PROTEOMICS AS A POSSIBILITY OF IMPROVING A HORSE REPRODUCTION METHODS

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Introduction. Spermatozoa are highly specialised cells that evolved for the particular purpose of fertilisation and left with only the most essential structures. Having eliminated most of their cytoplasm they lost the ability to transcribe genes. The functional transformation of spermatozoa, which occurs after they are released from the germinal epithelium of the testes, depends almost entirely on changes in their proteome composition (Swagen et al., 2015). Understanding the biochemical processes associated with an oocyte fertilization and knowledge about the structure and function of specific substances participating in these processes are crucial for the development of biotechnological methods of improving the animals and humans reproduction (Mogielnicka-Brzozowska, Kordan, 2011).

Aim of the study. The aim of our study was to isolate and identify phosphoproteins derived from stallion semen preserved in a liquid state.

Proteome of mammals seminal plasma. Seminal plasma components are considered to be vital factors involved in capacitation (Kratz, Achcińska, 2011). The differences in seminal plasma composition are results of individual proteins profiles and changes in gene expression. Moreover, these factors are especially taken into consideration during evaluating of semen quality. Total protein content in semen of different mammalian species ranged from 10 to 60 mg/ml. Most of the seminal plasma proteins regulate the functioning of sperm cells, increase tolerance of female reproductive system on presence of spermatozoa and participate in implantation process (Rodriguez-Martinez et al., 2011). The result obtained by Frazer and Bucci (1995) demonstrate the positive correlation between seminal plasma composition and the success of fertilization with semen obtained from chosen individuals. Proteome of seminal plasma is mainly determined in boar, bull, mice, rat and human.

Proteome of stallion semen. The total protein content in stallion semen amounts to 10 mg/ml on average (Topfer-Petersen et al., 2005). Ascertained proteins of stallion semen are designated with abbreviations from HSP-1 to HSP-8. Mentioned proteins are compounds in the range of 14 kDa to 30 kDa of molecular weights. All, except HSP-4, are bound to the sperm surface, and form multi-protein aggregates. Low molecular weight proteins

HSP-1 and HSP-2 are synthesized in the seminal vesicles and comprise about 70-80% of total proteins found in seminal plasma. Low molecular weight polypeptides belong to a relatively small group of proteins called Fn-2 and they are equivalents of heparin binding proteins (BSP) in bull. HSP-1 and HSP-2 exist as an oligomers with molecular weight of 90 kDa which can link to heparin or as a monomer which cannot. Furthermore, mentioned compounds have a capability of interacting with plasmalemma phospholipids and thus playing an important role in capacitation process (Frazer and Bucci, 1996). To the Fn-2 proteins family there is also included a high molecular weight protein called EQ-12 which originates from epididymis. During the epididymal passage and the time of ejaculation spermatozoa are ,,coated'' with all above-mentioned proteins (Topfer-Petersen et al., 2005). Another group of proteins normally occur in stallion seminal plasma are these from CRISP (cysteine-rich secretory proteins) family. These proteins with molecular weight of 25 kDa contain 16 cysteine residues and are located in the central part of the acrosome and sperm tail. Their main task is to protect the spermatozoa from premature capacitation in the female reproduction duct. The study made by Reinke et al. (1999) showed a positive correlation between presence of CRISP-3 protein and stallion fertility. It also exhibited a lack of protein CRISP-2 expression among infertile stallions. Form the third group of proteins present in stallion semen. They are multifunctional proteins, consisting of 110-133 amino acids and at least 2 disulfide bonds. Spermadhesins have been identified in human, boar, bull and stallion semen. So far these proteins indicate capability of binding to heparin, proteinase inhibitors, phospholipids and carbohydrates (Topfer-Petersen et al., 2005).

Research conducted by Swagen and Aitken (2014) demonstrated 67 proteins which can act as potential players in the interactions between spermatozoa and the extracellular environment. Among these polypeptides there can be found number of receptor/signalling proteins, chaperones, phosphoproteins and possible decapacitation factors; selected proteins of interest are listed in Table 1.

Mammals phosphoproteome. The reaction of phosphorylation and dephosphorylation of proteins is one of the most common posttranslational modification and it affects approximately 30% of all proteins of animal organisms. Phosphorylation regulates the activity and function of the polypeptides. Phosphorylated proteins acts as the regulators of cell activity, control their metabolism, growth or the “death path”, called apoptosis (Delom and Chevet, 2006).

Table – Swagen and Aitken (2014)

A selection of proteins and corresponding genes identified in stallion spermatozoa

chaperonins	chaperonin-containing complex/TRiC	t-complex 1	TCP1
		chaperonin containing TCP1, subunit 2 (beta)	CCT2
		chaperonin containing TCP1, subunit 3 (gamma)	CCT3
		chaperonin containing TCP1, subunit 4 (delta)	CCT4
		chaperonin containing TCP1, subunit 5 (epsilon)	CCT5
		chaperonin containing TCP1, subunit 6A (zeta 1)	CCT6A
		chaperonin containing TCP1, subunit 6B (zeta 2)	CCT6B
		chaperonin containing TCP1, subunit 7 (eta)	CCT7
		chaperonin containing TCP1, subunit 8 (theta)	CCT8
	heat shock protein	heat shock 10kDa protein 1 (chaperonin 10)	HSPE1
		heat shock 60kDa protein 1 (chaperonin)	HSPD1
		heat shock protein, alpha-crystallin-related, B9	HSPB9
		stress-70 protein, mitochondrial	HSPA9
		heat shock-related 70 kDa protein 2	HSPA2
		heat shock cognate 71 kDa protein	HSPA8
		heat shock 70kDa protein 5	HSPA5
		heat shock 70kDa protein 4-like	HSPA4L
		heat shock 70kDa protein 1-like	HSPA1L
		Heat shock protein HSP 90-alpha	HSP90AA1
Receptor proteins	receptor	G protein-coupled receptor, family C, group 5, member A	GPRC5A
		hyaluronan-mediated motility receptor (RHAMM)	HMMR
		parathyroid hormone 2 receptor	PTH2R
		peroxisome proliferator-activated receptor γ	PPARG
		protein tyrosine phosphatase, receptor type, G	PTPRG
		protein tyrosine phosphatase, receptor type, T	PTPRT
		olfactory receptor, family 5, subfamily W, member 2	OR5W2
	associated proteins	adrenergic B receptor kinase 2	ADRBK2
		low density lipoprotein receptor-related protein associated protein 1	LRPAP1
		regulator of G-protein signaling 22	RGS22
		transforming growth factor B associated protein 1	TRAP1
Candidate decapacitation factor proteins		phospholipase C, zeta 1	PLCZ1
		aspartate aminotransferase	GOT2
		phosphatidylethanolamine binding protein 1	PEBP1
		phosphatidylethanolamine-binding protein 4	PEBP4
		cysteine-rich secretory protein-2	CRISP-2

Special attention in the field of phosphorylation and dephosphorylation processes occurring in the male reproductive system is paid to their role in capacitation, acrosome reaction and sperm maturation. Phosphorylation of tyrosine residues of the sperm tail induces the hiperactivation of sperm as a result of which the cells acquire the ability to move and penetrate the oocyte. Phosphorylation of tyrosine residues of sperm proteins in other species like human, rabitt and rat was ascertained in 1991 (Naz et al., 1991). Previous studies ascertained also a positive correlation between phosphorylation of tyrosine, threonine and serine residues and spermatozoa activity, their ability to undergo a capacitation, acrosomal reaction and binding to zona pellucida (Naz 1996, Kalab et al., 1998).

For the first time the presence of phosphoproteins in mammals semen was demonstrated by Leyton and Saling (1989) in mouse sperm. In mentioned research by use of antiphosphotyrosine antibodies there were identified phosphoproteins with molecular weights of 52, 75 and 95 kDa. There was also demonstrated correlation between 95 kDa protein and the process of sperm capacitation and interaction with zona pellucida. Research carried out on mouse sperm showed that the proteins that act as chaperonines, locat-

ed in the plasmalemma of sperm tail, such as endoplasmin (erp99) and heat shock protein 60 (Hsp 60) are phosphorylated. These proteins enable the spermatozoon to recognize zona pellucida of an oocyte, and thus enhance the success of fertilization (Kalab et al,. 1998). Some research have confirmed the relationship between the tyrosine residues phosphorylation of the spermatozoa proteins and the sperm capacitation in mouse. It has been shown that CAMP-dependent tyrosine kinase is involved in this process. Similar relationships were found in the sperm of a human, bull, hamster and stallion (Bailey et al. 2005).

Studies concerning phosphorylation and dephosphorylation processes of proteins present in male reproductive system will determine biochemical changes during semen preservation. Because of growing interest in the usage of storage semen in mares artificial insemination there is a vital need for analysing biochemical parameters of preserved spermatozoa as well as interactions declining between sperm cells and seminal plasma.

Material and methods. Semen was collected from 4 warmblood stallions using a Missouri artificial vagina (Minitube Australia, Ballarat, VIC, Australia) in breeding and non-breeding season. Individuals were Polish Half Bred Horses, aged between 10 and 20 years. Samples of spermatozoa were retrieved both from fresh and diluted in EquiPro (MiniTube, Germany) semen extender.

Semen at first was diluted to 30 x 10^6 spermatozoa and stored in 5^0C temperature for three days (approx. 72 h). Samples of 1 ml volume from each day of preservation (D0, D1, D2, D3) were centrifuged at 8 000 x g for 10 min at 24ºC. Obtained supernatants were transferred into new tubes whereas the pellets of spermatozoa were diluted in RIPA buffer (Radio Immunoprecipitation Assay Buffer, 50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, pH 8.0). After 24 hours of incubation at +5^0C sperm extracts were centrifuged at 10 000 x g for 10 min at 24ºC, supernatants were collected and frozen for further analysis at -20^0C.

Phosphoproteins isolation. Phosphoproteins were isolated both from seminal plasma and sperm extracts using affinity chromatography with immobilised Fe^{3+} ions on PhosSelect Iron affinity gel (Sigma-Aldrich, USA) according to the manufacturer's protocol.

1D-PAGE electrophoresis. Probes were separated on a 12% polyacrylamide gel in the presence of sodium dodecyl sulphate (SDS) using Tris-glycine-SDS buffer (pH 8.3) according to Laemmli (1970) in Mini-Protean II apparatus (Bio-Rad, USA). At first, samples were pre-heated at 95ºC for 5 min in the presence of loading buffer (2% SDS, 5% β-mercaptoethanol, 125 mM Tris-HCl, pH 6.8) in a thermostat Thermo Block

TDB-125 (Biosan, Poland). On a single gel path 20 µl of sample solution was applied. Precision Plus Protein Standard (Bio-Rad, USA) served as molecular weight standards. After electrophoresis gels were stained with Coomassie Brilliant Blue R-250 for 24h, then destained with a buffer (5% CH_3COOH, 7% CH_3OH). Gels were then analysed in Multi Analyst programme (Bio-Rad, USA). Selected fractions were cut out with a scalpel and hydrolysed using Trypsin Gold, Mass Spectrometry Grade (Promega, USA) according to the manufacturer's protocol.

Mass spectrometry analysis. Digested polypeptides were spotted (1.0µl) onto MTP 384 Polished Steel TF Targets and overlaid with 1.0 µl of matrix solution containing 5 mg ml^{-1} α-cyano-4-hydroxycinnamic acid (Bruker Daltonics, Bremen, Germany) in 50% acetonitrile and 3% trifluoroacetic acid. Proteins were identified with the Autoflex III Smartbeam MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen Germany) equipped with a laser SmartBeam II (355 nm, Bruker Daltonics, Bremen, Germany) and FlexControl software v. 3.3. (Bruker Daltonics, Bremen, Germany) using technique PMF (Peptide Mass Fingerprinting) and partial sequencing method LIFT. To identify the proteins obtained mass spectra were compared with those from the database using Mascot v. 2.4 (Matrix Science, Boston, MA, USA).

Results. Our studies showed individual variation in the phosphoproteins profiles isolated from sperm extracts and seminal plasma of stallion semen. It has been demonstrated that storage of stallion semen in the liquid state induces variations in the phosphoprotein content in both seminal plasma and spermatozoa. Our survey also affirmed the presence of 20 phosphoproteins fractions isolated from seminal plasma and sperm extracts with molecular weight from 10 to 70 kDa. The mass spectrometry analysis of all peptides obtained from seminal plasma showed their similarity to: kallikrein (21,9 kDa), equine serum albumin (70,3 kDa) and paladin (25 kDa). Phosphoproteins isolated from sperm extracts showed similarity to: endoplasmin (32 kDa), Hsp 90 β member 1 (76,4 kDa) and cytochrome b-c1 complex (23 kDa). All of these proteins are involved in the proper functioning of the male reproductive system and play an important role in the processes of capacitation and acrosome reaction. Compatibility factor of kallikrein chain was 25% and obtained score was 93. Studies conducted by Calvete et al. (1994) showed similarity of HSP-6 and HSP-8 to various isoforms of kallikrein, which is a protein homologous to human PSA (prostate specific antigen). Compatibility factor of equine serum albumin chain amounted to 16% and obtained score was 114. Kasperczyk et al. (2014) confirmed a positive correlation between calcium concentration and higher levels of seminal plasma albumin in human semen, which resulted in better sperma-

tozoa motility, capacitation and response of sperm antioxidant system. Compatibility factor of paladin chain was 11% and obtained score was 91. Studies carried out on rats and mice demonstrated the presence of paladin in the testes. It occurred nearby Sertoli cells. The probably main task of paladin is to ensure proper transport of spermatocytes to the light of the seminal tubules during their maturation in epididymis (Niedenberger et al., 2013). Endoplasmin's chain compatibility factor was 21%, obtained score was 132. This protein is linked to the sperm surface. Tyrosine residues phosphorylation of mentioned polypeptide along with heat shock protein 60 (HSP 60) ends in spermatozoa ability to recognize the zona pellucida. Endoplasmin was localized by immunofluorescence on the surface of the mouse spermatozoa (Asquith et al. 2004). Cytochrome bc-1 complex showed 18% of adequacy and score was 87. Protein which is also known as cytochrome c reductase or complex III is a main component of the respiratory system that is localised in the inner mitochondrial membrane (Berry et al. 2000). Cytochrome b-c1 is a phylogenetically diversificated group of complexes responsible for transferring electrons from ubiquinol to cytochrome c. This process is oxygen dependent (Crofts, 2004). The complex consists of two identical subunits, each composed of 11 protein subunits and three cytochromes: cytochrome c1, two cytochromes b and iron-sulfur center (Iwata et al., 1998). Compatibility factor of heat shock protein 90 chain was 21% whereas an obtained score was 95. HSP protein family is responsible for protecting other polypeptides from denaturation or aggregation. Studies showed that HSP proteins are involved in a variety of immune responses. Under physiological conditions HSP90 is responsible for regulating the cell death pathway (necrosis). This protein may bind to ligand-activated transcription factors (i.e., androgens) as well as ligand-independent transcription factors. In addition, it can interact with tyrosine kinases, serine-threonine kinases and cell cycle kinases (Kaźmierczuk and Kilkiańska, 2009).

In stallion proteome profile three of proteins were identified as a correlated with fertility: SP-2 (75 kDa, pI 6.0), SP-3 (18 kDa, pI 4.3), and SP-4 (16 kDa, pI 6.5) (Brandon et al. 1999). Novak et al. (2010) confirmed negative influence of some seminal plasma proteins on stallion male fertility: SP1 (14 kDa), SP2 (19.2 kDa), kallikrein (1E2) and clusterin. But also found that cysteine-rich secretory protein 3 (CRISP3) is a good positive marker of stallion fertility.

Conclusion. Understanding the molecular mechanisms of reproduction is considered paramount for rational advances in assisted reproduction techniques. Levels of protein expression, their locations, isoforms produced by alternative splicing and posttranslational modifications, as well as functional protein interactions with biomolecules and the resulting signalling

cascades are basic of protein analysis called proteomics. It has allows us to understand what is actually express in the cell and beginning to expand understanding of the mammalian reproduction (Lee, 2001).

Proteomics give us a possibility to define specific proteins as a molecular fertility/infertility markers of individuals used in reproduction. Present of specific peptides or their isoforms can be correlated with reproduction indicators and exclude sick or infertile male from reproduction plan.

REFERENCES

1. Asquith K. L., Baleato R. M., McLaughlin E. A., Nixon B., Aitken J. R. 2004. Tyrosine phosphorylation activates surface chaperones facilitating sperm-zona recognition. Journal of Cell Science. 117. 3645-3657
2. Bailey J. L., Tardif S., Dube C., Beaulieu M., Reyes-Moreno C., Lefievre L., Leclerc P. 2005. Use of proteomics to study tyrosine kinase activity in capacitating boar sperm kinase activity and capacitation. Theriogenology. 63. 599-614
3. Berry E., Guergova-Kuras M., Huang L., Crofts A., Structure and function of cytochrome bc complexes. 2000. Annual Review of Biochemistry. 69. 1005–75
4. Brandon C.I., Heusner G.L., Caudle A.B., Fayrer-Hosken R.A. 1999. Two-dimensional polyacrylamide gel electrophoresis of equine seminal plasma proteins and their correlation with fertility. Theriogenology. 52. 863-873.
5. Calvete J.J., Nessau S., Mann K., Sanz L., Sieme H., Klug E., Topfer-Petersen E. 1994. Isolation and biochemical characterization of stallion seminal plasma proteins. Reproduction in Domestic Animals. 29. 411-426
6. Crofts AR., The cytochrome bc1 complex: function in the context of structure. 2004. Annual Review of Physiology. 66. 689–733
7. Delom F., Chevet E. Phosphoprotein analysis: from protein to proteomes. 2006. Proteome Science. 4.15
8. Frazer G. S., Bucci D. M., Characterization of the major polypeptides of equine seminal plasma by two-dimensional polyacrylamide gel electrophoresis. 1996. Theriogenology. 46. 1389-1402
9. Iwata S., Lee JW., Okada K., Complete structure of the 11-subunit bovine mitochondrial cytochrome bc1 complex. 1998. Science. 281 (5373). 64–71
10. Kalab P., Peknicova J., Guessova G., Moos J., Regulation of protein tyrosine phosphorylation in boar sperm through a cAMP-dependent pathway. 1998. Molecular Reproduction and Development. 51(3). 304-14
11. Kasperczyk A., Dobrakowski M., Zalejska-Fiolka J., Horak S., Machoń A., Birkner E., The role of calcium in human sperm in relation to the antioxidant system. 2014. Environmental medicine. 17. 34-40
12. Kaźmierczuk A., Kilkiańska Z. M., The pleiotropic activity of heat-shock proteins. 2009. Advances in Hygiene and Experimental Medicine. 63. 502-521
13. Kratz A. M., Achcińska M. K. Molecular mechanisms of fertilization: the role of male factor. 2011. Advances in Hygiene and Experimental Medicine. 65. 784-795
14. Laemmli, U.K. 1970. Nature. 227. 680-685
15. Lee K.H., Proteomics: a technology-driven and technology-limited discovery science. 2001. Trends in Biotechnology. 19. 217-222
16. Leyton L., Saling P., 95 kDa sperm proteins bind ZP3 and serve as tyrosine kinase substrates in response to zona binding. 1989. Cell. 57. 123-130
17. Mogielnicka-Brzozowska M., Kordan W., Characteristics of selected seminal plasma proteins and their application in the improvement of the reproductive processes in mammals. 2011. Polish Journal of Veterinary Sciences. Vol. 14. No. 3. 489-499

18. Naz RK., Ahmad K., Kumar R., Role of membrane phosphotyrosine proteins in human spermatozoa function. 1991. Journal of Cell Science. 99. 157-165
19. Naz RK., Involvement of protein tyrosine phosphorylation of human sperm in capacitation/acrosome reaction and zona pellucid binding. 1996. Frontiers in Bioscience. 1. 206-213
20. Niedenberger B. A., Chappell V. K., Kaye E. P., Renegar R. H., Geyer C. B. Nuclear localization of the actin regulatory protein Palladin in sertoli cells. 2013. Molecular Reproduction and Development. 80(5). 403-13
21. Novak S., Smith T.A., Paradis F., Burwash L., Dyck M.K., Foxcroft G.R., Dixon W.T. 2010. Biomarkers of in vivo fertility in sperm and seminal plasma of fertile stallions. Theriogenology.74. 956-967
22. Reineke A., Hess O., Schambony A., Petrunkina A.M., Bader H., Sieme H., Topfer-Petersen E., Sperm associated seminal plasma proteins—a novel approach for the evaluation of sperm fertilizing ability of stallions? 1999. Pferdeheilkunde. 6. 531–537
23. Rodriguez-Martinez H., Kvist U., Ernerudh J., Sanz L., Calvete J. J. Seminal plasma proteins: what role do they play? 2011. American Journal of Reproductive Immunology. 66. 11-22
24. Swegen A., R.J. Aitken, Characterisation of the stallion sperm proteome. 2014. Journal of Equine Veterinary Science. 34. 35-37
25. Swegen A., Curry B.J., Gibb Z., Lambourne S.R., Smith N.D., Aitken R.J., Investigation of the stallion sperm proteome by mass spectrometry. 2015. Reproduction. 149. 235–244
26. Topfer-Petersen E., Ekhlas-Hundrieser M., Kirchhoff C., Leeb T., Sieme H., The role of stallion seminal proteins in fertilization. 2005. Animal Reproduction Sciences. 89. 159-170

THE EFFECT OF FITODOCTOR APPLICATION ON YIELDING ABILITY AND MINERAL COMPOSITION OF WHITE MUSTARD (SINAPIS ALBA L.) ABOVE GROUND BIOMASS

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Introduction. White mustard is grown as oil and spice crop but it also used as fodder, green manure or mulch. It is applied in modern or in traditional medicine. Comparing to other oil crops grown in temperate climate zones (oil seed rape or sunflower) it has minor importance but it is grown on larger area than black or Indian mustard together. When used as a cover crop beneficial effects of white mustard (i.e. amount of available nutrients and organic matter) can be compared with full rate of farm yard manure of good quality [Harasimowicz-Hermann and Hermann 2006]. White mustard can produce high yield of biomass capable to fix nutrients. In some reports it is pointed out phytosanitary, soil protection role of this crop as well as the fact that white mustard can be applied as supporting crop for plants of soft stems in mixed stands [Szymczak-Nowak and Nowakowski 2000; Ceglarek