



Effect of scrotal insulation on sperm quality and seminal plasma proteome of Brangus bulls

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ABSTRACT

Seminal plasma (SP) contributes to sperm physiology and metabolism, prevents premature capacitation, and protects sperm against oxidative stress. In order to evaluate the impact of heat stress in the semen of tropically adapted Brangus breed and in their seminal plasma proteome, we studied the effects of scrotal insulation for 72 h. Semen samples from six bulls, between 7 and 8 years of age, were collected prior to scrotal insulation (pre-insulation), and at 4 and 11 wk after insulation. Seminal plasma samples were analyzed by 2D SDS-PAGE and liquid chromatography coupled with mass spectrometry (LC-MS/MS). Insulation caused decrease in vigour, gross and total motility after 4 wk of scrotal insult ($P < 0.001$). Total defects in sperm were higher after 4 wk compared to pre-insulation and 11 wk after scrotal insulation ($P < 0.001$). The analysis of the 2D protein profile of the SP resulted in the identification 183 unique protein spots in all gels evaluated. There was no difference in mean number of protein spots amongst time points. Eight protein spots were more abundant in SP after scrotal insulation, returning to the same expression level at 11 wk post-insulation. One spot had higher abundance at 11 wk post-insulation, and one spot had decreased abundance 4 wk after insulation. The ten protein spots with differential abundance amongst time points were identified as Seminal plasma protein PDC-109, Seminal plasma protein A3, Seminal plasma protein BSP-30 kDa, Spermadhesin-1 and Metalloproteinase inhibitor 2. The validation of these five proteins as biomarkers for thermal testicular stress in Brangus breed would allow the development of new biotechnologies that could improve bovine semen analysis in breeding systems in tropical and subtropical conditions. A close association between the identified BSP and Spermadhesin-1 was evidenced in protein-protein interaction analysis. Based on gene ontology analysis, variation in sperm function after insulation could be explained by variation in the expressed proteins in the SP. Further studies are required to verify if these proteins could be used as biomarkers for the identification of bulls with increased seminal resistance to heat stress in Brangus breed.

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1. Introduction

In beef cattle breeding systems, mating takes place during the

hottest months of the year, and bulls are exposed to environmental variations that may interfere with their fertility and reproductive effectiveness [1]. Seasonality may influence not only the animal's behavior and welfare, but also their sperm production and quality, which are essential for the effective production of livestock [2]. High summer temperatures decreased semen quality in bulls [3] because testicular temperature must not exceed 33–34.5 °C for normal spermatogenesis [4]. The production of viable sperm

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depends on testicular physiological mechanisms, especially thermoregulation, so much so that the temperature of the scrotum should be maintained at 2 °C to 6 °C below body temperature [5,6].

The Brangus breed (5/8 Angus × 3/8 Nelore) is synthetic cattle bred specifically to improve hybrid vigour and has a significant adaptation to tropical and subtropical regions and presents higher genetic potential to improve productivity efficiency [7,8]. Therefore, the identification of bulls with higher resistance to heat stress could improve the reproductive evaluation and management in breeding systems located in tropical and subtropical areas. Consequently, there is an increasing need to develop emerging tools and technologies that are able to better assess seminal parameters in bulls and, therefore, to correctly predict changes in seminal quality.

Seminal plasma has a key role in fertilization as it contributes to sperm capacitation, acrosome reaction and sperm-oocyte interaction [9,10]. In the last two decades, proteomic studies allowed a broader understanding of the seminal plasma composition, evidencing the presence of a myriad of transcription and growth factors that nourish and protect sperm as they travel within the male and female reproductive tracts [11]. To date, 1159 proteins were identified in the bovine seminal plasma, with several proteins already suggested as biomarkers for fertility [12]. The presence of tissue-specific seminal plasma proteins derived from the testis, epididymis and accessory glands can serve as potential biomarkers for male fertility assessment. These reproductive organs are disturbed during heat stress [13,14], which corroborates the potential for analyzing seminal plasma for molecular markers associated with spermatogenesis, sperm maturation, and seminal traits.

Scrotal insulation (SI) has become the method of choice to study the nature and magnitude of sperm defects in bulls [15–17]. Scrotal insulation for 72 h induces thermal insult to the testes and epididymides that interfere with testicular thermoregulation, predisposing to degeneration of the gonadal germinal epithelium and changes in seminal quality [17–19]. Previous works demonstrated significant changes in sperm protein profiles after SI in bulls [20,21]. However, the effects of SI in the bovine seminal plasma proteome are poorly understood. In addition, the identification of seminal plasma proteins can be used to identify possible testicular damage by heat stress factors or an acute inflammatory process that may lead to tissue modifications in the scrotum. Therefore, the purpose of this work was to investigate the effects of SI in the seminal plasma proteome of Brangus bulls in order to identify potential seminal biomarkers for heat stress.

2. Material and methods

2.1. Animals and experimental design

The experiment was conducted at the Experimental Station located between 30°04'30"S and 30°07'30"S latitude, 51°39'18"W and 51°42'18"W longitude, 46 m altitude, and climate classified as Cfa type (humid subtropical climate), which is characterized by four well-defined seasons, being very hot during the summer and potentially dry during the winter, according to Köppen–Geiger [22]. All the procedures involving animals during this study were approved by the Ethics Committee for care and use of experimental animals of the Universidade Federal do Rio Grande do Sul (Project 26,250; CEUA/UFRGS).

Animals were kept outdoors in the same environmental conditions and fed with a diet system based on cultivated (*Pennisetum americanum*) and natural (*Paspalum dilatatum*, *P. pauciliatum*, *P. modestum*, *P. pumilum*, and *Panicum aquaticum*) pasture. Stocking rate used for animals was 1.2 AU/ha, and all bulls received micro and macrominerals supplementation containing 40 g per kg recommended for this animal category. In addition, animals had free

access to water and shelter. The bulls' body condition score was 3.5 on a scale ranging from 1 to 5 (1 = very lean and 5 = very fat) during the entire experiment [23].

For the experiment, six Brangus bulls between seven and eight years of age were subjected to the Bull Breeding Soundness Evaluation (BBSE) at the beginning of the experiment, which consisted of a comprehensive general clinical examination, special clinical examination, and seminal evaluation [24]. Bulls were properly vaccinated against infectious bovine rhinotracheitis, bovine virus diarrhea, foot and mouth disease, and leptospirosis, according to Brazilian regulations. Seminal collections and analyses were performed from March 21st to May 2nd 2015.

2.2. Scrotal insulation

At the beginning of the experiment, ejaculates were collected using an electroejaculator. Then, in the next week, all bulls were submitted to SI for 72 h and were subjected to semen collection weekly for 13 consecutive wk [18]; however, only ejaculates collected prior to SI, and at 4 and 11 wk after SI removal were kept for seminal proteome analyses (Supplementary Fig. 1).

Thermal insult of the testes was induced by enclosing the entire scrotum, including the neck, using plastic diapers containing two layers of cotton, covering the entire scrotum surface. In addition, diapers were covered by a brown cotton bag, fixed with adhesive tape and carefully placed at the spermatic cord part of the scrotum. The insulation bag was placed with adhesive tape in the spermatic cord region and tightly enough to keep in place but not to restrict blood flow during the insult period. After scrotal being insulated, scrotum was monitored every 2 h for the first 8 h of insulation and observed 24, 48 and 72 h later after insulation removal. The use of this technique was consistent across bulls and for the entire period of 72 h [18,21].

2.3. Semen collection and evaluation

Bulls were restrained in the standing position in a chute suitable without sedation or tranquilization, and feces were removed manually prior to semen collection. A total of three ejaculates were collected from each bull using an automatic operated electroejaculator Pulsator IV (Lane Manufacturing Denver, CO, USA). An electroejaculator probe with three ventrally oriented longitudinal electrodes with 300 mL of a lubricant gel was used to deliver a sequence of electrical impulses to each bull. The preputial area was clipped to reduce the amount of dirt and foreign material which otherwise may interfere with the semen analysis.

Then, samples were collected using a semen collector attached to the collection bag, the ejaculated semen was immediately transferred to collecting tubes placed in a water bath at 37 °C. Seminal samples were immediately assessed for sperm motility, gross motility and vigour, within 2 h until collection. The same veterinarian examined samples under bright-field microscopy during the entire experiment.

Gross motility activity, or the amount of swirling present in an undiluted semen sample, was determined by placing a 10 µL drop of semen on a pre-warmed microscope slide, and the edge of the drop was examined using an optical microscope at 400x magnification, receiving a score ranging from 0 to five: 0 = no swirl; + = no swirl with generalized oscillation of individual sperm only; ++ = very slow, distinct swirl; +++ = slow, distinct swirl; ++++ = moderately fast, distinct swirl and eddies; and +++++ = fast, distinct swirls and eddies with the appearance of good quality semen [24,25]. Vigour (VIG) was evaluated using a scale from 0 to five based on progressive sperm movement, where 0 = none; 1 = very weak; 2 = weak; 3 = intermediate; 4 = strong;

and 5 = very strong [26].

Sperm total motility was assessed using 400x magnification under a bright-field microscope (NIKON, Eclipse E200, Melville, NY, USA) with a 5 μ L aliquot of semen placed on a warmed (37 °C) slide and covered with a coverslip. At least ten widely spaced fields were examined to provide an estimate of the percentage of motile sperm. Sperm motility was evaluated as the percentage of sperm movement (0–100%). Semen samples must have a minimum of 30% total motility to be considered satisfactory for a potential breeder, according to the BBSE minimum standards.

Sperm morphology was accessed using eosin-nigrosin staining using 1,000 x magnification under a bright-field microscope. Major sperm defects were considered as following: acrosome defect, abnormal head, double head, abnormal small head, proximal protoplasmic droplet, midpiece defect, accessory tail, strongly coiled or folded tail (DAG defect), and strongly bent tail. Minor sperm defects included distal protoplasmic droplet, abbatial implantation, bent tail, and detached head. The total defects were analyzed in 200 sperm cells from each animal, and a sperm classification was performed as previously described by the BBSE of the Western Canadian Association of Bovine Practitioners [24].

2.4. Seminal plasma processing and protein quantification

After collection, semen aliquots of 1,000 μ L were centrifuged at 800 \times g for 10 min at room temperature, followed by a second centrifugation at 12,000 \times g for 60 min at 4 °C, aiming to remove all cells and debris. To the supernatant, protease inhibitors (Protease Inhibitor Cocktail, Sigma, St. Louis, USA) at a final concentration of 1% v/v were added and samples were stored at –80 °C until proteome analysis. Protein quantification was performed with the BCA Protein Assay Kit (Pierce, Waltham, USA) according to the manufacturer's instructions.

2.5. 2D-SDS-PAGE

Seminal plasma proteins were analyzed in triplicates by 2D SDS-PAGE [27,28]. Briefly, isoelectric focusing (IEF) was carried out using 7-cm IPG Strips (pH 3–10, Bio-Rad, Hercules, USA). Seminal plasma protein extracts (300 μ g per sample) were mixed well with rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 50 mM DTT, 0.2% Bio-Lyte 3/10 ampholyte, 0.001% Bromophenol Blue) to a final volume of 125 μ L and 300 μ g of total protein. Isoelectric focusing was carried out using the PROTEAN i12 IEF System (Bio-Rad) according to the following program: passive hydration for 60 min, followed by active hydration for 12 h, linear gradient at 250 V, 50 μ Amp for 20 min; linear gradient at 4,000 V, 50 μ Amp for 120 min; and fast gradient at 4,000 V, 50 μ Amp up to a total of 10,000 Vh. After focusing, IPG strips were incubated for 15 min in equilibration buffer I (6 M urea, 30% glycerol, 2% SDS, 1.5 M Tris-HCl, pH 8.8 and 1% DTT) and re-equilibrated for an additional 15 min in equilibration buffer II (6 M urea, 30% glycerol, 2% SDS, 1.5 M Tris-HCl, pH 8.8). Strips were then placed on the top of the SDS-PAGE gels (15%), sealed with agarose (5% in SDS-PAGE running buffer), and ran at 80 V with 20 mA per gel. Gels were stained in colloidal Coomassie Blue R-250 (Sigma), 0.1% in methanol (50%), and acetic acid (7%) for 16 h. Then, gels were bleached in methanol solution (50%) and acetic acid (7%) for 2 h [29].

2.6. Image analysis

2D-SDS gels were scanned as TIFF file extensions at 300 dpi using Image Scanner III (GE Life Sciences, Pittsburg, USA) and analyzed using PDQuest 8.1 (Bio-Rad). Each sample was analyzed in duplicate, resulting in 12 gels per time point. A total of 36 gels were

performed during the experiment. A master gel was constructed, which included spots of a reference gel and spots consistently present in the other 2D maps. Landmark spots, present in key regions of the gels, matched in every member of the match set. Also, 2D SDS-PAGE Standards (Bio-Rad) were used as internal controls and for gel landmark references. Quantification of protein spots in the gels was given as parts per million (ppm) of the total integrated optical density of spots [30].

2.7. Protein identification by LC-MS/MS

Protein spots presenting significant differences between time points were investigated by liquid chromatography coupled to mass spectrometry (LC-MS/MS). Sections of each differently expressed protein spots were excised and subjected to in-gel trypsin digestion [31]. For quality control, a blank area of the gel and a known protein spot were also excised. Trypsinized digests were separated on an in-house made 20 cm reverse-phase column (5 μ m ODSAQ C18, Yamamura Chemical Lab, Japan) using a nano-UPLC (nanoLC Ultra 1D plus, Eksigent, USA) and eluted directly to a nanospray ion source connected to a hybrid mass spectrometer (LTQ-XL and LTQ Orbitrap Discovery, Thermo, USA). Mobile phases were water/acetonitrile/formic acid (95:5:0.1) as buffer A, and water/acetonitrile/formic acid (10:90:0.1) as buffer B. The flow rate was set to 300 nL min⁻¹ in a 60 min reverse-phase gradient. The mass spectrometer was operated in a data-dependent mode, with full MS1 scan collected in the Orbitrap, with *m/z* range of 400–1,600 at 30,000 resolution. The eight most abundant ions per scan were selected to CID MS2 in the ion trap, with dynamic exclusion applied. Mass spectra were analyzed using PatternLab platform [32]. MS/MS spectra were searched with COMET [33] using a non-redundant database proteome. The validity of the peptide-spectra matches (PSMs) generated by COMET was assessed using Patternlab's module SEPro [32] with a false discovery rate of 1% based on the number of decoys.

2.8. Bioinformatics analysis

All identified proteins were categorized by Gene Ontology (GO) annotation [34] according to biological process and molecular function using the Blast2GO 4.0 tool [35]. Blast2GO is a bioinformatics tool for the automatic functional annotation of DNA or protein sequence data mainly based on the GO vocabulary. In addition, protein-protein interactions were evaluated using STRING v.10 (<http://string-db.org/>), generating an interaction network based in physical and functional associations. The associations in STRING include direct (physical) interactions, as well as indirect (functional) interactions, as long as both are specific and biologically meaningful [36].

2.9. Statistical analysis

All seminal parameters were tested for normality of distribution using the Shapiro–Wilk test. Results are presented as mean \pm standard deviation (SD). Seminal variables were analyzed by Two-way repeated measures ANOVA followed by Tukey's test, assuming the animal as dependent variable and time point as independent variable. Protein concentration in seminal plasma samples and spot densities of each experimental group (before and after 4 and 11 wk post SI) were subjected to One-way ANOVA and means were compared between groups by Tukey test. Statistical significance was assumed when $P < 0.05$. Statistical analyses were carried out using GraphPad Prism 6 (La Jolla, USA).

3. Results

Scrotal insulation had a significant impact in seminal traits as described in Table 1. Insulation decreased in gross motility, motility and vigour after 4 wk of scrotal insult ($P < 0.001$). Total defects in sperm were higher after 4 wk of insulation compared to samples collected 1 wk prior and 11 wk after SI ($P < 0.001$). In addition, major defects had a pattern similar to total defects. However, minor defects were lower 4 wk after insulation compared to the other time points ($P < 0.001$). Seminal quality data for all collection times are presented as Supplementary Figs. 2, 3 and 4.

Seminal plasma total protein concentration did not differ statistically between time points (prior SI: $11.32 \pm 3.23 \mu\text{g}/\mu\text{L}$; 4 wk after SI: $12.07 \pm 2.25 \mu\text{g}/\mu\text{L}$; 11 wk after SI: $10.92 \pm 4.72 \mu\text{g}/\mu\text{L}$). The analysis of the seminal plasma 2D protein profile resulted in the identification of 183 unique protein spots. There was no difference in the mean number of protein spots in the 2D gels of seminal plasma collected before SI (61 ± 27 spots/gel) and after 4 (72 ± 17 spots/gel) and 11 (52 ± 18 spots/gel) wk of the testicular insult. Ten protein spots had differential abundances between time points (Fig. 1) and were identified by LC-MS/MS (Table 2). Eight protein spots were more abundant ($P < 0.05$) in seminal plasma 4 wk after SI (2101, 3803, 4201, 4501, 4701, 4702, 5603 and 6501), returning to pre-SI levels after 11 wk of insult (Fig. 2). Spot 3101 was the only spot which demonstrated higher expression at 11 wk of insult ($P < 0.05$), while spot 3203 had the lower abundance 4 wk after SI ($P < 0.05$, Fig. 2).

Six protein spots were identified as Binder of Sperm Proteins (BSP): Seminal plasma protein PDC-109 (PDC-109; spots 3203, 3803, 4201 and 4701), Seminal plasma protein A3 (BSP-A3; spot 6501) and Seminal plasma protein BSP-30 kDa (BSP-30K; spot 5603). Moreover, Spermadhesin-1 (SPADH1; spots 2101, 3101 and 4702) and Metalloproteinase inhibitor 2 (TIMP2; spot 4501) had their expression altered during the experiment. A close interaction between the identified BSP and SPADH1 was evidenced in protein-protein interaction analysis. All three BSPs interact with TIMP2, which is directly associated with eight matrix metalloproteinase (MMP) family members (MMP1, MMP2, MMP3, MMP7, MMP9, MMP13, MMP15 and MMP16) and hemopexin, an important heme binder. An enriched interaction network is presented in Fig. 3. Based on gene ontology analysis, the differentially expressed proteins in the seminal plasma due to scrotal insulation participate mainly in reproduction and reproductive process (14%), cellular process (12%), biological regulation (12%), and regulation of biological process (12%), among other metabolic events. As for biological functions, such proteins present binding properties (e.g.: to anions, heparin, proteins, enzymes, signaling receptors, and

glycosaminoglycans; 74%) and enzyme inhibitor activity (7%).

4. Discussion

In the present study, we used SI for 72 h as a model for heat stress in Brangus bulls to compare sperm traits and the seminal plasma proteome before and after the testicular insult. The increase in testicular temperature caused by thermal insult determines changes impair spermatogenesis and sperm maturation in bulls [37,38]. As expected, bulls had a significant decrease in seminal quality after 4 wk of SI, with semen traits being reestablished to pre-SI values after 11 wk of testicular insult. Studies using the SI approach to evaluate heat stress have been reported in domestic animals with comparable findings [16,37,39]. In a similar experiment with Brangus bulls, our group also had that sperm progressive motility and morphology returns to pre-insult values within approximately 9 wk after the thermal insult [18].

Changes in the seminal plasma proteome induced by SI indicate that seminal plasma is a satisfactory source of information to provide new insights regarding bovine spermatogenesis. Although some studies evaluated SI consequences in semen quality during a shorter period [40–42], our findings are in agreement with data obtained on other crossbreds bulls [3,21] and in rams [19,43]. To identify candidates as biomarkers for heat stress-induced impaired spermatogenesis, we evaluated the changes in the seminal plasma proteome before and after SI. Since the seminal plasma proteome obtained by electroejaculated bulls has already been separated in the 2-D maps and SDS-PAGE and identified by mass spectrometry [44], our focus in this study was to identify the protein spots that had different abundances between time points. We observed ten protein spots that were differentially expressed: eight had higher optical density in seminal plasma 4 wk after insulation, one was more abundant 11 wk after SI, and another presented with the lowest abundance 4 wk after SI. The potential roles of the identified proteins and the possibility of their use as biomarkers will be discussed herein. In order to avoid protein nomenclature ambiguity, protein names are presented as recommended by UniprotKB/Swiss-Prot.

The BSP proteins were the most prevalent protein superfamily that demonstrated increased abundance in seminal plasma after four week after testicular insulation. Extensively studied by the Manjunath group in the last 30 years, BSP are key players in sperm maturation in bulls [45]. Seminal plasma protein PDC-109, Seminal plasma protein A3 and Seminal plasma protein BSP-30 kDa - also known as BSP1, BSP3, and BSP5, respectively [46], are secreted by seminal vesicles and make up approximately 50–60% of the total proteins in seminal plasma from Brahman bulls [44], similar to what has been found in the seminal fluid of *Bos taurus* bulls [47–50].

Recently, comparative proteomics and bioinformatics analysis was performed to identify fertility markers of crossbred bulls frozen semen and observed 12 different proteins expressed in high-fertile and only three proteins in low-fertile sperm. The authors validated two selected proteins in spermatozoa, and inferred that ENO1 and BSP1 may acts as protein biomarker for high and low fertility, respectively [51]. In addition, high concentration of BSP5 in seminal plasma was associated Holstein bulls with proven superior fertility [12]. Since fertility data were based on at least 674 breeding outcomes per bull using artificial insemination with frozen semen, the influence of BSP on sperm membrane composition might explain the association.

BSP proteins are known to induce an initial cholesterol and phospholipid efflux from the sperm membrane, and to promote bovine sperm capacitation after ejaculation. In addition, part of the BSP protein binds to the sperm membrane to avoid free

Table 1
Seminal traits of the ejaculates collected from six Brangus bulls submitted to scrotal insulation for 72h.

Seminal traits	1-wk prior SI	4-wk after SI	11-wk after SI	P-value
Ejaculate volume (mL)	3.10 ± 1.46^{ab}	4.70 ± 1.84^a	2.43 ± 0.86^b	<0.01
Total sperm motility (%)	77.50 ± 21.88^a	7.50 ± 5.80^b	81.60 ± 7.89^a	<0.0001
Gross sperm motility (1–5)	2.40 ± 1.40^a	0.50 ± 0.50^b	3.57 ± 0.49^a	<0.0001
Vigour (1–5)	3.00 ± 1.60^a	0.83 ± 0.69^b	3.00 ± 0.76^a	<0.0001
Total sperm defects (%)	27.29 ± 14.00^b	87.71 ± 8.84^a	37.43 ± 13.76^b	<0.0001
Major sperm defects (%)	13.71 ± 7.30^b	84.71 ± 10.52^a	23.86 ± 8.84^b	<0.0001
Minor sperm defects (%)	13.57 ± 7.85^a	3.00 ± 2.67^b	13.57 ± 5.39^a	<0.001

SI: Scrotal Insulation, Wk: week. Means followed by different common letters within a row are statistically different. Data showed as mean \pm standard deviation.

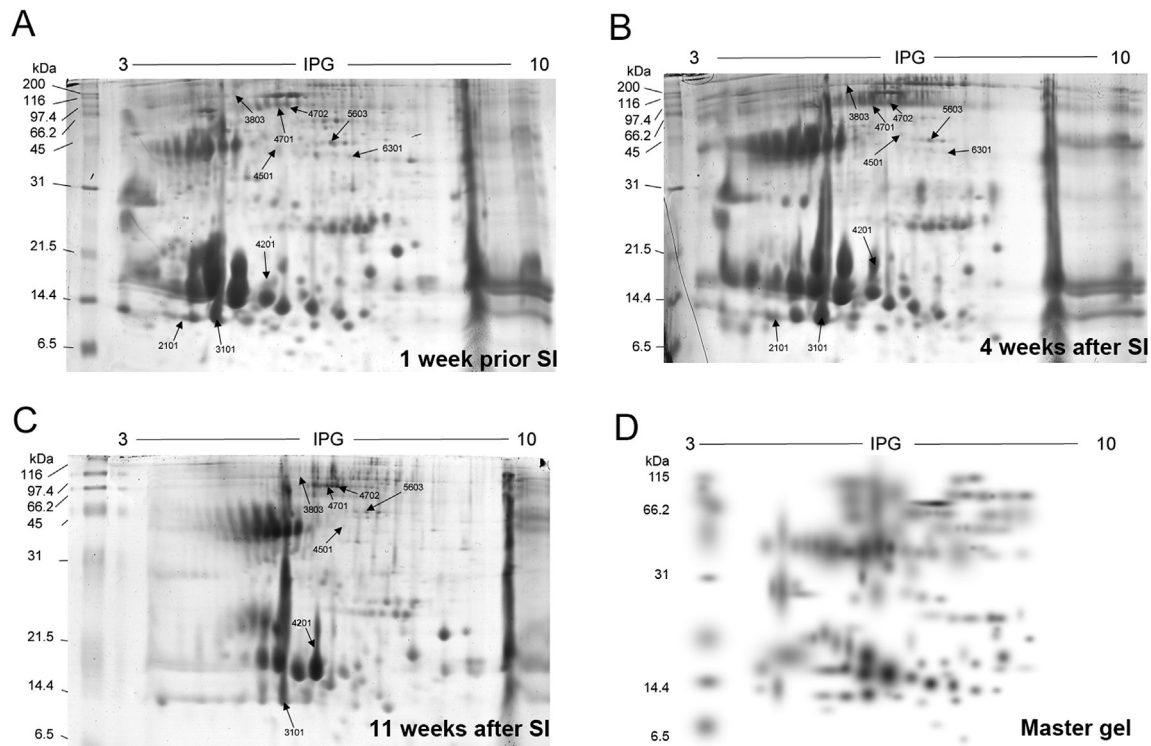


Fig. 1. Representative 2D SDS PAGE gels of seminal plasma proteins of Brangus bulls before and after scrotal insulation. (A) Sample collected 1 wk prior scrotal thermal insult; (B) Sample collected 4 wk after scrotal insulation; and (C) Sample were obtained after 11 wk of scrotal insulation; (D) Master gel generated by PDQuest software based in all spots used for statistical analysis. Arrows indicate the protein spots differentially expressed between time points. IPG; immobilized pH gradient (3–10).

phospholipid movement, thus protecting sperm until they reach the oviduct [52–54]. Inside the oviduct, high-density lipoproteins (HDL) and glycosaminoglycan (GAGs) found in luminal and follicular fluids interact with BSP proteins to promote sperm capacitation by means of a second phospholipid and cholesterol efflux that destabilizes the membrane [55–57]. Indeed, more studies to evaluate the purpose of the heat insult to the testes throughout a long period of semen recovery, is necessary to improve methods of interpretation of scrotal temperature patterns and to confirm the relationship with of seminal proteomics and fertility in bulls.

The increase of BSP proteins analogues, known as RSVPs (ram seminal vesicle proteins) spots were maximum 8 days after insulation started [43]. In addition, variation in RSVP seminal plasma expression as a result of scrotal insulation was mostly coincident with changes in sperm parameters. In bulls, however, seminal plasma protein A3 was decreased after SI in sperm protein extracts from animals insulated for 3 days [21].

It is noteworthy to mention that heat stress was directed towards the scrotum, not affecting the seminal vesicles directly. One possible explanation for the proteomic changes in the seminal plasma could be the effect of Leydig cells on testosterone synthesis, resulting in disruption of tissues that respond to androgens, such as accessory glands [58]. It was previously described that scrotal insulation for 96 h was associated with a decrease in testosterone and an increase in luteinizing hormone (LH) concentrations during the time of severe degeneration [59]. Interestingly, an opposite trend in hormonal concentrations was observed during the regenerative phase (15 wk after thermal insult) [59]. Also, scrotal insulation in the ram led to reduced testosterone serum concentration [60]. Based on these data, one can assume that a hypogonadal state could alter protein expression in the vesicular gland, affecting the composition of the seminal plasma proteome. In fact, this was already described for heparin-binding proteins secreted by

bovine accessory glands, in which its concentrations are regulated by androgens [61]. Nonetheless, a possible androgenic regulation of BSP genes and protein secretion in the male reproductive tract is yet to be determined.

Spermadhesin-1 (SPADH1), also known as acidic Seminal Fluid Protein (aSFP), is a 12.9 kDa acidic, non-glycosylated protein synthesized mainly by the ampulla and seminal vesicle epithelium [62–64], but that can also be detected in the epididymal fluid [65]. It is mainly formed by a CUB domain, a widely occurring structural motif found almost exclusively in extracellular and plasma membrane-associated proteins [66]. The members of the spermadhesin family participate in a wide range of biological functions, including complement activation, tissue repair, cell signaling, inflammation, and receptor-mediated endocytosis [67]. The comparison between the seminal plasma proteomes of bulls with high and low semen freezability suggests that SPADH1 could be a marker for high resistance to cryopreservation [29]. Studies with recombinant SPADH1 evidenced its binding ability to the sperm mid-piece [68], supporting its known influence on sperm mitochondrial activity. This effect was described by Schöneck et al. who observed a decrease in bovine sperm motility and mitochondrial activity after the addition of purified SPADH1 [69]. Together with its remarkable antioxidant behavior as a result of its redox equilibrium exhibited by cysteine residues Cys54 and Cys75 [70], SPADH1 may play an important role in protecting sperm against ROS during sperm storage in the epididymis prior to ejaculation. In this study, three protein spots (isoforms) were identified as SPADH1. Two were highly abundant in ejaculates collected after 4 wk of testicular insult, and one was more abundant in seminal plasma collected 11 wk after insulation removal. The identification of different isoforms of SPADH1 was already described in bulls [71,72], however a possible association between isoforms and sperm traits was not tested.

Table 2

Seminal plasma proteins differentially expressed in Brangus bulls after scrotal insulation. Protein spots identified by 2D SDS-PAGE and LC-MS/MS.

Protein name (UniProtKB entry) ^a	Theoretical Mass/pi [#]	Unique Peptide Count	Peptide Count	Spectral Count	Sequence coverage (%)	Matched peptides
Spermadhesin-1 (P29392 – SPAD1_BOVIN)						
Spot 2101	15.03 kDa/5.06	2	5	53	46	(36)EESGVIATYYGPK ⁽⁴⁸⁾ (65)VSIIQLQLNCNK ⁽⁷⁶⁾ (77)ESLEIIDGLPGSPVLGK ⁽⁹³⁾ (104)SSGSIMTVK ⁽¹¹²⁾ (116)EPEHPASFYEVLYFQDPQA ⁽¹³⁴⁾
Spot 3101	15.03 kDa/5.06	1	7	138	72	(27)NTNCGGILKEESGVIATYYGPK ⁽⁴⁸⁾ (36)EESGVIATYYGPK ⁽⁴⁸⁾ (49)TNCVWTIQMPPEYHVR ⁽⁶⁴⁾ (65)VSIIQLQLNCNK ⁽⁷⁶⁾ (77)ESLEIIDGLPGSPVLGK ⁽⁹³⁾ (94)ICEGSLMDYR ⁽¹⁰³⁾ (116)EPEHPASFYEVLYFQDPQA ⁽¹³⁴⁾
Spot 4702	15.03 kDa/5.06	2	2	7	27	(77)ESLEIIDGLPGSPVLGK ⁽⁹³⁾ (116)EPEHPASFYEVLYFQDPQA ⁽¹³⁴⁾
Seminal plasma protein PDC-109 (P02784 – SFP1_BOVIN)						
Spot 3203	15.48 kDa/4.91	10	10	24	57	(39)GPAELPEDEECVFPFVYR ⁽⁵⁶⁾ (59)KHFDTVHGSLFPWCSLDADYVGR ⁽⁸²⁾ (60)HFDCTVHGSLFPW ⁽⁷²⁾ (60)HFDCTVHGSLFPWCSLDADYVGR ⁽⁸²⁾ (67)GSLFPWCSLDADYVGR ⁽⁸²⁾ (73)CSLDADYVGR ⁽⁸²⁾ (74)SLDADYVGR ⁽⁸²⁾ (94)CVFPIYGGK ⁽¹⁰³⁾ (104)KYETCTK ⁽¹¹⁰⁾ (111)IGSMWMSWCSLSPNYDK ⁽¹²⁷⁾
Spot 3803	15.48 kDa/4.91	4	4	6	18	(59)KHFDTVHGSLFPWCSLDADYVGR ⁽⁸²⁾ (60)HFDCTVHGSLFPWCSLDADYVGR ⁽⁸²⁾ (73)CSLDADYVGR ⁽⁸²⁾ (104)KYETCTK ⁽¹¹⁰⁾
Spot 4201	15.48 kDa/4.91	6	6	17	48	(26)DQDEGVSTEPTQDQGAELPEDEECVFPFVYR ⁽⁵⁶⁾ (39)GPAELPEDEECVFPFVYR ⁽⁵⁶⁾ (60)HFDCTVHGSLFPW ⁽⁷²⁾ (60)HFDCTVHGSLFPWCSLDADYVGR ⁽⁸²⁾ (67)GSLFPWCSLDADYVGR ⁽⁸²⁾ (94)CVFPIYGGK ⁽¹⁰³⁾
Spot 4701	15.48 kDa/4.91	7	7	11	53	(26)DQDEGVSTEPTQDQGAELPEDEECVFPFVYR ⁽⁵⁶⁾ (39)GPAELPEDEECVFPFVYR ⁽⁵⁶⁾ (60)HFDCTVH ⁽⁶⁷⁾ (60)HFDCTVHGSLFPWCSLDADYVGR ⁽⁸²⁾ (67)GSLFPWCSLDADYVGR ⁽⁸²⁾ (94)CVFPIYGGK ⁽¹⁰³⁾ (104)KYETCTK ⁽¹¹⁰⁾
Metalloproteinaseinhibitor 2 (F1N430 – F1N430_BOVIN)						
Spot 4501	24.39 kDa/7.44	4	7	26	37	(27)CSCSPVHPQAFNCADIVIR ⁽⁴⁶⁾ (81)GPDQDIEFIYTAPSSAVCGVSLDIGGK ⁽¹⁰⁷⁾ (89)IYTAPSSAVCGVSLDIGGK ⁽¹⁰⁷⁾ (124)ITLCDFIVPWDTLSATQK ⁽¹⁴¹⁾ (127)CDFIVPWDTLSATQK ⁽¹⁴¹⁾ (165)YISSPDECLWMDWVTEK ⁽¹⁸¹⁾ (212)QEFLDIED ⁽²²⁰⁾
Seminal plasmaprotein BSP-30 kDa (P81019 – SFP4_BOVIN)						
Spot 5603	21.27 kDa/5.73	2	2	6	17	(85)ANDLNAVFEFGPACAFPTYK ⁽¹⁰⁴⁾ (138)DEPECVFPFIYR ⁽¹⁴⁹⁾
Seminal plasmaproteinA3 (P04557 – SFP3_BOVIN)						
Spot 6501	16.14 kDa/4.90	5	5	10	31	(26)DEQLSEDNVILPK ⁽³⁸⁾ (41)KDPASGAETK ⁽⁵⁰⁾ (42)DPASGAETK ⁽⁵⁰⁾ (54)CVFPIYGNK ⁽⁶³⁾ (99)CVFPIYEGK ⁽¹⁰⁸⁾

^a Full name as recommended by the UniProt consortium. Please refer to www.uniprot.org for alternative names. # Theoretical MW and pi obtained using ExPasy server (https://web.expasy.org/cgi-bin/compute_pi/pi_tool).

Another highly abundant protein in the seminal plasma of insulated bulls was metalloproteinase inhibitor-2, also known as TIMP-2. Differently from BSPs and SPADH1, TIMP-2 is expressed in the testis and epididymis, being found in the rete testis and epididymal fluid of several species [73–75]. It complexes with metalloproteinases (such as collagenases) and irreversibly inactivates them by binding to their catalytic Zn cofactor. Protein-protein interaction networks confirmed the interaction with 8

matrix metalloproteinases, key proteins in modulating the extracellular matrix (ECM), in spermatogenesis, and in bovine sperm viability during capacitation [76]. Interestingly, an increase of TIMP-2 in sperm from Holstein bulls was observed 28 days after SI, with a subsequent decrease to pre-SI level by the end of the experimental period [20]. An increase in the expression pattern of TIMP-2 in sperm proteins may be an indication of increased complex formation of this protein with membrane-type 1 (MT1)-MMP

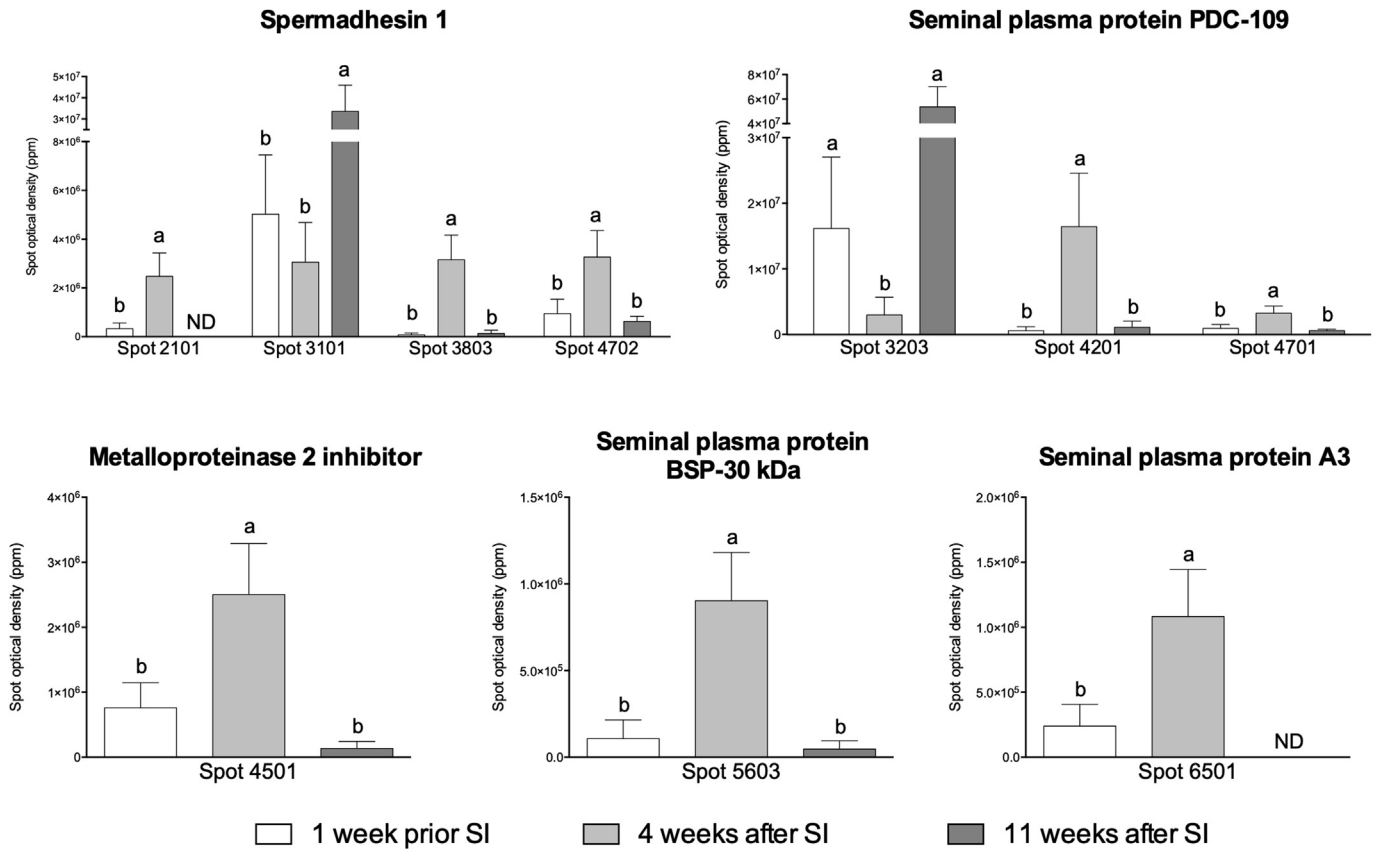


Fig. 2. Relative abundance of protein spots differentially expressed in 2D SDS-PAGE maps of seminal plasma from Brangus bulls submitted to scrotal insulation for 72 h. Data presented as mean ± SD. Letters indicate that means are statically different (Repeated measures ANOVA, Tukey test; P < 0.05); Asterisks indicate statistical difference between means (Student’s t-test; P < 0.05).

and activation of MMP-2 as a compensatory response for re-establishing normal spermatogenesis [20].

The most important function of the ECM is to maintain tissues with their specific mechanical and biochemical properties. In the

testis, Sertoli cells are responsible for the biosynthesis of ECM components [77]. Cell-matrix interactions are the result of the physical association between cell-surface receptors and ECM components. Pericellular matrix forms a microenvironment for

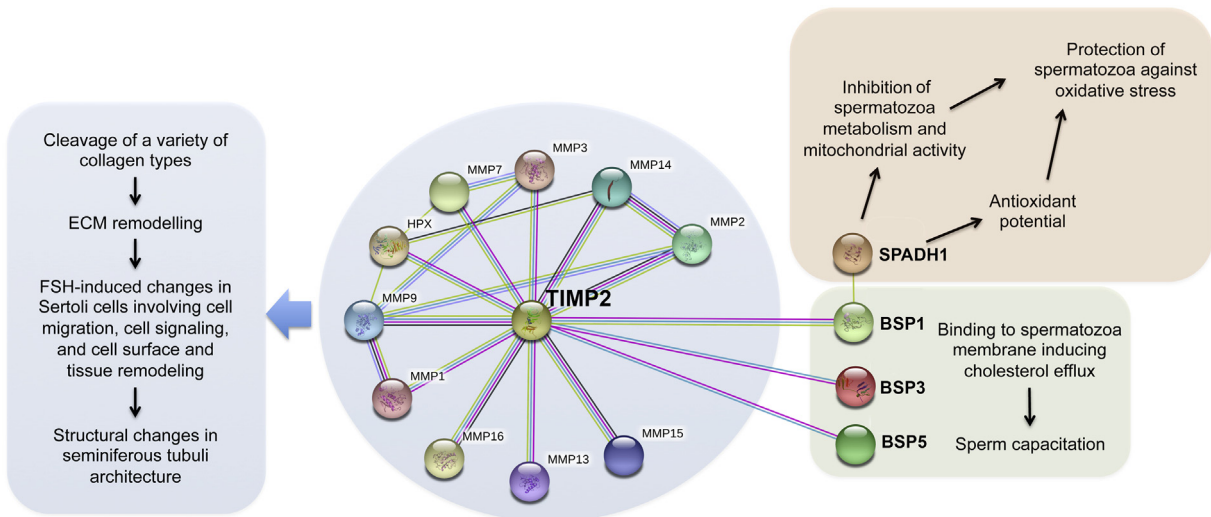


Fig. 3. Enriched protein-protein interaction network of 5 proteins differentially expressed in seminal plasma of Brangus bulls subjected to scrotal insulation for 72h. Color boxes indicate mutual roles for a given set of proteins. Network generated by String server using the following differentially expressed protein names: TIMP - metalloproteinase inhibitor-2; SPADH1 - Spermadhesin-1; BSP1 - Seminal plasma protein PDC-109; BSP3 - Seminal plasma protein A3; BSP5 - Seminal plasma protein BSP-30 kDa. Lines between nodes (proteins) represent different levels of interaction. MMP – matrix metalloproteinases; BSP – Binding of Sperm proteins; HPX – hemopexin.

protection against damaging physical factors, and facilitates cell signaling [78]. In fact, TIMP-2 is involved in Sertoli cell changes induced by the follicular stimulating hormone (FSH) [79]. These changes include cell migration, cell signaling, and cell surface and tissue remodeling, pivotal physiological processes for spermatogenesis [80]. Therefore, testicular TIMP-2 could be involved in tissue degeneration induced by scrotal insulation. Metalloproteinases are deeply involved in germ cell apoptosis triggered by *Fas* receptor signaling (extrinsic pathway) [81], which could be induced by hormonal disruption, like the one induced by scrotal insulation. In Holstein, this protein was suggested as a molecular indicator of bull fertility, since it was present in higher concentrations in seminal plasma of sires with elevated fertility scores [12]. In this regard, the use of seminal plasma TIMP-2 as a biomarker for monitoring spermatogenesis could improve bull and ejaculate selection for artificial insemination programs.

5. Conclusions

Scrotal insulation for 72 h induced a severe detrimental change in the seminal traits of Brangus bulls after 4 wk of insult; however, 11 wk after insulation removal, seminal parameters were reestablished to values prior to insulation. Seminal plasma protein PDC-109, Seminal plasma protein A3, Seminal plasma protein BSP-30 kDa, Spermadhesin-1, and Metalloproteinase inhibitor 2 were differentially expressed in bulls submitted to scrotal insulation. Based on their molecular function, regulation, and protein-protein interactions, we conclude that they are directly associated with cellular and molecular events that drive spermatogenesis. The validation of these five proteins as biomarkers for thermal testicular stress in Brangus breed would allow for the development of new biotechnologies that could improve bovine semen analysis in breeding systems in tropical and subtropical conditions.

Declaration of competing interest

The authors declare that there are no conflicts of interest that may have influenced that discussion presented herein.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.theriogenology.2020.01.014>.

Author contribution section

Gabriel Ribas Pereira: Conceptualization, Methodology, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition. Franciele Lucca de Lazari: Methodology. Pedro Ferrari Dalberto: Methodology. Cristiano Valim Bizarro: Methodology, Writing - original draft. Elistone Rafael Sontag: Methodology. Celso Koetz Junior: Methodology. Silvio Renato Oliveira Menegassi: Methodology. Júlio Otavio Jardim Barcellos: Project administration, Funding acquisition. Ivan Cunha Bustamante-Filho: Conceptualization, Writing - review & editing, Supervision, Funding acquisition.

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