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Differential abundances of four forms of Binder of SPerm 1 in the seminal plasma of *Bos taurus indicus* bulls with different patterns of semen freezability

Marcos Jorge Magalhães Jr [a], Leonardo Franco Martins [b], Renato Lima Senra [a], Thaís Ferreira dos Santos [a], Denise Silva Okano [b], Paulo Roberto Gomes Pereira [c], Alessandra Faria-Campos [d], Sérgio Vale Aguiar Campos [d], José Domingos Guimarães [b], Maria Cristina Baracat-Pereira [a,*]

[a] Department of Biochemistry and Molecular Biology, Universidade Federal de Viçosa, Viçosa, MG, Brazil
[b] Department of Veterinary, Universidade Federal de Viçosa, Viçosa, MG, Brazil
[c] Department of Plant Science, Universidade Federal de Viçosa, Viçosa, MG, Brazil
[d] Department of Computer Science-ICEX, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

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ABSTRACT

The Binder of SPerm 1 (BSP1) protein is involved in the fertilization and semen cryopreservation processes and is described to be both beneficial and detrimental to sperm. Previously, the relationship of BSP1 with freezability events has not been completely understood. The objective of this work was to determine the differential abundance of the forms of the BSP1 protein in cryopreserved seminal plasma of *Bos taurus indicus* bulls with different patterns of semen freezability using proteomics. A wide cohort of adult bulls with high genetic value from an artificial insemination center was used as donors of high quality, fresh semen. Nine bulls presenting different patterns of semen freezability were selected. Two-dimensional gel electrophoresis showed differential abundance in a group of seven protein spots in the frozen/thawed seminal plasma from the bulls, ranging from 15 to 17 kDa, with pI values from 4.6 to 5.8. Four of these spots were confirmed to be BSP1 using mass spectrometry, proteomics, biochemical, and computational analysis (Tukey's test at $P < 0.05$). The protein spot weighing 15.52 ± 0.53 kDa with a pI value of 5.78 ± 0.12 is highlighted by its high abundance in bulls with low semen freezability and its absence in bulls presenting high semen freezability. This is the first report showing that more than two forms of BSP1 are found in the seminal plasma of Nelore adult bulls and not all animals have a similar abundance of each BSP1 form. Different BSP1 forms may be involved in different events of fertilization and the cryopreservation process.

1. Introduction

Selection of bulls with high genetic value is an essential tool to improve farmers' herds in regard to the production of beef cattle [1]. At artificial insemination (AI) centers, it is necessary to assess a variety of breeding bulls to accurately select animals that possess high quality and sufficient freezability of the semen [2]. Bulls presenting high reproductive capacity in natura can produce semen sensitive to cryopreservation [3]. Routine assessments of semen [4] and systems for computer-assisted sperm analysis [5], which are widely used by AI centers, do not accurately determine the potential freezability of semen.

After cryopreservation of bull semen, the spermatic viability may be reduced by up to 50%, but cryopreservation is a poorly understood process at the molecular level [3,6].

* Corresponding author. Tel.: (+55) 31 3899 2919; fax: +55 (31) 3899-2873.
E-mail address: baracat@ufv.br (M.C. Baracat-Pereira).

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The presence of Binder of SPerm (BSP) proteins [7] is known to influence the success of cryopreservation [8], along with other biomolecules present in the complex mixture of the seminal plasma [9]. BSP proteins belong to a family of heparin-binding proteins and represent approximately 70% of the total protein content of the plasma [10]. They are involved in different steps of bovine fertilization, including sperm capacitation. BSPs promote a cholesterol efflux from sperm membranes, which increases their fluidity and modulates membrane stability [11]. During cryopreservation, the cholesterol efflux can lead to a decrease in sperm resistance to cold [3].

One of the most abundant BSP proteins, BSP1, is described to be a mixture of the BSP-A1 and BSP-A2 proteins [9] and is multifunctional in the seminal plasma [3,12]. In an attempt to unravel the physiological events involved in sperm fertility and the cryopreservation of the sperm of bulls, BSP1 has been widely studied [3,12–14]. BSP1 plays a role in forming an oviductal sperm reservoir by enabling the sperm to bind to the oviductal epithelium, maintaining bull sperm motility. In addition, BSP1 participates in the changes in the sperm plasma membrane by stimulating the cholesterol efflux, causing cryoinjuries to the frozen semen that arise from premature semen capacitation and acrosome reaction [3,11,12]. Therefore, BSP1, alongside other BSP proteins, may be both beneficial and detrimental to sperm [8], supporting the idea that this singular protein has multiple functions.

Despite the many studies concerning cryoinjuries in semen, information about the presence of different forms of BSP1 and the probable involvement of these forms in different events in reproduction and cryopreservation is scarce. Two-dimensional electrophoresis (2-DE) has proven to be helpful in the study of glycoproteins that have small differences in mass and charge [15]. The objective of this work was to use proteomics to determine the differential abundance of the forms of the BSP1 protein in cryopreserved seminal plasma of *Bos taurus indicus* bulls with different patterns of semen freezability.

2. Materials and methods

2.1. Materials

All materials were of the highest grade available. Chemicals and reagents were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA) or from Bio-Rad Laboratories (Hercules, CA, USA), unless otherwise stated. Specific materials for electrophoresis procedures were purchased from GE Healthcare (Life Sciences, USA).

2.2. Animals, sample collection, and semen analysis

Nine healthy fertile *Bos taurus indicus* adult bulls with an average age of 7 years (ranging from 3 to 10) were selected from a subset of a wide cohort of recurrent donors of semen from an AI center in the city of Magda, São Paulo, Brazil (20°38′38″ S, 50°13′34″ W, 526 m). All bulls were kept under similar handling and feeding conditions and were confirmed to have low-freezability semen for more than a year. Semen collection was performed using an artificial vagina. Semen samples were assessed as routinely performed at the AI center.

In this work, the physical and morphologic evaluations of the fresh (F) semen were performed immediately after collection while maintained at 37 °C, as recommended by the Brazilian Animal Reproduction College [4]. For semen cryopreservation, the sperm concentration was determined and adjusted to 25 million sperm per dose by the addition of a cryoprotector on the basis of citrate, egg yolk, and glycerol [16]. Semen samples were maintained at 4 °C following preservation inside of thin 250-μL cryovials in liquid nitrogen, as regularly performed at the AI center. Frozen samples were thawed in an ice-bath, and the physical evaluation of the cryopreserved semen was performed. Bull selection focused on animals that produced ejaculates of high sperm quality for F semen but did not present similar values for straight progressive sperm motility in the same semen after a frozen/thawing (FT) process. The accepted parameter values in F semen included greater than 70% straight progressive sperm motility, greater than 3 (scale from 0 to 5) sperm vigor, less than 15% major sperm defects, less than 15% minor sperm defects, and less than 30% of sperm abnormalities for total sperm defects. For FT semen, the minimum accepted value for straight progressive sperm motility was 30%, while the other parameter values were maintained.

The straight progressive sperm motility and the sperm vigor were assessed by placing a 10-μL aliquot of semen between a slide and a coverslip preheated to 37 °C and analyzing the sample under an optical microscope at a magnification of 200 to 400 times [4]. The spermatozoa defects were evaluated on a wet preparation. An aliquot of semen diluted in buffered formol-saline was used for analysis by phase-contrast microscopy at a magnification of 1000 times under a drop of immersion oil [17]. For each ejaculate, a set of 400 cells were examined, and the sperm defects were measured as a percentage according to the classification criteria adopted by Blomm [18], as recommended by the CBRA [4]. All analyses were performed in at least three technical replicates.

2.3. Seminal plasma preparation

Immediately after collection, a 6.0-mL aliquot of F semen from each bull was centrifuged at 150 × *g* for 10 minutes to remove the spermatozoa and again centrifuged at 700 × *g* for 10 minutes to remove cellular debris. Each recovered supernatant was filtered at 0.22 μm to obtain seminal plasma, followed by cryopreservation inside of thin 250-μL cryovials in liquid nitrogen. The 24 frozen cryovials were transferred to an ultra-freezer at −80 °C.

2.4. Determination of soluble protein concentration

Quantification of soluble proteins in FT seminal plasma samples was performed by three procedures as follows: (1) the Coomassie Brilliant Blue (CBB) method [19], (2) the CBB method for samples precipitated with pure acetone, and (3) the bicinchoninic acid (BCA) method [20]. Three FT samples from each bull were singly thawed and re-centrifuged (10,000 × *g* for 60 minutes at 4 °C). Then, the samples