Differences in the proteome of the stallion spermatozoa explain stallion to-stallion variability in sperm quality post thaw

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Abstract

Although cryopreservation is becoming a common practice in modern equine breeding, there are still a number of unresolved issues limiting their use. One of the them is the stallion to stallion variability in the freeze-ability (post thaw quality) of their ejaculates. The identification of ejaculates of better freeze-ability before cryopreservation will be of great interest avoiding the waste of time and resources freezing ejaculates that will no reach enough quality to be marketed. Our hypothesis was that the study of the stallion

sperm proteome in poor and good freezers can provide, after bioinformatic analysis, discriminant variables able to predict the freeze-ability of the ejaculate. We froze at least 3 ejaculates from 10 different stallions following a split sample design. Half of the ejaculate was investigated as fresh aliquot and the other half was frozen and then analyzed as frozen-thawed aliquot. Computer assisted sperm analysis and flow cytometry were used to analyze sperm quality. A detailed proteomic analysis was performed in fresh and frozen and thawed aliquots, and a bioinformatic analysis was used to identify discriminant variables. We selected those with a fold change >3, a P= 8.2e-04 and a q=0.074 (equivalent to FDR), and identified the following proteins in fresh samples as discriminant variables of good motility post thaw: F6YTG8, K9K273, A0A3Q2I7V9, F7CE45, F6YU15 and F6SKR3. Other discriminant variables were also identified to predict good membrane potential and good viability post thaw. Is concluded that proteomic approaches are a powerful tool to improve current sperm biotechnologies.

Key words: spermatozoa, UHPLC/MS/MS, proteins, artificial insemination, horse, flow cytometry

Introduction

Cryopreservation is still the most widely used technology for the long-term preservation of male gametes. This technique is applied both to human medicine and animal breeding. Particularly, in horse breeding supports an important international trade of the genetics of stallions of higher value, due to their morphological traits and/or performance in sports[1]. In spite that the technique has been in use since the second half of the past century[2], there are a number of unresolved problems, including, among others, the important breed and stallion to stallion variability in the capacity of their ejaculates to maintain acceptable sperm quality after thawing [3]. During the cryopreservation process, spermatozoa are exposed to the toxicity of the cryoprotectants [4], to hyperosmotic shock during the process of freezing and to hypoosmotic shock at thawing [5,6]. As consequence many spermatozoa succumb during the procedure due to osmotic induced necrosis. A high percentage of the surviving population experience damage in their flagellar machinery, plasma membranes, and mitochondria leading to reduced functionality [1]. The molecular mechanisms behind cryodamage are an osmotic induced necrosis and alteration of the redox regulation and metabolism in the surviving population of spermatozoa that leads to accelerated sperm senescence and finally death. Osmotic stress disturbs mitochondrial membranes, leading to increased superoxide production. Once the redox equilibrium is lost, the excess of Reactive oxygen species (ROS) attacks the lipids of the membranes increasing the production of lipoperoxides that leads to oxidative damage in DNA and proteins causing sperm malfunction and/or demise. Moreover, non-lethal damage to spermatozoa may impact the offspring [7]. The last decade has been witness of the incorporation of new techniques to the study of the sperm biology, particularly the application of proteomics is allowing a rapid advance in the knowledge of the molecular biology of these cells [8-11]. Proteomic analysis of sperm proteins has allowed to unveil numerous aspects of sperm biology, expanding our understanding of these particular cells. These studies have revealed numerous changes in the sperm proteome related to different conditions, including fertile vs infertile patients [12], identified new roles of sperm proteins controlling early embryo development [13], new endogenous metabolic pathways [14] and also have identified differences in the proteome of fractions of the ejaculate with high and low motility [15, 16]. Changes in

the proteome in relation to cryopreservation have been described in different species [11, 17-19]. However, there is no data on how the proteome of the ejaculated spermatozoa is related to the ability of the ejaculates to withstand cryopreservation. The identification of ejaculates with good freeze-ability will be of great interest for the equine industry, freezing ejaculates with poor freeze-ability causes a big waste of time and money that ideally should be avoided if adequate markers of freeze-ability can be developed. Since proteomics is a powerful tool, our hypothesis is that cryopreservation may impact differently in the proteome of good and poor freezers, and that the study of the proteome can provide specific proteins that can be used as discriminant variables in fresh samples to identify ejaculates with superior capacity to survive the cryopreservation process.

MATERIAL AND METHODS

Reagents and media

All chemicals were purchased from Sigma-Aldrich (Madrid, Spain), unless otherwise stated. Hoechst 33342, Ethidium homodimer, JC-1 and CellEvent™ reagents for flow cytometry were purchased from Thermofisher (Carlsbad, Ca USA). DRAQ7 was purchased from Beckman Coulter (Brea, Ca USA).

Semen collection and processing

Semen was collected from 10 Purebred Spanish horses (PRE) maintained as indicated in specific institutional and European regulations for animal care (Law 6/2913 June 11th and European Directive 2010/63/EU). The ethical committee of the University approved this study. Ejaculates were collected using a warmed and lubricated Missouri model artificial vagina. The gel was removed with an inline filter. Semen was immediately transported to the laboratory after collection for evaluation and processing. The experimental design employed a split sample approach, with single ejaculates divided in two subsamples (fresh and frozen thawed experimental groups). Upon arrival at the laboratory, the semen was processed through colloidal centrifugation [20, 21] to remove dead spermatozoa and seminal plasma, and then one aliquot was re-suspended in modified BWW media 95 mM NaCl, 4.7 mM KCl, 1.7 mM CaCl2·2H2O, 1.2 mM KH2PO4, 1.2 mM MgSO4·7H2O, 25 mM NaHCO3, 5.6 mM D-glucose, 275 µM sodium pyruvate, 3.7 µl/ml 60% sodium lactate syrup, 50 U/ml penicillin, 50 µg/ml streptomycin, 20 mM HEPES, and 0.1% (w/v) polyvinyl alcohol, 290 and 310 mOsm/kg and pH 7.4 [22](fresh extended semen), and the other aliquot was re-suspended in freezing media and frozen using standard procedures that have been previously described by our laboratory (frozen thawed semen) [23], In brief the aliquot was diluted in the freezing medium Cáceres (University of Extremadura Cáceres, Spain) containing 2% egg yolk, 1% glycerol, and 4% dimethylformamide to 100 $x10^6$ spermatozoa/ml. After loading the extended semen into 0.5-mL straws (IMV, L'Aigle, France), the straws were ultrasonically sealed with UltraSeal 21® (Minitube of America MOFA, Verona, Wisconsin, USA) and immediately placed in an IceCube 14S (SY-LAB Neupurkersdorf, Austria) programmable freezer. The following freezing curve was used. Straws were kept for 15 min at 20ºC, and they were then slowly cooled from 20ºC to 5ºC at a cooling rate of 0.1 ºC/min. Thereafter the freezing rate was increased to -40ºC/min from 5ºC to -140ºC. The straws were then plunged into liquid nitrogen and stored until analysis. Frozen samples were thawed in a water bath at 37°C for at least 30 sec.

Experimental design

Three independent ejaculates from 10 different stallions were collected and processed as follows. Half of the ejaculate was frozen using standard protocols in our laboratory (Frozen/thawed), the other half was processed as fresh spermatozoa (Fresh).

Sperm preparation

The spermatozoa (fresh and frozen thawed samples) were washed three times in PBS (600gx 10') and fresh and FT samples pelleted and kept frozen at -80ºC until analysis.

Protein solubilization

Isolated spermatozoa (200 x10^6 spermatozoa) were solubilized in lysis buffer (C7:C7Bz0 [3-(4-heptyl) phenyl-(3-hydroxypropyl) dimethylammoniopropanesulfonate], 7M urea, 2M thiourea and 40 mM Tris (pH 10.4); 20 microliters of lysis buffer was added per each 10 x 10^6 spermatozoa vortexed and incubated under constant rotation at -4ºC for 1 hour.

Protein Quantification

Protein quantification was performed using the 2-D Quant Kit (GE Healthcare, Sevilla Spain) following the instructions of the manufacturer: https://www.gelifesciences.co.jp/tech_support/manual/pdf/80648622.pdf. All samples were normalized to obtain a final concentration of 100 µg of protein per sample.

In-solution Trypsin digestion.

200 µL of solution obtained from the previous stage were mixed with 100 µl of 25 mM ammonium bicarbonate buffer pH 8.5 (100µg of protein in 300µL of solution). In this solution, the proteins were reduced by adding 30 µL of 10 mM DTT and incubated at 56 °C for 20 min. Then, the proteins were alkylated by adding 30 µL of 20 mM IAA and incubating 30 min at room temperature in the dark. Finally, digestion was performed by adding 1 µL of Trypsin Proteomics Grade (Sigma) (Trypsin solution: 1 µg/µL in 1mM HCl) during at least 3 h to overnight at 37 °C. Reaction was stopped with 10 µL of 0.1% formic acid and filtered through 0.2 µm (hydrophilic PTFE) to 2 mL dark glass vial. Finally, samples were dried using a nitrogen current with the vial in a heating block at 35ºC. The dry samples were resuspended in 20 µl of buffer A, consisting in water/acetonitrile/formic acid (94.9:5:0.1)

UHPLC-MS/MS analysis.

The separation and analysis of the samples were performed with a UHPLC/MS system consisting of 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, and 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. The control of the HPLC and Q-TOF were made by the MassHunter Workstation Data Acquisition software (Agilent Technologies, Rev. B.06.01). The sample was injected onto an Agilent AdvanceBio Peptide Mapping HPLC column (2.7 µm, 150 × 2.1 mm, Agilent technologies), thermostatted at 55 °C, at a flow

rate of 0.4 ml/min. This column is suitable for peptide separation and analysis. The gradient program started with 2% of B (buffer B: water/acetonitrile/formic acid, 10:89.9:0.1) that stayed 5 min in isocratic mode and then it increased linearly up to 45 % B in 40 min, and then it increased up to 95 % B in 15 min and it remained constant for 5 min. After this 70 min of run, 5 min of post-time followed using the initial condition for the conditioning of the column for the next run. The mass spectrometer was operated in the positive mode. The nebulizer gas pressure was set to 35 psi, whereas the drying gas flow was set to 10 l/min at a temperature of 250 °C, and the sheath gas flow was set to 12l/min at a temperature of 300 ºC. The capillary spray, fragmentor and octopole RF Vpp voltages were 3500 V, 340 V and 750 V respectively. Profile data were acquired for both MS and MS/MS scans in extended dynamic range mode. MS and MS/MS mass range were 50-1700 m/z and scan rates were 8 spectra/sec for MS and 3 spectra/sec for MS/MS. Auto MS/MS mode was used with precursor selection by abundance and a maximum of 20 precursors selected per cycle. A ramped collision energy was used with a slope of 3.6 and an offset of -4.8. The same ion was rejected after two consecutive scans.

Data processing

Data processing and analysis was performed using Spectrum Mill MS Proteomics Workbench (Rev B.04.01, Agilent Technologies, Santa Clara, CA, USA). Briefly, raw data were extracted under default conditions as follows: non fixed or variable modifications were selected; [MH]+ 50–10000 m/z; maximum precursor charge +5; retention time and m/z tolerance ± 60 seconds; minimum signal-to-noise MS (S/N) 25; finding ^{12}C signals. The MS/MS search against the appropriate and updated protein database (in this case: Uniprot/Horse) was performed with the following criteria: non fixed modifications were selected and as variable modification: carbamidomethylated cysteines was selected; tryptic digestion with 5 maximum missed cleavages; ESI-Q-TOF instrument; minimum matched peak intensity 50%; maximum ambiguous precursor charge +5; monoisotopic masses; peptide precursor mass tolerance 20 ppm; product ion mass tolerance 50 ppm; and calculation of reversed database scores. The autovalidation strategy used was Auto-threshold, in which the peptide score is automatically optimized for a target % FDR (1.2%). Then the protein polishing validation was performed in order to increase the sequence coverage of validated results with the restriction of a new maximum target protein FDR (0 %).

Computer-assisted Sperm Analysis (CASA)

Sperm motility and velocity were assessed in fresh and frozen thawed samples using a Computer-Assisted Sperm Analysis (CASA) system (ISAS Proiser, Valencia, Spain) according to standard protocols used at our center [24]. Semen samples were loaded in a Leja® chamber with a depth of 20 µm (Leja, Amsterdam, The Netherlands) and placed on a stage warmed at 37 °C. Analysis was based on an evaluation of 60 consecutive digitized images obtained using a 10x negative phase-contrast objective (Olympus CX 41). At least 500 spermatozoa per sample were analyzed in random fields. Spermatozoa with a VAP > 35 µm/s were considered motile. Spermatozoa deviating < 45% from a straight line were classified as linearly motile.

Flow cytometry

Flow cytometry analyses were conducted using a Cytoflex® flow cytometer (Beckman Coulter, Brea Ca USA) equipped with violet, blue, yellow and red lasers. The instrument was calibrated daily using specific calibration beads provided by the manufacturer. A compensation overlap was performed before each experiment. Files were exported as FCS files and analyzed using FlowjoV 10.6.1 Software (Ashland, OR, USA). Unstained, single-stained, and Fluorescence Minus One (FMO) controls were used to determine compensations and positive and negative events, as well as to set regions of interest as described in previous publications by our laboratory [25, 26]

Measurement viability and mitochondrial membrane potential in stallion spermatozoa

The mitochondrial membrane potential and sperm viability were simultaneously assessed. Sperm aliquots (1-5 x 10^6 sperm/mL) were stained with JC-1 1μM, (30 minutes in the dark at r.t.) and DRAQ7 3μM (10 minutes in the dark at r.t). Briefly, after assessment of the quality of the flow, doublets and debris were gated out, JC-1 was detected at a peak excitation of 488 and emission 525 nm (monomers) and excitation of 511 nm and emission of 596 nm (aggregates) and DRAQ7, at a peak excitation of 640, and emission of 690 nm.

Assessment of viability and caspase 3 activity

Samples were loaded with Hoechst 33342 (0.3 μM) and CellEvent™ (2 μM) and incubated at room temperature for 25 minutes. To gate dead spermatozoa samples were stained with 0.3 μM of Eth-1 and incubated for further 5 minutes before they were immediately evaluated in a flow cytometer (Cytoflex® flow cytometer, Beckman Coulter). CellEvent™ staining was validated as previously described [30]

Variance filtering and PCA

Data were normalized and $\log_2$ transformed using Qlucore Omics Explorer (https://qlucore.com). 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 [31] was used to determine the optimal filtering threshold.

Identifying discriminating variables

Qlucore Omics Explorer (https://qlucore.com) was used to identify the discriminating variables that are most highly significantly different between the two subgroups fresh and frozen thawed spermatozoa in good and poor freezers. The identification of significantly differential variables between the subgroups of good and poor freezers from each single ejaculate was performed by fitting a linear model for each variable with condition proteins in fresh samples of good freezers as a predictor, including the stallion nuisance covariate. P-values were adjusted for multiple testing using the Benjamini-Hochberg method [32, 33] and variables with adjusted p-values below 0.1 were considered significant.

Statistical analysis

The normality of the motility, sperm velocity and flow cytometry data were 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).

Results

Sperm characteristics in good and poor freezers

Good or bad freezers were classified according to its total motility (CASA) post thaw, with those showing values > 35% considered as good freezers and those showing values < 35% considered as poor freezers (Fig 1). Highly significant differences were observed between good and poor freezers in all the motility and velocity parameters (Fig 1). Additionally, we established two additional categories. The first depending of the viability at thawing, good showing > 40% intact membranes post thaw and poor <40 % intact membranes. The third category considered the mitochondrial activity at thawing, with good freezers showing > 40% of spermatozoa showing high mitochondrial membrane potential at thawing, and poor freezers those showing less that 40% of spermatozoa with high mitochondrial membrane potential at thawing. Average values for the groups of good and poor freezers in these categories are given in fig 2.

Motility in fresh samples does not predict the freeze-ability of the ejaculate

Motility in fresh semen was not different in good and poor freezers (Fig 3). Total motility in the good freezers group of stallions was 84.3 ± 1.8 % and in the poor group 84.8 ± 2.6% (Fig 3 B). The percentage of linear motile spermatozoa was higher in the group or poor stallions 66.6 ± 2.2% that in the group of good stallions 56.3 ± 4.1 % (P<0.05) (Fig 3 C). Sperm velocities were not different between both groups of stallions (Fig 3 D-F). However, differences in the proteome in good and poor freezers were evident in the Volcano plot (Fig 3 A). Moreover, we constructed a Venn diagram showing changes between fresh and frozen thawed sperm, and those present in good freezers in each of the three categories defined (Fig 4).

The spermatozoa of good and por freezers differ in the amount of specific proteins

Motility post thaw

We initially identified how cryopreservation differently modifies the proteome of good and poor freezers. We used Qlucore Omics explorer to identify the discriminant variables that are most highly significantly different between the subgroups of fresh and frozen thawed samples in good and poor freezers in terms of motility post thaw. With a q-value cut-off of 0.1 and a fold change >2, significant differences in the response to cryopreservation between the groups of good and poor freezers were observed (Fig 5 A-B). Using Venn diagrams, we identified 73 proteins present in both groups (good and poor freezers), 24 proteins present only in good freezers and 22 proteins present only in poor freezers (Fig 5 C). Then, in order to reduce the number of proteins and obtain a few proteins with the highest discriminant power, we selected the most significant variables in fresh samples able to discriminate between good and poor freezers. We selected those with a fold change >3, a P= 8.2e-04 and a q=0.074 (equivalent to FDR), and identified the following proteins as discriminant variables between good and poor freezers in terms

of motility post thaw: F6YTG8, K9K273, A0A3Q2I7V9, F7CE45, F6YU15 and F6SKR3 (Fig. 6), corresponding to *Mannosidase alpha class 2C member 1, Mitochondrial NADH dehydrogenase (Ubiquinone) 1 alpha subcomplex subunit 9-like protein, Isoleucyl-tRNA synthetase 2, mitochondrial, Acetyl-CoA acetyltransferase 1, Latherin, and Ubiquitin specific peptidase 43*.

Mitochondrial activity post thaw

The response to cryopreservation was studied in good and poor freezers in terms of mitochondrial potential post thaw. We identified changes between fresh and frozen samples in these two groups with p=0.01 and q= 0.1 and a fold change > 2 between both conditions. Both good freezers (Fig 7A), and poor freezers (Fig 7B) showed increased amounts in some proteins and reduced amounts in others. The Venn diagram showed that 94 proteins were present in both groups, but 34 proteins were only present in good freezers and 35 proteins were only present in poor freezers (Fig 7 C). Next we searched discriminant variables in fresh semen able to forecast good mitochondrial membrane potential post thaw. Variables with a fold change> 3, p=4.38e-04 and q=0.099 were identified in fresh samples showing better percentages of active mitochondria post thaw. The discriminant proteins were, F7A616, K9KDP8, A0A3Q2HAZ2, A0A3Q2L2U8, corresponding to *Phosphoglycerate mutase*, *Peroxiredoxin 6 like protein*, an uncharacterized protein similar to *actin-1* and a second uncharacterized protein similar to the transmembrane protein named *GRAM domain containing 1A*. (Fig 8)

Viability post thaw

Samples showing percentages over or below 40% intact membranes post thaw (good and poor freezers) showed different changes in the sperm proteome as consequence of cryopreservation. Differences in fresh and frozen thawed samples in both groups were observed with a fold change > 2 p=0.01 and q=0.1. (Fig 9 A-B). Venn diagrams revealed 92 proteins present in both good and bad freezers, with 19 proteins only present in poor freezers and 64 were present only in good freezers (Fig 9 C). Next we searched for discriminant variables in fresh semen able to the identification of good and poor freezers in term of viability post thaw. We selected variables with a fold change >3, a p= 0.01 and q= 0.08, retrieving two proteins serving as discriminant variables, *Angiotensin I converting enzyme* and *Phosphoglycerate mutase* (Fig 10).

Discussion

In the present study we investigated changes in the proteome in good and poor freezers and how the proteome can be used to discriminate between them. These were classified under three categories, motility, viability and mitochondrial membrane potential post thaw. We studied whether the impact of cryopreservation on the proteome differed between groups and whether specific proteins in fresh spermatozoa can be used as discriminant variables between good and poor freezers. Noteworthy, sperm motility and velocities in fresh samples did not predict motility post thaw, and unexpectedly, even the percentage of linear motility in fresh samples was higher in poor freezers (Fig 1). However, differences in the amount of proteins in fresh samples were evident between poor and good freezers. Also, cryopreservation had a different impact in the proteome of the stallion spermatozoa in both groups.

Although cryopreservation had a similar impact in good and poor freezers, causing reduction of the levels of some proteins and increases in others, there were specific proteins associated to good freezers. We identified six proteins in fresh samples, able to identify the group of good freezers showing better motility post thaw. Three of them were mitochondrial proteins (K9K273, A0A3Q2I7V9 and F7CE45), stressing the importance of these organelle for sperm function [34, 35], and particularly producing ATP for sperm motility through oxidative phosphorylation. The F6YTG8 (*alpha mannosidase*) is a protein with a role in the catabolism of oligosaccharides [36]. The alpha mannosidase activity prevents accumulation of oligosaccharides. More recently a role preventing mitochondrial dependent apoptosis has been proposed [37]. Since an important proportion of the damage occurring during cryopreservation involves a mitochondrial apoptotic pathway [29, 38], the aforementioned function of this protein will provide an explanation for our findings. Moreover, a study in ovine semen found a positive correlation between alpha mannosidase and freeze-ability [39]. The F7CE45, *Acetyl- CoA acetyltransferase 1*, catalyzes the last step in the mitochondrial beta oxidation pathway [40], and also plays a major role in ketone body metabolism [41]. Spermatozoa are able to obtain energy for motility using the beta oxidation pathway [10, 14] providing an explanation for the link between a major presence of this protein in fresh samples and better motility post thaw. Finally, we described for the first time the presence of *Latherin* (F6YU15) in the spermatozoa. This is present in the saliva and sweat of horses and has strong surfactant properties [42, 43]. This activity is responsible of the foam formed in the skin of horses during vigorous exercise. It is not clear the possible function of this protein in the spermatozoa, although antibacterial properties inhibiting the growth of biofilms [44] have been attributed to latherin. A potential contribution of sperm latherin to endometrial health is a tempting possibility that warrants further research.

Cryopreservation also caused a different impact in ejaculates showing good and poor mitochondrial activity post thaw. The Venn diagram revealed 94 common proteins, 34 proteins specific for the group showing high mitochondrial membrane potential post thaw, and 35 for the group showing poor mitochondrial membrane post thaw. We next searched for discriminant variables in fresh semen, and four proteins were potent discriminant variables for the prediction of good mitochondrial membrane potential post thaw. The *peroxiredoxin like 6 protein* was more abundant in the ejaculate of good freezers; peroxiredoxin 6 is considered as one of the major antioxidant defenses of the spermatozoa[45, 46], and taking into account that a high percentage of cryodamage come from oxidative stress[47, 48], is not surprising that samples richer in this antioxidant protein sustain better the process of cryopreservation. A glycolytic enzyme, *Phosphoglycerate mutase (PGAM)*, was also more abundant in good freezers. This enzyme is upregulated in many cancer cells [49]. This enzyme catalyzes the conversion of 3-phosphoglycerate (3-PG) to 2-phosphoglycerate (2-PG) during the glycolysis. In cancer cells that overexpress this protein, there is an increase of 2-PG and a decrease in 3-PG. Also, these cells express higher levels of lactate and increased flux through the pentose phosphate pathway[49], thus producing more reducing power in the form of NADPH. This mechanism may also explain the enhanced cryo-survival of ejaculates with higher levels of PGAM in stallions and warrants further research on the interaction between redox metabolism and redox regulation in the spermatozoa. The *GRAM domain containing 1A* was also more abundant in the spermatozoa of good freezers; this is a cholesterol transfer protein, with a role in the early steps of the autophagosome formation [50]. These functions may explain the major presence of this protein in good freezers

since mitophagy has recently been related to sperm quality [51]. Finally, an uncharacterized protein similar to *actin-1* was also more abundant in good freezers; a tempting possibility is that this protein is also related to mitophagy. Actin structures cages damaged mitochondria during mitophagy [52], however further research is warranted to characterize this protein and identify its roles in the spermatozoa.

Cryopreservation also had a different impact in the groups showing good and poor membrane integrity post thaw. The Venn diagram revealed 92 common proteins in both groups, 64 specific of the group showing high viability post thaw and 19 specific of the group showing poor motility post thaw. Next we performed bioinformatic analysis to reveal discriminant variables in fresh semen able to predict the viability after thawing.

The proteins *Angiotensin I converting enzyme* and *Phosphoglycerate mutase* were more abundant in samples showing better membrane integrity post thaw. *Phosphoglycerate mutase* was also present in samples showing better mitochondrial activity post thaw, and the reasons previously discussed are applicable. Moreover, this is a candidate protein of overall freeze-ability, since was discriminant in two groups of good freeze-ability, viability and mitochondrial membrane potential. *Angiotensin I converting enzyme* was a discriminant variable for samples showing good viability post thaw. This protein has a potential role in capacitation[53], being released during this process[54] and has been observed that cryopreservation reduces the presence of this protein in bull spermatozoa[55]. Angiotensin 1 converting enzyme in addition to its role as regulator of blood pressure has important roles in fertilization, through its glycosylphosphatidylinositol (GPI)-anchored protein releasing activity (GPIase activity)[56, 57]. The presence of higher amounts of this enzyme in good freezers is of interest and has potential rapid applications in the field of reproductive technologies.

In conclusion, cryopreservation impacts in a different manner different sperm functions and structures in good and poor freezers. Changes in specific proteins occur in these groups. On the other hand, specific proteins in fresh samples can be used as discriminant variables to potentially predict the response of specific ejaculates to cryopreservation. It is noteworthy that many of these proteins were mitochondrial, stressing the importance of these organelle for spermatozoa functionality. Also, proteins related to redox regulation and energetic metabolism were more abundant in good freezers. The data reported here provide a strong basis for further research in the molecular damage occurring during cryopreservation and paves the way for the development of simple assays to assess the freeze-ability of an ejaculate prior to cryopreservation.

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Figure legends

Fig 1.- Layout 1. 30 stallion ejaculates were frozen and thawed as described in material and methods. Based in their post thaw motility were classified in good (> 35% motility) or poor freezers (<35 % motility post thaw). Computer assisted sperm analysis (CASA) was used to analyze sperm quality after thawing. a) percentage of total motile spermatozoa in good and poor freezers b) percentage of linear motile spermatozoa in good and poor freezers. Layout 2. A) Circular velocity (VCL) B) Straight line velocity (VSL) and C) Average path velocity (VAP) in good and poor freezers. Data are means ± s.e.m. **** $P<0.001$, ** $P<0.01$

Fig 2.- Viability and mitochondrial membrane potential in good and poor freezers. 30 stallion ejaculates were frozen and thawed as described in material and methods. Samples were processed for flow cytometry analysis as described in material and methods. A) Viability (intact membranes and negative for caspase 3 spermatozoa) B) Caspase 3 positive spermatozoa C) Spermatozoa depicting high mitochondrial membrane potential in good and poor freezers. a-b) representative cytograms of the viability analysis, c-d) representative cytograms of the mitochondrial membrane potential analysis. Data are means ± s.e.m. **** $P< 0.001$.

Fig 3.- Computer Assisted Sperm and shot gun proteomic analysis in pre-freezing samples. A) Volcano plot showing differences in protein expression in good versus bad freezers B) Percentages of total motile spermatozoa in fresh samples of good and poor freezers C) Percentages of linear motile spermatozoa in fresh samples of good and poor freezers D) Circular velocity E) Average path velocity E) Straight line velocity. Data are means ± s.e.m. * $P<0.05$

Fig 4.- Venn diagram showing proteins significantly enriched in fresh spermatozoa and those in each category of good freezers, good motility, good mitochondrial membrane potential and good viability post thaw.

Fig 5. Layout 1- Heat map showing the impact of cryopreservation in the proteome of stallions showing good or poor motility post thaw. Proteins are classified following a hierarchical clustering. Fresh samples correspond to the blue marks, frozen thawed samples correspond to the yellow marks. The heat map code is present with red areas representing more amount of protein and green areas less amount of protein. Proteins were normalized, filtered by a fold change >2 with p=0.01 and q=0.1 A) good freezers B) poor freezers Layout 2- Venn Diagram showing significant changes caused by cryopreservation (q-value cut-off of 0.1 and a fold change >2) in amounts of proteins of stallions showing good and poor motility post thaw. 73 proteins were present in both

groups 24 proteins were only present the group of good freezers and 22 proteins were present only in the group of poor freezers.

Fig 6.- Discriminant variables between good and poor freezers in terms of motility post thaw F6YTG8, K9K273, A0A3Q2I7V9, F7CE45, F6YU15 and F6SKR3 corresponding to *Mannosidase alpha class 2C member 1, Mitochondrial NADH dehydrogenase (Ubiquinone) 1 alpha subcomplex subunit 9-like protein, Isoleucyl-tRNA synthetase 2, mitochondrial, Acetyl-CoA acetyltransferase 1, Latherin and Ubiquitin specific peptidase 43*. Proteins showing a fold change >3, a P= 8.2e-04 and a q=0.074 (equivalent to FDR)

Fig 7 Layout 1.- Heat map showing the impact of cryopreservation in the proteome of stallions showing good or poor mitochondrial membrane potential post thaw. Proteins are classified following a hierarchical clustering. Fresh samples correspond to the blue marks, frozen thawed samples correspond to the yellow marks. The heat map code is present with red areas representing more amount of protein and green areas less amount of protein. Proteins were normalized, filtered by a fold change >2 with p=0.01 and q=0.1 A) good freezers B) poor freezers. Layout 2 Venn Diagram showing significant changes caused by cryopreservation (q-value cut-off of 0.1 and a fold change >2) in amounts of proteins of stallions showing good and poor mitochondrial membrane potential post thaw. 94 proteins were present in both groups 34 proteins were only present the group presenting good mitochondrial membrane potential post thaw and 35 proteins were present only in the group presenting poor mitochondrial membrane potential post thaw.

Fig 8.- Discriminant variables between good and poor freezers in terms of mitochondrial membrane potential post thaw. The discriminant proteins were, F7A616, K9KDP8, A0A3Q2HAZ2, A0A3Q2L2U8, corresponding to *Phosphoglycerate mutase, Peroxiredoxin 6 like protein,* an uncharacterized protein similar to actin-1 and a uncharacterized protein similar to the transmembrane protein named *GRAM domain containing 1A.* Variables showing a fold change> 3, p=4.38e-04 and q=0.099.

Fig 9 Layout 1.- Heat map showing the impact of cryopreservation in the proteome of stallions showing good or poor membrane integrity post thaw. Proteins are classified following a hierarchical clustering. Fresh samples correspond to the blue marks, frozen thawed samples correspond to the yellow marks. The heat map code is present with red areas representing more amount of protein and green areas less amount of protein. Proteins were normalized, filtered by a fold change >2 with p=0.01 and q=0.1 A) good freezers B) poor freezers. Layout 2.- Venn Diagram showing significant changes caused by cryopreservation (q-value cut-off of 0.1 and a fold change >2) in the amounts of proteins of stallions showing good and poor membrane integrity post thaw. 92 proteins were present in both groups 64 proteins were only present the group presenting good membrane viability post thaw and 19 proteins were present only in the group presenting poor membrane viability post thaw.

Fig 10.- Discriminant variables between good and poor freezers in terms of viability post thaw, *Angiotensin I converting enzyme* and *Phosphoglycerate mutase*. Proteins with a fold change >3, a p= 0.01 and q= 0.08