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Research Article

Binder of Sperm Proteins 1 and 5 have contrasting effects on the capacitation of ram spermatozoa

Taylor Pini[1,*], Simon P. de Graaf[1], Xavier Druart[2], Guillaume Tsikis[2], Valerie Labas[3], Ana Paula Teixeira-Gomes[3], Barend M. Gadella[4] and Tamara Leahy[1]

[1]School of Life and Environmental Sciences, Faculty of Science, University of Sydney, Sydney, New South Wales, Australia; [2]Physiologie de la Reproduction et des Comportements, Institut National de la Recherche Agronomique, Le Centre National de la Recherche Scientifique, Institut Francais du Cheval et de L'Equitation, Université de Tours, Nouzilly, France; [3]Plate-forme de Chirurgie et Imagerie pour la Recherche et l'Enseignement, Pôle d'Analyse et d'Imagerie des Biomolécules, Institut National de la Recherche Agronomique, Nouzilly, France and [4]Department of Farm Animal Health and Biochemistry and Cell Biology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

*__Correspondence:__ School of Life and Environmental Sciences, Faculty of Science, University of Sydney, Sydney, New South Wales 2006, Australia. E-mail: taylor.pini@sydney.edu.au

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Abstract

Binder of Sperm Proteins (BSPs) are the most abundant seminal plasma protein family in the ram and bull. They have been extensively studied in the bull but less is known about their function in ovine seminal plasma and current knowledge suggests that BSPs may have different effects in these two species. In the bull, they facilitate capacitation and destabilize the sperm membrane during in vitro handling, whereas in the ram, they appear to stabilize the sperm membrane and prevent cryopreservation-induced capacitation-like changes. Further investigation into the effects of BSPs on ram spermatozoa under capacitating conditions is required to further clarify their physiological roles in the ram. We investigated the effects of Binder of Sperm Proteins 1 and 5 on epididymal ram spermatozoa in conditions of low, moderate, and high cAMP. BSPs had minimal effects on sperm function in low-cAMP conditions, but caused significant changes under cAMP upregulation. BSP1 stabilized the membrane and qualitatively reduced protein tyrosine phosphorylation, but significantly increased cholesterol efflux and induced spontaneous acrosome reactions. BSP5 slightly increased spontaneous acrosome reactions and caused sperm necrosis. However, BSP5 had minimal effects on membrane lipid order and cholesterol efflux and did not inhibit protein tyrosine phosphorylation. These findings demonstrate that under maximal cAMP upregulation, BSP1 affected ram spermatozoa in a manner comparable to bull spermatozoa, while BSP5 did not.

Summary Sentence

Binder of Sperm Proteins originating from seminal plasma play both pro- and decapacitating roles in ram spermatozoa.

Key words: sperm capacitation, ovine, seminal plasma.

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Introduction

Bedford and Chang described the "decapacitation" effect of seminal plasma over 50 years ago [1, 2], and a significant amount of research since has focused on how this fluid can be exploited to prevent detrimental capacitation-like changes in bull, boar and ram spermatozoa, caused by semen handling and storage [3, 4]. We now know that seminal plasma contains proteins which are able to suppress or reverse capacitation (e.g., murine Serpin Family E Member 2 (SERPINE2) [5]) and proteins which promote capacitation (e.g., human CD38 molecule [6]). Proteomic studies have highlighted the complex makeup of ram seminal plasma, identifying over 700 proteins and the most abundant protein families (e.g., Binder of Sperm Proteins [BSPs] and spermadhesins) [7]. BSPs 1 and 5 are particularly interesting, as they are highly abundant in ram seminal plasma, bind to the sperm membrane in large amounts at ejaculation [8], are well conserved across a range of species [9], and their homologs play roles in the capacitation of bull [10], mouse [11], human [12], and boar [13] spermatozoa.

BSPs account for over 50% of bull seminal plasma proteins [14], and their effects have been well characterized in this species. BSPs have powerful cholesterol efflux potential [15], interact with other capacitation promoters such as high-density lipoprotein [16], and promote the acrosome reaction [17], making them an important stimulator of capacitation for bull spermatozoa. However, because of these roles in capacitation, extended exposure of bull spermatozoa to BSP rich seminal plasma during in vitro handling can be detrimental [18]. Our knowledge of how BSPs affect ram spermatozoa is more limited; however, previous research suggests that they are abundant in ram seminal plasma [7], and the main constituent of seminal plasma which significantly protects ram spermatozoa during in vitro handling [19] and cold shock [20], by stabilizing the sperm membrane. One previous study has looked at the effect of BSPs on ram spermatozoa under capacitating conditions, and suggests that they act as decapacitation factors [21], a potential benefit during transit through the ewe's convoluted cervix [22]. Thus, there is potentially a considerable divergence in the "natural" roles of BSPs in the ram and bull, and their effects during in vitro sperm processing. These differences may reflect species-specific sperm membrane makeup [23] and post-translational protein modifications (e.g., glycosylation [24]); however, the effects of BSPs are not yet well characterized enough in the ram for a fair comparison. A more in-depth assessment of capacitation-related parameters is required in order to fully elucidate the in vitro and likely in vivo roles of ram BSPs.

Capacitation involves a swathe of changes, including promotion of hyperactivated motility, increased disorder of membrane lipids, cholesterol efflux, changes to sperm glycoconjugates, and development of tyrosine phosphorylation [25–27]. These changes can be replicated in vitro using a medium which mimics oviductal fluid [28], and typically contains bicarbonate, calcium, and delipidated albumin. This base medium is then further modified to include species-specific capacitation stimuli, which in the ram includes cyclic AMP analogs (e.g., dibutyryl (db) cAMP) and phosphodiesterase inhibitors (caffeine, theophylline). These chemicals serve to significantly upregulate cAMP levels, a phenomenon observed in response to physiological capacitating agents such as oviductal fluid [29], and allow for the development of capacitation-associated high molecular weight protein tyrosine phosphorylation and increased lateral fluidity of membrane phospholipids [30, 31]. Ram spermatozoa do not display these capacitation hallmarks without such additional cAMP upregulation when compared to other mammalian species (for instance pigs, cattle, rodents, and humans) [32] and the reason for this is not well understood. The moieties responsible for the promotion of ram sperm capacitation in vivo are unknown. Seminal plasma proteins may be a key factor affecting the responsiveness of ram spermatozoa to capacitation induction under low cAMP stimulatory conditions; however, this requires further investigation.

BSPs appear to have contrasting effects on ram and bull spermatozoa [19], but information on their action in ram spermatozoa is limited. Further, profiling of the effects of BSPs on ram spermatozoa may provide avenues to improve in vitro capacitation and to better understand their roles in vivo. Consequently, we have investigated the effects of isolated BSPs 1 and 5 on ram sperm functional parameters in both basal and stimulatory conditions.

Materials and methods

Chemicals

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (Castle Hill, Australia). Fluorescent probes were purchased from Life Technologies (Scoresby, Australia). Primary rabbit IgG antibody against gelatin affinity purified ram BSP proteins [33] (RRID AB_2715559, Supplementary Table S1) was kindly provided by P. Manjunath (Departments of Biochemistry and Medicine, Faculty of Medicine, University of Montreal).

Animals and semen collection

Rams used for seminal plasma collection (n = 50) were housed at the commercial CEDEO AI center in Ordiarp, France, and maintained on pasture. Semen for seminal plasma isolation was collected from mature rams (n = 50) via artificial vagina (1 ejaculate per ram). All ejaculates were assessed for wave motion and were of sufficient quality (≥4 out of 5). Ejaculates were pooled across rams and centrifuged twice (14 000× *g*, 20 min, 4°C) to isolate seminal plasma, which was stored at –80°C until further use.

Testes with epididymides were collected from a local slaughterhouse, transported to the laboratory on ice, stored at 4°C and flushed within 24 h. A set of epididymides from a single ram (n = 3) was considered a biological replicate, with each epididymis acting as a technical replicate, giving a 3 × 2 experimental design. Epididymal spermatozoa were collected by retrograde flushing of the cauda epididymis via the vas deferens using warm Tyrode lactate pyruvate (TLP) medium (10 mM HEPES, 0.4 mM $MgCl_2$, 100 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 0.3 mM NaH_2PO_4, 25 mM $NaHCO_3$, 2 mM sodium pyruvate, 5 mM glucose, 21.6 mM sodium lactate, pH 7.3) and assessed as above.

BSP isolation

Three hundred and twenty milligrams of ethanol precipitated seminal plasma proteins were loaded onto a 12 mL gelatin affinity column (gelatin was previously coupled to Affi-Gel 15 resin (Bio-Rad, Marnes-la-Coquette, France)). Bound proteins were eluted with phosphate-buffered saline (PBS) containing 5 M urea. One-milliliter fractions were collected and pooled relative to absorbance at 280 nm, desalted three times with a PD10 column, and lyophilized. A total of 64 mg of the gelatin-absorbed proteins were subjected to multiple runs of reversed-phase high-performance liquid chromatography (RP-HPLC) on a Waters XBridge BEH C18 OBD Prep column (250 mm × 10 mm i.d., particle size 5 μm, pore size 130 Å; Waters, Guyancourt, France). A linear 28–45% acetonitrile gradient with 0.1% trifluoroacetic acid was used at a constant flow rate of 3 mL/min for 29 min. BSP1 and BSP5 were isolated in two separate

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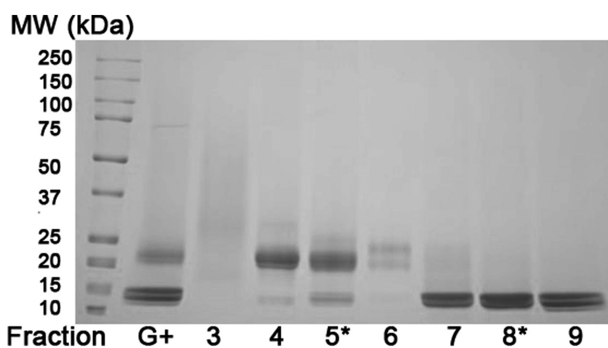

Figure 1. Fractions collected after gelatin affinity chromatography, followed by RP-HPLC of ethanol precipitated ram seminal plasma proteins. Coomassie brilliant blue stained 8–16% SDS PAGE of gelatin binding fraction (G+) (10 μg) and sequentially eluted RP-HPLC fractions (fractions 3 and 6; 5 μg, fractions 4, 5, 7, 8, 9; 10 μg). The fractions employed as "purified BSP1" (8) and "purified BSP5" (5) are indicated*.

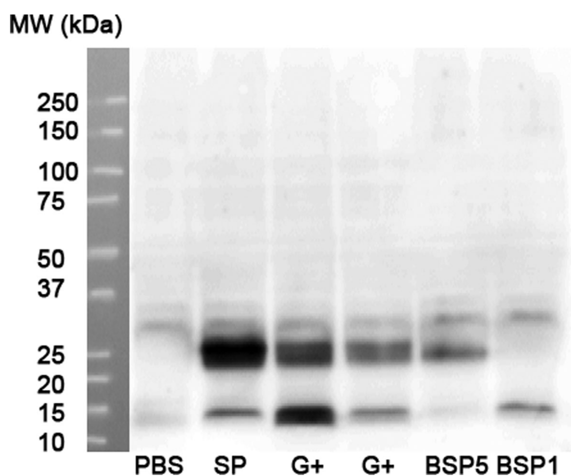

Figure 2. Binding of purified BSP1 and BSP5 to epididymal ram spermatozoa (1 h, 37°C). Western blot of epididymal spermatozoa (490 × 10^6 total) incubated 1:1 (v/v) with (1) PBS, (2) seminal plasma, (3, 4) gelatin binding fraction (13 or 1 mg/mL), (5) purified BSP5 (0.4 mg/mL), (6) purified BSP1 (1 mg/mL), probed with anti-BSP.

fractions (Figure 1), which were desalted by dialyzing against 50 mM ammonium bicarbonate and lyophilized. Western blotting confirmed binding of the isolated proteins to epididymal spermatozoa (Figure 2). Purified proteins were resuspended in physiological saline at high concentration (>2 mg/mL) and stored at –80°C. Prior to use, proteins were thawed on ice and warmed to 37°C.

Liquid chromatography tandem mass spectrometry of purified proteins

Six reversed-phase chromatography fractions (Figure 1) were in-solution digested with bovine trypsin (sequencing grade, Roche Diagnostics, Germany). Briefly, proteins in 50 mM NH_4HCO_3 were reduced in 5 mM dithiothreitol (30 min, 56°C) and alkylated in 12.5 mM iodoacetamide (20 min, room temp in the dark). Proteins were digested overnight with 12.5 ng/μL trypsin (sequencing grade, Roche, France) with a ratio of 1:40 enzyme:substrate. Five microliters of peptides were directly injected onto a trap column and separated on a nano-column as previously described [34], using a 4–55% B 90 min gradient at a flow rate of 300 nL/min on an Ultimate 3000 RSLC UHPLC system (Dionex, Netherlands). Eluate was ionized using a Thermo Finnigan Nanospray Ion Source 1 and tandem mass spectrometry (MS/MS) was carried out on an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Germany). Data were acquired in positive data-dependent mode, with sequential isolation (isolation width 2 *m/z*) and fragmentation (collision induced dissociation) of the 20 most intense peptide ions (charge state 2+, *m/z* range 300–1800). Proteins were identified by Mascot search engine (version 2.3, Matrix Science) against the NCBInr database with mammalian taxonomy (downloaded December 2016). Database search criteria included trypsin as a protease with two missed cleavages allowed, variable modifications (carbamidomethylcysteine, methionine oxidation, acetylation of N-terminal proteins) and 5 ppm/0.8 Da parent/fragment ion match tolerance. Scaffold software (version 3.6, Proteome Software, USA) was used to validate protein identifications using the Peptide and Protein Prophet algorithims. Protein identifications were accepted if they contained at least two peptides and had >95% probability. The abundance of identified proteins was estimated by calculating the emPAI using Scaffold Q+ software (Proteome Software, USA).

Treatment with capacitation stimulants and isolated protein

All experiments were repeated six times, including three biological replicates (rams) and two technical replicates (epididymides). Epididymal spermatozoa were diluted with TALP (TLP with 0.3% (w/v) fatty acid free bovine serum albumin (BSA), fraction V) to 58 × 10^6 spermatozoa/mL. Aliquots were then further diluted to 50 × 10^6 spermatozoa/mL with TALP containing 0, 75, or 150 μg/mL of isolated BSP1 or BSP5 and cAMP upregulators, as appropriate. The concentrations of BSPs used are slightly lower than the total concentration of BSPs in a ram ejaculate (roughly 200 μg per 50 × 10^6 spermatozoa as per Manjunath et al. [18], assuming ejaculate concentration of 4 × 10^9 spermatozoa/mL). Three different cAMP stimulation levels were used across three independent experiments; basal (TALP alone), moderate (TALP with 1 mM caffeine), and high (TALP with cAMP upregulators 1 mM caffeine, 1 mM theophylline, 1 mM db cAMP). All treatments were incubated for 20 min at 37°C immediately after protein/cAMP upregulator addition to allow for protein binding to the sperm membrane. Samples were then held at 37°C, with assessment at 0, 3, and 6 h. For flow cytometry and motility analyses, aliquots taken at each time point were treated with 1 mM D-penicillamine to prevent agglutination and allow for accurate analysis [31].

Motility analysis

Objective computer-assisted motility analysis was performed using an IVOS II (Hamilton Thorne, operating Animal Breeder software, version 1.8), with settings appropriate for ram spermatozoa (head size 10–42 μm^2, progressive motility thresholds of straightness 80%, and average path velocity 75 μm/s). Samples were diluted to a final concentration of 25 × 10^6 spermatozoa/mL immediately prior to assessment and loaded onto a CELL-VU slide. Eight screen captures recording ≥ 200 spermatozoa were obtained for each sample.

Flow cytometry

Flow cytometry analysis was performed using a C6 Accuri flow cytometer (Becton Dickinson, New Jersey, USA) with a 20 mW 488 nm laser source for scatter detection of spermatozoa and excitation of spermatozoa-associated fluorescent probes. Instrument calibration was performed each day using Spherotech 8-peak and 6-peak vali-

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Table 1. Total and progressive motility of epididymal ram spermatozoa incubated in TALP, TALP plus 1 mM caffeine, or TALP plus cAMP upregulators* at 0, 3, and 6 h of incubation at 37°C.

	Total motility (%)			Progressive motility (%)			Necrotic sperm**		
Time (hours)	0	3	6	0	3	6	0	3	6
TALP	68.1 ± 5.3[a]	66.9 ± 6.1[a]	55.8 ± 10.7[a]	50.7 ± 4.3[a]	53.3 ± 6.4[a]	44.7 ± 9.7[a]	12.6 ± 1.4[a]	15.7 ± 2.7[a]	23.5 ± 4.7[a]
TALP + 1 mM caffeine	78.4 ± 1.7[a]	78.0 ± 3.7[a]	66.8 ± 6.7[a]	65.7 ± 2.4[a]	67.0 ± 4.1[a]	56.0 ± 6.5[a]	14.9 ± 1.3[a]	18.2 ± 2.4[a]	26.1 ± 3.3[a]
TALP + cAMP upregulators*	63.8 ± 10.7[a]	30.9 ± 4.4[b]	22.9 ± 5.7[b]	51.4 ± 10.4[a]	17.1 ± 2.2[b]	12.5 ± 3.7[b]	24.0 ± 2.2[b]	33.8 ± 3.5[b]	48.6 ± 1.2[b]

[a]Different superscript letters denote significant differences ($P < 0.05$) within column.

*cAMP upregulators included 1 mM caffeine, theophylline, and db cAMP.

**"Necrotic" sperm were defined as nonviable (PI positive) but acrosome intact (FITC-PNA negative).

Data are presented as mean ± SEM.

dation beads (Becton Dickinson, New Jersey, USA). Probes were used to assay viability (propidium iodide, PI, 6 μM), acrosome integrity (fluorescein isothiocyanate conjugated to peanut agglutinin, FITC-PNA, 0.4 μg/mL), early changes in membrane permeability (YO-PRO-1, 25 nM), and membrane lipid disorder (merocyanine 540, M540, 0.83 μM). Stains were run in combination (FITC-PNA/PI and M540/YO-PRO-1) to allow for viability gating. Probes were incubated with samples in the dark for 10 min at 37°C prior to analysis. Fluorescence detection employed a 533/30 nm band pass filter for FITC-PNA and YO-PRO-1 and a >670 nm long pass filter for PI and M540. Forward/side scatter was used to eliminate debris and select spermatozoa, with further gating based on viability as measured by appropriate probes (PI or YO-PRO-1). A minimum of 10 000 events within the initial population of spermatozoa were analyzed and samples were either compared on the basis of percentage of probe-positive spermatozoa (FITC-PNA, PI) or median channel fluorescence of the relevant fluorophore within the viable population (M540).

Amplex Red cholesterol assay

Aliquots at 50 × 10^6 spermatozoa/mL were extended with TLP, washed (14 000× *g*, 10 min, room temperature), and the supernatant was retained. The supernatant was filtered (0.22 μm) to remove any contaminating spermatozoa and stored at –80°C. Thawed supernatants were assessed for cholesterol content using an Amplex Red cholesterol assay kit (Thermofisher, Waltham, USA), according to manufacturer's instructions. Briefly, cholesterol is oxidized by cholesterol oxidase, producing H_2O_2, which in turn reacts with the Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine). In the presence of horse radish peroxidase, this reaction produces fluorescent resorufin (ex/em maxima 571/585 nm). Fluorescence intensity was measured at 590 nm on an Infinite M-1000 pro spectrophotometer (Tecan, Mannedorf, Switzerland) and cholesterol concentration calculated against standards.

Tyrosine phosphorylation western blotting

A total of 7.5 × 10^6 spermatozoa were washed twice with TLP (14 000× *g*, 10 min and 600× *g*, 10 min). The supernatant was discarded and the pellet diluted 1:1 (v/v) with lysis buffer (cOmplete EDTA free protease inhibitor cocktail (Sigma), 1% (w/v) sodium dodecyl sulphate, 1 mM sodium orthovanadate, TLP). Spermatozoa were lysed at room temperature for 60 min with regular vortexing, and then centrifuged (7500× *g*, 15 min). The supernatant was retained and stored at –80°C. Cell lysates were measured for protein content using a Qubit Protein assay (Life technologies, California, USA), according to manufacturer's instructions. Ten microgram of protein was separated on a 10% TGX stain free gel (Bio-Rad) by SDS-PAGE (200 V, 40 min) using a mini-PROTEAN tetra cell (Bio-Rad) and blotted onto a PVDF membrane (100 V, 75 min, 4°C) using a mini Trans-Blot cell (Bio-Rad). The membrane was blocked with tris-buffered saline (TBS) with 0.1% Tween-20 (TW) and 1% (w/v) BSA at room temp for 60 min. The blocked membrane was probed with 1:2000 HRP-anti-phosphotyrosine (Merck-Millipore, Billerica, USA, RRID AB_310779, Supplementary Table S1) in TBS-TW with 0.1% (w/v) BSA at room temperature for 60 min and washed five times with TBS-TW. Signal was visualized using 1:1 (v/v) luminol and peroxidase (Bio-Rad Immun-star western chemiluminescence kit), incubated at room temperature for 5 min. Images were captured using a ChemiDoc XRS+ (Bio-Rad, California, USA) and qualitatively analyzed using Image Lab software (version 5.1, Bio-Rad, California, USA). Due to sample limitations for basal and moderate cAMP stimulation, only western blots of BSP1 treated spermatozoa were produced.

Statistical analysis

Statistical analysis was carried out using Genstat (version 18, VSNI International). Aliquots of collections from a single epididymis were considered an experimental unit, giving a total of six replicates per treatment. Data were assessed for normality using the Shapiro–Wilk test and homogeneity of variances by fitted value residual plots, and transformed if necessary (by $\log_{10}$, square root or power as appropriate) to meet the requirements of a linear mixed model. Outcomes were assessed using a linear mixed model incorporating treatment, time (as applicable), technical replicate and ram, with an α of 0.05. Means were compared on the basis of least significant difference, and all values are reported as the mean ± standard error of the mean, back-transformed if applicable.

Results

Confirmation of purity of isolated BSPs

Mass spectrometry confirmed that the isolated fractions of interest contained BSP1 and BSP5, at 98% and 89% purity, respectively (Supplementary Table S2).

Effects of BSP proteins under various levels of capacitation stimulation

The effects of BSPs on sperm motility and viability

Compared to plain TALP medium (basal cAMP stimulation), moderate cAMP stimulation (1 mM caffeine) did not alter total motility, progressive motility, or the proportion of necrotic spermatozoa (Table 1). In comparison, high cAMP stimulation significantly

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Table 2. Motility parameters and viability of epididymal ram spermatozoa incubated in TALP, TALP plus 1 mM caffeine, or TALP plus cAMP upregulators, with or without isolated BSP proteins, pooled over 6 h of incubation.

	TALP					TALP + 1 mM caffeine					TALP + cAMP upregulators*				
Treatment	Control	75 μg/mL BSP1	150 μg/mL BSP1	75 μg/mL BSP5	150 μg/mL BSP5	Control	75 μg/mL BSP1	150 μg/mL BSP1	75 μg/mL BSP5	150 μg/mL BSP5	Control	75 μg/mL BSP1	150 μg/mL BSP1	75 μg/mL BSP5	150 μg/mL BSP5
Total motility (%)	63.6 ± 7.9[a]	56.0 ± 4.0[a]	50.6 ± 3.3[a]	63.8 ± 5.2[a]	59.4 ± 4.7[a]	74.4 ± 2.8[a]	68.1 ± 3.6[b]	61.5 ± 4.5[c]	68.8 ± 3.1[a,b]	70.1 ± 4.1[a,b]	39.2 ± 5.9[a]	48.5 ± 5.8[a]	41.4 ± 6.0[a]	65.0 ± 5.0[b]	65.4 ± 4.3[b]
Progressive motility (%)	49.6 ± 3.8[a]	41.0 ± 3.4[a]	36.1 ± 2.9[a]	46.1 ± 5.5[a]	42.8 ± 4.9[a]	62.9 ± 2.8[a]	57.2 ± 3.3[b]	52.8 ± 4.0[b]	58.1 ± 3.1[b]	57.8 ± 4.0[b]	27.0 ± 5.5[a]	41.2 ± 5.4[b]	35.8 ± 5.4[b]	53.8 ± 4.9[c]	53.5 ± 4.6[c]
VAP (μm/s)	165.7 ± 7.5[a]	165.3 ± 11.7[a]	162.9 ± 11.5[a]	158.2 ± 11.3[a]	165.1 ± 10.3[a]	136.8 ± 2.6[a]	148.1 ± 3.1[b,c]	153.3 ± 3.8[c]	142.4 ± 3.6[a,b]	137.6 ± 3.1[a]	103.8 ± 4.8[a]	133.4 ± 4.3[b,c]	141.1 ± 3.5[b]	125.2 ± 4.5[c,d]	123.2 ± 5.0[d]
VCL (μm/s)	212.9 ± 10.5[a]	224.6 ± 16.2[a]	217.6 ± 12.7[a]	204.3 ± 12.4[a]	211.7 ± 11.4[a]	173.8 ± 4.9[a]	194.0 ± 6.6[b]	199.6 ± 7.1[b]	174.8 ± 6.1[a]	169.8 ± 5.9[a]	122.3 ± 5.3[a]	154.5 ± 5.5[b]	169.3 ± 5.0[c]	141.1 ± 5.0[d]	139.6 ± 5.6[d]
VSL (μm/s)	151.6 ± 7.0[a]	147.6 ± 10.6[a]	146.3 ± 11.3[a]	142.1 ± 11.3[a]	147.9 ± 10.9[a]	128.2 ± 2.3[a]	137.2 ± 2.8[b]	142.9 ± 3.4[b]	133.5 ± 3.4[a]	128.9 ± 2.6[a]	96.4 ± 5.0[a]	127.3 ± 4.3[b,c]	132.9 ± 3.4[b]	119.2 ± 4.5[c,d]	117.6 ± 4.8[d]
LIN (%)	71.5 ± 1.0[a]	66.5 ± 0.7[a]	67.2 ± 1.8[a]	69.5 ± 1.5[a]	69.5 ± 1.8[a]	75.9 ± 1.3[a]	72.8 ± 1.8[b]	73.4 ± 1.6[b]	78.5 ± 1.4[c]	78.1 ± 1.4[a,c]	81.9 ± 1.8[a,b]	83.7 ± 1.5[a,c]	80.0 ± 1.7[b]	85.6 ± 1.1[c]	85.5 ± 1.2[c]
ALH (μm)	6.3 ± 0.2[a]	7.0 ± 0.5[b]	6.8 ± 0.4[b]	6.2 ± 0.2[a]	6.3 ± 0.3[a]	5.2 ± 0.2[a]	5.9 ± 0.3[b]	5.8 ± 0.3[b]	4.8 ± 0.2[a]	4.8 ± 0.2[a]	4.0 ± 0.2[a,b]	4.4 ± 0.2[b,c]	4.6 ± 0.2[c]	3.8 ± 0.2[a]	3.8 ± 0.2[a]
BCF (Hz)	35.9 ± 0.9[a]	35.8 ± 0.8[a]	34.7 ± 0.6[a]	34.2 ± 1.0[a]	35.1 ± 1.0[a]	33.2 ± 0.8[a,b]	33.9 ± 0.6[a]	34.7 ± 0.7[a]	31.1 ± 1.1[b]	30.8 ± 1.3[b]	25.3 ± 1.2[a]	27.5 ± 1.4[a]	32.3 ± 1.3[b]	26.6 ± 0.8[a]	26.9 ± 1.1[a]
Necrotic sperm (%)**	17.3 ± 2.1[a]	15.2 ± 1.7[a]	12.5 ± 1.3[b]	20.9 ± 2.0[c]	22.4 ± 2.0[c]	19.7 ± 1.8[a]	14.1 ± 1.5[b]	15.8 ± 1.9[b]	28.1 ± 2.5[c]	25.9 ± 2.3[c]	35.5 ± 2.8[a]	37.6 ± 4.1[a]	30.8 ± 3.6[a]	34.2 ± 2.9[a]	37.3 ± 3.0[a]

[a]Different superscript letters denote significant differences ($P < 0.05$) across treatments, within media, for each kinetic parameter.

*cAMP upregulators included 1 mM caffeine, theophylline and db cAMP.

**"Necrotic" sperm were defined as nonviable (PI positive) but acrosome intact (FITC-PNA negative).

Data are presented as mean ± SEM.

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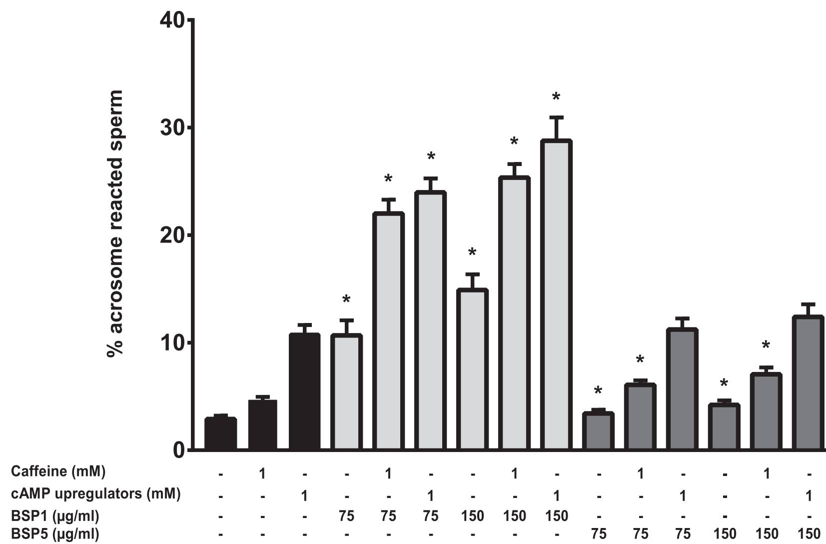

Figure 3. Acrosome integrity assessed by FITC-PNA fluorescence. Percentage of acrosome-reacted spermatozoa, pooled across a 6-h incubation with 0 or 1 mM of caffeine or all cAMP upregulators (caffeine, theophylline, dbcAMP), and with 0, 75, or 150 μg/mL of BSP1 or BSP5. * $P < 0.05$ relative to relevant control (with 0 or 1 mM of caffeine or cAMP upregulators).

altered these parameters, reducing motility and causing deterioration of spermatozoa, particularly after extended incubation (Table 1).

In plain TALP, motility parameters including total and progressive motility, velocity, linearity, and beat cross frequency were not significantly altered by the presence of 75–150 μg/mL of BSP1 or BSP5 (Table 2). BSP5, but not BSP1, caused some slight sperm deterioration. Several motility parameters were altered by the presence of 75–150 μg/mL of BSP1 or BSP5 under moderate cAMP stimulation (Table 2). While BSP1 reduced sperm necrosis at this level of stimulation, BSP5 promoted it. Interestingly, inclusion of either BSP1 or BSP5 under high cAMP stimulation diminished the negative effects of cAMP upregulators on a range of motility parameters (Table 2). However, addition of BSPs was unable to combat the significant deterioration of sperm viability under high cAMP stimulation. In general, compared to samples with no BSPs present, BSP1 consistently increased velocity, while BSP5 only showed improvements with all cAMP upregulators present. Linearity and straightness were decreased by BSP1 but increased by BSP5. BSP1 caused a consistent increase in the amplitude of lateral head displacement, and also increased beat cross frequency under high cAMP stimulation.

The effects of BSPs on the induction of acrosome reactions

Spontaneous acrosome reactions were measured following 0, 3 or 6 h of incubation in basal, moderate, and high cAMP-stimulating conditions. The acrosome reaction in the absence of BSPs was <5% in both basal and moderate cAMP-stimulating conditions and approximately 10% under high cAMP stimulation (Figure 3). BSP1 addition significantly increased acrosome reactions compared to the control under all cAMP stimulation levels, with a further significant effect of dose (75 versus 150 μg/mL). In contrast, BSP5 caused a slight but significant increase in acrosome reactions only under basal and moderate cAMP stimulation, with no effects of dose.

The effects of BSPs on sperm membrane lipid disorder responses

Membrane lipid disorder significantly increased in control samples with stimulation by exogenous cAMP upregulators. Membrane lipid disorder was not significantly different in BSP-exposed spermatozoa compared to the control without cAMP stimulation (Figure 4). However, when caffeine was introduced, all BSP treatments had slightly but significantly lower membrane lipid disorder than the control, and this effect was not dose dependent. When all cAMP upregulators were present, 150 μg/mL BSP1 significantly minimized membrane lipid disorder in relation to the control; however, membrane disorder was still significantly higher than that observed in basal conditions (Figure 4).

The effects of BSPs on protein tyrosine phosphorylation responses

In basal cAMP conditions, there was time-dependent phosphorylation of a 55-kDa band in the control, which was not observed in spermatozoa treated with 75–150 μg/mL BSP1 (Figure 5A). This 55 kDa band was present with moderate cAMP stimulation, but its development was not time dependent. However, this band was again not observed in spermatozoa treated with 75–150 μg/mL BSP1 (Figure 5B). There was no observable, time-dependent development of tyrosine phosphorylation of high molecular weight (>75 kDa) proteins in any treatments under basal or moderate cAMP stimulation. Presence of cAMP upregulators was required for the development of time-dependent tyrosine phosphorylation of high molecular weight proteins in the control (Figure 5C) as has been previously shown [30]. Both 75 and 150 μg/mL BSP1 appeared to inhibit to some degree the tyrosine phosphorylation of high molecular weight (>75 kDa) proteins in response to high cAMP stimulation, while also inducing somewhat stronger time-dependent protein tyrosine phosphorylation of an 18 kDa band compared to the control (Figure 5C). Such an inhibitory response was not evident for BSP5, which did not alter tyrosine phosphorylation of high molecular weight proteins at 75 or 150 μg/mL compared to the control (Figure 5D). However, at 150 μg/mL, BSP5 appeared to increase tyrosine phosphorylation of four moderate molecular weight protein bands (35, 37, 52, 55 kDa), as well as the same 18 kDa band, albeit at a slower rate than BSP1.

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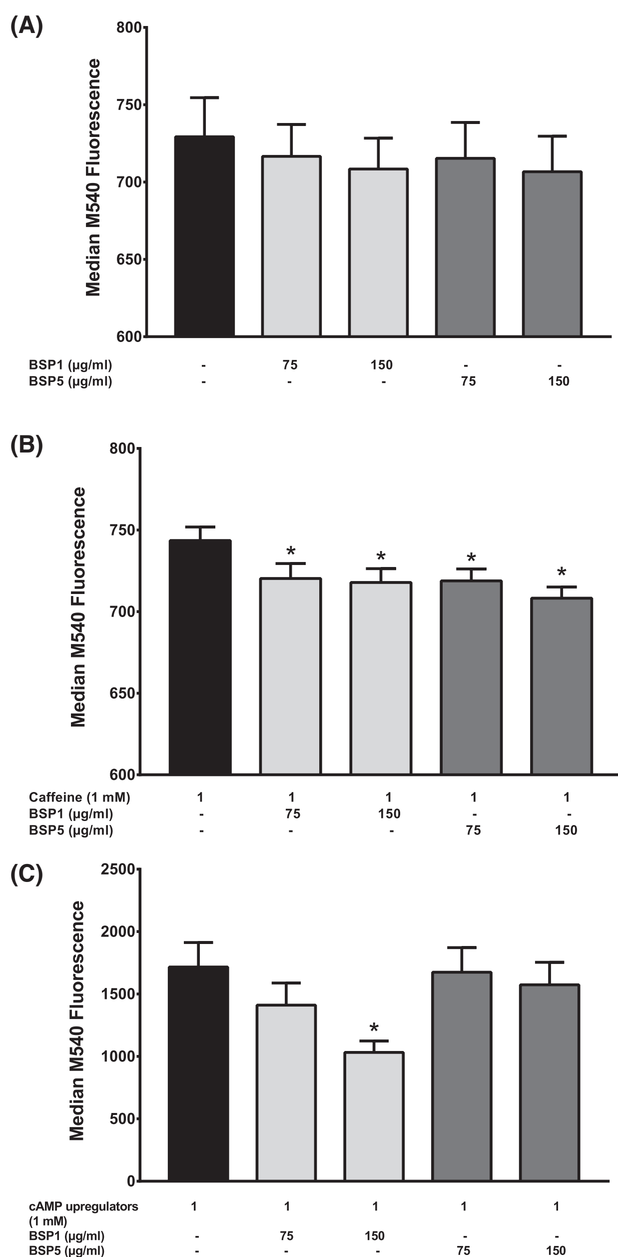

Figure 4. Membrane lipid disorder, assayed as median M540 fluorescence (arbitrary units) of the YO-PRO-1 negative ("viable") population, pooled across a 6 h incubation in basal TALP (A), or with 1 mM of caffeine (B) or all cAMP upregulators (caffeine, theophylline, dbcAMP, C), and with 0, 75, or 150 μg/mL of BSP1 or BSP5. $^{*}P < 0.05$ relative to relevant BSP free control.

Effects of BSPs on cholesterol efflux

There was no significant efflux of cholesterol from any treatment over 6 h of incubation with low or moderate cAMP stimulation (Figure 6A and B). With all cAMP upregulators present, the control showed minimal cholesterol efflux, compared to significant cholesterol efflux in the presence of 150 μg/mL BSP1 (Figure 6C).

Discussion

BSPs have been shown to play intricate roles in the capacitation of bull spermatozoa. In this study, we have investigated the effects of BSP1 and BSP5 on ram spermatozoa in environments ranging from minimal to maximal promotion of capacitation. Epididymal ram spermatozoa were used for these experiments, as they have had very little contact with BSPs, compared to ejaculated spermatozoa which contain high levels of BSPs originating from seminal plasma [8]. Epididymal spermatozoa were obtained from the most distal region of the cauda epididymis to ensure maximal maturity and adequate response to capacitation stimulants [35–37]. Epididymal spermatozoa has previously been used in investigations of capacitation [38–40] and for both in vitro [41, 42] and in vivo fertilization [43, 44], confirming that epididymal spermatozoa are fully capable of undergoing capacitation. Caffeine, theophylline, and db cAMP were used in this study to create varying levels of cAMP upregulation, a requirement of ram spermatozoa to demonstrate the classical hallmarks of capacitation in vitro [30, 32]. While the phosphodiesterase inhibitor activity of caffeine leads to significant increases in intracellular cAMP [45], addition of caffeine has also been shown to significantly increase intracellular calcium [45, 46]. However, as the main impact of a caffeine-driven increase in calcium appears to be hyperactivation [45, 46], which was not observed in this study, we suggest that the observed effects of caffeine are likely due to its role as a cAMP upregulator. The results presented in this report demonstrate that the two BSPs tested (BSP1 and BSP5) each affected specific responses to basal, moderate, and high cAMP-stimulating conditions. We have shown that in the ram, overall both BSPs have both pro- and decapacitating effects on epididymal spermatozoa. BSP1 caused significantly higher loss of acrosome integrity than BSP5, and only BSP1 induced the efflux of cholesterol. Under the highest level of cAMP stimulation, BSP1 was able to limit membrane lipid disorder, while BSP5 had no such effects. Finally, BSP1 qualitatively appeared to reduce the cAMP-dependent tyrosine phosphorylation response of high molecular weight proteins (>75 kDa), which was not observed in samples treated with BSP5. In contrast, BSP5 qualitatively appeared to increase the phosphorylation of moderate weight bands (35–55 kDa) compared to the control. Overall, the unique effects of BSP1 and BSP5 may be caused by differences in the structure of these two proteins. While their tandem fibronectin domains are very similar, BSP5 contains an extended N-terminal and is more highly glycosylated than BSP1 [47]. In addition, BSP5 has fewer hypothetical cholesterol interacting "CRAC" domains than BSP1 [48], which is in line with our observations.

BSPs are largely contributed by seminal plasma at ejaculation, after which they bind tightly to the sperm membrane [8]. There is a significant body of evidence which describes how BSPs interact with plasma membranes. BSP1 binds specifically to the phosphorylcholine head group of phosphatidylcholine (PC), while BSP5 also interacts with phosphatidylethanolamine (PE), phosphatidylserine (PS), and several other phospholipids [49]. BSPs are able to penetrate into the membrane, becoming partially embedded in the outer leaflet and remaining strongly adhered via PC [50]. While much research has focused on the interaction between BSPs and phospholipid head groups, there is evidence that BSP1 also interacts with the acyl chains of PC upon insertion [51]. Of most relevance to the current results are previous studies showing that insertion of BSPs into the sperm membrane or analogous lipid vesicles results in rapid and significant immobilization of phospholipids [52], as well as cholesterol [53]. Such interactions with the membrane can significantly influence lipid ordering of the outer leaflet. BSP1 was able to inhibit membrane lipid disorder to some degree, and significantly more so than BSP5, possibly because its structure allows for deeper penetration into the outer lipid leaflet, rendering it more lipid ordered. This

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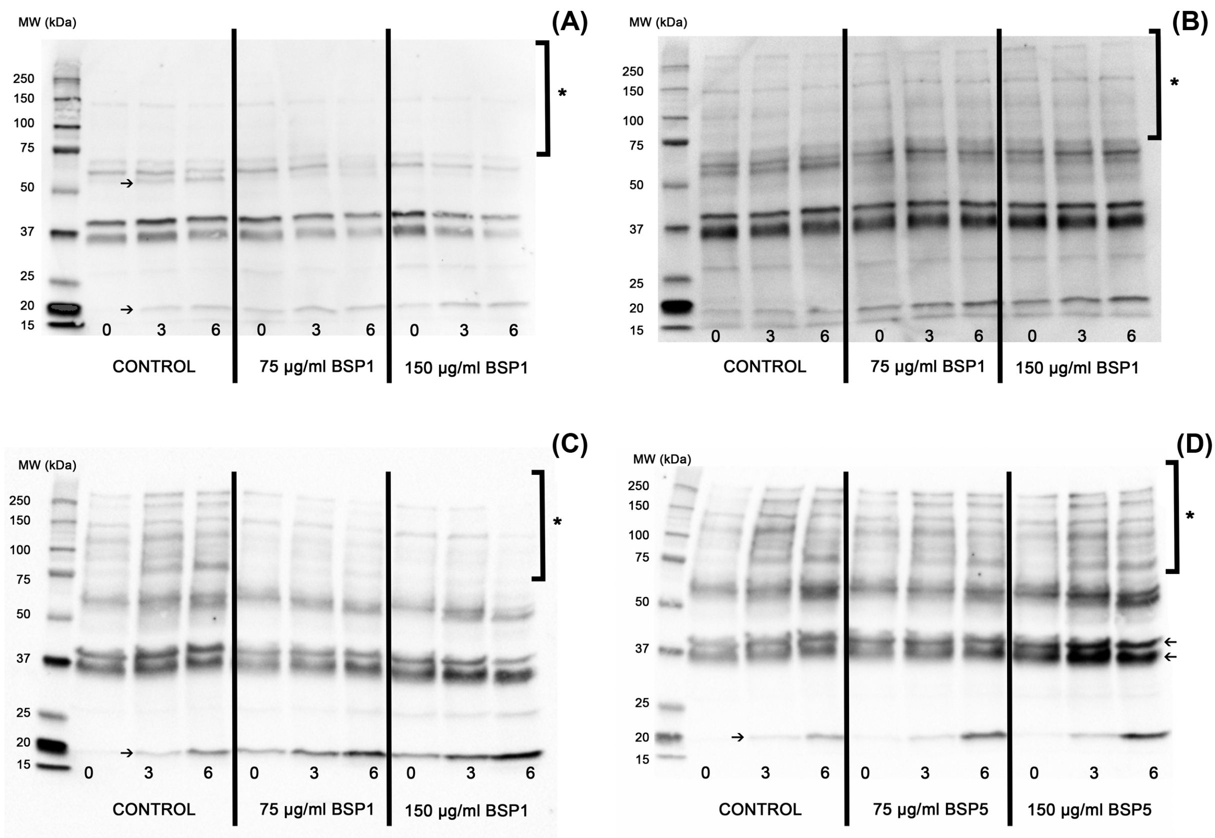

Figure 5. Western blots against tyrosine phosphorylation at 0, 3, and 6 h of incubation, from lysates of epididymal spermatozoa (10 μg total) exposed to 0, 75, or 150 μg/mL BSP1 (A, B, C) or BSP5 (D) in TALP (A), with 1 mM caffeine (B) or with 1 mM cAMP upregulators (caffeine, theophylline, db cAMP, C, D). *Indicates high molecular weight region of interest, arrows indicate other bands of interest.

is the first report of the effects of BSPs on membrane disorder using the fluorescent probe merocyanine 540, so there are no instances with which to compare our findings. However, our results may help to explain the previously observed beneficial effects of BSPs on ram spermatozoa in "challenging" in vitro conditions [20, 54, 55], and encourages further investigation into their potential exploitation to reduce handling induced changes to ram spermatozoa.

In contrast to its stabilizing effect, consistent, long-term exposure to BSP1 has been shown to cause significant phospholipid [17] and cholesterol efflux [15] from epididymal bull spermatozoa when incubated in basal capacitating medium (e.g., Tyrode medium). Interestingly, these processes lead to the formation of BSP-phospholipid and BSP-cholesterol efflux particles. While BSPs are not able to directly bind cholesterol [49], these authors have previously suggested that complexes may be formed between aggregated BSP proteins with hydrophobic pockets and effluxed sterols, effectively mitigating the requirement for a cholesterol acceptor (e.g., albumin, HDL). However, the only situation in which we were able to observe significant efflux of cholesterol was when epididymal ram spermatozoa were exposed to both BSP1 and cAMP upregulators (caffeine, theophylline, db cAMP) in a highly stimulatory environment. Bull spermatozoa do not require cAMP regulation to efflux cholesterol after exposure to BSP1 [15], and this difference is most likely due to the different conditions required to stimulate capacitation of ram spermatozoa [30, 31]. This aligns with the idea that while extended exposure to BSP rich seminal plasma may be beneficial for ram spermatozoa during in vitro handling (i.e., without cAMP upregulation), it is detrimental for bull spermatozoa [19]. Interestingly, while BSP1 caused significant cholesterol efflux, no such response was observed for BSP5. Apart from a lack of cholesterol interacting domains in BSP5 [48] and a slightly different structure to BSP1 [47], this could possibly be due to interference with concomitant cholesterol efflux caused by the phospholipid binding preferences of BSP5. Partial scrambling of phospholipids to the sperm surface during capacitation is suggested to facilitate cholesterol efflux [56, 57]; however, binding of BSP5 to external PE and PS may hinder this process.

Upregulation of protein tyrosine phosphorylation by a cAMP/PKA pathway was first linked to mouse sperm capacitation [58] and has since been documented in other species (bull [59], ram [30], human [60]). While some BSP homologs have been shown to play a role in the development of capacitation-associated protein tyrosine phosphorylation [11], others have no apparent effect [12]. There has been no investigation into the effects of BSPs on bull sperm tyrosine phosphorylation during capacitation. However, in vitro studies have demonstrated that bovine BSP1 has a strong, dose-dependent inhibitory action on protein tyrosine kinase (PTK) activity [61], a group of enzymes responsible for tyrosine phosphorylation. This inhibitory activity may explain why in the current study we only observed limited high molecular weight (>75 kDa) tyrosine phosphorylation when BSP1 was present in addition to cAMP upregulators, which drive the cAMP/PKA pathway, promoting PTK activation and subsequent tyrosine phosphorylation [59, 62]. Interestingly, however, ejaculated ram spermatozoa, which have had exposure to BSP1 through seminal plasma, are able to

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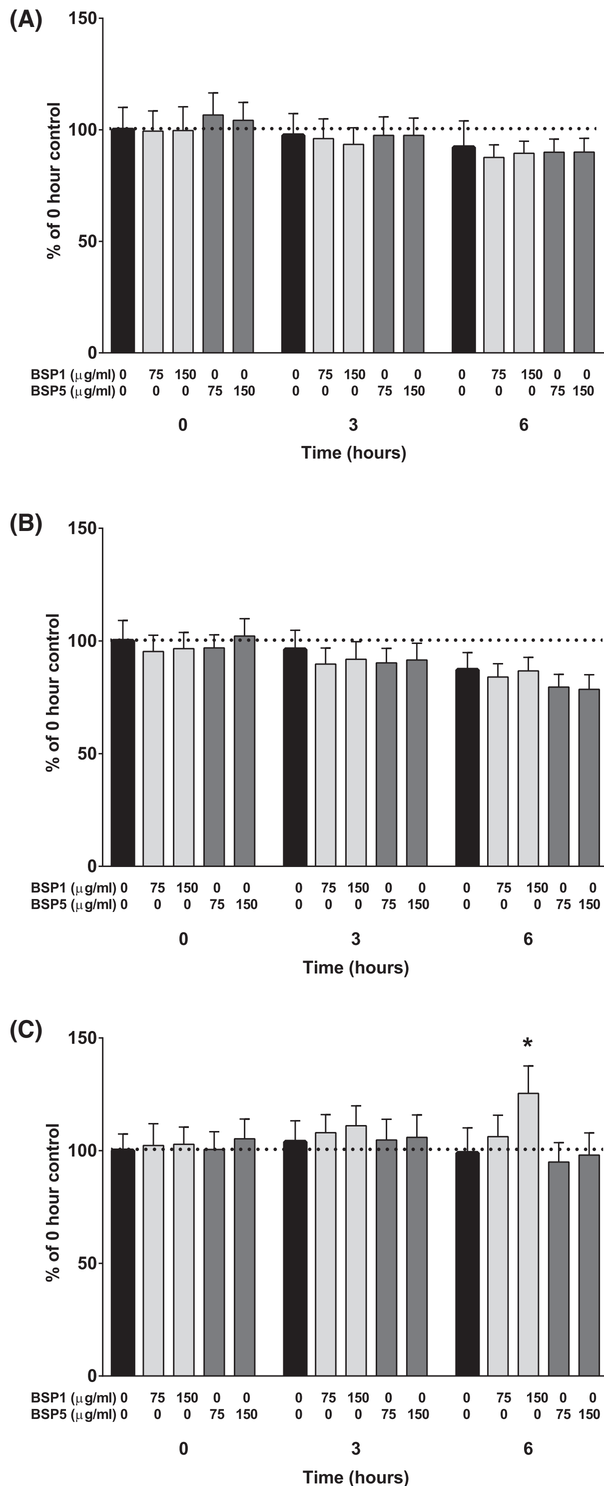

Figure 6. Supernatant cholesterol as a percentage of the 0 h control measurement (indicated by dotted line) from samples containing epididymal spermatozoa incubated in TALP (**A**), TALP with 1 mM caffeine (B), or TALP with cAMP upregulators (caffeine, theophylline, dbcAMP, C) and 0, 75, or 150 μg/mL of BSP1 or BSP5. Supernatant cholesterol was assessed using an Amplex Red assay. $^{*}P < 0.05$ relative to the control.

develop strong high molecular weight tyrosine phosphorylation in response to cAMP upregulators [30]. This suggests that in a physiological situation, the effects of BSP1 on this pathway are tempered by other seminal plasma constituents.

We found that BSP1, and to a lesser extent BSP5, significantly increased spontaneous acrosome reactions in ram spermatozoa, in a time, stimulation, and dose-dependent manner. BSP1 in particular caused a significant increase in spontaneous acrosome reactions compared to the control after just 20 min of incubation, and this value also rose significantly after 3 and 6 h of incubation. In line with our findings, prolonged exposure of epididymal bull spermatozoa in basal capacitation medium to purified bovine BSP1 or BSP5 has been shown to significantly increase both lyso-PC-induced and spontaneous acrosome reactions [17]. Similar results have been documented in humans, mice, and pigs, with isolated or recombinant BSP proteins promoting ionophore-induced acrosome reactions [12, 13, 63]. These results suggest that BSPs, particularly BSP1, are able to trigger a nonphysiological acrosome reaction (i.e., not preceded by capacitation) in ram spermatozoa, which involves a pathway that is sensitive to the direct or indirect effects of cAMP upregulators. Further investigation into how BSPs interact with other sperm membrane proteins and lipids is needed to guide our understanding of the exact biochemical physiology of this protein family. Until we understand more about their physiological relationship with acrosome integrity in ram spermatozoa, use of these proteins to improve in vitro handling outcomes should be treated with caution.

While cholesterol efflux and the acrosome reaction are important endpoints of sperm capacitation, the maintenance of motility is just as crucial for successful fertilization. There has been limited investigation into how BSPs impact sperm motility, particularly when sperm are challenged by stimulatory conditions to induce capacitation. While some studies report no changes in motility after BSP addition [12, 13], this is likely due to the use of washed, ejaculated spermatozoa. As BSPs physically insert into the sperm membrane [50], washing is unlikely to totally remove these proteins and as such, "control" spermatozoa would still have BSPs present. In addition, previous studies have largely carried out subjective motility analysis, which is not as comprehensive as CASA. While there were limited differences in basal conditions, we found that under high cAMP stimulation, BSPs could preserve total and progressive motility, with BSP1 significantly increasing velocity and the amplitude of lateral head displacement and BSP5 increasing linearity. These findings suggest that BSPs have a profound effect on the patterns and maintenance of motility exhibited by spermatozoa undergoing capacitation, as demonstrated previously in the presence of oviduct explants [64]. Epididymal spermatozoa exposed to BSP1 swim faster and more vigorously in stimulating environments, a potential advantage for penetrating cervical mucus, transiting the female tract and entering the cumulus-oocyte complex (COC). This may be a contributing factor in the improved ability of seminal plasma exposed ram spermatozoa to transit the cervix [44] and the increased cleavage rates observed when epididymal bull spermatozoa were exposed to BSP1 during in vitro fertilization of bovine COCs [65].

Finally, it is worth considering the likely responses to seminal plasma-derived BSPs in more physiological circumstances. Previous proteomic studies have confirmed that both BSP1 and BSP5 are present in ram seminal plasma [7, 66], and on ejaculated spermatozoa [8]. As this study investigated the effects of each protein in isolation, it would be interesting for future studies to use BSP1 and BSP5 in combination, which more closely resembles the physiological situation. Interestingly, immunofluorescence work has established that

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BSP1 and BSP5 show different patterns of localization on ejaculated ram spermatozoa [54]. Roughly, one-third of spermatozoa exhibit binding of BSP1 and BSP5 over the whole sperm surface. However, of the remainder, more spermatozoa show binding of BSP5 than BSP1 on the tail and more show binding of BSP1 than BSP5 within the surface area covering the acrosome. These differences in localization align with our findings of relatively different effects of BSP1 and BSP5, and suggest that in vivo, the two BSPs likely act synergistically, performing slightly different roles.

In conclusion, BSPs have been investigated for decades in the bull, and more recently in the mouse and human, highlighting their important roles in sperm capacitation. This is the first report of BSPs showing both pro- and decapacitation properties in ram spermatozoa. BSPs were shown to cause spontaneous acrosome reactions and could promote cholesterol efflux under capacitating conditions. Their maintenance of membrane lipid order, disruption of tyrosine phosphorylation and push towards higher motility and velocity under this stimulation shows their alternative potential for preservation of sperm quality. This study highlights the potential in vivo roles of BSPs in the ram, and clarifies the differences in their action on ram spermatozoa in comparison to their well-established effects on bull spermatozoa.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Supplementary Table S1. Antibodies used for western blotting. **Supplementary Table S2**. Relative normalized emPAI and contribution (%) of proteins identified by LC-MS/MS from fractions collected following gelatin affinity chromatography and RP-HPLC of ram seminal plasma proteins.

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Conflict of Interest: The authors have declared that no conflict of interest exists.

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