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Original article

Is stallion epididymal fluid phosphoproteome affected by the equine reproductive season?

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Abstract

Phosphorylation and dephosphorylation of proteins are considered to be the most important processes in sperm maturation during epididymal transit. The main aim of this study was to isolate and identify phosphoproteins from the epididymal milieu obtained from reproductively mature stallions during and out of the breeding season. With the use of 1D-PAGE and nanoLC-MS/MS, we identified phosphoproteins that fulfil various functions: regulatory, transport, motility, ubiquitination, chaperone, antioxidant, apoptotic and enzymatic. Moreover, we characterized tyrosine, serine and threonine phosphorylation patterns, taking into consideration the seasonal and epididymal segment variables. The intensity of phosphorylation and profiles of phosphoproteins varied in subsequent regions of the epididymis. With the use of western and immunoblot tests, we demonstrated that fourteen proteins underwent phosphorylation both during and out of the breeding season. However, significant differences ($p \leq 0.05$) in the phosphorylation status were demonstrated in the case of 44 kDa (glutamine synthetase), 38 kDa (malate dehydrogenase), 34 kDa (clusterin/inorganic pyrophosphatase), 31 kDa (clusterin/ /ubiquitin thioesterase), 29 kDa (14-3-3 protein/purine nucleotide phosphorylase) for the season factor and 55 (Rab GDP dissociation inhibitor alpha) and 31 kDa ((clusterin/ubiquitin thioesterase) proteins for the segment factor. The occurrence of the other phosphoproteins was spontaneous among individuals and in both seasons.

Key words: stallion, proteomics, epididymal fluid, phosphoproteins

Introduction

Contemporary knowledge of the complex, post testicular sperm maturation process in mammals is still quite scant. Spermatozoa mature and gain fertilization competency as they move through a specialized duct called the epididymis. After leaving the testis and entering the caput (the proximal segment) of the epididymis, spermatozoa are considered as immature and incapable of fertilization (Skerget et al. 2015). Other sperm functions associated with sperm capacitation, i.e. hyperactivation, binding to the zona pellucida, and exocytosis of the acrosome, are achieved in the cauda (distal segment) or even in the vas deferens regions (Aitken et al. 2007). During the epididymal transit, male gametes undergo changes in the degree of protein phosphoryla-

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tion. Reversible protein phosphorylation plays a key role as a molecular switch in many cellular processes, including transduction of extracellular signals, intracellular transport, and cell cycle progression. Sperm maturation in the epididymis arises as a result of sequential interactions with components of the milieu, particularly with the proteins secreted by the epididymal epithelium (Guyonnet et al. 2011). During the transit through the epididymis, sperm is bound by various fertilizing factors secreted by the epididymal epithelium. Phosphorylated proteins might serve as such factors. Unfortunately, the exact localization and identification of phosphorylated proteins in the epididymal epithelium and fluid have rarely been documented (Sawatpanich et al. 2018). A number of previous studies have revealed several proteins whose expression increased during epididymal maturation (Baker et al. 2005, Aitken et al. 2007). Amongst them were phosphorylated proteins, i.e. glucose-regulating protein, heat-shock protein 70, actin, β-tubulin, lactic acid dehydrogenase, aconitase and β-subunit F1 ATPase (Aitken et al. 2007).

Therefore, the main goals of this study were to characterize the degree of protein phosphorylation, to identify phosphoproteins that appear in stallion epididymal fluid, and to investigate the impact of the breeding season on the intensity of the phosphorylation process.

Materials and Methods

Epididymides were collected from mature, warmblood stallions at the age of 3-4 years during castration surgeries. Castrations were performed in the breeding season, i.e. from March to June (6 stallions), and out of the breeding season (off-season) from September to December (6 stallions). The castrated stallions were not used for breeding. To obtain epididymal fluid, the experimental material was first subjected to epididymectomy. The epididymides were dissected into three parts, i.e. the caput, cauda and corpus. These parts are organized into lobules that serve not only as internal support for the organ, but also as a functional separation which allows for selective expression of genes and proteins within each individual segment (de Souza et al. 2017).

The dissected segments were placed in sterile Eppendorf tubes and centrifuged (2000 x g, 10 min, 10°C). The supernatant was centrifuged once again (10000 x g, 15 min, 10°C) to remove the remaining spermatozoa. To avoid erythrocyte contamination, the Red Blood Cell Lysing Buffer (Sigma-Aldrich, USA) was added initially. Protease and Phosphatase Inhibitor Cocktail (Sigma-Aldrich, USA) was then added to every sample. Finally, all samples were frozen at -80°C for at least a week. The total protein content was determined in all samples according to the method of Lowry et al. (1951).

Phosphoproteins were isolated on PHOS-select Iron Affinity Gel (Sigma-Aldrich, USA). The isolation was conducted according to the manufacturer's manual. Samples containing 0.5 mg of total protein were applied to the columns and subjected to phosphoprotein isolation. Fractions of 100 µl volume were eluted from the columns. The phosphoprotein content in eluted fractions was estimated using the method of Lowry et al. (1951). Protein fractions were then precipitated from the eluates according to the DOC-TCA protocol (Bensadoun and Weinstein 1976) with modifications. One µl of 2% sodium deoxycholate was added to 100 µl of every sample. The solutions were incubated for 30 min at 4°C. After incubation, all samples were mixed with 10 µl of 100% trifluoroacetic acid. The mixtures were vortexed and incubated for 15 min at 4°C. The samples were then centrifuged (15000 x g, 15 min, 4ºC) and supernatants were removed; the pellets were suspended in SDS-loading buffer (2% SDS, 5% β-mercaptoethanol, 125 mM Tris-HCl, pH 6.8).

SDS-PAGE separations were conducted according to the Laemmli method (1970) on 12% polyacrylamide gels using Tris-glycine-SDS buffer (pH 8.3). 20 µl of every sample, containing 10 µg of proteins, were applied on a single gel path. Precision Plus Protein Standard (Bio-Rad, USA) served as molecular weight standards. After electrophoresis, the gels were stained with Coomassie Blue Silver for 12h according to the method of Candiano et al. (2004), and then destained with redistilled water. Molecular weights of phosphoprotein fractions were determined after analyses supported by Multi Analyst software (Bio-Rad, USA).

Epididymal fluid phosphoproteins were electro-blotted using a Semi-Dry Blotter (Sigma-Aldrich, USA), then transferred to a PVDF membrane (Millipore, USA) and maintained at room temperature for 60 min., with a constant current equal 1 mA/cm^2. The PVDF membrane was incubated in 2% bovine serum albumin in TBS (a blocking solution 10 mM Tris-HCl, 100 mM NaCl, 0.1% Tween 20; pH 7.5) for 1 h at 4°C. After blocking, the membranes were washed four times for 5 minutes in TBS buffer and then left for overnight incubation at 4°C in TBS with the addition of monoclonal biotinylated antibodies (Sigma-Aldrich, USA), i.e. either anti-phosphotyrosine (1:50000) or anti-phosphoserine (1:300000) or anti-phosphotreonine (1:60000) antibodies. On the following day, the membranes were washed with TBS buffer three times for 5 min and incubated for 2 h in TBS buffer (20 ml) with the addition of streptavidin (4 µl). The membranes were then washed four times in TBS buffer for 5 minutes and stained in 10 ml of buffer containing: 100 mM Tris-HCl, 100 mM NaCl, (pH 9.5)

with an addition of 200µl of nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) solution (Sigma-Aldrich, USA) until stained bands were visible. Finally, the membranes were dried in the open air. Molecular masses of visible fractions were determined after analyses supported by Multi-Analyst software (BioRad, USA).

Phosphoproteins isolated with SDS-PAGE which demonstrated differences in appearance (% of protein fraction content measured by Multi Analyst Software; BioRad, USA) between individual segments of the epididymis during both seasons were subjected to in-gel trypsin digestion. Excised gel pieces were washed once with 50% acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA). They were then destained twice with 200 µl of ammonium bicarbonate (100 mM), dehydrated with 100 µl of acetonitrile (50%) at 37 °C, and dried in speed vacuum. They were then subjected to a reduction and alkylation process by first using a 50 mM dithiothreitol solution and then 50mM iodoacetamide/50 mM NH_4 HCO_3 solutions. The samples were then incubated with 100 µl of 50mM NH_4 HCO_3 at room temperature for 5 min with gentle shaking. They were then centrifuged, dehydrated with 100 µl of 50% ACN and dried in speed vacuum. Finally, they were incubated overnight at 37°C with trypsin (12.5 ng/ µl in 25 mM ammonium bicarbonate) (Promega, USA). After the incubation period, peptides were transferred to new microcentrifuge tubes. Gel slices were covered with 100 µl of 50 mM NH_4 HCO_3 and incubated for 15 min at 37°C. The peptides were then extracted twice with 25 µl of formic acid (HCOOH) in 50% ACN for 30 min at 37°C in a water bath. The extracts were again dried in speed vacuum and resuspended in 20 µl of 5% ACN with 0.1% addition of HCOOH. Tryptic digests were analysed using the NanoLC-MS/MS technique.

The nanoLC-MS/MS analysis was carried out in a Proxeon EASY-nLC II nanoLC system (Thermo Fisher Scientific, Germany) connected online to an ESI-IT mass spectrometer AmaZon ETD (Bruker--Daltonics, Germany) operated in a positive-ion mode. 7 µl of samples were loaded on a two-column system: an RPC18 precolumn (2 cm, 5 µm particle size, 100 µm ID Thermo Fisher Scientific, USA) and an RP C18 separation column (10 cm, 3 µm particle size, 75 µm ID Thermo Fisher Scientific, USA). The flow rate was 300 nL/min. The peptides were eluted at 70 min long gradient of buffer A (0.1% FA in water) and B (0.1% FA in acetonitrile). Data Analysis software (version 4.0, Bruker-Daltonics, Germany) was used to convert raw data to mgf files suitable for protein identification using Mascot software. All mgf files were checked against the mammalian taxonomic group in the UniProt KB database. The search was done with Mascot software, (Matrix Science, London, UK). Scores ≥ 50 were regarded as significant in Mascot software.

Statistical analysis was performed using the Statistica programme (version 13.1, StatSoft Incorporation, USA). Based on immunoblotting results, the percentage of each protein fraction on a single path in every segment of the epididymis was assessed (Multi Analyst, Bio-Rad, USA). The data were analyzed using ANOVA, followed by non-parametric U Mann-Whitney (for season x antibody variables) and Kruskal-Wallis (for segment x antibody variables) tests. Results were shown as medians and interquartile ranges.

Results

Based on 1D-PAGE and Multi Analyst Software results, we noticed that the highest number of protein fractions during the breeding season were present in the cauda region (Fig. 1A). In contrast to that, out of the breeding season, most phosphoproteins were detected mainly in the caput and corpus segments (Fig. 1B).

Such observations might be associated with the function of the caput and corpus parts, which are regarded as regions of sperm maturation *per se*, whereas the cauda part is considered to act as a sperm reservoir (Sostaric et al. 2008). However, we did not demonstrate significant differences between phosphoprotein profiles during and out of the breeding season. During the breeding season, phosphoproteins of approximately 76, 66, 58, 55, 49, 47, 44, 34, 31 and 22 kDa were detected in all segments simultaneously, while those with a molecular weight of about 136 and 92 kDa were observed in the caput and corpus and those whose molecular weight was 116 kDa were noted mainly in the cauda segment (Fig 1A.). Electrophoretic profiles of phosphoproteins obtained out of the breeding season showed three phosphoproteins with molecular weights 136, 128 and 92 kDa in the caput and corpus. Moreover, proteins with molecular weights of 76, 66, 58, 55, 49, 44, 34, 31, 29, 25, 24, 22, 19 and 15 kDa were present in all segments of the epididymis. It should be emphasized that the greatest range of fractions (ranges from 100 to 40 kDa and from 30 to 20 kDa) appeared in the cauda segment (Fig. 1B). The occurrence of the remaining phosphoproteins was diverse in each segment of the epididymis, both in and out of the breeding season.

The above results were enhanced utilizing western- and immunoblotting methods. Proteins with molecular weights of approximately 58, 55, 53, 49, 47, 44, 38, 34, 31, 29, 25 and 24 kDa underwent phosphorylation in every segment of the epididymis both during and out of the breeding season. These proteins were subjected to phosphorylation on tyrosine, serine and threonine residues simultaneously (Fig. 2A, B).

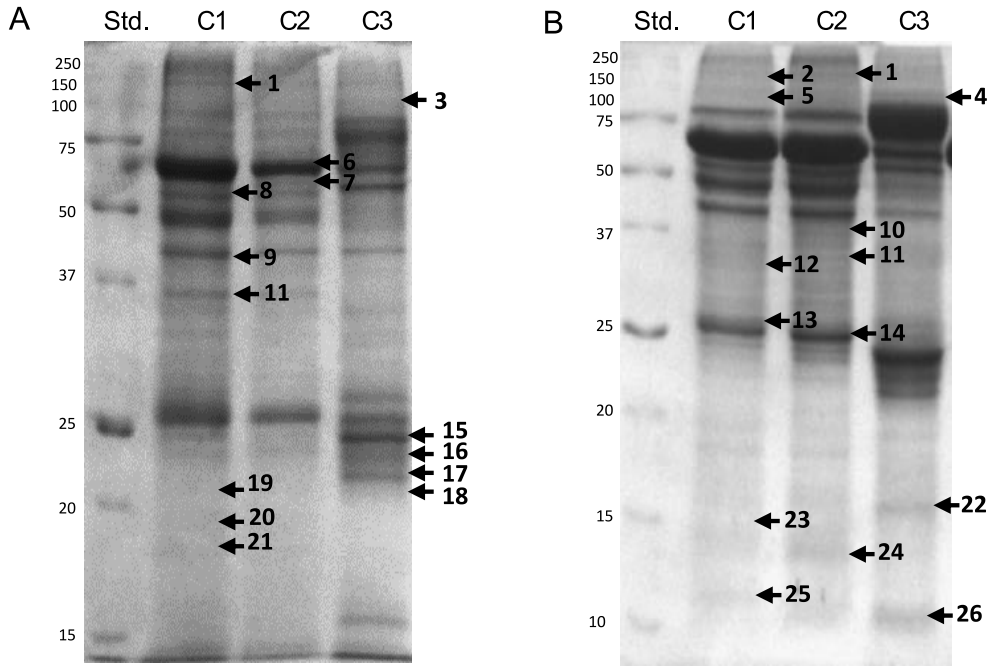

Fig. 1. SDS-PAGE profile of phosphoproteins obtained during the breeding season (A) and out of the breeding season (B). Identified proteins are marked by arrows (1-26). C1-caput, C2-corpus, C3-cauda, Std. – molecular weight standards.

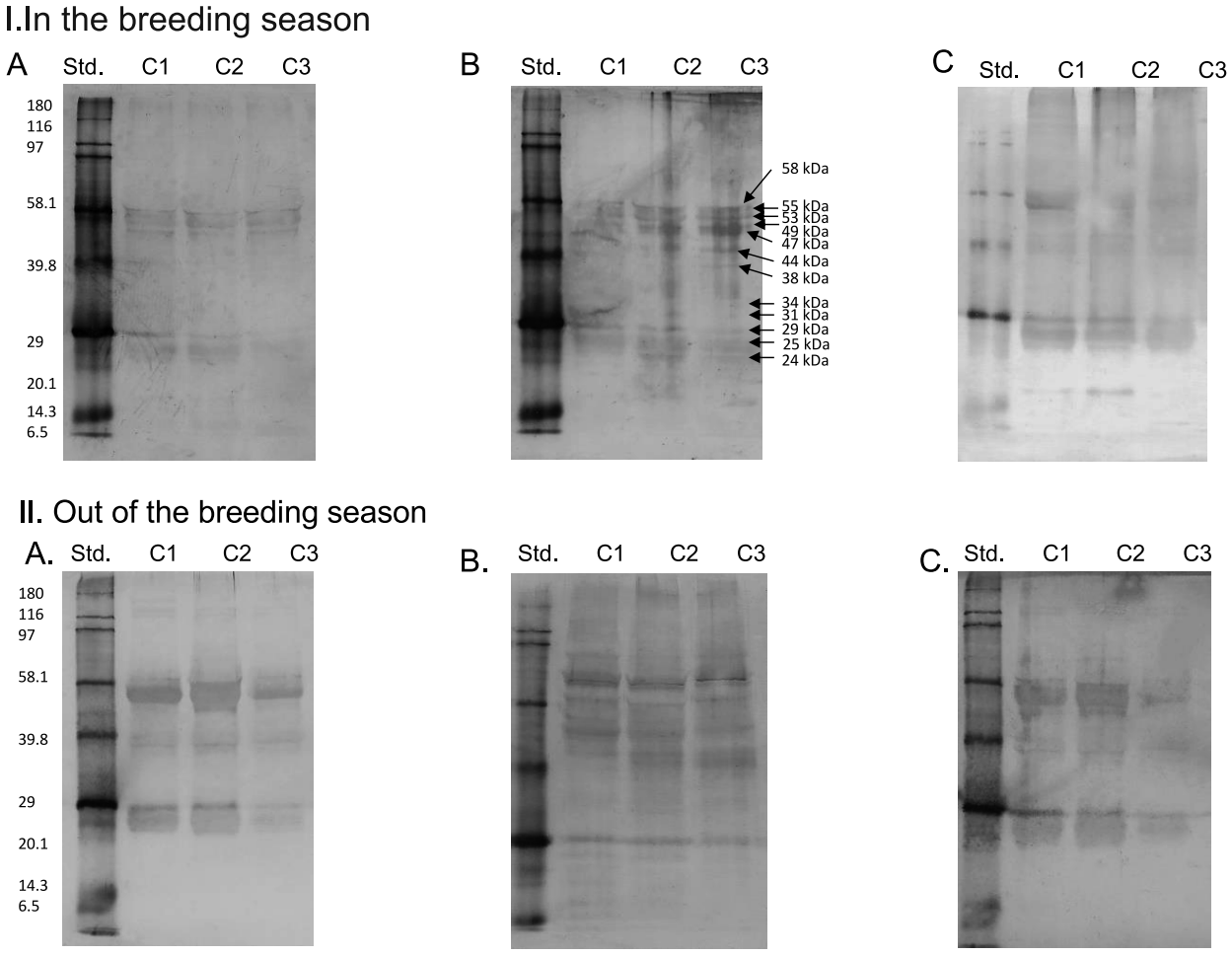

Fig. 2. Western Blot analysis of phosphoserine (A), phosphothreonine (B) and phosphotyrosine (C) residues obtained from sperm extracts during the breeding season (I) and out of the breeding season (II). Std.- protein standards (Sigma-Aldrich). C1-caput, C2-corpus, C3-cauda of stallion epididymis. Phosphoproteins identified by mass spectrometry are marked with arrows.

Proteins with molecular weights of approximately 136, 128 and 116 kDa underwent phosphorylation on every residue, mainly in the cauda and corpus regions, although their presence varied between individuals. Nonetheless, some proteins, such as 87, 73, 71, 69, 67 and 60 kDa, were more often subjected to phosphorylation on serine residues in the breeding season. Moreover, polypeptides with molecular weights of approximately 19, 18, 17, 16, 15, 14, 11, 10 kDa were more frequently phosphorylated both on serine and threonine than on tyrosine residues in the off-season period (Fig. 2B).

Based on the results obtained after conducting one-dimensional electrophoretic separations and immunoblotting resolutions (i.e. differences observed in the phosphoprotein profiles of various segments of the epididymis during and out of the breeding season), twenty-six protein bands were selected and subjected to nanoLC-MS/MS mass spectrometry (Table 1).

MS/MS spectrometry analyses have so far revealed hundreds of proteins with progressive composition changes throughout the epididymal tubule in several species (Cornwall 2014). We identified phosphoproteins that fulfil various functions: regulatory, transport, motility, ubiquitination, chaperone, antioxidant, apoptotic and enzymatic (Table 2).

Discussion

In our suvey selected proteins were found in epididymal fluids both during and out of the breeding season, although their occurrence was differentiated among individuals. The protein with a molecular weight of 136 kDa was predicted to be heat shock 70 kDa protein 1 (HSP70). We supposed that HSP70 could be a part of some protein complex on the surface of epididymal spermatozoa. Such a protein complex was documented by DIGE analysis in rats and included two heat shock 70-kDa proteins, HSP90-α, clusterin, and protein disulfide-isomerase A3 precursor (Baker et al. 2005). HSP70 is one of three sperm proteins which have been reported to increase in content during the passage from the caput to cauda epididymidis. HSP70 is regarded as a potential indicator of an animal's adaptation to environmental stress (Zhang et al. 2015). The 128 kDa protein showed similarity to ceruloplasmin, which acts as a copper and iron transporter as well as an antioxidant factor. Another peptide found in our survey, predicted to be the ubiquitin-like modifier-activating enzyme 1 (116 kDa), has been determined in the literature as an important regulator of spermatogenesis (Yi et al. 2012b). Within the band of the molecular weight of 92 kDa, an enzyme that showed similarity to mammalian transitional endoplasmic reticulum ATPase was identified. This is an evolutionarily conserved enzyme that participates in many intracellular mechanisms (Zhang et al. 2015). Serotransferrin (76 kDa), which was identified in this study is mainly produced by the epididymis (Moura et al. 2010). This protein protects spermatozoa against both oxidative stress and lipid peroxidation (Wakabayashi et al. 1999).

Rab GDP dissociation inhibitor alpha – GDI1 (55 kDa) underwent stronger phosphorylation in the caput and cauda, whereas ubiquitin thioesterase 1 (31 kDa) was phosphorylated more intensively in the caput segment. GDI proteins are present in secretory cells with both regulated and constitutive secretion pathways. They are important modulators of GDP/GTP exchange reactions (Shisheva et al. 1994). GDI1 is involved in several aspects of actin cytoskeleton organisation and also regulates cell morphology and cell motility (Matt et al. 2007). Its expression was demonstrated to be decreased among patients with asthenozoospermia (Curry et al. 2011).

Additionally, albumin (ALB) was identified phosphoprotein band with molecular weight of 66 kDa. Albumin seems to be a part of a high molecular mass glycoprotein complex (~260 kDa) that is involved in sperm penetration of the zona pellucida (Arroteia et al. 2014). In the male reproductive tract, albumin may be involved in the transport of other molecules to the sperm membrane during epididymal maturation, in the movement of proteins during the acrosomal reaction and in membrane remodeling during sperm-oocyte membrane fusion (Dacheux et al. 2009). In addition, the epididymal albumin-containing protein complex may be absorbed by the sperm acrosome, as occurs with other epididymal secreted proteins. On the other hand, clusterin (CLU) identified in 7 electrophoretic bands is a highly conserved protein which is expressed at increased levels in response to a broad variety of stress conditions. Clusterin can represent 30-40% of the all the proteins secreted (Fournier-Delpech et al. 1985). It is supposed to protects GST and catalase from heat-induced precipitation and α-lactalbumin and BSA from precipitation induced by reduction (Humphreys et al. 1999). This protein can be sequentially secreted under different isoforms in different parts of the epididymis. In the stallion and the ram CLU interacts with hydrophobic proteins to maintain their solubility (Cornwall 2009). It is of interest that both described proteins may be detected in various amounts throughout the duct (Fouchecourt et al. 2000). Some of the proteins identified in the course of this study i.e. HSP70, plastin, RALDH 1, 14-3-3 protein epsilon, annexin V, PEBP1 and RAP, which undergo phosphorylation in different parts of the stallion epididymis, have been previously found in epididymosomes of the boar, ram, bull and human (Aitken et al. 2007).

Table 1. Phosphoproteins identified by nanoLC-MS/MS spectrometry.

Band	Predicted protein	M.W. of excision location by Multi Analyst (kDa)	M.W. of predicted protein by Mascot (kDa)	pI	Score	Protein sequence cov. %	Peptide matches
1	Heat shock 70 kDa protein 1	136	70	5.53	70	2	2
2	Ceruloplasmin	128	122	5.44	139	6	10
	Retinal dehydrogenase		55	6.44	57	2	3
3	Ubiquitin-like modifier-activating enzyme 1	116	118	5.51	66	1	2
4	Transitional endoplasmic ATP-ase	92	90	5.13	63	2	2
	Heat shock protein 70		95	5.63	57	1	2
5	Serotransferrin	76	80	6.83	296	21	21
6	Serum albumin	66	70	5.89	452	26	23
	Heat shock 70 kDa protein 1		70	5.53	105	2	2
	Chaperone protein dnaK		67	4.67	105	3	2
	Plastin-2		71	5.29	62	3	3
7	Glucose-6-phosphate isomerase	58	63	7.08	66	2	2
8*	**Rab GDP dissociation inhibitor alpha**	**55**	51	5	72	4	3
	Tryptophan-tRNA ligase		54	5.49	70	4	2
	Serotransferrin		80	6.83	55	2	3
9*	**Glutamine synthetase**	**44**	43	6.28	131	9	4
	Adenosylhomo-cysteinase		48	5.88	81	5	4
	Serotransferrin		80	6.83	58	2	3
10*	**Malate dehydrogenase. cytoplasmic**	**38**	37	6.16	62	5	3
	Alcohol dehydrogenase		37	6.8	55	2	2
11*	**Clusterin**	**34**	53	5.77	123	15	12
	Inorganic pyrophosphatase		33	5.54	63	7	3
12*	**Clusterin**	**31**	53	5.77	123	15	13
	Ubiquitin thioesterase		31	4.85	57	5	2
	Annexin a5		36	5.6	53	2	2
13*	**14-3-3 protein epsilon**	**29**	29	4.63	95	10	4
	Purine nucleoside phosphorylase		32	5.92	85	3	2
	Glyceraldehyde-3-phosphate dehydrogenase		35	8.26	78	4	2
	Clusterin		53	5.77	74	4	4
	Guanine nucleotide-binding protein subunit beta-2-like1		36	7.6	59	5	2
14	Proteasome subunit alpha type-2	25	26	6.34	106	26	9
	Glutathione s-transferase mu5		17	5.51	105	26	5
	Triosephosphate isomerase		27	6.45	76	16	5
	Heat shock protein beta-1		22	5.98	74	4	4
	Ubiquitin carboxyl-terminal hydrolase isozyme L1		29	5.55	62	7	3
	Translin		26	6.01	58	8	3
	Putative high mobility group protein B1-like		24	5.92	54	7	2
15	Proteasome subunit alpha type-2	24	26	6.34	127	26	9
	Proteasome subunit type-1		26	8.26	95	14	5
	Heat shock protein beta-1		23	6.23	60	4	2
	Glutathione s-transferase mu5 (fragments)		17	5.51	56	11	2
16	Peroxiredoxin-1	23.5	22	8.59	100	21	5
	Peroxiredoxin-1		23	6.51	88	15	4
	Clusterin		52	5.65	64	2	2
17	Clusterin	23	53	5.77	134	11	8
	Ras-related protein Rab-7a		24	6.4	84	6	1
	Peroxiredoxin-2		22	5.66	79	9	2
	Transgelin		23	8.87	54	5	2

18	Clusterin	22	53	5.77	88	3	2
	Filaggrin-2		249	8.45	75	1	3
19	Phosphatidylethanolamine-binding protein 1	21	21	7.01	66	18	6
20	Cofilin	18	19	8.16	221	23	6
	ADP-ribosylation factor 4		21	5.91	133	31	10
21	Transgellin	17	23	8.87	63	11	3
22	Myosin light polypeptide 6	16	17	4.56	71	10	2
23	Profilin	13	15	8.46	118	20	4
24	Clusterin	11	53	5.77	107	8	6
	Transgellin		23	8.87	77	5	1

* Phosphoproteins which presented significant differences in band contents (%) during both seasons are marked in bold. Excision location of cut fractions based on Multi Analyst Software calculation. Predicted molecular weights that were provided by Mascot Software are based on the Uniprot source.

Table 2. Biological functions of identified phosphoproteins derived from stallion epididymal fluid.

Biological function	%
regulatory/enzymatic	52
ubiquitination	11
chaperone	11
antioxidant	10
transport	7
motion	7
apoptotic	2

Table 3. Phosphoproteins whose content (%) differed between seasons ($p \leq 0.05$). Results are presented as medians with an interquartile range. s – during the breeding season, os – out of the breeding season.

protein	season	antibody	median	interquartile range
glutamine synthetase (44 kDa)	s	ser	2.445	5.93
	os		6.980	13.66
malate dehydrogenase (38 kDa)	s	thr	7.510	12.19
	os		2.935	4.25
clusterin/inorganic pyrophosphatase (34 kDa)	s	tyr	0.000	3.65
	os		0.000	0.00
clusterin/ubiquitin thioesterase (31 kDa)	s	tyr	1.165	3.88
	os		0.000	0.00
14-3-3 protein/purine nucleotide phosphorylase (29 kDa)	s	tyr	16.030	19.07
	os		2.330	11.45

Significant differences ($p \leq 0.05$) in the phosphorylation status were noted in the case of 44, 38, 34, 31 and 29 kDa polypeptides (for season x antibody variables) (Table 3), and 55 and 31 kDa polypeptides (for segment x antibody variables) (Table 4).

Glutamine synthetase (GS) catalyzes the synthesis of glutamine, an amino acid that seems to influence sperm motility in mammals. One single band of approximately 44 kDa which was ascertained in our study by electrophoreses and immunoblotting as GS had been previously found in human (Francou et al. 2012) and buffalo (Mohanarao and Atreja 2011) sperm extracts. A product of this enzyme's action, i.e. glutamine, is a component of seminal plasma (Tomlins et al. 1998). Glutamine synthetase underwent significantly stronger phosphorylation in stallion epididymal fluid on serine residues out of the breeding season.

On the other hand, a significantly higher degree of phosphorylation affected polypeptides of approximately 34, 31 and 29 kDa (on tyrosine residues) and 38 kDa (on threonine residues) during the breeding season. Inorganic pyrophosphatase (PPA1; 34 kDa) can

Table 4. Phosphoproteins whose content (%) differed between epididymal segments (p≤0,05). Results are presented as medians with an interquartile range. 1 – caput, 2 – corpus, 3 – cauda of epididymis.

protein	segment of epididymis	antibody	median	interquartile range
Rab GDP dissociation inhibitor alpha (55 kDa)	1	ser	3.890	6.720
	2		0.000	6.110
	3		11.350	13.065
clusterin/ubiquitin thioesterase (31 kDa)	1	thr	3.135	2.570
	2		0.000	1.100
	3		0.000	0.000

decompose cellular PPi, which is yielded by various biosynthetic processes, and hydrolyze it to two inorganic phosphates (Pi). Sperm motility, capacitation and acrosome reaction may be inhibited by extrinsic and purified PPA1 addition (Yi et al. 2012a). Ubiquitin thioesterase 1 (OTUB1; 31 kDa) plays an important regulatory role at the level of protein turnover by preventing degradation. Moreover, OTUB1 knockdown promotes caspase- and TNF-dependent cell death (Goncharov et al. 2013). In the band with a molecular weight of 29 kDa, two interesting proteins were identified, i.e. 14-3-3 epsilon (ε) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The 14-3-3 isoform ε is expressed in testicular germ cells (Puri et al. 2011). The family of 14-3-3 proteins is known for interacting with various cellular phosphoproteins and regulating their localization and phosphorylation status as well as modulating the activity of enzymes (Bridges and Moorhead 2005). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which turned out to be phosphorylated, can be found along with hexokinase and lactate dehydrogenase in the sperm fibrous sheath of many mammalian species (Turner 2006). The phosphorylated form of GAPDH interacts with 14-3-3 regulatory proteins and consequently exhibits lower activity and enhanced sensitivity to regulation by adenylates and inorganic pyrophosphate (Bustos and Iglesias, 2003). Generally, little is known about the phosphoprotein which was identified as malate dehydrogenase (MDH; 38 kDa). More intensive phosphorylation of all the proteins mentioned above might be associated with the roles they fulfil in spermatogenesis during the breeding season.

Conclusions

Our results indicate that the majority of proteins present in the epididymal fluid are subjected to various degrees of the phosphorylation processes. This seems to be determined by the role that proteins play during spermatogenesis, as well as the season (in the case of 44,38,34,31 and 29 kDa proteins) and the region of the epididymis (in the case of 55 and 31 kDa proteins) from which samples were obtained.

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