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IDENTIFICATION AND CHARACTERIZATION OF NON-PHOSPHORYLCHOLINE-BINDING AND PHOSPHORYLCHOLINE-BINDING PROTEINS OF CANINE SEMINAL PLASMA*

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Abstract

Seminal plasma (SP) proteins participate in the process of fertilization by binding to the sperm membrane, particularly to the phosphorylcholine-containing lipids. This study aimed to identify and characterize non-phosphorylcholine-binding and phosphorylcholine-binding proteins (nPchBPs and PchBPs, respectively) of canine SP. The nPchBPs and PchBPs were isolated from canine SP by affinity chromatography. Electrophoretic studies revealed that the nPchBPs and PchBPs occurred in their native state as high-molecular-weight aggregates. Immunofluorescent staining showed preferential binding of nPchBPs to the sperm acrosome membrane, whereas PchBPs coating was uniformly distributed on the sperm post-acrosomal membrane, mid-piece and tail regions. Analysis with mass spectrometry confirmed that canine prostate specific esterase (CPSE) is a component of the nPchBPs and PchBPs, which is implicated in key mechanisms of protein-coating on the sperm plasma membrane surface. In addition, proteins of known binding properties such as prostaglandin-H2 D-isomerase and lipocalin-like 1 protein, identified in canine SP, might have a specific role in the fertilization-associated processes.

Key words: seminal plasma, non-phosphorylcholine-binding proteins, phosphorylcholine-binding proteins, canine

Seminal plasma (SP) comprises fluids secreted by the epididymis, accessory sex glands and male reproductive tract. Moreover, the SP is a carrier for spermatozoa, and it contains numerous substances that are implicated in the sperm maturation and development at different stages of the reproductive processes (Rodriguez-Martinez et al., 2011). Modern analytical methods have been used to study the structure and function of the SP proteins, and to determine their effect on sperm function (Niżański

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et al., 2016). Moreover, a special role is attributed to the SP proteins bound to the membrane structures of spermatozoa, which protect them against the adverse conditions in the female reproductive tract (Rodriguez-Martinez et al., 2011). Studies have shown that the SP proteins are also known for their ability to exert a diverse effect on sperm function (Rodriguez-Martinez et al., 2011; Mogielnicka-Brzozowska et al., 2014 a; Schäfer-Somi and Palme, 2016; Zelli et al., 2016). It should be emphasized that the function of SP proteins is dependent on the binding of various low-molecular-weight (LMW) ligands to the sperm membrane structures. Evidence has shown that the binding of phosphorylcholine-binding proteins (PchBPs) to choline phospholipids of the sperm plasma membrane at ejaculation induces cholesterol efflux, an essential step for the capacitation process (Therien et al., 1995; Manjunath and Therien, 2001). It should be noted that the PchBPs are implicated in the epididymal sperm maturation (Tannert et al., 2007) and in the formation of the sperm reservoir in the fallopian tube (Gwathmey et al., 2006). These proteins have been isolated from the SP of human, bull, boar, rat and hamster (Leblond et al., 1993; Calvete et al., 1997; Manjunath and Therien, 2001; Mogielnicka-Brzozowska et al., 2014 b). To date, the structure and function of the non-phosphorylcholine-binding proteins (nPchBPs) or the phosphorylcholine-binding proteins (PchBPs) of canine SP have not been reported. The aim of this study was to identify and characterize the nPchBPs and PchBPs from the SP of dogs with good semen quality.

Material and methods

All chemical reagents were of the highest purity grade and were purchased from Sigma Aldrich (St. Louis, MO, USA), unless stated otherwise.

Preparation of seminal plasma samples

Whole ejaculates were collected from five healthy crossbred dogs (aged 3–5 years), kept at the Cryopreservation Laboratory of the Department of Animal Biochemistry and Biotechnology, University of Warmia and Mazury in Olsztyn. The animals were kept in individual cages, with full access to water, and were fed with Purina Dog Chow® TMR (Nestle PURINA, St. Louis, MO, USA) with the addition of Can-Vit® mineral and vitamin supplement (Colmed, Łomianki, Poland). A total of 5 ejaculates were collected by digital manipulation (Linde-Forsberg, 1991; Strzeżek and Fraser, 2009), from each dog over a 6-week period (n=25). All experiments were carried out in accordance with the guidelines set out by the Local Ethics Committee for Experimentation with Animals (No. 90/2012). Sperm concentration was determined using a Bürker counting chamber (Equimed-Medical Instruments, Kraków, Poland). Only semen samples that had more than 80% total sperm motility and more than 80% morphologically normal spermatozoa were used in this study. Sperm motility characteristics were analyzed using a computer assisted sperm analysis (CASA) system (Hamilton-Thorne Research, HTR, IVOS version 12.3; Beverley, MA, USA). Aliquots of sperm samples (3µl) were placed on a prewarmed Makler

counting chamber (Sefi-Medical Instruments Ltd., Israel) and examined at 38ºC. The CASA sperm motility parameters were measured with the following settings: frame acquired 30, frame rate 60 Hz, minimum cell contrast 75, minimum cell size 6 pixels, straightness threshold 75%, low VAP cut-off 9.9 μm/s^{-1}, low VSL cut-off 20 μm/s^{-1}, static size gates 0.80–4.93, static intensity gates 0.49–1.68, static elongation gates 22–84. Total sperm motility (TMOT, %) was analyzed by the CASA system.

The percentages of spermatozoa with intact acrosome integrity were determined in fresh semen, according to a previously described method (Watson, 1975), with some modifications (Fraser et al., 2007). Spermatozoa with intact acrosomes exhibited a uniform Giemsa staining pattern overlying the acrosomal region, whereas spermatozoa with non-intact acrosomes displayed a clear patchy staining pattern, with damaged apical ridges or loose acrosomal caps.

Each ejaculate was centrifuged (1000 × g, 15 min at room temperature) to separate the spermatozoa from SP according to a previously described method (Rijsselare et al., 2002). The recovered SP was further centrifuged (10000 × g, 10 min at room temperature) and the supernatant was dialyzed against deionized H_2O for 24 h at room temperature. Following dialyses, the SP from the 5 dogs was pooled, giving a total of 5 different pooled SP samples, which were stored at –80°C until further analysis.

Freshly ejaculated canine spermatozoa were immediately analyzed to determine the attachment of biotin-labeled SP proteins.

Protein determination

Total protein content was measured in the fresh SP and protein fractions recovered by phosphorylcholine-dependent affinity chromatography (Lowry et al., 1951). Bovine serum albumin (BSA, IBSS BIOMED S.A., Poland) was used as the standard.

Phosphorylcholine affinity chromatography

The nPchBPs and PchBPs of the SP were isolated, using affinity chromatography on immobilized p-aminophenyl phosphoryl choline agarose (Thermo Fisher Scientific, Waltham, MA, USA). Chromatographic separations were performed according to a previously described procedure (Calvete et al., 1997). The equilibration buffer contained 50 mM Tris-HCl, 150 mM NaCl and 5 mM EDTA (pH 7.4). Dialyzed SP from the ejaculates of the five dogs was pooled five times (average protein content of 20 mg/mL) and diluted 1:1 with the equilibration buffer. Two replicates (5 ml) from each of the five pooled SP samples were added to the chromatography bed and incubated for 24 h at 4°C in a laboratory cradle. The nPchBPs were eluted from the bed with the equilibration buffer, whereas the PchBPs were eluted from the bed with another equilibration buffer comprising 10 mM phosphorylcholine and 10 M urea. The fractions were collected and their protein content was determined. The results were used to create a chart with protein peaks, using the Microsoft Office Excel 2007. The samples were pooled, desalted by dialysis against deionized water for 24 h at room temperature, and then they were concentrated in dialysis bags by submersion in dry polyethylene glycol (4°C). All samples were frozen at –80ºC until required for analysis.

Polyacrylamide gel electrophoresis (PAGE)

The isolated protein fractions of nPchBPs and PchBPs were subjected to polyacrylamide gel electrophoresis (PAGE) in non-denaturing conditions without sodium dodecyl sulphate (SDS), according to a previously described method (Laemmli, 1970). Aliquots of the protein samples were diluted (1:1) with 2-fold concentrated buffer containing 0.34 M Tris-HCl, 20% glycerol, 2% bromophenol blue (pH 6.8). Native electrophoresis was performed in 6% polyacrylamide gel. Electrophoresis was performed at a constant voltage (120V) in a buffer (0.025 M Tris, 0.192 M glycine, pH 8.3). After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 solution (0.025% Coomassie Brilliant R-250, 40% acetic acid, 7% methanol in deionized water). The molecular mass was estimated using the High Molecular Weight Calibration Kit for Native Electrophoresis (GE Healthcare Life Sciences, Chicago, Il, USA).

Preparation of biotin-labelled nPchBPs and PchBPs

Biotin-labelled nPchBPs and PchBPs were prepared according to a previously described method (Manaskova and Jonakova, 2008). One milliliter of *N*-hydroxysuccinimidobiotin, dissolved in 50 µL of *N,N*-dimethylformamide, was added to the protein solution (2 mg/mL) in 0.5 M $NaHCO_3$, pH 8.8 (4 mL). After stirring for 30 min at room temperature in the dark, the solution was dialyzed against phosphate-buffered saline (PBS) overnight at 4°C.

Attachment of biotin-labelled nPchBPs and PchBPs to ejaculated spermatozoa

Freshly ejaculated canine spermatozoa were washed four times with PBS according to a previously described method (Manaskova and Jonakova, 2008). Sperm suspension, diluted 1:10 with PBS, was incubated with biotin-labelled nPchBPs and PchBPs for 1 h at 37°C. After washing and centrifugation, avidin coupled with FITC (fluorescein isothiocyanate) (100 µg/ml) was added to the samples, which were incubated for 1 h. Following incubation and washing, the sperm suspension was diluted 1:10, smeared on slide and further incubated for 15 min with 1.5 µg/ml of DAPI. The stained samples were analyzed, using a confocal laser microscope (Zeiss LSM-700), with the aid of the Zeiss LSM Image Browser. As a control, washed sperm samples were incubated with avidin-FITC and no interaction was observed (data not shown).

Two-dimensional polyacrylamide gel electrophoresis (2-DE)

Two-dimensional polyacrylamide gel electrophoresis was performed, according to the method of O'Farrell et al. (1977), with some slight modifications. Seventy five micrograms of each protein fraction (nPchBPs, PchBPs) were resuspended in 145 µl of the sample buffer: 9.5 M urea; 2% Triton X-100; 0.065 M DTT; 2% ampholytes (pH 3–10); 0.1% bromophenol blue. The protein samples were then applied onto strips (ZOOM Strip, pH 3-10 NL, Invitrogen, Waltham, MA, USA), fixed in a cassette (ZOOM IPG Runner cassettes, Invitrogen, Waltham, MA, USA), and subjected to a 24-h strip rehydration. Following rehydration, the cassette with the strips was placed in the ZOOM IPG Runner apparatus (Invitrogen), and isoelectrofocusing (IEF) was carried out using deionized water. The voltage change used during IEF was as follows: 200 V (20 min.), 400 V (15 min.), 700 V (15 min.), and 1,000 V

(120 min.). After IEF the strips were equilibrated for 30 min in a buffer comprising 6 M urea, 2% SDS, 0.375 M Tris, 20% glycerol (Avantor Performance Materials Poland S.A.), 2% DTT and 0.2% Bromophenol Blue they were loaded onto 12% SDS PAGE gels (Laemmli, 1970) using Mini Protean II Cell (BioRad Laboratories, Hercules, CA, USA). Separation was carried out in a buffer containing 0.5 M Tris; 0.25 M glycine; 0.5% SDS (pH 8.3) at a voltage of 80 V (15 min) and 150 V (50 min). Precision Plus Protein Standards were used as the molecular weight standards (BioRad Laboratories, Hercules, CA, USA). All gels were stained with Coomassie Brilliant Blue R-250 solution. The polyacrylamide gels were destained in a solution of 5% methanol, 7% acetic acid and in deionized water, and were analyzed using PDQuest 7.2 (BioRad Laboratories, Hercules, CA, USA). The 2-DE digital images were analyzed, according to the PDQuest user guide regarding spot detection, optical density (ODU), matching, and calculating Mr/pI values for all of the spots.

Matrix assisted laser desorption/ionization time of flight/time of flight mass spectrometry protein identification (MALDI TOF/TOF MS)

Only protein spots showing good quality were excised from the gel, and were subjected to in-gel trypsin digestion, using sequencing-grade modified trypsin (Promega, Madison, WI, USA). Desalting was conducted with ZipTip μC18 (Merck Millipore, Billerica, MA, USA) (Zakharchenko et al., 2011). The peptides were eluted with 70% acetonitrile (ACN, Merck, Darmstadt, Germany), dried and stored at −80°C, until mass spectrometery (MS) analysis.

The matrix was prepared by dissolving 5 mg of α-cyano-4-hydroxycinnamic acid (Bruker Daltonics, Bremen, Germany) in 1 ml of 50% ACN and 0.1% trifluoroacetic acid (TFA, Fluka, Buchs, Sweden). The dried in-gel digests were reconstituted with 1.5 μl of 10% ACN and 0.1% TFA. The samples were spotted, using the dried-droplet method, where 0.5 μl of reconstituted in-gel digest sample was spotted initially on a MTP 384 target plate ground steel (Bruker Daltonics), followed by 0.5 μl of matrix, which were mixed on the spot by gentle up and down motion of the pipette tip. Additionally, a peptide calibration standard (Bruker Daltonics) was spotted with the matrix for the calibration of the MS.

Mass spectra were acquired in the range of 700–3500 m/z, using a MALDI-TOF autoflex speed TOF/TOF mass spectrometer equipped with a smartbeam II laser (355 nm, Bruker Daltonics). The operating conditions were as follows: ion source 1 = 19.00 kV, ion source 2 = 16.85 kV, lens voltage = 7.50 kV, reflector voltage = 21.00 kV, reflector 2 voltage = 9.59 kV, optimized pulsed ion extraction time = 120 ns, matrix suppression = 750 Da, and positive reflectron mode. The strongest precursors were selected for MS/MS analysis. Peak lists were generated from the MS spectra with flex Analysis version 3.3 (Bruker Daltonics). The spectra were then searched with a Mascot version 2.4 (Matrix Science, Boston, MA, USA). The database search criteria were as follows: SwissProt database, enzyme; trypsin, fixed modification; carbamidomethylation (C), and variable modifications; oxidation (M), peptide mass tolerance of 250 ppm, fragment mass tolerance of 0.7 Da, and one missed cleavage allowed. The search results were filtered with a significant threshold of $P<0.05$ and a MASCOT ion score cut-off of ≥30.

Results

The elution profile of the phosphorylcholine-dependent affinity chromatography revealed 2 different peaks of the isolated SP protein fractions. The higher curve was represented by the nPchBPs (A) and the lower curve by the PchBPs (B). The PchBPs and nPchBPs accounted for 8–10% and 90–92% of the total SP proteins, respectively (Figure 1).

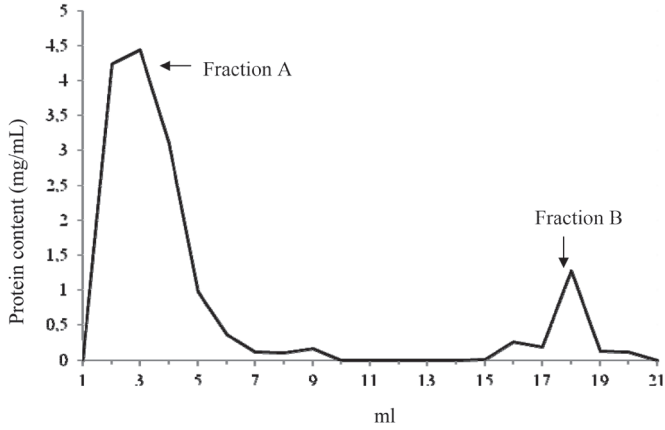

Figure 1. The non-phosphorylcholine-binding proteins, (nPchBPs) (A) and phosphorylcholine-binding proteins (PchBPs) (B) of canine seminal plasma separated by affinity chromatography on Immobilized p-Aminophenyl Phosphorylcholine Gel

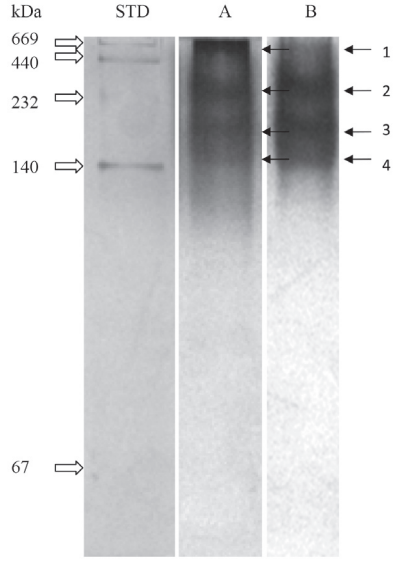

Figure 2. Polyacrylamide gel electrophoresis (PAGE) of the non-phosphorylcholine-binding proteins (nPchBPs) (A) and phosphorylcholine-binding proteins (PchBPs) (B) of canine seminal plasma. Each lane represented 122 µg protein. 1–4 fractions of native nPchBPs and PchBPs. STD – High Molecular Weight Calibration Kit for Native Electrophoresis

PAGE analysis of the canine SP showed that the nPchBPs and the PchBPs comprised four high molecular weight aggregates, ranging from 140 to 669 kDa (Figure 2).

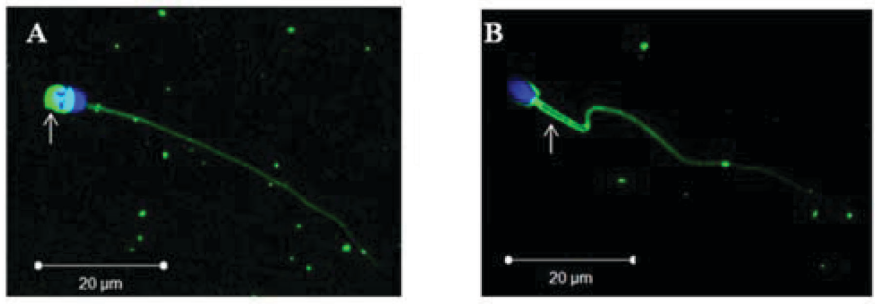

Figure 3. The attachment of biotin-labeled non-phosphorylcholine-binding proteins, (nPchBPs) (A) and phosphorylcholine-binding proteins, (PchBPs) (B) to canine spermatozoa, detected by the fluorescence staining technique. Green fluorescent indicates the binding of the nPchBPs and PchBPs (biotin/avidin-FITC system), whereas blue fluorescent indicates staining of the cell nucleus with DAPI. Scale bar 20µm

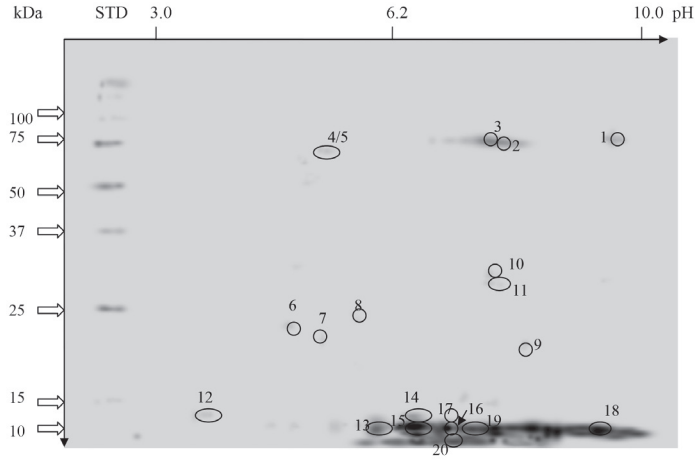

Figure 4. Two dimensional electrophoresis (2-DE) of the non-phosphorylcholine-binding proteins (nPchBPs) of canine seminal plasma. Numbers 1–20, spots were identified using MALDI TOF/TOF MS. STD – Precision Plus Protein Standard (BioRad)

Immunofluorescent staining showed that there were binding sites for the nPchBPs and PchBPs on the sperm plasma membrane (Figure 3 A, B). There was preferential binding of nPchBPs on the sperm acrosome membrane (Figure 3 A), whereas PchBPs coating was uniformly distributed on the post-acrosomal membrane, mid-piece and tail regions of the spermatozoa (Figure 3 B).

The results of the 2-DE analysis showed that the nPchBPs and PchBPs comprised numerous low molecular weight (LMW) polypeptides (<10 kDa) and polypeptides with molecular masses less than 80 kDa (Figures 4, 5).

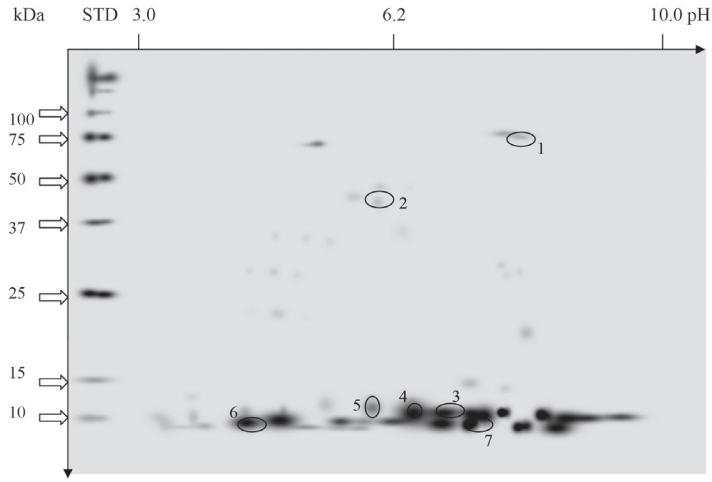

Figure 5. Two dimensional electrophoresis (2-DE) of the phosphorylcholine-binding proteins (PchBPs) of canine seminal plasma. Numbers 1–7, spots were identified using MALDI TOF/TOF MS. STD – Precision Plus Protein Standard (BioRad)

The nPchBPs comprised 64 to 76 protein spots, with the isoelectric points (pI) in the pH range of 3.1 to 9.9. Using mass spectrometry, the spots numbered 1 to 20 were identified as nPchBPs, comprising five protein fragments; lactotransferrin (LTF), albumin, prostaglandin H2 D-isomerase, arginine esterase (CPSE) and lipocalin-like 1 protein isoform (Figure 4). Table 1 shows the nPchBPs identified in canine SP by MALDI TOF/TOF MS.

The PchBPs revealed 42 to 56 protein spots with the pI in the pH range of 4.1 to 9.9. (Figure 5) Mass spectrometry analysis showed that the spots numbered 1 to 7 were identified as PchBPs, comprising only two protein fragments: LTF and CPSE (Figure 5). Table 2 shows the PchBPs identified in canine SP by MALDI TOF/TOF MS.

Discussion

It is a well known fact that canine spermatozoa contain large amounts of phosphatidylcholine, and that the binding sites for SP proteins on the plasma membrane of sperm cells have been identified mainly as choline phospholipids (Desnoyers and Manjunath, 1992). In this study, following the isolation and characterization of the PchBPs from the SP, we investigated whether these proteins could interact with the membrane structures of the spermatozoa. Despite the purported importance of the SP proteins in the fertilization-associated processes there are no reports about the characterization and role of the PchBPs in the canine semen.

Table 1. The non-phosphorylcholine-binding proteins (nPchBPs) of canine seminal plasma, identified by MALDI TOF/TOF MS. All peptides were identified for *Canis lupus familiaris*

Spot no	Identified protein	Accession no	Mascot score	Sequence coverage	Calculated pI/Nominal mass kDa	Number of peptides (ion score ≥30)	Peptide score	Peptide sequence
1	2	3	4	5	6	7	8	9
1nPchBPs	lactotransferrin precursor	gi|559767226	273	15%	8.62/79.2	3	96	K.KGTNFQLNQLQGVR.S
							67	R.YFGYTGAFR.C
							63	R.DSTILQNTNGGNPEPWAR.D
2nPchBPs	lactotransferrin precursor	gi|559767226	287	20%	8.62/79.2	3	103	R.DSTILQNTNGGNPEPWAR.D
							66	K.KGTNFQLNQLQGVR.S
							52	R.YFGYTGAFR.C
3nPchBPs	lactotransferrin precursor	gi|559767226	332	25%	8.62/79.2	3	96	R.DSTILQNTNGGNPEPWAR.D
							83	K.KGTNFQLNQLQGVR.S
							41	R.YFGYTGAFR.C
4/5nPchBPs	albumin	gi|3319897	130	13%	5.36/67.8	1	31	K.KAPQVSTPTLVEVSR.K
6nPchBPs	prostaglandin-H2 D-isomerase precursor	gi|50978842	45	7%	7.62/21.4	2	45	K.GLGQDFHMATLYSR.T + Oxidation (M)
							33	K.GLGQDFHMATLYSR.T
7nPchBPs	prostaglandin-H2 D-isomerase precursor	gi|50978842	31	7%	7.62/21.4	1	31	K.GLGQDFHMATLYSR.T + Oxidation (M)
8nPchBPs	arginine esterase precursor	gi|50979094	125	10%	7.96/29.4	2	52	R.KSFIHPLYK.T
							48	K.SFIHPLYK.T
9nPchBPs	arginine esterase	gi|163907	41	3%	7.96/29.4	1	41	K.SFIHPLYK.T

Table 1 – contd.

1	2	3	4	5	6	7	8	9
10nPchBPs	arginine esterase precursor	gi|50979094	133	15%	7.96/29.4	2	53	K.SFIHPLYK.T
							33	R.KSFIHPLYK.T
11nPchBPs	arginine esterase precursor	gi|50979094	135	17%	7.96/29.4	2	53	K.SFIHPLYK.T
							52	R.KSFIHPLYK.T
12nPchBPs	lipocalin-like 1 protein isoform X3	gi|928145697	168	28%	5.08/20.0	1	53	R.FAVLYLETEAKGTR.N
13nPchBPs	arginine esterase precursor	gi|50979094	98	10%	7.96/29.4	1	51	K.SFIHPLYK.T
14nPchBPs	arginine esterase precursor	gi|50979094	61	6%	7.96/29.4	1	48	K.SFIHPLYK.T
15nPchBPs	arginine esterase precursor	gi|50979094	226	7%	7.96/29.4	3	68	K.SFIHPLYKTK.V
							66	R.KSFIHPLYK.T
							64	K.SFIHPLYK.T
16nPchBPs	arginine esterase precursor	gi|50979094	226	18%	7.96/29.4	3	68	R.KSFIHPLYK.T
							57	K.SFIHPLYK.T
							57	K.SFIHPLYKTK.V
17nPchBPs	arginine esterase precursor	gi|50979094	221	7%	7.96/29.4	3	69	R.KSFIHPLYK.T
							64	K.SFIHPLYK.T
							57	K.SFIHPLYKTK.V
18nPchBPs	arginine esterase precursor	gi|50979094	98	15%	7.96/29.4	2	41	R.AVIRPGEDRSHDLMLLHLE-EPAK.I
							41	R.AVIRPGEDRSHDLMLLHLE-EPAK.I + Oxidation (M)
19nPchBPs	arginine esterase precursor	gi|50979094	203	18%	7.96/29.4	3	58	R.KSFIHPLYK.T
							53	K.SFIHPLYKTK.V
							48	K.SFIHPLYK.T
20nPchBPs	arginine esterase precursor	gi|50979094	101	12%	7.96/29.4	1	53	K.SFIHPLYK.T

Table 2. The phosphorylcholine-binding proteins (PchBPs) of canine seminal plasma, identified by MALDI TOF/TOF MS. All peptides were identified for *Canis lupus familiaris*

Spot no	Identified protein	Accession no	Mascot score	Sequence coverage	Calculated pI/Nominal mass kDa	Number of peptides (ion score ≥30)	Peptide score	Peptide sequence
1PchBPs	lactotransferrin precursor	gi|559767226	78	9%	8.62/79.2	1	37	R.YFGYTGAFR.C
2PchBPs	lactoferrin, partial	gi|84872712	99	15%	6.99/19.3	1	38	R.YFGYTGAFR.C
3PchBPs	arginine esterase precursor	gi|50979094	48	3%	7.96/29.4	1	40	K.SFIHPLYK.T
4PchBPs	arginine esterase precursor	gi|50979094	57	3%	7.96/29.4	1	50	K.SFIHPLYK.T
5PchBPs	arginine esterase precursor	gi|50979094	68	3%	7.96/29.4	1	64	K.SFIHPLYK.T
6PchBPs	arginine esterase precursor	gi|50979094	170	12%	7.96/29.4	4	93	R.SHDLMLLHLEEPAK.I
							97	R.SHDLMLLHLEEPAK.I + Oxidation (M)
							39	R.VMPHLMWIK.D + Oxidation (M)
							43	R.VMPHLMWIK.D + 2 Oxidation (M)
7PchBPs	arginine esterase precursor	gi|50979094	63	6%	7.96/29.4	1	46	K.SFIHPLYK.T

Our study revealed that the nPchBPs and PchBPs occurred in canine SP as high molecular weight (HMW) aggregates, with the molecular weights ranging from 140 to 669 kDa. It was shown that proteins of the canine SP with molecular weights greater than 200 kDa were associated with high sperm motility and velocity parameters (Ivanova et al., 2015). The HMW of the SP proteins were identified as zinc-binding proteins (ZnBPs), which exhibited protective effect on the sperm plasma membrane (Mogielnicka-Brzozowska et al., 2012; Mogielnicka-Brzozowska et al., 2014 a). A study in our laboratory demonstrated that the native complexes of ZnBPs and PchBPs in canine semen have similar molecular weight (Mogielnicka-Brzozowska et al., 2012), suggesting that the HMW protein aggregates of the canine SP could bind to zinc and phosphorylcholine, These protein complexes, with the involvement of phosphorylcholine moieties, are involved in the coating mechanisms of the sperm plasma membrane at ejaculation, and protect the structural and functional integrity of the spermatozoa against the adverse conditions in the female reproductive tract. It is likely that these protein complexes might confer protection to spermatozoa against cold shock during semen processing, indicating their potential benefits in sperm technologies.

To date, proteins with affinity for phosphorylcholine have been identified in the SP of bulls (Manjunath and Therien, 2001), horses, boars (Calvete et al., 1997; Mogielnicka-Brzozowska et al., 2014 b), rats and hamsters (Leblond et al., 1993). Even though the phosphorylcholine-binding proteins are associated with the fertilization-associated processes in different animal species, the mechanisms responsible for the sperm plasma membrane coating by canine SP proteins are still unclear.

The results of the present study showed that there was preferential binding of the nPchBPs on the sperm acrosome membrane. It is noteworthy that the nPchBPs do not have any affinity for phosphorylcholine, and the mechanism involved in their binding to the sperm membrane structures is somewhat unclear. However, since the SP proteins also have affinity for other substances, such as ethanolamine, sphingomyelin, phosphatidylserine and cholesterol (Manjunath and Therien, 2001; Tannert et al., 2007), it is possible that these substances might facilitate the binding of the nPchBPs to the sperm membranes, as observed in the current study.

The results of our study showed that PchBPs could bind simultaneously to the post-acrosomal membrane, mid-piece and tail regions of canine spermatozoa. It is well known that, in the case of PchBPs, coating on the sperm plasma membrane is mediated by phosphorylcholine moieties of phospholipids (Leblond et al., 1993).

The findings of the present study showed that the high molecular weights native nPchBPs and PchBPs aggregates were denatured and reduced under *in vitro* conditions (2-DE). The polypeptide maps revealed that the assayed fractions of the nPchBPs and PchBPs isolated from canine SP consisted mostly of LMW polypeptides, pI at basic pH. Previous studies showed similar findings in the SP of other animal species, indicating that the PchBPs could exist as LMW polypeptides after oligomer dissociation (Therien et al., 1995; Calvete et al., 1997).

The proteomic tool, such as mass spectrometry, has been used to identify and characterize specific protein associated with male fertility. Mass spectrometry analy-

sis of canine SP detected five PchBPs, such as lactotransferrin, albumin, prostaglandin H2 D-isomerase, arginine esterase and lipocalin-like 1 protein isoform. It should be emphasized that the role of some of these proteins in canine SP has not been fully elucidated as yet. Albumins are quite abundant in different animal species SP and they have been recently identified in canine SP (Schäfer-Somi and Palme, 2016). Albumins can absorb lipid peroxides, which contribute to their protective effect on the sperm motility apparatus and membrane structural integrity. Furthermore, albumins belong to the zinc-binding protein family and may regulate free zinc levels in the SP (Schäfer-Somi and Palme, 2016). Prostaglandin (H2) D-isomerase, expressed in different reproductive organs, bind to small non-substrate lipophilic molecules and may act as a scavenger for harmful hydrophobic molecules (Zhou et al., 2010). The prostaglandin (H2) D-isomerase is potentially involved in different biological processes, such as the vesicle-mediated transport and defense response (Zhou et al., 2010). Lipocalins are small extracellular proteins, which are characterized by a range of different molecular recognition properties. Moreover, lipocalins bind to specific cell-surface receptors and may form macromolecular complexes (Flower et al., 2000). In the fish lipocalins were identified in the sperm flagella, suggesting their potential role in motility (Nynca et al., 2011). The results of the present study showed that LTF belongs to the fractions of the nPchBPs and PchBPs It should be emphasized that the role of LTF in the biological fluids has not been elucidated as yet. However, it has been confirmed that LTF is an iron-binding protein and is involved in regulating the availability and catalytic activity of iron (Kiso et al., 2013). Furthermore, LTF has also been suggested to have antibiotic properties, conveyed by its ability to sequester iron and prevent the harmful effects of pathogens on spermatozoa (Adlerova et al., 2008; Kiso et al., 2013). The origin of LTF in canine SP is not yet known, but in other animal species (stallion, boar, mouse) it was shown to originate from the epididymis (Adlerova et al., 2008). Our results showed that the majority of the CPSE precursors were detected in the fractions of the nPchBPs and PchBPs. To date, this is the first study to show that the CPSE occurs in numerous proteoforms and is a phosphorylcholine-binding protein. The CPSE, identified in canine SP, is a multifunctional protein due to its zinc-binding properties, as confirmed in one of our recent studies (Mogielnicka-Brzozowska et al., 2015). Moreover, the CPSE accounts for more than 90% of the proteins secreted by the prostate, and about 30% of the canine SP proteins (Isaacs and Coffey, 1984). Under native conditions, the enzyme has a molecular weight of 29 kDa, and it can be dissociated into two sub-units with molecular masses of 12–14 kDa and 15 kDa, respectively (Isaacs and Coffey, 1984). Several isoelectric points, observed on the 2-DE gels of the CPSE spots, are probably differently glycosylated (Issacs and Coffey, 1984). The enzyme was detected in the post-acrosomal region and sperm tail of ejaculated spermatozoa, but not detected in the epididymal spermatozoa (Issacs and Coffey, 1984), suggesting that it can bind to phosphorylcholine of the sperm lipid membrane and coat ejaculated spermatozoa. Such binding characteristics suggest that CPSE, as a sperm-binding protein, could be implicated in the sperm fertilization-related events. However, further studies are still needed to clarify the specific role of the CPSE in the egg-sperm fertilization process.

Presently, only heparin and zinc-binding proteins have been isolated from canine SP (De Souza et al., 2006; Mogielnicka-Brzozowska et al., 2012). These proteins, and other identified protein components, such as lactoferrin, matrix metalloproteinase, superoxide dismutase, catalase and glutathione peroxidase, have been shown to be associated with canine semen quality (Aquino-Cortez et al., 2016). Furthermore, different studies indicate that prolactin, arginine esterase, acid phosphatase and alkaline phosphatase could be used as biomarkers of reproductive disorders (Aquino-Cortez et al., 2016).

The results of this study indicate that the binding of various phospholipids, including phosphorylcholine-containing phospholipids, is one of the key mechanisms of protein coating on the plasma membrane of canine spermatozoa. Moreover, the proteins identified in canine SP might influence various aspects of the sperm functions. It can be suggested that the recognition of the mechanisms in which canine SP proteins could interact with spermatozoa might be useful to modulate the fertilization-associated processes. Furthermore, the combination of different proteomic techniques could allow the identification of various potential markers for reproductive disorders in the dog. Further studies are warranted to unravel the role of canine SP proteins in the sperm-egg fertilization process.

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