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CHANGES IN THE EXPRESSION OF SELECTED ANTIOXIDATIVE PROTEINS IN ROE DEER (*CAPREOLUS CAPREOLUS*) EPIDIDYMIS IN DIFFERENT PERIODS OF THE RUTTING SEASON*

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Abstract

One of the factors determining the homeostasis of biological systems is the balance between the formation of reactive oxygen species (ROS) and the activity of the antioxidative defence system. Regarding seasonal variations in secretory activity in different parts of roe buck's (*Capreolus capreolus*) epididymis, the aim of present study was to compare the expression of selected antioxidative proteins in three periods of the rutting season. Using proteomics methods (2D PAGE, tandem mass spectrometry MS/MS) 5 antioxidative enzymes were identified for the first time in different segments of the epididymis of the roe buck. The highest expression of these enzymes was found in the rutting season. Findings indicated that the antioxidative function of the buck's epididymis, regardless of the period of the rutting season, but particularly in the rutting season, is maintained mainly because of the presence of three conservative polypeptides: glutathione S-transferase (GST), protein disulfide isomerase A3 (PDIA3), and PDIA3 precursor. Moreover, a protective role against the harmful products of redox reactions is played during the discussed periods by peroxiredoxin-2 (PRDX2), identified in the cauda of the epididymis. In the tissue of the corpus and caput of the epididymis its expression was only found in the rutting season. The expression of biliverdin reductase A (BRVA) in the epididymis was only observed in the rutting season.

Key words: roe deer (*Capreolus capreolus*), epididymis, antioxidant system, proteomics

The roe deer (*Capreolus capreolus*) is a typical species with seasonal breeding. The breeding season is defined by three periods: 1) pre-rut season (May–June); 2) rut season (July–August), which is synchronized with female oestrus; and 3) post-rut season (first fortnight of August). Changes in the composition of cells in individual segments of the epididymis corresponding with annual changes in spermatogen-

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esis were found in mature roe bucks (Shön and Blottner, 2009). Most of the research examining the epididymis to date has been limited to the study of the secreted proteins involved in the maturation of spermatozoa. It is important to understand the protein components, and the regulation and function of the tissue itself, since these are the basis for all of its physiological processes (Yuan et al., 2006).

Antioxidative proteins and peptides determining normal functions of the reproductive system are a subject of special interest. Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen. These include free radicals – molecules with one or more unpaired electrons, and hydrogen peroxide, hypochlorous acid and nitrous oxide (Sharma and Agarwal, 1996). Spermatozoa are able to generate ROS, whose low concentration plays an important role in sperm physiological processes, such as capacitation, acrosome reaction, and signalling processes to ensure fertilization (Agarwal et al., 2014). Overproduction of ROS or depletion of the compensatory potential of the antioxidative system in mammalian sperm leads to oxidative stress (Guthrie and Welch, 2012). Prolonged exposure of spermatozoa to ROS causes damage to plasma membranes, motility disorders, chromatin destabilisation, and consequently leads to loss of fertilizing capacity (Aitken et al., 2012). Mammalian spermatozoal membranes are rich in polyunsaturated fatty acids (PUFAs), and are sensitive to oxygen-induced damage mediated by lipid peroxidation. Spermatozoa are sensitive to ROS attack, which results in decreased sperm motility, probably by a rapid loss of intracellular ATP leading to axonemal damage, decreased sperm viability, and increased mid-piece sperm morphological defects with harmful effects on sperm metabolism (Sikka, 1996).

Regarding seasonal variations in secretory activity in different parts of roe buck's (*Capreolus capreolus*) epididymis, the aim of present study was to compare the expression of selected antioxidative proteins in three periods of the rutting season.

Material and methods

The reagents used in the experiment were supplied by Sigma-Aldrich (Saint Louis, MO, USA) unless stated otherwise.

Sampling of material and preparation of samples

Testes and epididymides were obtained from 15 hunted animals with the permission of the Regional Directorate of the State Forests in Krosno. Samples (n=5 animals per season) were taken in 3 periods of the rutting season: pre-rut season (May–June), rutting season (July–August), and post-rut season (first fortnight of August). Epididymides were dissected and subdivided into three anatomical regions (caput, corpus and cauda). Tissue samples were frozen in liquid nitrogen.

Proteins from tissue specimens were extracted using a TRI-Reagent Kit, according to a previously described method (Chomczynski and McKay, 1995). The protein content was determined according to a previously described method (Bradford, 1976).

Two-dimensional polyacrylamide gel electrophoresis (2D PAGE)

Two-dimensional polyacrylamide gel electrophoresis (2D PAGE) was performed according to the method of O'Farrel et al. (1975) with some modification, using 3–10 pH gradient. The extracted protein samples were applied onto strips (50µg protein per strip) (Zoom Strip, pH 3–10 NL, Invitrogen, Waltham, MA, USA) fixed in a cassette (Zoom IPG Runner cassettes, Invitrogen, Waltham, MA, USA). After isoelectrofocusing (IEF) and strip equilibration in buffer: 6 M urea, 2% SDS, 0.375 M Tris, 20% glycerol (Polish Chemicals Inc., Poland), 2% DTT and 0.2% Bromophenol Blue proteins were separated (SDS-PAGE) in 12% gels (Laemmli, 1970) using Mini Protean II Cell (BioRad Rockville, MD, USA). All gels were stained in Coomassie Brilliant Blue R-250 solution (0.025% Coomassie Brilliant R-250, 40% acetic acid, 7% methanol in deionized water). The polyacrylamide gels were destained in a solution of 5% methanol, 7% acetic acid and deionized water. Destained gels were analysed with a computer program PDQuest (BioRad, Rockville, MD, USA). 2D PAGE digital images were analysed according to the PDQuest user guide, regarding spot detection, optical density (ODU), matching, and calculating Mr/pI values for all of the spots.

Tandem mass spectrometry MALDI-TOF/TOF

Spots of interest were excised from gels, digested with trypsin, desalted and concentrated with a ZipTip® C₁₈ (Merck Millipore, Billerica, MA, USA). The prepared proteins were spotted (1.0µl) onto MTP 384 Polished Steel TF Targets and overlaid with a 1.0µl matrix solution containing 5 mg ml⁻¹ α-cyano-4-hydroxycinnamic acid (Bruker Daltonics, Bremen, Germany) in 50% ACN and 0.1% trifluoroacetic acid. Samples were analysed with the Autoflex III Smartbeam MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen Germany). Chosen peaks were fragmented using TOF/TOF Lift technology, and identified using BioTools software (Bruker Daltonics, Bremen, Germany). Identification was performed by searching the NCBI database using the MASCOT search engine (Matrix Science, Boston, MA, USA). In all searches performed protein scores greater than 77 were considered statistically significant (P<0.05).

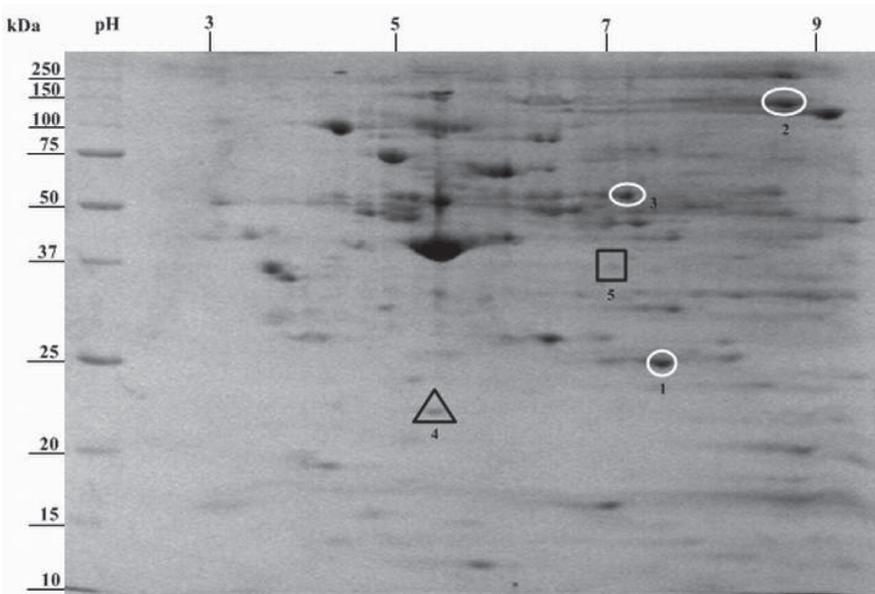
Statistical analysis

Differences in protein spots' optical density were analysed by Kruskal-Wallis test using Statistica software (StatSoft, Tulsa, OK, USA). Differences between means were considered significant at P≤0.05.

Results

Analysis employing proteomic methods, i.e. two-dimensional polyacrylamide gel electrophoresis (2D PAGE) and tandem mass spectrometry (MS/MS), identified 38 polypeptides from the roe bucks' epididymis having enzymatic, structural or cell signalling functions (data not shown) and 5 polypeptides with antioxidant properties.

An example electropherogram of the tissue from a buck's epididymis presents five identified antioxidative proteins (Figure 1).



- conservative proteins identified regardless of the period of the rutting season, in each segment of the epididymis,
- protein identified in post-rut and the rutting season,
- protein identified only in the rutting season.

Figure 1. Two dimensional electrophoresis (2D – PAGE) of proteins of the epididymis tissue homogenate

Regardless of the period of the rutting season three conservative polypeptides similar to: glutathione S-transferase (GST), protein disulfide isomerase A3 precursor (PDIA3) and protein disulfide isomerase A3 (PDIA3) were identified in all segments of the epididymis (caput, corpus, cauda). Moreover, in all periods of the rutting season the expression of peroxiredoxin-2 (PRDX2) was identified in the tissues of the cauda epididymis. In the caput and corpus tissues, only PRDX2 was identified in the rutting season, with traces of PRDX2 expression in post-rut season. Of all analysed electropherograms biliverdin reductase A (BRVA) was only identified in the tissues from the epididymides sampled in the rutting season. All analysed protein spots showed high protein sequence coverage and scores with predicted proteins from the NCBI database (Table 1).

Depending on the period of the rutting season there were statistically significant differences ($P \leq 0.05$) in the concentration of identified polypeptides expressed in optical density units (ODU). The highest values of ODU for all analysed proteins were observed in the rutting season. Findings from analyses were confirmed by statistical methods (Table 2).

Table 1. Protein name, NCBI database index number, calculated MW, pI, peptide coverage and Mascot score of identified polypeptides

Protein name	No of protein	NCBI database index number	Calculated MW (kDa)	pI	Protein sequence coverage %	Mascot score
Glutathione S-transferase	1	426216186	27.14	6.83	34	140
Protein disulfide-isomerase A3 precursor	2	251823897	57.37	6.23	25	105
Protein disulfide isomerase A3 (58 kDa glucose regulated protein)	3	729433	57.29	6.61	25	78
Peroxiredoxin-2	4	548472190	20.157	5.56	19	247
Biliverdin reductase A	5	426356116	34.49	6.27	20	103

Table 2. Changes in the optical density (mean±SD) of analysed proteins depending on the period of the rutting season (n=5)

Proteins	No of protein	Pre-rut season	Rutting season	Post-rut season
Glutathione S-transferase	1	0.254 b±0.065	0.462 a±0.067	0.262 b±0.087
Protein disulfide-isomerase A3 precursor	2	0.184 b±0.027	0.424 a±0.019	0.186 b±0.008
Protein disulfide-isomerase A3	3	0.220 b±0.015	0.486 a±0.019	0.250 b±0.029
Peroxiredoxin-2	4	0.062 b±0.042	0.194 a±0.035	0.074 b±0.027
Biliverdin reductase A	5	-	0.200±0.016	-

a, b – differences between means marked with different letters were statistically significant at $P \leq 0.05$.

Discussion

One of the factors determining the homeostasis of biological systems is the balance between the formation of reactive oxygen species (ROS) and the activity of the antioxidative defence system. This system includes many elements, both enzymatic and non-enzymatic, which by interaction ensure an appropriate level of antioxidative potential. It should be noted that activity of antioxidant enzymes in male reproductive system is determined by species, for example all basic antioxidant enzymes (superoxide dismutase – SOD, catalase – CAT, glutathione peroxidase – GPx) were identified in stallion (Baumer et al., 2002). By contrast in boar the superoxide dismutase was the dominant identified enzyme, meanwhile the activity of glutathione peroxidase was on very low level and the activity of catalase was not observed (Jelezarsky et al., 2008).

Although our previous studies here demonstrated the activities and mRNA expression of SOD, CAT and GPx in the tissues of the male roe deer reproductive tract (Koziorowska-Gilun et al., 2015), in this study there was no presence of these enzymes in the analysed samples. This result could be caused by the kind of method that we used for spots staining, which is too insensitive in the detection of polypeptides occurring in low concentrations.

In this study we used proteomic techniques and identified the presence of 5 antioxidative proteins in the epididymis of the roe buck, with the highest expression levels in the rutting season.

One element of an antioxidative defence system in human semen comprises glutathione S-transferases, from a family of isoenzymes catalysing the binding of electrophilic compounds and harmful products of lipid peroxidation by glutathione (Aydemir et al., 2007). GST were identified in the epididymal tissue from the rat (Hales et al., 1980) and the head of goat sperm cells (Gapalakrishnan and Shaha, 1998). It has been demonstrated that in goat spermatozoa, the sperm surface glutathione S-transferases are able to use extracellular glutathione to maintain motility, viability, energy status, and the ability to bind *zona pellucida* of oocyte during the exposure of sperm cells to H_2O_2 or lipid peroxidation products (Hemachand and Shaha, 2003). Studies by Ciszewska-Piłczyńska and Barańczyk-Kuźmiat (2000) revealed that GST present in each segment of bull and boar epididymis is likely to form a part of the enzymatic defence system in spermatozoa against the toxic effects of free radicals.

In our study we demonstrated the presence of GST in all segments of epididymis of the roe deer, regardless of the period of the rutting season, which may suggest the major role of GST in eliminating products of oxidative stress.

Similar expression was found for other enzymes identified in our studies – protein disulfide isomerase A3 (PDIA3) and PDIA3 precursor. Protein disulfide isomerases (PDIs) include the CXXC sequence with a cysteine residue, involved in thiol-disulfide exchange reactions (Ellgaard and Ruddock, 2005). PDIA3 has the activity of thioredoxin, one of enzymes from the mammalian sperm antioxidant system (Zhao et al., 2013).

PDIA3 was identified in sheep, goat and human testes (Zhang et al., 2007; Lv et al., 2011). In ram sperm cells PDIA3 was observed in the entire spermatozoa, but mainly at the equatorial segment and the forepart of the flagellum (Lv et al., 2011). Rat PDIA3 was also observed to some extent in acrosome; most were evenly distributed on the entire sperm cell membrane, except in the equatorial segment (Ellerman et al., 2006). PDIA3 binds *in vivo* to specific DNA targets, which are mainly present in intronic sequences having the features of regulatory regions. The nature of the genes involved leads to the hypothesis that the regulation of their expression is part of the mechanism of action of PDIA3 as a stress-response protein (Huang et al., 2009).

Another identified protein was peroxiredoxin-2, and this had the highest expression level in the rutting season. Relatively recently identified peroxiredoxins belong to the class of SH-dependent peroxidases. To date, 6 isoforms of PRDX have been identified, with transcripts located in the whole mammalian reproductive system. The key function of peroxiredoxins is to protect spermatozoa from oxidative stress (Gong et al., 2012) by neutralizing the toxic effects of hydrogen peroxide. Immunocytochemistry studies revealed a broader localization of PRDX2 in the plasma membrane, mitochondrial sheath, flagellum and head of human spermatozoa (O'Flaherty, 2014). Using proteomic techniques and immunocytochemistry Manandhar et al. (2009) investigated the presence of PRDX2 in mouse and boar spermatozoa and in

mouse spermatids. PRDX2 is an enzyme involved in redox reactions, and neutralizes H_2O_2 more efficiently than glutathione peroxidase or catalase. Studies on boar and mouse spermatozoa revealed that PRDX2 follows a similar path to glutathione peroxidase 4 (GPX4). It is a soluble enzyme in spermatids but turns into an insoluble structural protein that colocalizes with the GPX isoform, becoming part of the mitochondrial sheath (Manandhar et al., 2009). Biliverdin reductase A was also identified in the epididymal tissues. However, the expression of BVRA was only observed during the rutting season. BVRA and the epididymal sperm binding protein 1 (ELSPBP1) are involved in the reduction of biliverdin to bilirubin (Sullivan et al., 2015). In reaction with free radicals bilirubin is oxidized to biliverdin, which is again reduced by BVR. Because of these cyclic transformations one bilirubin molecule can quickly react with multiple ROS (Aprioku, 2013). The activity of BVR in tissues increases under conditions of oxidative stress (Bełtowski et al., 2004), which may explain the presence of this enzyme only in specimens dissected during the rutting season.

Conclusions

For the first time, using proteomics methods, antioxidative proteins were identified in different segments of the epididymis of roe deer. Findings indicated that the antioxidative performance of the roe buck's epididymis, particularly in the rutting season, is maintained mainly because of the presence of three conservative enzymes: glutathione S-transferase, protein disulfide isomerase A3 (PDIA3), and PDIA3 precursor. Peroxiredoxin-2 and biliverdin reductase A also have a protective function against the harmful products of redox reactions during the rutting season.

References

- Agarwal A., Virk G., Ong C., du Plessis S.S. (2014). Effect of oxidative stress on male reproduction. *World J. Mens Health*, 32: 1–17.
- Aitken R.J., Jones K.T., Robertson S.A. (2012). Reactive oxygen species and sperm function in sickness and in health. *J. Androl.*, 33: 96–106.
- Aprioku J.S. (2013). Pharmacology of free radicals and the impact of reactive oxygen species on the testis. *J. Reprod. Infertil.*, 14: 158–172.
- Aydemir B., Onaran I., Kiziler A.R., Alici B., Akyolcu M.C. (2007). Increased oxidative damage of sperm and seminal plasma in men with idiopathic infertility is higher in patients with glutathione S-transferase Mu-1 null genotype. *Asian J. Androl.*, 9: 108–115.
- Baumer J., Sauber K., Vo K., Ball B.A. (2002). Generation of reactive oxygen species by equine neutrophils and their effect on motility of equine spermatozoa. *Theriogenology*, 57: 1025–1033.
- Bełtowski J., Jamroz A., Borkowska E. (2004). Heme oxygenase and carbon monoxide in the physiology and pathology of the cardiovascular system. *Postep. Hig. Med. Dosw.*, 58: 83–99.
- Bradford M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, 72: 248–254.
- Chomczynski P., MacKay K. (1995). Modification of the TRI reagent procedure for isolation of RNA from polysaccharide- and proteoglycan-rich sources. *Biotechniques*, 19: 942–945.

- Ciszewska-Piłczyńska A., Barańczyk-Kuźma A. (2000). Glutathione conjugation in male reproductive system: studies on glutathione-S-transferase of bull and boar epididymis. *Acta Biochim. Pol.*, 47: 223–231.
- Ellerman D.A., Myles D.G., Primakoff P. (2006). A role for sperm surface protein disulfide isomerase activity in gamete fusion: evidence for the participation of ERp57. *Dev. Cell*, 10: 831–837.
- Ellgaard L., Ruddock L. (2005). The human protein disulphide isomerase family: substrate interactions and functional properties. *EMBO Rep.*, 6: 28–32.
- Gapal Krishnan B., Shaha C. (1998). Inhibition of sperm glutathione S-transferase leads to functional impairment due to membrane change. *FEBS Lett.*, 422: 296–300.
- Gong S., San Gabriel M., Zini A., Chan P., O'Flaherty C. (2012). Low amounts and high thiol oxidation of peroxiredoxins in spermatozoa from infertile men. *J. Androl.*, 33: 1342–1351.
- Guthrie H.D., Welch G.R. (2012). Effects of reactive oxygen species on sperm function. *Theriogenology*, 78: 1007–1008.
- Hales B., Hachey C., Robaire B. (1980). The presence and longitudinal distribution of the glutathione S-transferases in rat epididymis and vas deferens. *Biochem. J.*, 189: 135–142.
- Hemachand T., Shaha C. (2003). Functional role of sperm surface glutathione S-transferases and extracellular glutathione in the haploid spermatozoa under oxidative stress. *FEBS Letters*, 538: 14–18.
- Huang T.S., Olsvik P.A., Krøvel A., Tung H.S., Torstensen B.E. (2009). Stress-induced expression of protein disulfide isomerase associated 3 (PDIA3) in Atlantic salmon (*Salmo salar* L.). *Comp. Biochem. Physiol. Biochem. Mol. Biol.*, 154: 435–442.
- Jelezarsky L., Vaisberg C., Chaushev T., Sapundjiev E. (2008). Localization and characterization of glutathione peroxidase (GPx) in boar accessory sex glands, seminal plasma, and spermatozoa and activity of GPx in boar semen. *Theriogenology*, 69: 139–145.
- Koziorowska-Gilun M., Fraser L., Gilun P., Koziorowski M., Kordan W. (2015). Activity of antioxidant enzymes and their mRNA expression in different reproductive tract tissues of the male roe deer (*Capreolus capreolus*) during the pre-rut and rut seasons. *Small Rumin. Res.*, 129: 97–103.
- Laemmli U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227: 680–685.
- Lv L.X., Ujisguleng B., Orhontana B., Lian W.B., Xing W.J. (2011). Molecular cloning of sheep and cashmere goat PDIA3 and localization in sheep testis. *Reprod. Domest. Anim.*, 46: 980–989.
- Manandhar G., Miranda-Vizuete A., Pedrajas J.R., Krause W.J., Zimmerman S., Sutovsky M., Sutovsky P. (2009). Peroxiredoxin 2 and peroxidase enzymatic activity of mammalian spermatozoa. *Biol. Reprod.*, 80: 1168–1177.
- O'Farrell P.H. (1975). High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.*, 250: 4007–4021.
- O'Flaherty C. (2014). The enzymatic antioxidant system of human spermatozoa. *Adv. Androl.*, 24: 1–15.
- Sharma R.K., Agarwal A. (1996). Role of reactive oxygen species in male infertility. *Urology*, 48: 835–850.
- Shön J., Blottner S. (2009). Seasonal variations in the epididymis of the roe deer (*Capreolus capreolus*) *Anim. Reprod. Sci.*, 111: 344–352.
- Sikka S.C. (1996). Oxidative stress and role of antioxidants in normal and abnormal sperm function. *Front. Biosci.*, 1: 78–86.
- Sullivan R., D'Amorus O., Caballero J., Belleannee C. (2015). The sperm journey in the excurrent duct: functions of microvesicles on sperm maturation and gene expression along the epididymis. *Anim. Reprod.*, 12: 88–92.
- Yuan H., Liu A., Zhang L., Zhou H., Wang Y., Zhang H., Wang G., Zeng R., Zhang Y., Chen Z. (2006). Proteomic profiling of regionalized proteins in rat epididymis indicates consistency between specialized distribution and protein functions. *J. Proteome Res.*, 5: 299–307.
- Zhang J., Wu J., Huo R., Mao Y., Lu Y., Guo X., Liu J., Zhou Z., Huang X., Sha J.

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- (2007). ERp57 is a potential biomarker for human fertilization capability. *Mol. Hum. Reprod.*, 13: 633–639.
- Zhao X.J., Tang R.Z., Wang M.L., Guo W.L., Liu J., Li L., Xing W.J. (2013). Distribution of PDIA3 transcript and protein in rat testis and sperm cells. *Reprod. Domest. Anim.*, 48: 59–63.

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