MAJOR PROTEINS OF THE SEMINAL PLASMA OF NEW ZEALAND WHITE RABBITS AND ASSOCIATION WITH SEMEN CRITERIA

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ABSTRACT

In rabbits, there are only few studies relating rabbit seminal plasma (SP) proteins and semen criteria and all those have been conducted in temperate regions. Therefore, information on the proteome of rabbit seminal plasma is crucial for the understanding on how these proteins regulate sperm attributes. The objective of this research was to describe the identities of the major rabbit seminal plasma proteins and associations between protein spots and semen criteria. Semen samples from 12 mature New Zealand White rabbit bucks were collected and evaluated, and SP was subjected to 2D-electrophoresis. Major protein spots were identified by mass spectrometry. Correlation coefficients were calculated to estimate associations between spot intensities and semen traits. Rabbit bucks ejaculated an average of 363.5±69.7 million sperms, with 82.0±6.3% motile cells, vigor of 3.8±0.2 and 67.8±4.1% of morphologically normal cells. Most spermatozoa (56.5±4.2%) had both intact acrosome and functional membrane. On average, each seminal plasma protein map had 244.3±47.2 spots, with 63 consistently present on all gels, representing 62.7±7.1% of the combined intensity of all spots. The seven major spots of the gels, representing 37.8±4.1% of all spot intensities were identified as Annexin 5, while a single spot matched a zeta globin-like protein. Annexin 5 spot intensities were positively associated with semen criteria, including sperm vigor, concentration, and morphology, and negatively related to the percentage of sperm with head abnormalities. Zeta globin-like spot, in turn, was correlated with the percentage of cells with both membrane and acrosome damaged. Associations among protein spots and semen traits suggest that, in rabbits, seminal proteins are involved in the regulation of important aspects of sperm function.

Key words: Rabbits, seminal plasma, protein profile, semen.

INTRODUCTION

Seminal plasma contains a diverse cohort of proteins, originating mostly from epididymis and accessory sex glands. These proteins participate in various events related to sperm function, such as epididymal sperm maturation, sperm capacitation, acrosome reaction and formation of the oviduct reservoir (Topfer-Petersen *et al*., 1998; Gwathmey *et al*., 2006). Some of these proteins have been related to fertility indexes of sires as well (Killian *et al*., 1993; Bellin *et al*., 1994).

In rabbits, seminal plasma has a positive effect in maintaining sperm motility and viability during *in vitro* storage (Castellini *et al*., 2000), and this effect has been associated with seminal plasma antioxidant properties. However, there are only few studies relating rabbit seminal plasma proteins and semen criteria (Davis and Davis, 1983; De Lamirande *et al*., 1983; Okabe *et al.*, 1993; Minelli *et al*., 2001), and all those have been conducted in temperate regions, with a lack of information regarding animals raised in the tropics. Therefore, information on the proteome of rabbit seminal plasma is crucial for the understanding on how these proteins regulate sperm attributes, and thus, serve as basis for the search of biomarkers of reproductive efficiency.

The objective of this research was to describe the identities of the major rabbit seminal plasma proteins and associations between protein spots and semen criteria.

MATERIALS AND METHODS

Animals and experimental design

The experiment was performed between October and November 2011, in the Animal Science Department of the Federal University of Ceara (Fortaleza, Ceara, Brazil). Twelve mature New Zealand White rabbit bucks, weighing an average of 3.5 kg were used in the study. The animals were reared under intensive system, housed in individual cages, arranged in flat-deck system, fed a commercial diet and water *ad libitum*. Semen samples were weekly collected and evaluated. Seminal plasma was separated from sperm and subjected to 2D-electrophoresis. Major protein spots were excised, digested and identified by tandem mass spectrometry. Correlation coefficients were calculated to estimate associations between spot intensities and semen traits.

Semen collection and evaluation

Two ejaculates per male were collected weekly by artificial vagina (containing water at 45ºC), with an interval of 15-30 min between successive ejaculates, as described by Mocé *et al*. (2000). Before semen collection, the gel mass was removed (IRRG, 2005) and ejaculate volume (mL) was measured and only the second ejaculate was used. A small aliquot of semen was covered by a cover slip and immediately examined subjectively under the high power magnification (X 400) to assess vigor and motility. The vigor evaluation was based on the progressive rectilinear quality movement and speed of sperm, on a scale from 1 to 4. The motility was evaluated by the percentage of motile spermatozoa. The concentrations of spermatozoa was estimated using a Neubauer counting chamber, after dilution of 20 µL of semen in 2 mL of formalin-saline solution (1:100), using optic microscopy under 400 x magnification. All semen samples were treated with a protease inhibitor cocktail before the collection. This cocktail was added as a volume of 10 µL to each 1 mL of seminal plasma. The cocktail was prepared by diluting a protease inhibitor stock (4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), pepstatin A, E-64, bestatin, leupeptin, and aprotinin – Sigma-Aldrich, USA) by a factor of ten times (v/v) in distilled water. The remaining of semen samples was immediately centrifuged at 700 × *g* (15 min., 4°C) to separate seminal plasma from sperm cells. The supernatant plasma was pipetted out into a clean tube, subjected to another centrifugation at 5,000 × *g* (60 min., 4°C), aliquoted in sterile tubes and stored at – 80°C until further use.

Hypo-osmotic swelling test (HOST)

The functionality of sperm membrane was evaluated according to the method described by Ducci *et al.* (2002), with adaptations. Briefly, an aliquot of semen (40 µL) was mixed with 460 µL of hypo-osmotic solution (60mOsmol of fructose) containing 1% (w/v) of eosin Y, and incubated for 15 minutes in water bath at 37°C. After incubation, 10 µL of semen suspension were added to 20 µL of fixative solution (1% formaldehyde) and evaluated by optical microscopy under 400X magnification. A total of 200 cells were counted and divided in the following categories: 1) stained head and curly tail (damaged acrosome and intact membrane), 2) unstained head and curly tail (intact acrosome and membrane), 3) stained head and straight tail (damaged acrosome and membrane), and 4) unstained head and straight tail (intact acrosome and membrane damaged).

Two-dimensional gel electrophoresis of seminal plasma proteins

Total protein concentration was determined in aliquots of seminal plasma according to Bradford's method (Bradford, 1976), in triplicates, using bovine serum albumin (Sigma-Aldrich, USA) as standards (Souza *et al.*, 2010). Two-dimensional electrophoresis of proteins from seminal plasma was carried out as described before (Souza *et al.*, 2010, 2011), with modifications. Samples containing 700 µg of total proteins were mixed with re-hydration buffer sufficient to make 340 µL, incubated with 18-

cm IPG strips (pH 3-10NL) and allowed to rehydrate for 20 hours. Isoelectric focusing was carried out for a total of 43,100 Vh. After focusing, IPG strips were equilibrated, and separated on SDS-PAGE gels (17.5% T/0.8% C). Gels were stained using colloidal Coomassie blue for 72 hours, after which they were rinsed with water and scanned. According to a strategy previously reported (Moura *et al*., 2010; Souza *et al*., 2010), maps of seminal plasma proteins were evaluated using PDQuest software and a synthetic master gel generated, based on a reference gel. Additional spots consistently present in the remaining gels were also added to the master so that they could be matched to all samples. Final spot matching was achieved after several rounds of comparisons and checking each spot in each gel with the respective pattern in the master gel. For spot quantification, gel images were normalized using the total intensity of valid spots, and protein quantities were given as parts per million of the total integrated optical density of spots in the gels.

Identification of the major seminal plasma proteins by tandem mass spectrometry and database search

Major spots detected after 2-D PAGE after PDQuest analysis were cut from the seminal plasma gels, subjected to in-gel trypsin digestion (Rocha *et al*., 2009, Moura *et al*., 2010) and analyzed by capillary liquid chromatography/nanoelectrospray ionization tandem mass spectrometry (CapLC-MS/MS), using a Micromass Q-ToF API US mass spectrometer, coupled with a Waters CapLC high-performance liquid chromatography (HPLC) unit. The product ion spectra collected were processed using Protein Lynx Global Server 2.1 and were converted to peak list text files for database searching. In order to identify the proteins, MS/MS ion searches were performed on the processed spectra against the NCBInr and Swissprot databases using MASCOT Daemon search engine, allowing a maximum of one missed trypsin cleavage and using partially oxidized methionine residues, and completely carbamidomethylated cysteine residues. Peptide mass tolerance and fragment mass tolerance were initially set to 0.5 and 0.1 Da, respectively, for MS/MS ion searching. However, candidate peptide IDs were only accepted if the *m/z* values were observed within 0.1 Da (typically less than 0.05 Da) of the theoretical mass of the candidate ID, as determined when manually reviewing MASCOT search results.

Statistical Analysis

The experiment followed a completely randomized design, where each animal was considered as a random factor. Semen parameters and spot intensities in the protein maps were tested for normality, using Shapiro-Wilk's test, and for asymmetry and kurtosis, using the UNIVARIATE procedure with NORMAL and PLOT options of Statistical Analysis System. Normally distributed variables were compared between groups using the Student's t test after analysis of variance using the GLM procedure ($P<0.05$). Non-parametric data were analyzed by the Mann-Whitney test (Sas, 2003).

RESULTS AND DISCUSSION

Rabbit bucks ejaculated an average of 364±70 million sperms, with 82.0±6.3% motile cells, vigor of 3.8±0.2 and 67.8±4.1% of morphologically normal cells. Among sperms with morphological defects, most were in the midpiece (16.8±2.0%), followed head (9.6±2.4%) and tail (5.7±1.1%), and only 0.1% of the cells had proximal cytoplasmic droplets. Most spermatozoa (56.5±4.2%) had both intact acrosome and functional membrane.

On average, each seminal plasma protein map had 244.3±47.2 spots, with 63 of them consistently present on all gels, representing 62.7±7.1% of the combined intensity of all spots. The most intense spots had low molecular weight (below 30 kDa) and acidic isoelectric points (pIs below 5). The seven major spots of the gels, representing 37.8±4.1% of all spot intensities were cut and identified by tandem mass spectrometry as Annexin 5, while a single spot matched a zeta globin-like protein (Figure 1). Annexin 5 was positively associated with several semen criteria, including sperm vigor, #1104; $r = 0.78$; $p = 0.0076$) and #2405 ($r = 0.64$; $p = 0.0639$), concentration, #1223 ($r = 0.63$; $p = 0.0494$), #1104 ($r = 0.71$; $p = 0.0226$), and percentage of morphologically normal sperm, #1222 ($r = 0.74$; $p = 0.0140$) and #2122 ($r = 0.73$; $p = 0.0263$), and negatively related to the percentage of sperm with head abnormalities, #1222 ($r = -0.64$; $p = 0.0457$). Zeta globin-like spot, in turn, was correlated

with the percentage of cells with both membrane and acrosome damaged (r = 0.77; p = 0.0256). Annexin 5 is a calcium-dependent phospholipid-binding protein (Vadnais and Althouse, 2011) and, in humans, it has been shown to protect sperm membrane functionality and DNA integrity against oxidative stress (Lu *et al*., 2011). In rabbits, Annexin 5 has been reported as a decapacitating factor (Okabe *et al*., 1993). These findings are especially important because rabbit ovulate induced by mating. Thus, sperm spend a long time in the female reproductive tract before fertilization, and premature capacitation would decrease sperm viability and reproductive efficiency (Giojalas *et al*., 2004). Therefore, it is interesting that a decapacitating and sperm protection protein is the major protein spot in seminal plasma.

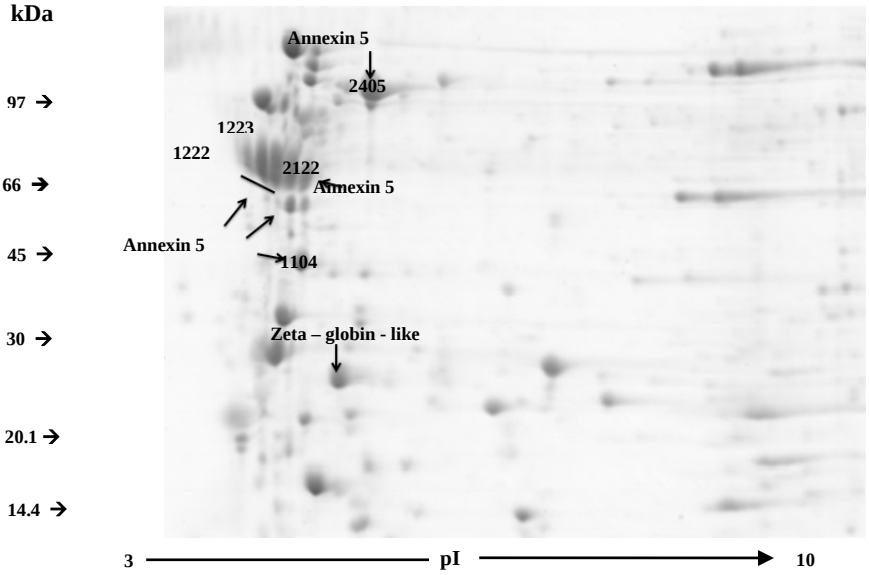

Figure 1: Two-dimensional map of seminal plasma proteins from New Zealand White rabbits. Spot numbers refer to those described in the text .

CONCLUSION

Taken together, associations among protein spots and semen traits suggest that, in rabbit bucks raised in a tropical environment, seminal plasma proteins are involved in the regulation of important aspects of sperm physiology. Annexin 5, the major seminal plasma protein, appears to have a beneficial effect in maintaining sperm viability, given the correlations with several semen parameters. Identification of other seminal plasma proteins associated with semen criteria, will bring important clues about the mechanisms underlying such relationships, as well as serve as basis for the discovery of new biomarkers of rabbit reproductive efficiency.

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