Theriogenology 95 (2017) 178–186

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Proteomic characterization of canine seminal plasma

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ARTICLE INFO

Article history:
Received 21 October 2016
Received in revised form
16 March 2017
Accepted 18 March 2017
Available online 20 March 2017

Keywords:
Proteins
Proteomic
Canine semen
Prostatic fraction
Sperm-rich fraction
Dog

ABSTRACT

The present study was conducted to identify the major proteome of the sperm-rich fraction and prostatic fraction of canine seminal plasma. Three semen samples from four healthy dogs were obtained by digital manipulation. The pre-sperm fraction, sperm-rich fraction and prostatic fraction were separated from each ejaculate. Immediately after sperm analysis, a protease inhibitor was added to the sperm-rich fraction and prostatic fraction, and the fractions were separately centrifuged and frozen at −80 °C. The samples were thawed, re-centrifuged, and the total protein concentration was determined. Samples were subjected to 1D SDS-PAGE and Coomassie-blue stained gels, were analyzed by Quantity One 1D Analysis Software. Bands detected in the gels were excised and proteins subjected to digestion with trypsin. Proteins were identified by nano-HPLC-MS and tools of bioinformatics. Tandem mass spectrometry allowed the detection of 268 proteins in the gels of sperm-rich fraction and prostatic fraction of canine ejaculate. A total of 251 proteins were common to the sperm-rich and prostatic fractions, while 17 proteins were present in the sperm-rich fraction and absent in the prostatic fraction. The intensity of the bands detected in range 1 and 2 represented 46.5% of all of the band intensities detected in the 1D gels for proteins of the sperm-rich fraction and 53.0% of all bands in the prostatic fraction. Arginine esterase and lactotransferrin precursor were the protein with the highest intensity observed in the both fractions. Among the proteins present only in the sperm-rich fraction, the proteins UPF0764 protein C16orf89 homolog and epididymal-specific lipocalin-9 were the most abundant. In conclusion, canine sperm-rich fraction and prostatic fraction express a very diverse set of proteins, with unique biochemical properties and functions. Moreover, although most proteins are common to both sperm-rich fraction and prostatic fraction, there are some exclusive proteins in sperm-rich fraction.

1. Introduction

As assisted reproductive technologies evolve, a great deal of attention has been dedicated to the composition of seminal plasma, and reports are currently available about this topic in stallions [1], bulls [2,3], rams [4,5], goats [6], boars [7,8] and dogs [9]. Additionally, knowledge about the biochemical characteristics of seminal fluid will help to better understand the physiological mechanisms by which sperm function is modulated before and after ejaculation. The general concept that seminal plasma components regulate important sperm attributes has long been advocated, and this seems to be true for numerous species, from humans [10] to farm and wild animals [4,11]. Analysis of the seminal plasma proteome may also reflect the physiological state of the epididymides as well as the accessory sex glands [10] and may contribute to the identification of molecular markers of fertility.

Canine semen is composed of the pre-sperm fraction, sperm-rich fraction (SRF) and prostatic fraction (PF) [12]. The first and last fractions of the ejaculate are from the prostate gland and appear clear and translucent [13]. The SRF, the second fraction,

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http://dx.doi.org/10.1016/j.theriogenology.2017.03.016
0093-691X/

contains spermatozoa and is easily differentiated from the others because it is milky and dense [14,15]. Studies on canine seminal plasma proteins are still limited, and initial investigations were able to detect 20–37 bands [9,16] using one-dimension electrophoresis of canine seminal plasma. However, neither of these studies described the actual identities of the proteins. Individual proteins have also been identified in seminal plasma samples from dogs, such as alkaline and acid phosphatase [17], arginine esterase [18], lactoferrin [19], superoxide dismutase [20], catalase [21], osteopotin [22], matrix metalloproteinase [23], glutation peroxidase [24], zinc-binding protein [25] and canine prostate specific esterase [26]. These pieces of information bring important knowledge about the diversity and composition of canine seminal secretions. Thus, the present study was conducted to determine the proteome of seminal plasma obtained from SRF and PF of canine semen.

2. Materials and methods

2.1. Animals

Two healthy Rottweiler and two healthy German Shepherd dogs (4–9 years old; 30 to 40 Kg) were selected for this study. These dogs belonged to the 4th Regiment of the Military Police of the State of Ceará in the city of Fortaleza, Brazil (3° 43′ 02″ S; 38° 32′ 35″ W), and were accommodated in individual kennels. The dogs had free access to water and were fed twice daily with a commercial dry feed (26% protein supplemented with 0.5% methionine, 0.12% taurine, 50 mg/kg chondroitin sulfate, 600 mg/kg of glucosamine sulfate, PremieR Medium Breed Formula Adults®). The present study was approved by the Ethics Committee for Animal Use of Ceara State University (protocol number 6591178/2014).

2.2. Semen collection and analysis

Semen samples were collected by manual stimulation, three times from each dog, at weekly intervals. Each ejaculate was carefully separated into its three distinct fractions as follows: pre-sperm fraction, sperm-rich fraction and prostatic fraction (Fig. 1). A 15-ml tube and plastic funnel were used to collect each individual fraction of the ejaculate to prevent mixing of fractions. The pre-sperm fraction was discarded immediately after collection, and the two others were used for the study [27].

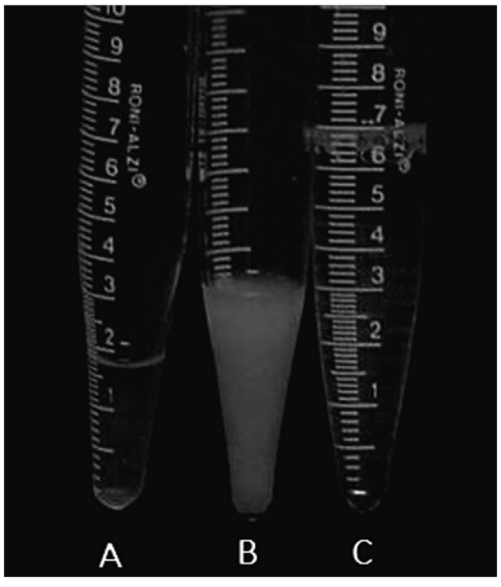

Fig. 1. Aspects of the three fractions of canine semen: pre-sperm fraction (A), sperm-rich fraction (B) and prostatic fraction (B).

Immediately after collection, total sperm motility (%) and vigor (0–5) were assessed through light microscopy. Total motility and vigor were evaluated subjectively at 100× magnification. Spermatozoa concentration was determined using a Neubauer chamber and a 10 μL aliquot of semen diluted in 2 mL of a saline solution with 1% formalin (1,000X). The percentage of live spermatozoa was evaluated using bromophenol blue stain, counting 100 cells/ejaculate with a light microscope, at 400× magnification [28]. A wet smear stained with Rose Bengal was prepared from determination of percentage of spermatozoa with normal morphology (1,000× magnification), where cells were defined as morphologically normal or with primary or secondary alterations [14]. To assess the percentage of spermatozoa with integral plasma membrane, 10 μL of each ejaculate was incubated at 38 °C for 45 min in 90 μL of a hyposmotic solution (distilled water) to perform a hyposmotic swelling test (HOST). For each sample, 100 sperm were examined with phase contrast microscopy (400× magnification) and those with a swollen tail were considered to have a functional membrane [29].

2.3. One-dimensional electrophoresis

For one-dimensional electrophoresis, the pool of three SRF from each animal and pool of three PF from each animal were analyzed separately (Fig. 2). Immediately after semen collection, 1 μL of protease inhibitor cocktail (Sigma-Aldrich, catalog # P8340; USA) containing AEBSF (4-(2-aminoethyl) benzenesulfonyl fluoride), pepstatin A, E-64, bestatin, leupeptin, and aprotinin, was added to 1 mL of SRF and 1 mL of PF of all animals as described [30], with modifications. Then, the two fractions separately were centrifuged at 1840 g for 20 min (Centribio/Daikki 80-2B®; Brazil) at room temperature. The clear supernatant from the two fractions obtained after centrifugation was pipetted out into clean tubes, examined microscopically to confirm the absence of sperm cells and stored at −80° C [27]. After thawing, samples were re-centrifuged at 5,000g for 60 min at 4 °C (Centrifuge 5804 R, Eppendorf®; EUA), and the supernatant was used for the quantification and characterization of proteins.

Total soluble protein in the SRFand PF from each pool was determined by Bradford's reaction [31], using bovine serum albumin (BSA) as standards. Seminal plasma samples were then subjected to SDS-PAGE according to the protocol described [32] with modifications. For the gel runs, the four columns from left consisted of the SRF of dogs A, B, C, D, respectively (Fig. 2). The four columns from right represented the PFs of the four animals, following the same sequence (Dog A, B, C and D; Fig. 2). In summary, a volume of sample containing 30 μg of protein was mixed with sample buffer (0.125 M Tris–HCl, pH 6.8, 4% SDS, 20% (v/v) glycerol, 0.2 M DTT, 0.02% bromophenol blue), boiled for 90 s, and loaded into the wells of a stacking gel (4% acrylamide), laid on top of a 8–16% gradient polyacrylamide resolving gel (GE Healthcare®, USA)In one well of stacking gel, 10 μL of 225–12 kDa molecular weight standard mix (GE Healthcare®, Piscataway, NJ, USA) was loaded to allow molecular weight estimation of the protein bands. An initial current at 160 V, 45 mA and 20 W for approximately 40 min to make proteins slowly migrate through the stacking gel [30]. The gel was stained with Coomassie Blue R-350 (GE Healthcare®, USA) for 12 h, and destained after several washes in a solution containing ethanol (40%), acetic acid (10%) in double distilled water. Gels were scanned at 300 dpi (ImageScanner II; GE Lifesciences, USA) and were analyzed using Quantity One 1D Analysis Software, version 4.6.3 (Bio-Rad Laboratories, USA).