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Characterization of the porcine seminal plasma proteome comparing ejaculate portions

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ABSTRACT

Full identification of boar seminal plasma (SP) proteins remains challenging. This study aims to provide an extensive proteomic analysis of boar SP and to generate an accessible database of boar SP-proteome. A SP-pool (33 entire ejaculates/11 boars; 3 ejaculates/boar) was analyzed to characterize the boar SP-proteome. Twenty ejaculates (5 boars, 4 ejaculates/boar) collected in portions (P1: first 10 mL of sperm rich ejaculate fraction (SRF), P2: rest of SRF and P3: post-SRF) were analyzed to evaluate differentially expressed SP-proteins among portions. SP-samples were analyzed using a combination of SEC, 1-D SDS PAGE and NanoLC-ESI-MS/MS followed by functional bioinformatics. The identified proteins were quantified from normalized LFQ intensity data. A total of 536 SP-proteins were identified, 409 of them in *Sus scrofa* taxonomy (374 validated with ≥99% confidence). Barely 20 of the identified SP-proteins were specifically implicated in reproductive processes, albeit other SP-proteins could be indirectly involved in functionality and fertility of boar spermatozoa. Thirty-four proteins (16 identified in *S. scrofa* taxonomy) were differentially expressed among ejaculate portions, 16 being over-expressed and 18 under-expressed in P1–P2 regarding to P3. This major proteome mapping of the boar SP provides a complex inventory of proteins with potential roles as sperm function- and fertility- biomarkers.

Biological significance: This proteomic study provides the major characterization of the boar SP-proteome with >250 proteins first reported. The boar SP-proteome is described so that a spectral library can be built for relative 'label free' protein quantification with SWATH approach. This proteomic profiling allows the creation of a publicly accessible database of the boar SP-proteome, as a first step for further understanding the role of SP-proteins in reproductive outcomes as well as for the identification of biomarkers for sperm quality and fertility.

1. Introduction

Artificial insemination (AI) is used worldwide in the swine industry [1]. Nowadays, commercial semen AI-doses are used as liquid state, but increasing research is done for the practical incorporation of frozen-thawed semen and even sex-sorted spermatozoa into commercial swine AI-programs [2]. Boar ejaculates are selected if most relevant sperm attributes, such as motility, morphology or membrane sperm integrity, are within acceptable limits when evaluated using innovative technologies such as computer-assisted analysis and flow-cytometry based procedures [3]. Despite this pre-screening, not all selected ejaculates perform well, neither fertilizing nor overcoming technological handling [4]. Consequently, complementary criteria for boar ejaculate selection are currently being explored, specially focusing on seminal plasma (SP) a composite fluid that surrounds and interacts with sperm from ejaculation to deposition into the uterus of the sow.

Seminal plasma is a complex mixture composed of exocrine secretions from the testis, epididymis and the male accessory sexual glands, containing a wide variety of both organic and inorganic components, being proteins the main one and probably the major conditioners of the reproductive success involving the use of processed semen [5,6]. Certainly, in different mammalian species some SP-proteins have been evidenced to stabilize sperm membrane [7], regulate sperm motility [8] and capacitation [7,9], and further facilitate the colonization of the sperm reservoirs in the female reproductive tract [10]. Moreover, some SP-proteins, once into the female genital tract after natural mating or AI, modulate the uterine immune response against the foreign semen, facilitating sperm transport [11], sperm-oocyte fusion [12] and the development of a healthy embryo [13]. These findings provide clear evidence of the relevance of SP-proteins for sperm functional performance and further highlight the possible use of SP-proteins as potential markers for ejaculate selection. As indicated by Park et al. [14],

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extensive and comprehensive knowledge of SP-proteome is an essential prerequisite before SP-proteins could be used as biomarkers of sperm functionality, or even fertility. Unfortunately, the boar SP-proteome is still far from being completely decoded, requiring further research [5,15]. Despite the economic relevance, porcine is currently the livestock species with the fewer SP-proteins identified, <100 [15,16], which contrasts with other livestock species, such as poultry or ovine with >600 SP-proteins identified [17,18]. This shortage in porcine is surprising, considering boar ejaculates yield a large SP-volume, usually above 150 mL. Consequently, the first aim of the current study will be to improve the knowledge of the boar SP-proteome. To achieve this, SP-samples from boars with proven fertility would be examined using 1-D SDS PAGE and Nano LC-ESI-MS/MS followed by bioinformatics of Gene Ontology (GO) to evaluate cellular location, molecular function and biological process of the identified proteins. In addition, the boar SP-proteome will be described so that a spectral library can be built for relative 'label free' protein quantification with SWATH approach [19].

The boar ejaculate is fractionally emitted, alike other mammalian species such as equine and even human [5]. Then, it is possible to visually differentiate two main fractions during the ejaculation, a first fraction named sperm rich fraction (SRF), containing most of the total sperm, and a second named post-SRF, largest in volume but containing very few sperm. As boar ejaculates are traditionally hand-collected, the SRF is the only fraction sampled, discarding the post-SP and thereby most of the total SP. However, the ejaculate collection method is currently shifting from manual to semi-automated, for hygienic and labour reasons, moving from collecting just the SRF to the entire ejaculate [20]. The change increases the relevance of SP, as the large SP-volume of post-SRF fraction will now become a part of the collected ejaculate. There are yet no studies evaluating how this procedural change can affect functional sperm performance. However, in this regard, it is well known that sperm from the SRF freezes better than those from the entire ejaculate, suggesting that the SP from the post-SRF, owing to its rich protein composition, impairs sperm freezability [21]. Furthermore, large proportions of SP in boar semen samples impair sperm sortability and sorting efficiency in ejaculates subjected to flow cytometry for sperm sex-sorting [22]. In addition, [23] demonstrated that sperm from the first 10 mL of the SRF sustain cryopreservation better than those from the rest of the ejaculate, which was attributable to differences in SP-proteins between the ejaculate fractions. Accordingly, this study also aims to identify possible qualitative and quantitative differences in SP-proteome among the most relevant boar ejaculate portions: P1: the first 10 mL of SRF, P2: the rest of SRF and P3: the post-SRF.

2. Material and methods

2.1. Boars and ejaculates

All procedures involving boars were performed according to international guidelines (Directive 2010/63/EU), following the approval of the Bioethics Committee of Murcia University (research code: 639/2012).

Healthy and sexually mature Large White and Landrace boars, with proven fertility and undergoing regular semen collection for commercial AI (AIM Iberica, Spain), were used as ejaculate donors. Boars were subjected to diet and housing conditions characteristics of an AI-center. All the ejaculates used fulfilled the standards of sperm quantity and quality thresholds for the preparation of semen doses for use in AI programs. Thirty-three entire ejaculates collected using the semi-automatic system Collectis® (IMV Technologies, L'Aigle, France) from 11 boars (3 ejaculates per boar), were used to characterize the boar SP-proteome. In order to evaluate differences in SP-protein composition among ejaculate portions, twenty ejaculates were collected fractionated (P1–3) using the gloved hand method, from 5 boars (4 ejaculates per boar). All ejaculates used meet the minimum quality requirements for AI-semen doses production.

2.2. Preparation of seminal plasma samples for proteome analysis

Immediately after collection, fully filled 15 mL tubes of semen from each ejaculate/portion were centrifuged twice at 1500 ×*g* for 10 min (Rotofix 32A; Hettich Zentrifugen, Tuttlingen, Germany). The second SP-supernatant recovered was microscopically verified as sperm free. A protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) was added to the SP-samples (1% vol/vol) and then they were stored at −80 °C (Ultra Low Freezer; Haier, Schomberg, Ontario, Canada) until proteomic analysis.

The proteomic analyses were carried out in the Proteomics Unit of the University of Valencia, Valencia, Spain (member of the PRB2-ISCIII ProteoRed Proteomics Platform). Seminal plasma samples were thawed at room temperature, centrifuged at 16,100 ×*g* at 4 °C for 1 min. The protein concentration of the SP-sample was measured using a Qubit fluorometer (Invitrogen, Carlsbad, CA, USA) according to manufacturer instructions. Before starting the proteome analysis, the 33 SP-samples collected for characterization of boar SP-proteome were mixed in a single pool. Similarly, the 4 SP-samples of each ejaculate portion collected from each boar to identify and quantify differentially expressed proteins among ejaculate portions were also mixed in a single pool, thereby generating a total of 5 SP-pools for each ejaculate portion, one per boar.

A SEC was carried out in an ETTAN LC system (GE Healthcare Life Sciences, Little Chalfont, United Kingdom) using a Superdex 200 5/150 GL column (GE Healthcare Life Sciences) controlled by an AKTA ETTAN LC system (GE Healthcare Life Sciences), which furthermore would ease a large-scale proteomic study. 50 μL of the SP-pool were injected into the column, equilibrated with 200 mM ammonium bicarbonate and 1 mM DTT (Sigma-Aldrich) at a flow rate of 0.18 mL/min at 4 °C. The eluent was collected in 0.2 mL fractions. It was immediately evident that it was not possible to achieve only one fraction with all proteins except the dominant ones. Therefore, 2.5 to 5 μg of protein (depending of the total amount of the sample) from the fractions were collected after SEC step (A3-A10), dried in a rotatory evaporator and loaded onto different wells of 12% Tris-HCl precast 1-D SDS PAGE (Bio-Rad, Richmond, CA, USA). The gel was run at a constant voltage of 200 mV for 30 min at room temperature including a molecular weight marker (ECL Plex Fluorescent Rainbow Marker, GE Healthcare Life Sciences), and Coomassie Brilliant R250 Blue stain (Bio-Rad) was used to visualize protein bands on the gel. Thereafter, the gel was sliced at 38 kDa, as indicated in Fig. 1, and the top of the gel used to analyze less abundant proteins by in-gel digestion processing. The 1-D Gel fraction between 38 kDa and 20 kDa was discarded for the analysis because in a previous analysis (data not shown) this gel area was highly contaminated by the more abundant SP-proteins, providing little information to the global study of the boar SP-proteome. The more abundant proteins were identified from an aliquot of the mixed SP-sample analyzed by in-solution processing.

2.3. Building a MS/MS library for SWATH analysis of boar SP

2.3.1. Complete proteome. In-solution digestion processing

The more abundant proteins were analyzed using in-solution digestion. 3 μL of the pooled SP, containing 10 μg of proteins, were digested with Sequencing Grade Trypsin (Promega Corporation, Madison, USA) to generate peptides of each individual protein according to the following steps: (1) the proteins were reduced using 2 mM DTT reducing reagent in 50 mM NH_4HCO_3 (Sigma-Aldrich) to a final volume of 25 μL and the mixture incubated for 20 min at 60 °C; (2) the proteins were alkylated using 5.5 mM iodoacetamide (IAM) (Sigma-Aldrich) alkylating reagent in 50 mM NH_4HCO_3 to a final volume of 30 μL and incubated for 30 min in the dark; (3) 10 mM DTT in 50 mM NH_4HCO_3 were added to a final volume of 60 μL and sample was vortex and