox regulation, and the loss of redox equilibrium rapidly leads to sperm malfunction [5, 14]. Interestingly, our findings may also suggest a different regulation of different aspects of sperm kinematics, suggesting

that motility, circular velocity, and average path velocities, may have similar regulation, while straight line velocity may have distinctive particularities.

In sum, seminal plasma proteins may have a major impact of sperm functionality, and specific seminal plasma proteins may be used as discriminant variables for poor motility, VCL and VAP, while discriminant variables for good VSL were also identified. Validation of these data and further research may help to develop potent biomarkers of sperm functionality which are rapidly applicable in clinical settings. These findings also underpin the role of seminal plasma in sperm functionality.

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Table 1.- The Biomarker Workbench in the bioinformatic software Qlucore Omics Explorer 3.7. (https://qlucore.com) was used to identify seminal plasma proteins able to identify poor and good samples in terms of motility and velocity. The number of variables identified specifically for each trait with a q value <0.05 are given. Variables (seminal plasma proteins SPP) able to discriminate between stallions were found, and after correction for FDR 3 were able to differentiate between stallions with poorer motility, VCL and VAP and 2 proteins were able to identify stallions with better VSL.

Explanatory Variable	Explanatory Variable	Eliminated Factors	SPP p<0.05	SPP q<0.05
Type	Details			
Multi Group Comparison	STALLION		19	0
Two Group Comparison	motility [POOR >All]		17	3
Two Group Comparison	linear motility [POOR >All]		11	0
Two Group Comparison	VAP [POOR >All]		17	3
Two Group Comparison	VCL [POOR >All]		17	3
Two Group Comparison	VSL [GOOD>All]		84	2

Figure legends

Figure 1.- Computer assisted sperm analysis in 10 stallions (3 ejaculates each, n=30). Ejaculates were obtained as described in the material and methods and diluted to $50x10^6$ spermatozoa in Tyrode's media. Aliquots were then loaded into a Leja chamber and at least 500 spermatozoa were analyzed. Stallions were classified into three categories according to their performance in total and linear motility, with stallions showing ejaculates with median percentages of total motility over 90% classified as "good", those with total motility over 80% classified as average and those with percentages below 80% classified as poor. The values for good, average, and poor percentages of linear motile spermatozoa were 70% 60% and 40% respectively. Violin plots were used showing the data distribution in each group. A) Percentage of total motile spermatozoa B) Percentage

391 of linear motile spermatozoa C) Circular velocity (VCL; µm/s) D) Average path velocity
392 (VAP; µm/s) E) Straight line velocity (VSL; µm/s) * $P<0.05$** $P<0.01$, ns=non-
393 significant.
394
395 Figure 2.- Volcano plots showing differentially expressed proteins in the ejaculates of 10
396 stallions, different colors show different values of each protein represented by the z-
397 scores represented in the heat map. The difference of protein content (log_2 fold change)
398 is plotted against the significance of the difference $-log_{10}(p)$ between the two conditions
399 (poor motility vs good and average for % total motility, VAP and VCL, or good versus
400 poor and average in the case of VSL. Three independent ejaculates from 10 different
401 stallions, in addition to two technical replicates (n = 60 samples) were used to derive
402 results from.- A) Differential amounts of proteins in stallions showing poor values of %
403 motility; these stallions showed increased amounts of proteins as seen in the upper right
404 quadrant of the plot (in red). B) Stallions showing poorer values of VCL showing
405 increased amounts of proteins depicted in the upper right quadrant of the plot (in red) C)
406 Stallions showing poorer values of VAP showing increased amounts of proteins in the
407 upper right quadrant (in red). D) Stallions showing better values of VSL had higher
408 amounts of proteins depicted in the upper right quadrant (in green).
409
410 Figure 3.- Discriminant variables retrieved after bioinformatic analysis able to identify
411 stallions with poor total motility, defined as stallions with motility within normal ranges,
412 but below 80% total motility. Qlucore omics explorer bioinformatic software (Lund
413 Sweden) was used to identify these variables, through the comparison of relative amounts
414 of proteins based on spectral counts among stallions classified as good average or poor in
415 terms of total motility. Proteins were Log_2 transformed and normalized, then variables
416 with a corrected p value, $q < 0.05$ were considered as biomarkers; 3 independent
417 ejaculates from 10 different stallions in addition to two technical replicates (n = 60
418 samples) were used to derive results from.
419
420
421 Figure 4.- Discriminant variables for stallions with poor VCL, these were normal fertile
422 stallions but showed a VCL in the lower rank in our group of stallions with an average
423 VCL of 141.7 ± 10.8 µm/s. Qlucore Omics Explorer bioinformatic software (Lund
424 Sweden) was used to identify these variables through the comparison of the relative

amounts of proteins based on spectral counts among stallions classified as good, poor and average in terms of VCL. Proteins were Log_2 transformed and then variables with a corrected p value, $q \leq 0.05$ were considered as biomarkers; 3 independent ejaculates from 10 different stallions in addition to two technical replicates (n = 60 samples) were used to derive results from.

Figure 5.- Discriminant variables for stallions with poor VAP, consisting of those stallions with VAP of 70.3 ± 7.6 8 µm/s. Qlucore Omics Explorer bioinformatic software (Lund Sweden) was used to identify discriminant variables measuring the relative amounts of proteins based on spectral counts among stallions classified as good, poor and average. Proteins were Log_2 transformed and normalized and then variables with a corrected p value, $q \leq 0.05$ were considered as biomarkers; 3 independent ejaculates from 10 different stallions in addition to two technical replicates n = 60 samples were used to derive results from.

Figure 6.- Discriminant variables identifying stallions with good VSL, (mean value of 81.5 ±3.7 µm/s). Qlucore Omics Explorer (Lund Sweden) bioinformatic software was used to compare relative amounts of proteins based on spectral counts among stallions classified as good, poor and average for VSL. Proteins were Log_2 transformed and normalized and then variables with a corrected p value, $q \leq 0.05$ were considered as biomarkers; 3 independent ejaculates from 10 different stallions in addition to two technical replicates (n = 60 samples) were used to derive results from.