Proteomics is advancing the understanding of stallion sperm biology.

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Abstract

The mammalian ejaculate is very well suited for proteomics studies. As such, investigations on the sperm proteomics are offering a huge amount of new information on the biology of the spermatozoa. Among domestic animals, horses represent a special interest species, in which reproductive technologies and an important market of genetic material has growth exponentially in the last decade. Investigations using proteomic approaches have been conducted in recent years, showing that proteomics is a potent tool to dig into the biology of the stallion spermatozoa. The aim of this review is to present an overview of the research conducted, and how these studies have improved our knowledge of the stallion sperm biology. The main outcomes of the research conducted so far have been an improved knowledge of the metabolism, and its importance of sperm functions, the impact of different technologies in the sperm proteome, and the identification of potential biomarkers. Moreover, proteomics of the seminal plasma and phosphoproteomics are identified as areas of major interest.

INTRODUCTION

The equine industry contributes significantly to the economy in many different regions of the world. This is an animal breeding industry with a great degree of diversification and high added value. In this context, reproductive technologies are an important component of this industry. Commerce of seminal doses of highly valuable stallions is at the centre of the equine industry, with artificial insemination being the most widely used reproductive technology, which, in addition, constitutes the basis for embryo transfer and in vitro production of embryos. Spermatozoa are cells which are especially suited for proteomics and other omics analysis since they are cells in suspension, easy to isolate, and barely contaminated with other cell types in the case of equines. Moreover, the independent study of the two major components of sperm anatomy, the nucleus, and tails, can be performed easily after separating these components. The separation of specific subpopulations of the ejaculate allows the independent study of sperm fractions with different functionality [1]. The last decade has witnessed an explosion of proteomics, and other omics studies of the mammalian spermatozoa, including equines. This effort in research has provided a huge amount of new information regarding the biology of the stallion spermatozoa, mutually reinforcing other mechanistic studies, and opening new lines of research leading to significant improvements to our understanding of stallion sperm biology. Another aspect of major importance is the fact that stallions, in addition to reaching advanced ages, are exposed to similar environmental contaminants as humans, thus the study of the equine ejaculate in addition to the importance for the equine industry may also provide clues applicable to human andrology.

As far as the authors know, the first comprehensive study of the proteome of the stallion spermatozoa was conducted by Australian researchers at the University of Newcastle [2]. Since this first study, proteomic and phospho-proteomic approaches have been introduced to the study of stallion sperm biology [3-11]. This

review aims to provide an updated overview of the major findings and advances in the comprehension of the stallion spermatozoa obtained thanks to the introduction of proteomics to the study of stallion sperm biology, and the impact of these findings on sperm biotechnology in equines.

Proteomics

Nowadays the approach to the study of sperm and seminal plasma proteomics is the UHPLC/MS/MS, an approach in which the peptides are separated using liquid chromatography, followed by tandem mass spectrometry [1]. The equine ejaculate is a mixture of a liquid component, the seminal plasma, and a cellular component, the spermatozoa. Although the main component of the cellular fraction is the spermatozoa, other cell types may be present, such as epithelial cells, immature cells from the germinal epithelium, and leucocytes, although the latter are not common in stallions in comparison to humans. Moreover, different populations of spermatozoa are in different physiological states. Although, as previously mentioned, the ejaculate is a cellular suspension especially suitable for proteomics studies, careful experimental design and sample pre-processing must be conducted. Ejaculates can be pre-processed using colloidal centrifugation, to separate seminal plasma, and to remove dead spermatozoa and potentially contaminating cells (Figure 1). The nature of the spermatozoa with highly compacted and transcriptionally silent chromatin, mean spermatozoa are a transcriptionally silent cell, apart from limited transcriptional activity in the mitochondria, and this makes these cells highly dependent on post-transcriptional modifications of proteins and/or protein trafficking for cellular regulation. This fact makes the study of post-translational modifications of special interest in these cells[12]. Among the most frequent post-translational protein modifications are, phosphorylation, acetylation, and O/N-linked glycosylation[12-16].

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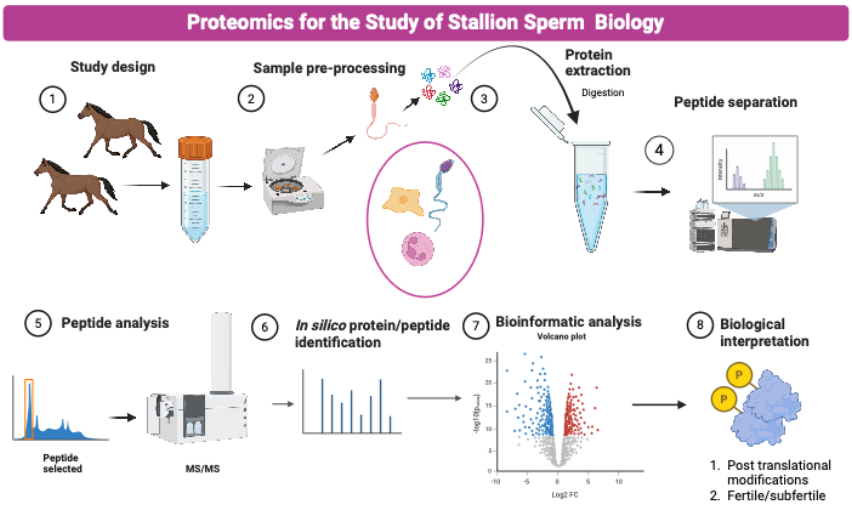

Figure 1.- Basic principles for the use of sperm proteomics in the study of stallion sperm biology. The initial step is the study design based on a well-formulated hypothesis. Once the sample size is determined, samples are collected, and a pre-processing step is necessary. Although the equine ejaculate is not usually contaminated by many other cells, epithelial cells and defective spermatozoa may be found that must be removed by colloidal centrifugation (purple circle). The next step involves protein extraction, digestion, and peptide separation using liquid chromatography, then peptides are analyzed by MS/MS and identified based on their relation mass/charge (m/z) using specific software like Spectrum Mill or MASCOT to perform "in silico" searches in proteomics databases. Bioinformatics analysis is then performed using different options, including commercial software like Qlucore Omics Explorer or free software like ProteoMill, or R-based packages.

Finally, biological interpretation and further confirmatory experiments are conducted. Cautious interpretation of increases in protein content must be performed due to the transcriptionally silent characteristics of the spermatozoa. Since sperm physiology is highly regulated by post-transcriptional modification of sperm proteins, the study of these modifications is of special interest in spermatology.

Descriptive sperm proteomics

The stallion spermatozoa is acknowledged as having a particular physiology concerning its strategies to manage and generate energy and the regulation of redox homeostasis. Early studies on stallion sperm biology through proteomic approaches disclosed particular aspects of energy metabolism, and regulation of the redox homeostasis in these cells. The pioneering work of Swegen et al. [2], identified 1030 proteins, with a predominance of proteins associated with metabolism, with proteins belonging to the gene ontology (GO) term cellular metabolic processes being the largest group. All the major energy generation pathways were identified (oxidative phosphorylation, glycolysis, and fatty acid metabolism), and mitochondrial proteins predominated. This work supported previous research pointing out the intense metabolism of these cells, and the paradoxical production of reactive oxygen species in the spermatozoa of the most fertile stallions[17], a fact that linked fertility to the intense generation of energy by the spermatozoa. Guasti et al. [18] studied protein extracts from stallion spermatozoa and seminal plasma using a combination of in-gel digestion and tandem mass spectroscopy and LC-MS/MS, identifying 24 proteins in seminal plasma and 33 proteins in sperm membrane extracts. The number of proteins identified was low, a fact that may relate to the methods used, in-gel digestion, and the use of only membrane extracts, which may have missed many mitochondrial proteins that are especially abundant in the stallion spermatozoa.

Proteomics in the improvement of stallion sperm biotechnologies

The introduction of proteomics to the study of stallion spermatozoa was rapidly applied to the investigation of major issues faced by the equine industry. In this regard, the study of molecular lesions induced by cryopreservation has been the subject of several proteomic studies. Cryopreservation of spermatozoa is a major component in the equine industry and the commerce of semen in most breeds. It offers numerous advantages such as the international commerce of spermatozoa, and the conservation in the long term of the genetics of superior stallions. It is quite common for the economic value of seminal doses of superior stallions to increase over the time, as they perform exceptionally well in sports or morphological competitions. Also, if the progeny of the stallion (foals) perform well in sports and/or morphology competitions, the value of the stallion and its semen further increases. In this sense semen doses stored by freezing behave like stocks in the markets, increasing their value over time.

However, cryopreservation is still a suboptimal procedure, and not all stallions provide spermatozoa that can be successfully stored frozen in liquid nitrogen with acceptable quality. Cryopreservation is a stressful procedure for the spermatozoa, that are exposed to toxic cryoprotectants, and experience a massive hypoosmotic shock during freezing, and a dramatic hypoosmotic stress at thawing[19-21]. This causes on average, 50% of the spermatozoa entering the procedure to succumb to necrosis induced by the osmolarity changes that cause membrane rupture[22]. In addition, a large percentage of the surviving population of spermatozoa, are not completely functional[22, 23]. The nature of these functional alterations of the spermatozoa surviving cryopreservation is not fully understood, although it is known that oxidative stress and alteration of redox homeostasis are major factors involved[23]. Proteomics studies have contributed to shedding light on the molecular alterations induced by cryopreservation. Our laboratory has used proteomic approaches to study these modifications[3, 4]. We report that cryopreservation impacts numerous proteins involved in metabolism regulation (mainly mitochondrial proteins involved in the TCA cycle, and oxidative phosphorylation), and affects proteins with oxidoreductase activity. Moreover, specific proteins with important roles in the sperm-oocyte interaction are also affected by the procedure[3]. In addition, we identified proteins which were most significantly reduced after cryopreservation[4], which are the Aldo-keto reductase family 1 member B and Superoxide dismutase (Cu-Zn). SOD1 is a discriminating variable identified using bioinformatic analysis. The relative amount of this protein is significantly reduced after cryopreservation. This finding strongly supports the theory that alteration in redox regulation and oxidative stress is a major factor

involved in cryodamage and suggests that control of redox regulation should be a major target to improve current cryopreservation procedures.

Another approach involved establishing the relationship between the proteome of the stallion spermatozoa and its ability to provide high-quality cryopreserved spermatozoa. The hypothesis behind these studies was that if cryopreservation alters predominantly metabolic and redox proteins, those ejaculates enriched in these groups of proteins will likely better tolerate the stress of cryopreservation.

Cryopreservation causes reduction in the levels of some proteins and increases in others but, there are specific proteins associated with stallions producing ejaculates with better motility post thaw. Six proteins were identified in fresh samples, capable of identifying the group of stallions showing better motility post thaw. Three of them are mitochondrial proteins (K9K273, A0A3Q2I7V9 and F7CE45), stressing the importance of these organelles for sperm function [24, 25], and in particular producing ATP for sperm motility through oxidative phosphorylation. F6YTG8 (*alpha mannosidase*) is a protein with a role in the catabolism of oligosaccharides [26]. Alpha mannosidase activity prevents accumulation of oligosaccharides. More recently a role in preventing mitochondrial dependent apoptosis has been proposed [27]. Since an important proportion of the damage occurring during cryopreservation involves a mitochondrial apoptotic pathway [19, 28], the function of this protein provides an explanation. F7CE45,*Acetyl- CoA acetyltransferase 1* , catalyzes the last step in the mitochondrial beta oxidation pathway [29], and also plays a major role in ketone body metabolism [30]. Spermatozoa can obtain energy for motility using the beta oxidation pathway [2, 31] providing an explanation for the link between a major presence of this protein in fresh samples and better motility post thaw. Finally, the presence of*Latherin* (F6YU15) was described for the first time in the spermatozoa. This is present in the saliva and sweat of horses and has strong surfactant properties [32, 33]. Its activity is responsible for the foam formed on the skin of horses during vigorous exercise. It is not clear what the possible function of this protein is in the spermatozoa, although antibacterial properties inhibiting the growth of biofilms [34] 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. Four proteins are potent discriminant variables for the prediction of good mitochondrial membrane potential post thaw. *Peroxiredoxin like 6 protein* is more abundant in the ejaculates showing better mitochondrial activity post thaw. Peroxiredoxin 6 is considered as one of the major antioxidant defenses of the spermatozoa [35, 36], and taking into account that a high percentage of cryodamage come from oxidative stress [37, 38], it is not surprising that samples richer in this antioxidant protein are able to better withstand the cryopreservation process. A glycolytic enzyme, *Phosphoglycerate mutase (PGAM)* , is also more abundant in good freezers. This enzyme is upregulated in many cancer cells [39] and catalyzes the conversion of 3-phosphoglycerate (3-PG) to 2-phosphoglycerate (2-PG) during glycolysis. In cancer cells that overexpress this protein, there is an increase in 2-PG and a decrease in 3-PG. Also, these cells express higher levels of lactate and increased flux through the pentose phosphate pathway [39], 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. *GRAM domain containing 1A* is also more abundant in the spermatozoa of stallions showing good mitochondrial membrane potential post thaw; this is a cholesterol transfer protein, with a role in the early stages of autophagosome formation [40]. These functions may explain the major presence of this protein in good freezers since mitophagy has recently been related to sperm quality [41]. Finally, an uncharacterized protein similar to *actin-1* is also more abundant in good freezers; a tempting possibility is that this protein is also related to mitophagy. Actin structures cage damaged mitochondria during mitophagy [42], however further research is warranted to characterize this protein and identify its role in spermatozoa.

Cryopreservation also had a different impact in the groups showing good and poor membrane integrity post thaw. The proteins *Chaperonin containing TCP1 subunit 8 and testis expressed 101* are more abundant in samples showing better membrane integrity post thaw. The chaperonin containing TCP1 complex plays a role

in mediating sperm-oocyte interaction [43-45], thus playing a major part in the early stages of fertilization. The testis expressed 101 also plays a role in fertilization, mediating binding of sperm to the zona pellucida, as well as in the migration of spermatozoa within the oviduct [46, 47]. The presence of these proteins with direct and major roles in fertilization in the ejaculates of stallions showing better viability post thaw, underpins the need for proper assessment of sperm membranes in the andrological evaluation of stallions.

A noteworthy finding of recent proteomic studies is a molecular explanation for the link between sperm motility and velocities, metabolism, and ability to fertilize[5]. In stallions showing better percentages of motility, circular and average velocity mitochondrial proteins with roles in the citric acid cycle, pyruvate metabolism and oxidative phosphorylation predominate. Interestingly, in stallions with better percentages of total motility, sperm proteins are also enriched in proteins within the gene ontology (G0) terms, single fertilization (G0: 0007338), fertilization (G0: 0009566), and zona pellucida receptor complex (GO:0002199). The enrichment of this protein in samples with better percentages of total motility may offer a molecular explanation for the link between this parameter and fertility.

Post-translational modifications in the sperm proteome

Although some reports suggest that the translation of proteins in the spermatozoa may occur in the mitochondria, the consensus is that spermatozoa are translationally/transcriptionally silent cells. Changes in protein abundance reported may be due to many reasons not related to new synthesis, such as post-translational modifications of the proteins that may facilitate protein/peptide identification.

Due to the transcriptionally silent nature of these particular cells, post-translational modifications are of major importance in the physiology of the stallion spermatozoa. These modifications have been recognized concerning capacitation, however detailed proteomic studies, focused on identifying post-translational modifications to the proteome are scarce. Due to the high dependence of the spermatozoa on post-translational modifications, the investigation of the sperm phospho-proteome and other post-translational modifications is warranted. In our laboratory, we investigated changes to the stallion sperm proteome caused by cryopreservation[11].

Spermatozoa are terminal and highly differentiated cells with very little or no capacity for transcription of new proteins. Due to these characteristics, spermatozoa are highly dependent on the incorporation of new proteins through micro-vesicle trafficking and post-translational modifications of existing proteins. Protein phosphorylation is a post-translational modification (PTM) involved in regulating most biological processes [48]. Phosphorylation of sperm proteins plays a major role in many important functions such as the acquisition of motility upon ejaculation, capacitation, and fertilization [49-52]. Interestingly, many phosphorylation events in the spermatozoa are redox regulated, including phosphorylation of proteins essential for motility, capacitation, and viability [53]. Cryopreservation significantly reduces the presence of the phosphoproteins;*Ca^{2+} binding tyrosine phosphorylation regulated, protein kinase cAMP-activated catalytic subunit beta* (CABYR)*, mitochondria eating protein* (SPATA18)*, A kinase anchoring protein 4* (AKAP4), *A kinase anchoring protein 3* (AKAP3) and *family with sequence similarity 71 member B,* (FAM71B). The proteins dephosphorylated as a result of cryopreservation, have important roles in the functionality of the stallion spermatozoa. Cryopreservation significantly reduces phosphoproteins involved in mitochondrial maintenance like *SPATA18* . This protein has previously been described in rat, mouse, and boar spermatozoa [54] [55, 56]. This protein shows a 79.9% homology with the human ortholog (Q8TC71). This is a recently described product of the *p53* gene [57, 58], with an important role in mitochondria quality control. This finding goes in line with the current consensus on the pivotal role of mitochondria in stallion sperm functionality. Mitophagy has been described in spermatozoa as a potential quality control system [41], the presence of SPATA18 in spermatozoa supports this previous finding, and in addition, is a good candidate for further support of the fundamental role of mitochondria and the development of new methods for sperm diagnosis. Phosphorylation of this protein appears to be necessary for its activation in stallion spermatozoa. *SPATA 18* or*Mieap* induces the accumulation of lysosomal proteins within mitochondria in response to mitochondrial damage and eliminates oxidized proteins to repair unhealthy mitochondria [57-60]. In this context, it is well known that cryopreservation causes oxidative stress and that mitochondrial proteins are a

preferential target for electrophilic aldehydes that originate after lipid peroxidation, like 4- hydroxynonenal (4-HNE) [61, 62]. It is likely that the stress of cryopreservation may lead to the dephosphorylation of this phosphoprotein, further contributing to mitochondrial damage. Potential kinases regulating the phosphorylation of this protein were identified.*In silico* analysis identified that this protein was phosphorylated by PKCβ. Functional kinase assays using PKC beta inhibition showed that inhibition of the phosphorylation sites of this protein lead to apoptotic changes in the stallion spermatozoa. Interestingly it is well described that cryopreservation induces apoptotic changes [63-66], as did PKCβ inhibition, further supporting the link between dephosphorylation of this protein during cryopreservation and apoptotic changes.

Two phosphoproteins with important roles in sperm functions such as capacitation, the acrosome reaction, and motility are reduced after cryopreservation, the *A-kinase anchoring proteins* (AKAP) 3 and 4. These proteins, and particularly AKAP4 have been proposed as biomarkers of sperm quality in stallions [67], moreover, AKAP4 has also been identified as highly susceptible to adduction by 4-HNE [67].

Ca^{2+} binding tyrosine phosphorylation regulated(CABYR) is another protein affected by cryopreservation. This protein plays a major role in the regulation of capacitation and the acrosome reaction [68], and it is phosphorylated in tyrosine upon capacitation. Studies observed a decrease in the general phosphorylation of this protein after cryopreservation, another finding that does not support the cryocapacitation theory. The predicted kinases revealed that this protein is regulated by GSK3ß, and the kinase inhibition assay performed supported the role of this protein in stallion sperm function since incubation in the presence of inhibitors of GSK3ß increased the percentage of apoptotic spermatozoa. Although the main known role of this protein so far is capacitation, it may have important roles in other aspects of sperm functionality, which have also been proposed in mouse spermatozoa [69].

Proteomics of seminal plasma

Seminal plasma (SP) has a major role in sperm function and regulates the interactions between the spermatozoa and the mare's genital tract. The roles of seminal plasma have recently been reviewed in detail[70]. The important role of specific SP proteins is already well known, such as the case of lactoferrin or cysteine-rich secretory proteins (CRISPs). Cysteine-rich secretory protein- 3 (CRISP- 3) suppresses the binding of PMNs to live sperm[71], while lactoferrin appears to increase the number of dead sperm bound to PMNs *in vitro[72].* However, shot-gun proteomic studies on equine seminal plasma are scarce. Although the few existing studies have provided interesting findings [6, 7, 73], reinforcing the idea of the important role of seminal plasma in sperm functionality and the interaction with the mare's reproductive tract.

In addition to cryopreservation, commerce of seminal doses is performed in the liquid state; ejaculates are diluted in extenders which maintain an acceptable level of fertility for a variable period, normally up to 48 h refrigerated at 5^{o} C. Although this technology is simple, less damaging for the spermatozoa, and less expensive, there are important drawbacks. Some stallions do not tolerate refrigeration, and there is a huge variability in the quality of semen after refrigeration and storage.

In our laboratory using seminal plasma from 10 different stallions, we identified and matched 3544 different proteins to the equine proteome database, that are mainly related to the gene ontology terms, cell response to hydrogen peroxide, metabolism, extracellular vesicle transport, and immune response. Analysis of the seminal plasma proteome has shed light on important issues related to reproductive technologies in the equine industry. One of them is the existence of stallions whose ejaculates did not tolerate conservation by refrigeration. These stallions were identified by the presence of high amounts of the protein Annexin A2 in seminal plasma. These stallions tolerated refrigeration after the removal of the seminal plasma [73].

Conclusions and future directions

Recent advances in stallion sperm biology have been possible thanks to the introduction of proteomics to the study of these cells. Significant advances on the importance of the metabolism and redox regulation of the spermatozoa have been sped up through proteomics, with results that have shown to be readily applicable, i.e. through the modification of semen extenders adapted to the metabolic requirements of the

stallion spermatozoa. Also, these studies have improved our comprehension of the links among different sperm characteristics, such as mitochondrial activity, redox regulation and energetic metabolism or the special presence of proteins with roles in fertilization preferentially present in more motile and vigorous spermatozoa. Finally, the proteome of seminal plasma, primarily vehiculated in microvesicles, and the importance of the study of post translation modifications in the proteome of the stallion spermatozoa are areas of special interest for the near future.

Conflict of interest

All authors declare no potential conflict of interest.

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