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EXPERIMENTAL STUDY

The presence of glutathione peroxidase 8 (GPx8) in rat male genital organs

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ABSTRACT

OBJECTIVES: The aim of our work was to determine the presence of GPx8, the latest discovered member of glutathione peroxidase family, in rat male genital organs.

BACKGROUND: The oxidative stress is considered as one of the most important causalities of male infertility. To defend itself, the organism comprises many different antioxidants.

METHODS: We assessed the GPx8 presence in tissues of genital organs from adult rat Sprague-Dawley males by mRNA expression, Western Blot analysis, and immunohistochemistry.

RESULTS: The highest mRNA and protein levels were detected in the testis, followed by seminal vesicle. Within testis the enzyme was observed predominantly in the Leydig and Sertoli cells, residual and Hermes bodies. In other organs, such as epididymis, seminal vesicle and prostate gland, the GPx8 was seen in the cytoplasm of epithelial cells. The enzyme was also observed in the muscular layer of hollow organs, in blood plasma and extracellular matrix.

CONCLUSIONS: The antioxidant enzyme GPx8 was detected in all examined male genital organs. The fact, that the enzyme was released into lumen of genital organs probably means, that GPx8 is also a component of the semen. To our knowledge, this is the first paper describing GPx8 presence in male genital organs of mammals *(Fig. 8, Ref. 63)*. Text in PDF *www.elis.sk*

KEY WORDS: antioxidant enzyme, oxidative stress, male infertility, immunohistochemistry, Western Blot.

Introduction

Oxidative stress is the result of an imbalance between the systems generating and eliminating ROS (1). Such a balance is carefully regulated through large numbers of different enzymatic or non-enzymatic antioxidants (2). Sperm are extremely sensitive to oxidative injury since the elevated polyunsaturated fatty acids (PUFA) content in their plasma membrane makes them particularly susceptible to ROS attacks, with consequences such as lipid peroxidation, DNA damage, or impaired sperm motility (3). Moreover, being transcriptionally and translationally silent, without expelled cytoplasm containing antioxidants, spermatozoa depend on protective substances situated in their environment (4). Interestingly, although sperm are susceptible to ROS damage, they are also producers of ROS themselves (5). However, beside their detrimental effects, ROS are vital as they participate as the second messenger in response to various stimuli, modulating intracellular signal transduction pathways. It has been also found that ROS are the key players in signal transduction leading to sperm capacitation, acrosome reaction and sperm-oocyte fusion, the events that are essential for fertilization. From this point of view ROS represent a double-edged sword, as on the one hand they are beneficial for the sperm maturation and capacitation, but on the other hand they are harmful whereas depending on their concentration and length of exposure they could result in sperm damage or even their death (6). That is the reason, why it is very important for male genital system to control ROS in order to maintain the right balance between their production and degradation.

The cells contain a lot of antioxidant systems to protect against ROS toxicity. One of them are superoxide dismutases (SODs), which are universal enzymes of living organisms that catalyze the conversion of superoxide anions into oxygen and hydrogen peroxide (7). SOD1 was detected in spermatids, SOD2 in spermatocytes, and both proteins in Leydig cells of rat (8). The resulting hydrogen peroxide is further disarmed employing enzymes such as catalase (CAT) or glutathione peroxidases (GPxs). CAT can use as a substrate only H_2O_2. Moreover, it is activated when H_2O_2 concentrations are large above its physiological levels under stress

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conditions, during so-called oxidative burst (9). On the other hand, GPxs are considered to be more versatile and more important protective enzymes under physiological conditions since they are activated for a small H_2O_2 rise concentrations and besides H_2O_2 they can also recycle organic peroxidized molecules, including those in PUFA (10). Several members of GPxs family, such as GPx1, GPx3, GPx4, and GPx5 occupy a special position in mammalian sperm development, because they are present in large amounts either on a sperm or in their immediate neighborhood during sperm journey through the male genital organs (11). GPxs comprise eight enzymes (GPx1 - GPx8) whereas each of them possesses unique features, tissue distribution, substrate specificity, and biological function (12).

GPx7 and GPx8, which presumably developed from GPx4, have very similar amino acid composition. Both enzymes are monomeric proteins located in the endoplasmic reticulum but unlike GPx7, completely situated inside this compartment, GPx8 is transmembrane protein anchored by one end in the endoplasmic reticulum membrane. GPx7 and GPx8 are probably involved in correct protein folding, prevention of endoplasmic reticulum stress and subsequent H_2O_2 leakage from endoplasmic reticulum to the cytosol (13).

The aim of our work was to determine the presence of GPx8 in rat male genital organs, since the oxidative stress is considered to be one of the main factors of male infertility. The lack of information about whether GPx8, the latest discovered members of GPxs family, is present in any form and any amount in some male genital organ prompted us to start into this investigation. Recently we found, that GPx8 is expressed in rat embryos and female genital organs during preimplantation period of pregnancy (14). It was therefore reasonable to assume that this protein may be involved also in sperm development.

Material and methods

Ethical statement

The experiment was approved by the Committee for Ethical Control of Animal Experiments at Pavol Jozef Safarik University (UPJŠ) and the State Veterinary and Food Administration of the Slovak Republic (permission no. Ro-11557/18-221/3, permission granted from 01/07/2018). A reduced number of animals was used in the experiment to minimize their suffering.

Animals

The Laboratory of Research Biomodels of the UPJS provided five adult Sprague Dawley rat males aged 90-100 days weighting approximately 410 g. Standard diet and water were available to the animals ad libitum all the time and the animals were exposed to a 12 h light/12 h dark cycle. Males were killed by a lethal dose of anesthetic.

RNA isolation, reverse transcription and quantitative RT-PCR

Total RNA was isolated using Trizol (Gibco/BRL, MD, USA) and purified using RNasy Mini Kit (Qiagen, Germany). The RNA concentration was quantified at 260 nm and 1µg of RNA was transcribed using Superscript II (Invitrogen, UK) reverse transcriptase and oligodT primers (Invitrogen). Quantitative RT-PCRs were performed in duplicates by iCycler iQ Real-Time PCR Detection System (BioRad, CA, USA) in 30 µl reaction volume containing: 1x iQTM SYBR Green Supermix (0.2 mM dNTP, 3 mM MgCl2), 0.5 µM forward and reverse primer and 2 µl of cDNA. The reaction conditions were as follows: 95 °C 30 s, 40 cycles (95 °C 5 s, 55.5 °C 30 s, 72 °C 30 s), 72 °C 2 min followed by melting curve analysis to confirm amplification of the desired single and specific product. The relative expression levels of GPX8 and Beta-Actin genes were evaluated using the standard curve method. Standard curves for GPX8 and Beta-Actin were obtained by amplification of serially-diluted mixtures of cDNA samples (four-fold dilutions), with four to five dilution points, each in duplicate. The calculated resulting relative expression of GPX8 gene was normalized to relative Beta-actin expression (GPX8/Beta-Actin). The results were evaluated as a ratio to untreated control and are presented as mean ± standard deviation of three independent experiments.

Western blot

The tissue was washed twice with ice-cold PBS and homogenized into the RIPA buffer (1xPBS, 1 % Nonidet P-40, 0.5 % sodium deoxycholate, 0.1 % SDS, Thermo Fisher Scientific, Inc, Waltham, MA, USA) in the presence of protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Inc). Lysates were incubated for 45 min on ice and sonicated for 30 s at 30 V (Sonopuls HD 2070; Bandelin electronic GmbH & Co. KG, Berlin, Germany) to shear the DNA. After the centrifugation at 10.000x g for 10 min at 4 °C, the supernatant was transferred into a new microcentrifuge tube. Protein samples were separated on 12 % SDS-polyacrylamide gel, electroblotted onto Immobilon-P transfer membrane (Millipore Co, Billerica, MA, USA) and detected using anti-GPX8 (#orb183909, 1:200; Biorbyt Ltd. Cambridge, United Kingdom) and anti-β-actin (clone AC-74, 1:10.000; Sigma-Aldrich, Saint-Louis, MO, USA) primary antibodies. Then the membranes were incubated with secondary horseradish peroxidase-conjugated antibodies (Goat anti-Rabbit IgG F(AB') 2, 1:10.000, PI-31461 and Goat anti-Mouse IgG F(AB') 2, 1:10.000, PI-31436, Pierce, Rockford, IL, USA) for 1 h and the antibody reactivity was visualized with ECL Western blotting substrate (PI-32106, Pierce) using Kodak Biomax film (#1788207, Sigma – Aldrich, Saint-Louis, MO, USA). The films were scanned using GS-800 Calibrated Densitometer, and for protein quantification Image J software version 1.52 (NIH, National Institute of Health, Bethesda, MD, USA) was used.

Immunohistochemistry

Testes were fixed in a modified Davidson's fluid (mDF) (15), while other organs were fixed in the formalin. In the next step male genital organs were embedded into blocks of paraffin using standard procedures. Sections of 5 µm were cut, deparaffinized, and rinsed in EnVision Flex Wash Buffer (#K800721-2, Agilent Dako, Santa Clara, CA, USA, later in the text referred to as wash buffer). Endogenous peroxidase activity was blocked by incubation of slides in a mixture of methanol and hydrogen peroxide. After another rinsing in wash buffer, antigens were revitalized in the microwave. Slides were rinsed in wash buffer, and 2% milk blocking solution in Tris buffer was added. The primary anti-GPx8 rabbit polyclonal anti-

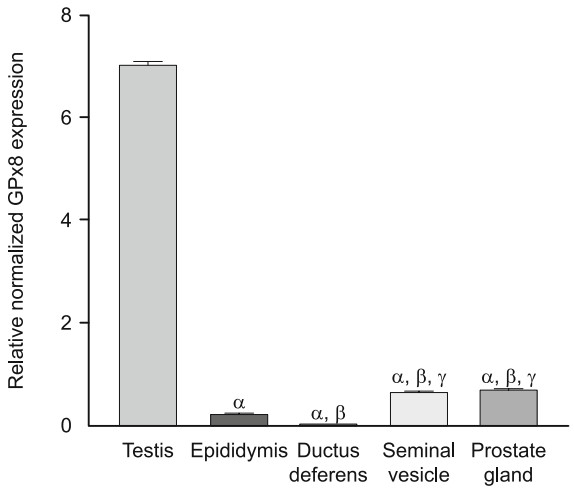

Fig. 1. One-way ANOVA test revealed that GPx8 gene expression differs between rat male genital organs ($p < 0.001$). Moreover, α shows organs which significantly differed from testis, β means organs which significantly differed from epididymis, and γ shows organs which significantly differed from ductus deferens in post hoc Tukey test ($p < 0.01$). The values of seminal vesicle and prostate gland did not differ between each other ($p > 0.05$)

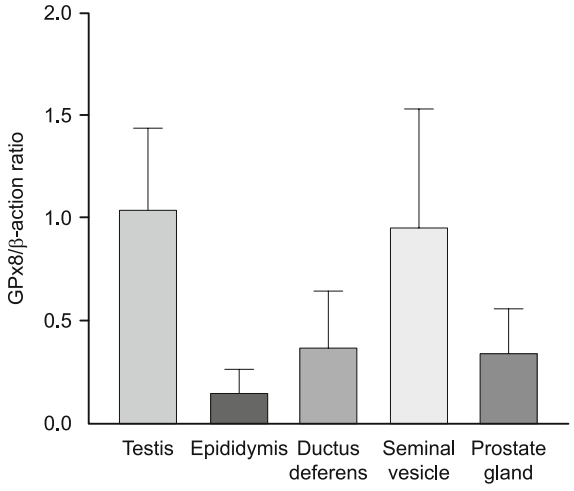

Fig. 2. Western blot analysis of GPx8 from three representative samples of each organ to demonstrate variabilities between individual rat males in amount of produced protein. In all organs the band at approximately 65 kDa was observed, and in testis, epididymis and prostate gland also band at 48 kDa. On the other hand, the band of 24 kDa molecular weight was present only in seminal vesicles

body (#16846-1-AP, Proteintech Group Inc, Rosemont, IL, USA) in 1:200 dilution was applied overnight at 4°C, followed by rinsing in wash buffer. Subsequently, Biotinylated Link (#K0675, Agilent Dako, Santa Clara, CA, USA) was used, then slides were rinsed with wash buffer, and Streptavidin-HRP (#K0675, Agilent Dako, Santa Clara, CA, USA) was applied. The tissue sections were again washed in wash buffer, and 3,3-diaminobenzidine (DAB) (#K5207, Agilent Dako, Santa Clara, CA, USA) was applied. The slides were rinsed in tap water, counterstained by hematoxylin, and embedded into Pertex. The negative controls were created by omitting the primary antibody. Two observers independently evaluated the results of immunostaining under a light microscope.

Statistical analysis

The results are given as mean ± S. D. Data were firstly analyzed by Shapiro-Wilk test for normal distribution. Because all data sets passed normality test, they were subsequently analyzed by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test.

Results

GPx8 gene expression

The results are depicted in Figure 1. The highest relative normalized gene expression of GPx8 was detected in testis (7.00 ± 0.05), followed by prostate gland (0.72 ± 0.084), and seminal vesicle (0.67 ± 0.011). The lowest values were observed in epididymis (0.24 ± 0.0041), and predominantly in ductus deferens (0.045 ± 0.00026). One-way ANOVA test revealed that GPx8 gene expression between examined organs differs significantly ($p < 0.001$). Moreover, post hoc Tukey test confirmed that also differ values between individual organs ($p < 0.01$). The only exception were seminal vesicle and prostate gland, which did not differ between each other ($p > 0.05$).

WB analysis

The results are shown in Figure 2. The band of 24 kDa molecular weight, which supposed to be GPx8 enzyme, was detected only in seminal vesicles. However, in all examined organs was observed the band also at approximately 65 kDa, and in testis, epididymis and prostate gland the band at 48 kDa, as well. The highest mRNA level of GPx8, revealed in the testis, correlates with 48 kDa form of this protein. On the other hand, relatively low mRNA level of GPx8 in the seminal vesicles does not correlate with large amount of the 24 kDa protein detected in this organ.

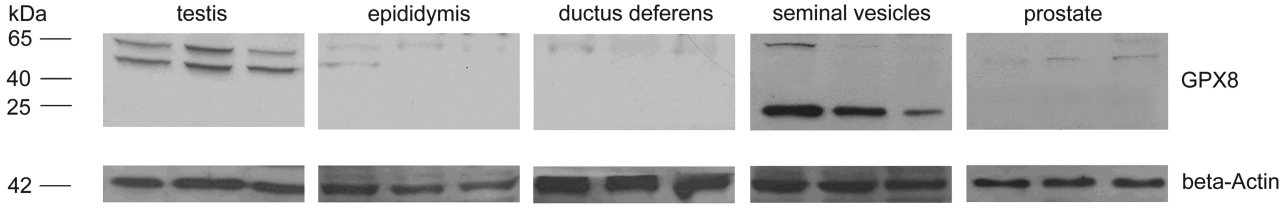

Fig. 3. Densitometry of Western blot results. One-way ANOVA test revealed that GPx8/β-actin ratio differs between rat male genital organs significantly ($p < 0.05$).

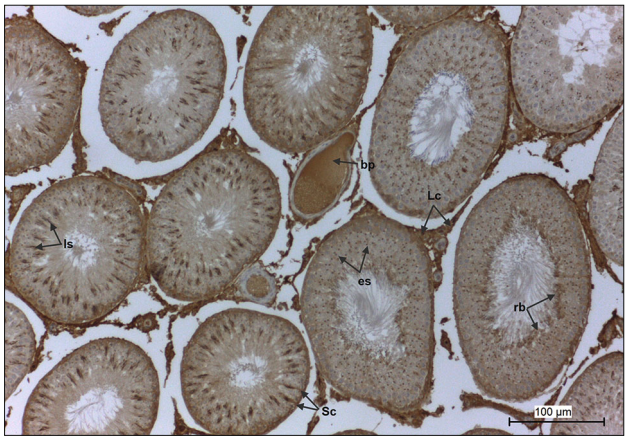

Fig. 4. GPx8 presence in the rat testis. The enzyme was detected predominantly in the cytoplasm of Leydig cells (Lc), Sertoli cells (Sc), in the forming "acrosomal cap" in the early spermatids (es), in the cytoplasm of late spermatids (ls), in the residual bodies (rb), and in the blood plasma (bp). Note, that muscular wall of vessels and erythrocytes inside lumen of vessels are free of the enzyme

Densitometry

The results are shown in Figure 3. The highest GPx8/β-actin ratio was observed in testis (1.00 ± 0.40), followed by seminal vesicle (0.96 ± 0.58), ductus deferens (0.37 ± 0.27), and prostate gland (0.34 ± 0.21). The lowest value was detected in epididymis (0.15 ± 0.11). One-way ANOVA test revealed that the amount of the protein between examined organs differs significantly ($p < 0.05$). On the other hand, post hoc Tukey test did not confirm differences between individual organs ($p > 0.05$), probably because of large variance detected mainly in seminal vesicles.

Immunohistochemistry

GPx8 was observed in the testis in both seminiferous tubules and interstitial tissue. Within seminiferous tubules the protein

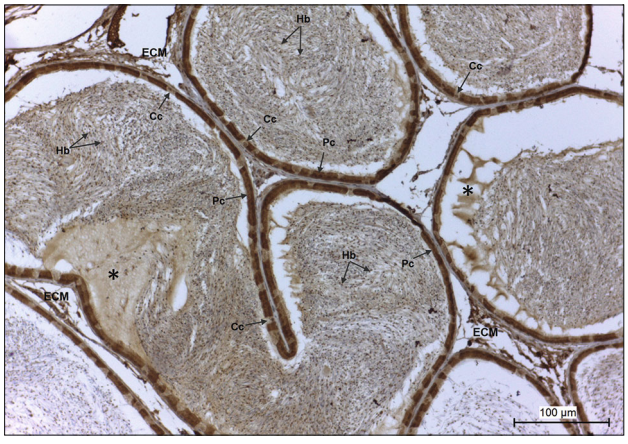

Fig. 5. GPx8 presence in the rat epididymis. The enzyme was observed in the cytoplasm of principal cells (Pc), in the fluid inside epididymal lumen (asterisk), in the extracellular matrix (ECM), and in the Hermes bodies (Hb). Note, that clear cells (Cc) are free of the GPx8.

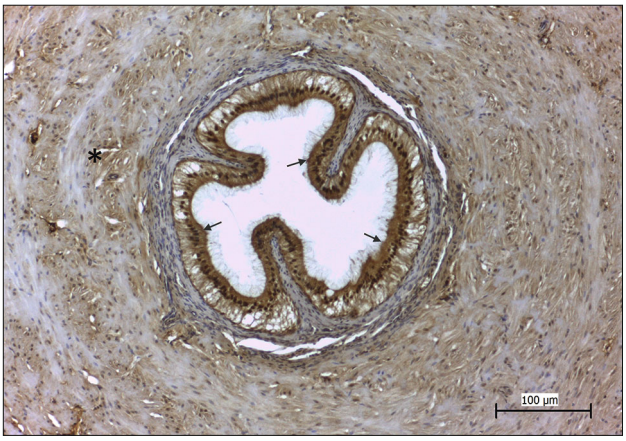

Fig. 6. GPx8 presence in the rat ductus deferens. GPx8 was detected in apical part of the cytoplasm of the epithelial cells (arrows), and in the smooth muscles (asterisk)

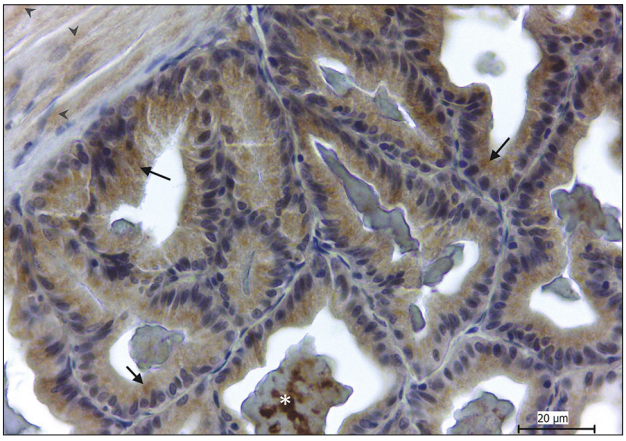

Fig. 7. GPx8 presence in the rat seminal vesicles. The enzyme positivity was visible in the cytoplasm of epithelial cells (arrows), in the fluid inside the lumen (white asterisk), and also in the surrounding muscular layer (arrowheads)

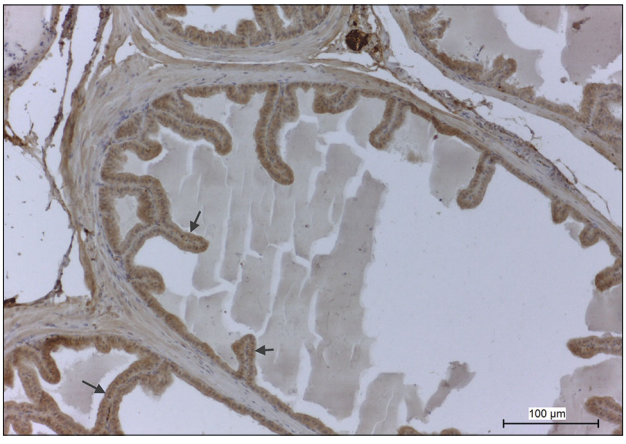

Fig. 8. GPx8 presence in the rat prostate gland. The enzyme positivity was observed in the cytoplasm of epithelial cells (arrows).

was detected predominantly in the cytoplasm of Leydig and Sertoli cells, early and late spermatids and in residual bodies. In the interstitial space the enzyme was observed in the blood plasma (Fig. 4). In the epididymis was the enzyme detected in the cytoplasm of principal cells. On the other hand, clear cells were free of GPx8. The enzyme was also detected in the fluid inside the lumen of the epididymal duct, inside Hermes bodies, and in the extracellular matrix (ECM) (Fig. 5). In the deferent duct the enzyme was observed in the apical part of the cytoplasm of the epithelial cells and in smooth muscles forming muscular wall of this organ (Fig. 6). In the seminal vesicle the GPx8 positivity was very often seen in the fluid secreted into lumen of the gland, but mainly inside the cytoplasm of both epithelial cells and smooth muscle cells (Fig. 7). In the prostate gland the Gpx8 was detected in the cytoplasm of epithelial cells and in the fluid inside lumens (Fig. 8).

Discussion

The presence of GPx8 was confirmed in all examined male genital organs. The highest amount of the mRNA and also the protein was detected in the testis, followed by seminal vesicle. Employing immunohistochemistry, the GPx8 was observed in the Leydig and Sertoli cells, in the residual bodies detached from the late spermatids, in the Hermes bodies, in the blood plasma and ECM, and in epithelial cells of extratesticular ducts and accessory male glands.

Still a little is known about the real GPx8 function, but according to the current knowledge, the enzyme, which is anchored in the plasmatic membrane of endoplasmic reticulum (ER), is involved in protein disulfide isomerase (PDI) peroxide-mediated oxidative protein folding (16), and in subsequent prevention of the peroxides leakage from the ER into cytoplasm (17). Therefore, it was no surprise that we detected the GPx8 in both Sertoli and Leydig cells. Sertoli cells are known to synthesize a large number of many different proteins, such as extracellular matrix proteins, occludin, and TGFβ family proteins. Some of them act as the transport proteins for "nourishing" of germinal cells, some of them they have enzymatic activity, and some of them are proteins without defined function yet (18 – 20). Leydig cells, besides of testosterone synthesis, are also known for protein production. For example, steroidogenic acute regulatory protein (StAR), a member of the START domain protein family, which mediates cholesterol transport from the outer to the inner mitochondrial membrane (21). Such StAR-mediated cholesterol transport is a key step in testosterone formation (22).

Rounded (early) spermatids are the first germinal cells expressing the GPx8, namely in their acrosomal cap. The acrosome develops from detached vesicles of the Golgi apparatus that join together to form "cap" at one pole of the nucleus of the round spermatid (23), subsequently forming proper acrosome, a lysosome-like structure required for sperm penetration into egg (24). One can ask why is the ER-enzyme located in the former part of the Golgi complex. Here we have to keep in mind that newly synthesized proteins travel from ER to Golgi apparatus in anterograde traffic, where are further modified and subsequently are transported to their destination. Proteins, which reside in the ER leave the Golgi complex and return back to the ER in retrograde traffic (25). Nevertheless, the question if the GPx8 is active as the antioxidant enzyme also in the acrosomal cap is opened and needs further investigation. Activity of the enzyme was continuously detected in the very next developmental stage, which are elongated (late) spermatids. Unlike early spermatids, where the GPx8 was detected only in the forming acrosome, here was the positivity seen in the whole cytoplasm. It could means that the enzyme is again produced in the ER. The last structures of the testis with the GPx8 positivity are residual and Hermes bodies.

During transformation of the late spermatid into the sperm is the vast majority of the cytoplasm expelled into the residual body. It contains packed organelles, including parts of ER and Golgi apparatus, which were used earlier by the spermatid but are no longer necessary for sperm survival (26). These cytoplasmic fragments are phagocytized and subsequently digested by the Sertoli cells (27), where in the supranuclear region their decomposition can be observed (28). The phagocytosis of residual bodies is essential to maintain testicular homeostasis, including elimination of apoptotic components, and is also needed for recycling of degenerated substrates as a source of energy for Sertoli cells (29). From this point of view, it makes sense that the antioxidant enzyme is present in the residual body, where it probably prevents both the leakage of peroxides into the environment of the seminiferous tubule as well as prevents from the excessive oxidation of residual body's components, because for their subsequent detoxification Sertoli cells would have to spend a large amount of their internal resources.

Smaller portion of the spermatid's cytoplasm persists as the Hermes body (former name cytoplasmic droplet) along midpiece of the newly created sperm. This organelle is made also by elements of the ER and by flattened cisternae of the Golgi apparatus (30, 31). Hermes body is typical for sperm during its passage through the epididymal duct. It contains more than one thousand different types of proteins, including protective enzymes, such as heat shock proteins HSP70.3 or HSP86 (32). According to our finding, the GPx8 is another enzyme inside Hermes body, which could here provide the protection against oxidative stress.

In the process of the passage through the epididymis still immature spermatozoa undergo a series of modifications thanks to which they acquire motility and fertilizing capacity. The epididymal environment supply H_2O_2 to allow extensive disulfide bridging formation of various sperm proteins which complete the fine shaping of these highly differentiated cells (33). Peroxide can be efficiently utilized to make native disulfide bonds in a folding protein with no significant oxidative side reactions so long as the peroxide concentration is kept at submillimolar concentrations (34). For this reason, epididymal cells express a lot of antioxidant enzymes, including several members of GPx family to defend spermatozoa and also to defend themselves. GPx1, GPx3, and cytoplasmic GPx4 (cGPx4) are expressed in the epithelial cells, whereas GPx5 is secreted into lumen and acts as an important luminal scavenger that directly protects sperm (35, 10). We found that GPx8 is another member of this family, present in principal cells of epididymal epithelium. The enzymatic presence here could be the consequence of the residual bodies uptake, since the principal cells of the epi-

didymis phagocytose the remaining residual bodies that were not removed by the Sertoli cells inside the testis (36). Principal cells, besides of protein endocytosis from the epididymal lumen, synthesize a large number of proteins that are either retained within the cells or are actively secreted into the luminal compartment (37). Thus, the Gpx8 here probably serves for the proper folding of many proteins synthesized and secreted by these cells. On the other hand, the enzyme was not observed in clear cells, which are fulfilled by lysosomes and participate in luminal acidification (38).

Compared to other parts of the male reproductive system, a significant lesser attention is devoted to the ductus deferens function, since it is considered basically as a tube for the transport of sperm from the epididymis to the male urethra. On the other hand, there are some evidences that the ductus deferens could be involved also in the secretion and resorption of fluid and various substances (39). For example, aquaporin 2 (AQP2), the vasopressin-regulated water channel, originally identified in renal collecting ducts was discovered as the apical membrane protein in rat ductus deferens (40). We observed GPx8 in similar places within the cytoplasm of the epithelial cells. Moreover, on the luminal surfaces of epithelial cells the GPx8 enzyme was often seen within many buds and blebs, which were finally released into lumen of ductus deferens to form extracellular vesicles (EVs). GPx5, another member of the GPx family, is secreted into the luminal compartment of the epididymis in the similar manner (41). In male genital system, EVs comprise of heterogeneous vesicles of different size and content (lipids, proteins, RNA), and they are secreted mainly by epididymis (epididymosomes), and prostate gland (prostasomes) (42). Some evidence suggests that ductus deferens could also produce populations of EVs, currently with unknown functions (43).

The seminal vesicles are male accessory sex gland that produce majority of seminal plasma. Components of seminal plasma play many important functions in reproduction, such as formation of vaginal plug, nutritive support for sperm and regulation of the immune environment in the lumen of female reproductive tract to improve chances for conception and later embryo development (44). So far 126 different proteins were identified in mouse seminal vesicle fluid, whereas most of them are members of seminal vesicle secretory (SVS) protein family (45). Taking into account that all proteins are formed in the endoplasmic reticulum, one cannot be surprised that we observed the presence of the monitored enzyme in the cytoplasm of the epithelial cells of this organ. In addition, seminal vesicle was the only organ, in which we detected the presence of the GPx8 employing WB analysis at the level of a protein transcript with the proper molecular size of 24 kDa. Moreover, also very high protein amounts of GPx8 have been detected in this organ using densitometry. These facts confirm the importance of GPx8 occurrence for the correct production of large amounts of proteins secreted by the gland.

Under light microscope was the enzyme observed also within secreted fluid inside the lumen of the seminal vesicles, prostate gland, and the luminal compartment of epididymis. The reason of GPx8 presence in fluids is probably explained by results of in vitro experiment, where the GPx8 addition to a mixture of peroxide and reduced denatured protein PDI, which acted as a reductant instead of glutathione (GSH), resulted in rapid oxidative folding of protein (46).

Moreover, we detected the GPx8 presence in the blood plasma and extracellular matrix (ECM). A recently published study alleges that PDI is present in human blood plasma at important levels (47). Thus, one could suppose that GPx8 could employ it, since GPx8 enzyme accepts PDI as a reductant more efficiently than glutathione (16). ECM is a supramolecular material that fulfills the interstitial spaces of all mammalian tissues. It is not only building network among cells, but it also contains a lot of functional proteins that regulate cellular activity, migration, division, remodeling and differentiation (48, 49). The ECM is degraded by the matrix metalloproteinase enzymes (MMPs), which are activated by ROS (50). The GPx8, a member of the antioxidant family that degrades H_2O_2, could be one of such tools needed for the achievement of fine equilibrium, which preserves tissue stability from destructive degradation and remodeling of ECM visible in many pathological condition, such as for example cancer (51). Recent studies confirmed the crucial role of ECM in supporting of Sertoli and germ cell function in the seminiferous epithelium, including the blood-testis barrier (52) or in enhancing the human spermatogonial stem cells survival under in vitro condition (53). Besides that, ECM could serve as an indicator of healthy status, because under pathological condition the composition of ECM in mouse testis and epididymis differs from the control animals (54).

Another tissue with positive GPx8 reaction was the smooth muscle layers of hollow organs, such as the seminal vesicles, prostate gland, and ductus deferens. Here is the enzyme probably needed for proper protein folding because these muscles also synthesize proteins, for example members of actin family involved in cell motility (actin alpha2, actin gamma2), or proteins involved in the regulation of muscle contraction (caldesmon1, caponin1) (55).

Prostate gland is male accessory gland which constitutes a lesser but still very important component of the seminal fluid. Its epithelial cells secrete several exocrine proteins, as for example transglutaminase 4 (TGM4). This highly specialized enzyme is exclusively produced in prostate (56), and it is involved in the copulatory plug formation by catalysis of cross-ridges between some SVS proteins (57, 58). In rat was TGM4 detected in all 3 parts of prostate gland, namely anterior (coagulating gland), ventral and dorsolateral lobes (59). In our work we found the presence of the GPx8 in the cytoplasm of epithelial cells of all three mentioned prostate lobes, and this event could be related to the production of corresponding prostate proteins. On the other hand, the amount of GPx8 detected by WB in prostate gland was three times lesser than amount of this enzyme in the seminal vesicle.

Concerning WB analysis, we found that GPx8 is present in all observed organs as the band of molecular weight approximately of 65 kDa. In the testis, epididymis, and prostate gland also a band of 48 kDa was detected. GPx8 of the proper molecular weight 24 kDa was seen only in the seminal vesicle. Changes in molecular weight (65 kDa) could be due to the post-translational modification, such as phosphorylation or glycosylation, which both cause increasing of the protein sizes, as was presumed in rat females (14). Glycosylation is the most common posttranslational modification of proteins

(60). N-glycosylation is the attachment of the oligosaccharide to a nitrogen atom. The resulting proteins, called N-glycans, are for example, essential for spermatogenesis, since the spermatids were not transformed to the sperm and males became infertile, when the synthesis of N-glycans was blocked in mice (61). Moreover, glycosylation improves protein properties, such as stability and solubility (62). The possibility, that the GPx8 forms dimer (48 kDa) also cannot be excluded, as it was already described in GPx (63).

In conclusion, the antioxidant enzyme GPx8 was detected in all examined male genital organs. The highest mRNA level and the protein amount was observed in the testis. Here was the enzyme found in the cells that possess high protein production, such as Leydig and Sertoli cells. The participation of the GPx8 in the acrosomal cap creation in the round spermatids was phenomenon, which needs to be further investigated. The fact, that the enzyme is released into lumen of excretory ducts and accessory male glands means that GPx8 could be a component of the semen. To our knowledge, this is the first paper describing GPx8 presence in male genital organs of mammals.

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