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EXPERIMENTAL STUDY

Glutathione peroxidase 1 and 2 during preimplantation period of pregnancy in mouse

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ABSTRACT

OBJECTIVES: This study describes the presence of glutathione peroxidase (GPx) 1 and 2 in oocytes, preimplantation embryos, and mouse female genital organs.

BACKGROUND: GPx1 and 2 are antioxidant enzymes, which play important roles in protection of cells against oxidation, which may be formed during cell physiological processes.

METHODS: After superovulation of female mice, oocytes and preimplantation embryos (O/PE) were isolated for immunofluorescent analysis, female genital organs were removed for immunohistochemical and Western blot analyses.

RESULTS: Using immunofluorescence, GPx1 was detected in all OP/E, where it formed clusters near nuclei, or under the cytoplasmic membrane. GPx2 was not detected in any O/PE. Using immunohistochemistry, GPx1 was observed in corpus luteum and oocytes, and in oviductal and uterine epithelium. GPx2 was detected in corpus luteum, but not in oocytes. Oviductal and uterine epithelium was mostly negative. Using Western blot, two GPx1 bands of different molecular weights were detected in uterus. One GPx2 band was observed in all investigated organs.

CONCLUSION: These results show that both enzymes may be important during preimplantation period of pregnancy in genital organs, GPx1 also in O/PE. GPx2 may play roles in embryo after the implantation, when gastrointestinal tract is formed *(Fig. 10, Ref. 75).* Text in PDF *www.elis.sk*

KEY WORDS: embryos; oxidative stress; preimplantation; reactive oxygen species; superovulation.

Introduction

Oxidative stress (OS) is caused by reactive oxygen species (ROS), which are formed during aerobic processes in cells. To the ROS belong for example hydroxyl radical (OH•), hydrogen peroxide (H_2O_2) or superoxide anion radical ($\cdot O_2^-$) (1). OS occurs from two reasons, either ROS levels are elevated, or an antioxidant amount is insufficient. This imbalance may lead to the cell damage (2), such as proteins, lipids and DNA (3), which may result into many pathologies, for example the infertility (4) or an oncogenesis (5).

Antioxidants are significant for cells to prevent a damage caused by OS. Enzymatic antioxidants are important in the first line of a cell protection against OS. A superoxide dismutase generates O_2 and H_2O_2 from a superoxide anion. Then, a hydrogen peroxide is transformed into O_2 and a water by for example a catalase (6). The glutathione peroxidase (GPx) generates a water from H_2O_2 using a GSH (7).

In mammals, to the GPx group belong eight enzymes (GPx1-8). GPx4, 7 and 8 are monomers, GPx1, 2, 3, 5, and 6 are homotetramers (8). According to the amino acid contents, selenocysteine is part of GPx1, 2, 3, 4 and 6 in human. GPx5, 7 and 8 contain cysteine (9).

GPx1 was found in the cytoplasm and mitochondria (10), and in epithelial cells nuclei in the prostate (11) and mammary epithelial cells (12). GPx1 protects DNA against OS in the mitochondria (13), reduces H_2O_2 (14), and removes intracellular peroxides (15). GPx1 is a selenium-dependent enzyme (16), which may be important in the follicle maturation. This is related with female reproductive hormones synthesis, because ROS and potential OS are formed (17). GPx1 mRNA was detected in bovine oocytes (18), and in mice oocytes after an antioxidant treatment were observed

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high expression levels of GPx1 gene (19). GPx1 gene expression was investigated in bovine preimplantation embryos, where GPx1 gene expression was upregulated (20). Interestingly, de Haan et al (21) did not observe any health or reproduction abnormalities in GPx1 knockout mice. This indicates reduced role of GPx1 in development processes, but on the other hand, GPx1 is not replaceable in the organism in the protection against OS (22), because GPx1 is localized in almost all cells and tissues (23). In the case of female genital organs, genes for GPx1 and 2 were found in the bovine oviduct, where may play the important role in the protection against OS (24).

GPx2 is a cytoplasmic enzyme found predominantly in the gastrointestinal tract (GIT) (25), where may neutralize ROS and protects against an inflammation (26, 27). GPx1 and 2 are very similar enzymes, structurally and according to the substrate specificity. Both enzymes use H_2O_2 and hydroperoxides of fatty acids (23). GPx2 was detected in mice embryos after an implantation, and in extraembryonic tissues (28). GPx2 knockout mice did not show development disorders before delivery (27), but GPx1/GPx2 knockout mice had abnormal phenotype (29).

Materials and methods

Animals

All procedures in this experiment involving animals were performed according to the Ethical committee for Animal Experimentation at the Institute of Animal Physiology and the State Veterinary and Food Administration of the Slovak Republic. The experiment was carried out according to the Slovak legislation based on EU Directive 2010/63/EU, which protects animals used for experiments or other scientific purposes. In this experiment were minimized suffering of the animals and reduced their numbers.

Outbred ICR (CD-1 IGS) mice were used for this experiment. All the time were available water and standard diet ad libitum to the animals. The daily cycle consisted of 12h of the dark and 12h of the light. 4–5 weeks old female mice were administrated by an intraperitoneal application of 5 IU pregnant mare's serum gonadotropin (PMSG; Folligon, Intervet International Bv. Boxmeer, Holland) with subsequent administration of 5 IU human chorionic gonadotropin (hCG; Pregnyl, Organon, Oss, Holland) 46h after PMSG. To obtain metaphase II unfertilized oocytes, mice under-

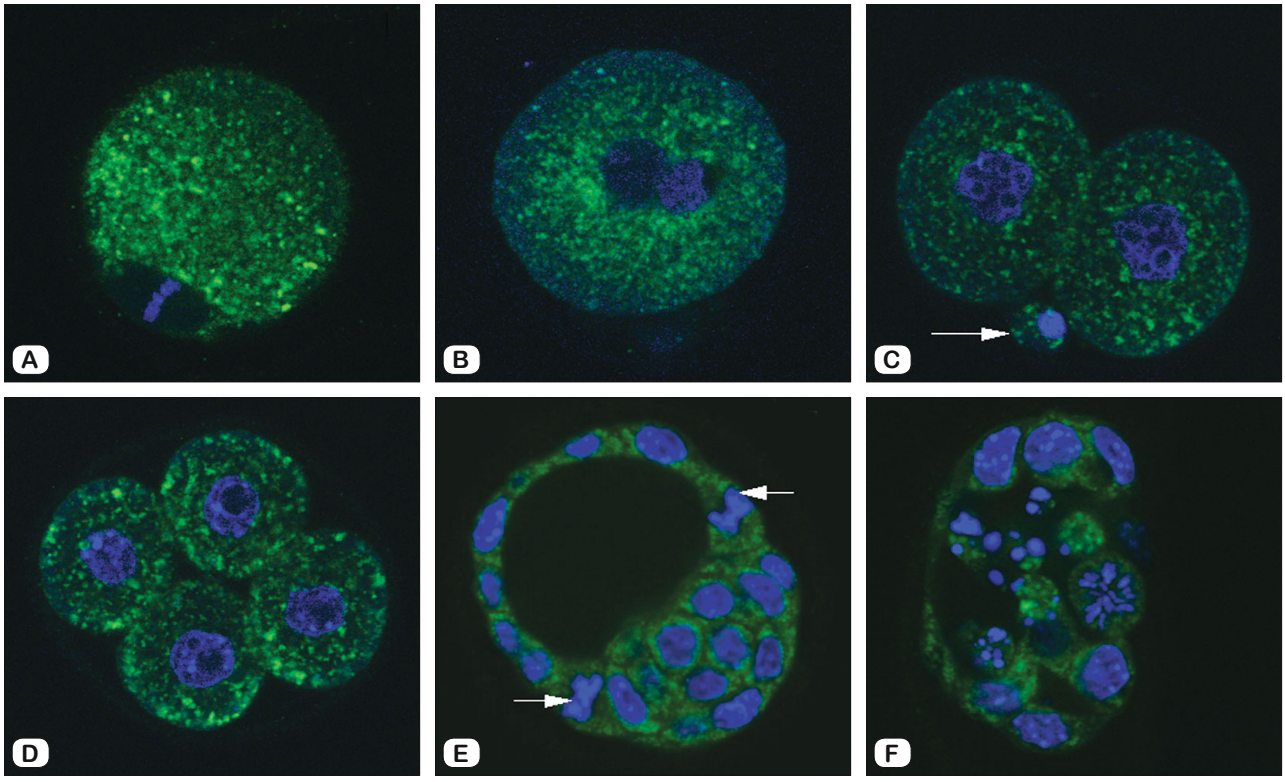

Fig. 1. Presence of GPx1 in a mouse oocyte and preimplantation embryos under a confocal microscope using an immunofluorescent analysis. (A) shows the mouse oocyte on D1. Clusters of GPx1 were localized in the cytoplasm mainly near the nucleus. (B) shows the mouse zygote on D1. GPx1 clusters were formed in the cytoplasm mostly around pronuclei. (C) shows the mouse 2-cell embryo with a pole body on D3. Clusters of the enzyme were observed in the cytoplasm mainly in a perinuclear space and in a pole body (arrow). (D) shows the mouse 4-cell embryo on D3. Clusters of GPx1 were in the perinuclear space and under a cytoplasmic membrane. (E) shows the mouse blastocyst on D5. GPx1 clusters were localized around nuclei and under a cytoplasmic membrane. In blastomers with mitosis (arrows), the enzyme was situated on a periphery of the cell. (F) shows the mouse degenerated blastocyst on D5. GPx1 formed clusters in the cytoplasm or was localized homogeneously. DNA (blue) was visualized by Hoechst 33342; GPx1 (green) was identified by the anti-GPx1 anti-body conjugated with FITC (fluorescein isothiocyanate); GPx1 is a glutathione peroxidase 1; D1, D3, and D5 are corresponding days of a pregnancy.

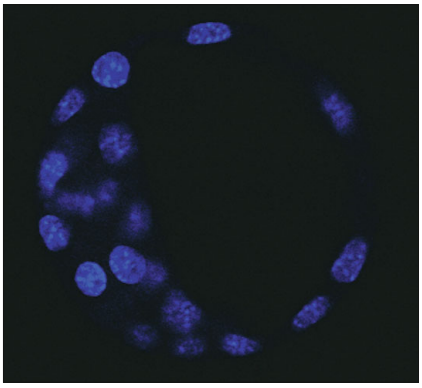

Fig. 2. Absence of GPx2 in the mouse blastocyst under a confocal microscope using an immunofluorescent analysis. Picture shows the mouse blastocyst on D5. No GPx2 was observed in blastomers. DNA (blue) was visualized by Hoechst 33342; GPx2 (red-not presented) was identified by the anti-GPx2 antibody conjugated with CF594; GPx2 is a glutathione peroxidase 2; D5 is a fifth day of a pregnancy.

went a cervical dislocation 20h after an hCG application. Oocytes were flushed out from the oviducts. Zygotes, 2-cell embryos, 4-cell embryos and blastocysts were obtained after overnight mating with males of the same strain. The presence of the vaginal plug was the evidence of the successful mating. Females underwent the cervical dislocation 20, 45, 52 or 97 hours after an hCG administration, respectively, and their oviducts and uterine horns were removed for the isolation of oocytes and embryos.

Isolation of oocytes and preimplantation embryos (O/PE)

Oocytes, zygotes, 2- and 4-cell embryos were obtained by flushing from the oviducts, and blastocysts were flushed out from uterine horns. The isolated O/PE were washed in the FHM medium drops (30), which contained 1 % bovine serum albumin (BSA). For cumulus cells removing, 0.1 % hyaluronidase was used (Sevac, Prague, Czech Republic). A stereomicroscope and a rinse solution phosphate buffer solution (PBS) + BSA (3 mg/mL) were used for O/PE isolation.

Immunofluorescent analysis (IFA)

O/PE were fixed in PBS with 4 % paraformaldehyde (PFA; Merck, Darmstadt, Germany) for 1h at a room temperature (RT) and stored for a maximum of 1 week in 1 % PFA at 4 °C. O/PE were washed three times using PBS/BSA (0.1 % BSA) with a subsequent permeabilization using PBS containing 0.5 % Triton X-100 (Sigma-Aldrich, Germany) for 1h at RT. Cells were washed two times for 5 min in PBS/BSA. One part of O/PE was incubated overnight at 4°C in the rabbit polyclonal anti-GPx1 antibody conjugated with FITC (bs-3882R-FITC, Bioss, Woburn, Massachusetts, USA), the second part of O/PE in the rabbit polyclonal anti-GPx2 antibody conjugated with CF594 (orb157377-CF594, Biorbyt, Cambrige, UK). Then, cells were washed in PBS/BSA six times for 5 min at RT. Hoechst 33342 (10 µL-1 in PBS; Sigma-Aldrich, Saint-Louis, MO, USA) was used for 5 min at RT for DNA staining. Another washing in PBS/BSA was performed. O/PE were mounted on slides using of Vectashield (Vector Laboratories, Burlingname, CA, USA) and were examined and photographed under the confocal microscope (FV-1000 BX61; Olympus, Tokyo, Japan), according to Baran et al 2013 (31).

Immunohistochemistry (IHC)

Animals were divided into four groups: D0 (without mating with males), D1, D3, and D5 (first, third, fifth day of pregnancy, respectively). Each group consists of five animals. After O/PE removing, female genital organs (ovaries, oviducts, and uterine

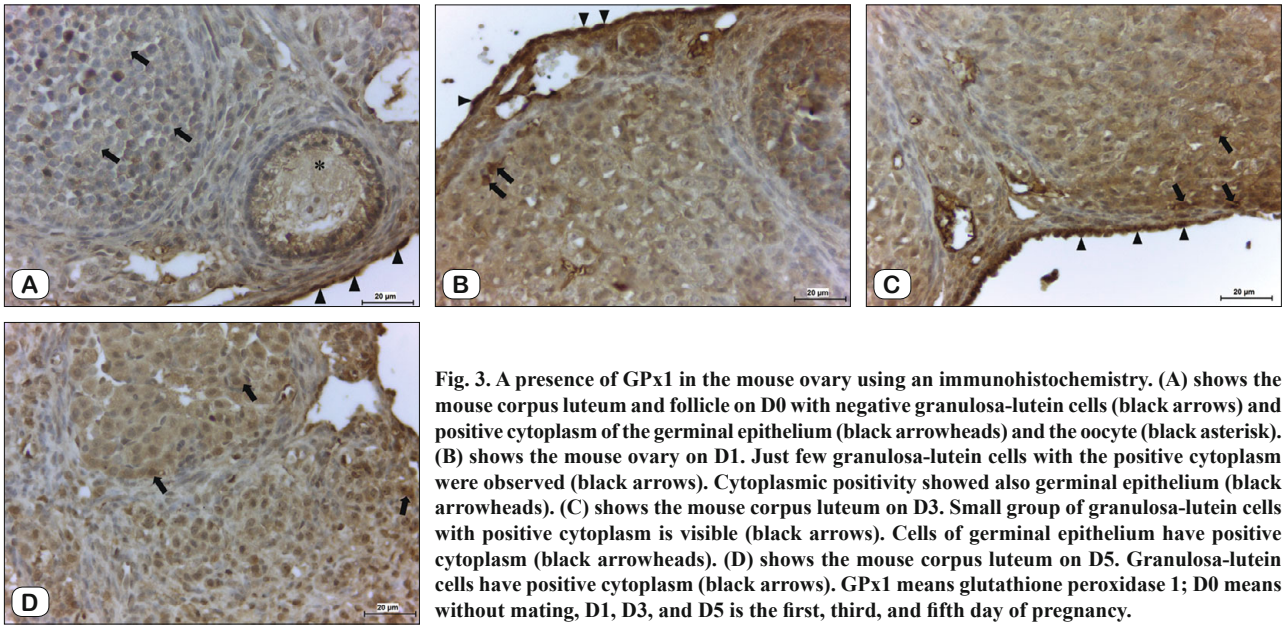

Fig. 3. A presence of GPx1 in the mouse ovary using an immunohistochemistry. (A) shows the mouse corpus luteum and follicle on D0 with negative granulosa-lutein cells (black arrows) and positive cytoplasm of the germinal epithelium (black arrowheads) and the oocyte (black asterisk). (B) shows the mouse ovary on D1. Just few granulosa-lutein cells with the positive cytoplasm were observed (black arrows). Cytoplasmic positivity showed also germinal epithelium (black arrowheads). (C) shows the mouse corpus luteum on D3. Small group of granulosa-lutein cells with positive cytoplasm is visible (black arrows). Cells of germinal epithelium have positive cytoplasm (black arrowheads). (D) shows the mouse corpus luteum on D5. Granulosa-lutein cells have positive cytoplasm (black arrows). GPx1 means glutathione peroxidase 1; D0 means without mating, D1, D3, and D5 is the first, third, and fifth day of pregnancy.

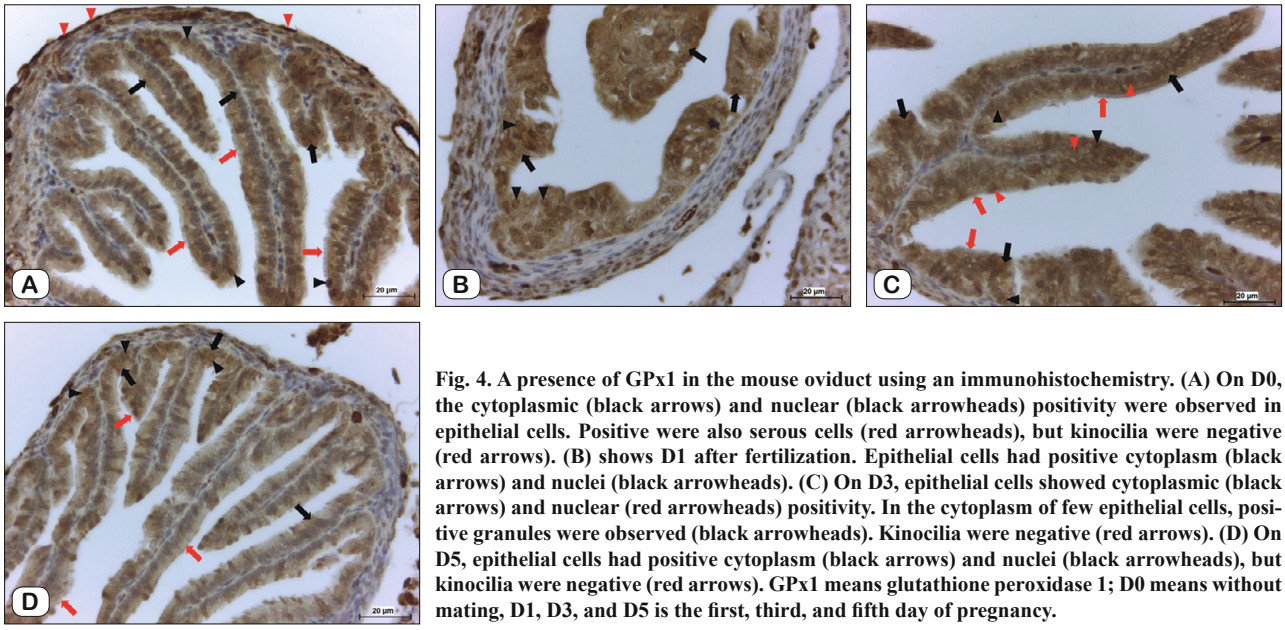

Fig. 4. A presence of GPx1 in the mouse oviduct using an immunohistochemistry. (A) On D0, the cytoplasmic (black arrows) and nuclear (black arrowheads) positivity were observed in epithelial cells. Positive were also serous cells (red arrowheads), but kinocilia were negative (red arrows). (B) shows D1 after fertilization. Epithelial cells had positive cytoplasm (black arrows) and nuclei (black arrowheads). (C) On D3, epithelial cells showed cytoplasmic (black arrows) and nuclear (red arrowheads) positivity. In the cytoplasm of few epithelial cells, positive granules were observed (black arrowheads). Kinocilia were negative (red arrows). (D) On D5, epithelial cells had positive cytoplasm (black arrows) and nuclei (black arrowheads), but kinocilia were negative (red arrows). GPx1 means glutathione peroxidase 1; D0 means without mating, D1, D3, and D5 is the first, third, and fifth day of pregnancy.

horns) were embedded in paraffin by the standard procedure. Blocks of paraffin were cut into 5 µm thick sections. For slides deparaffinization, rehydration, and antigen retrieval was used PT-Link (Dako PT100) together with EnVisionTM FLEX Target Retrieval Solution Low (pH 6.0). After an incubation in 3 % H_2O_2 for 10 min, GPx1 and 2 visualization was performed using a rabbit primary anti-GPx1 (bs-3882R, Bioss, Woburn, MA, USA) and anti-GPx2 (MBS9140671, MyBioSource, San Diego, CA, USA) antibodies, respectively. Slides were incubated with primary antibodies for 30 min at RT following incubation with EnVisionTM FLEX+ Rabbit/Mouse LINKER for 15 min at RT. Incubation with a Universal LSA B2 KIT/HRP visualization reagent was performed for 20 min at RT. Next step was an addition of EnVision Flex 3,3´-diaminobenzidine tetrahydrochloride (DAB) for 5 min at RT for GPx1 and 2 visualization. Slides were washed using tap water, then they were stained using hematoxylin and mounted into Pertex. After each step, buffer wash for 5 min was performed using EnVisionTM FLEX Wash Buffer 20x (32).

The whole procedure was performed omitting the primary antibody to obtain negative controls. All slides were evaluated by two observers using a light microscope and compared to each other, if specimens had the same pattern.

Western blot (WB)

Female genital organs were stored in Eppendorf tubes at –80 °C. After that, ice-cold PBS was used for specimens washing. Then, homogenization of organs was performed using protease and phosphatase inhibitor cocktail, and RIPA buffer (1x PBS, 1 % Nonidet P-40, 0.5 % sodium deoxycholate, 0.1 % sodium dodecyl sulfate-SDS) (all chemicals from Thermo Fisher Scientific, Inc., Waltham, MA, USA). After incubation of obtained lysates on ice for 45 min, sonication was performed for 30 s at 30 V (Sonopuls HD 2070; Bandelin electronic GmbH & Co. KG, Berlin, Germany). Next step was centrifugation (10.000× g) at 4 °C for 10 min. Obtained supernatant was put into a microcentrifuge tube and samples were separated using 10 % SDS-polyacrylamide gel. Immobilon-P transfer membrane (Millipore Co., Billerica, MA, USA) was used for electroblotting. For enzyme detection, primary antibodies anti-GPx1 (bs-3882R, Bioss, Woburn, MA, USA), anti-GPx2 (MBS9140671, MyBioSource, San Diego, CA, USA), and anti-β-actin (clone AC-74, 1:10.000; Sigma-Aldrich, Saint-Louis, MO, USA) were used. Membranes incubation 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 one hour was performed. Reactivity of antibody was visualized using ECL Western blotting substrate (PI-32106, Pierce, Rockford, IL, USA) and Kodak Biomax film (#1788207, Sigma-Aldrich, Saint-Louis, MO, USA). After then, scanning of films was performed using GS-800 Calibrated Densitometer. For protein quantification, Image J software version 1.52 (NIH, National Institute of Health, Bethesda, MD, USA) was used, similarly to Feckova et al, 2019 (33).

Results

Immunofluorescent Analysis (IFA)

We detected GPx1 in the cytoplasm of all obtained O/PE. In the unfertilized oocyte (Fig. 1A), GPx1 formed clusters of different sizes in the cytoplasm mainly near nuclei. In the zygote similarly to the oocyte, GPx1 formed clusters localized mostly near pronuclei, less on the periphery of the cell (Fig. 1B). GPx1 was detected in the cytoplasm of the polar body. GPx1 clusters were situated in the 2-cell embryo (Fig. 1C) mainly around nuclei, and in the polar body. In the 4-cell embryo (Fig. 1D), GPx1

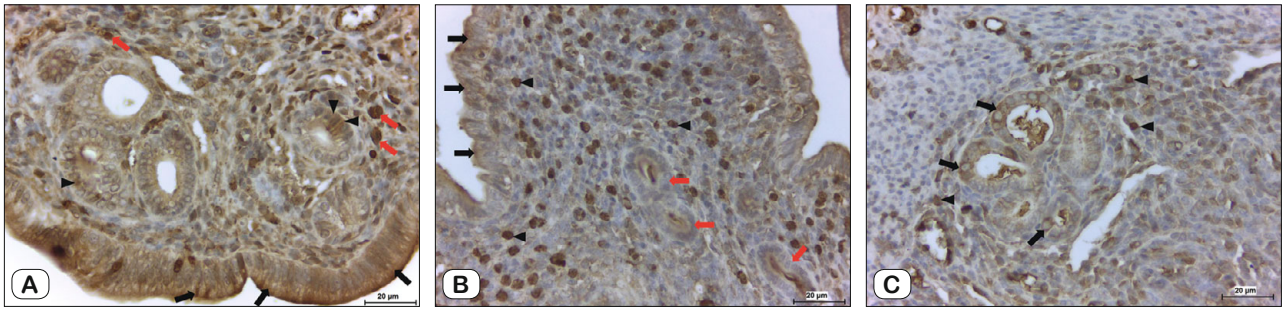

Fig. 5. A presence of GPx1 in mouse uterine horns using an immunohistochemistry. (A) shows the endometrium on D0. Epithelial cells lining the lumen had positive cytoplasm (black arrows). Positivity showed also few epithelial cells of uterine glands (black arrowheads) and fibroblasts (red arrows). (B) shows the endometrium on D3. Epithelial cells lining the lumen were almost negative, positive were only few cells (black arrows). Epithelial cells of uterine glands were mostly negative (red arrows). Several fibroblasts were positive (black arrowheads). (C) On D5, positive cytoplasm showed few epithelial cells of uterine glands (black arrows). Positive were also several fibroblasts (black arrowheads). GPx1 means glutathione peroxidase 1; D0 means without mating, D1, D3, and D5 is the first, third, and fifth day of pregnancy.

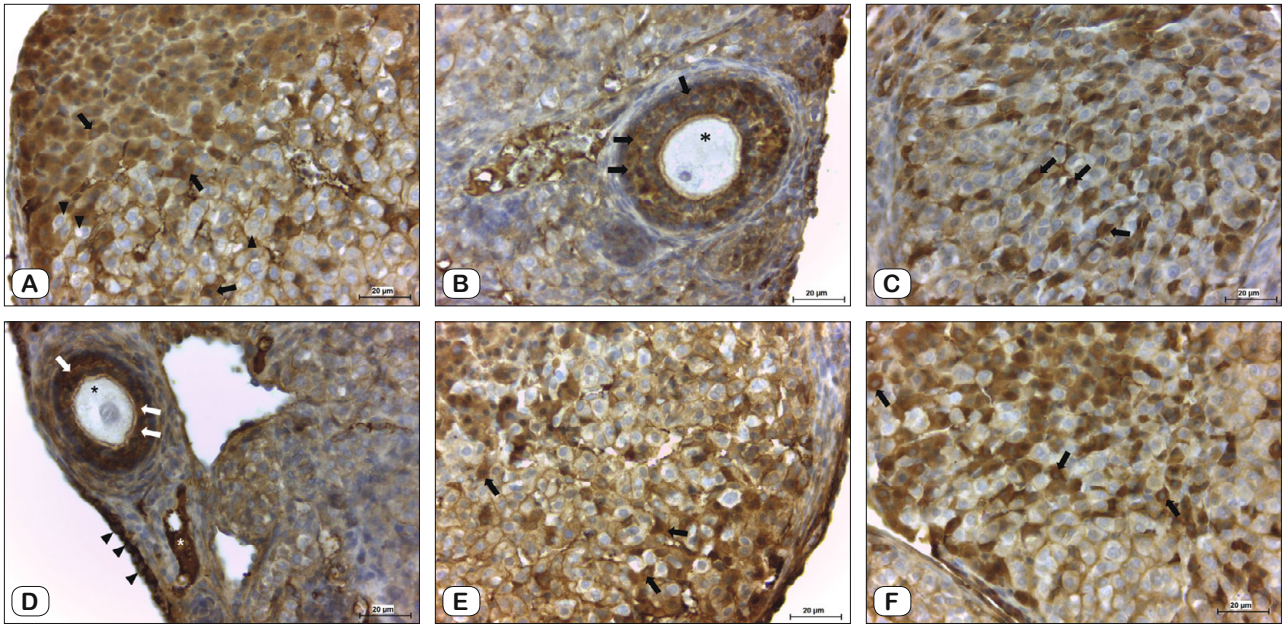

Fig. 6. A presence of GP2 in the mouse ovary using an immunohistochemistry. (A) shows the corpus luteum on D0. Several granulosa-lutein cells had positive cytoplasm (black arrows), but part of the corpus luteum was negative (black arrowheads). (B) In the ovary on D0, the blood plasma was positive (white asterisk). Positivity also showed the cytoplasm of follicular cells (black arrows). The oocyte cytoplasm was negative (black asterisk). (C) On D1, in the corpus luteum were observed several cytoplasmic positive granulosa-lutein cells (black arrows). (D) On D1, positivity also showed the blood plasma (white asterisk), cytoplasm of follicular cells (black arrows) and cells of germinal epithelium (black arrowheads). The cytoplasm of the oocyte was negative (black asterisk). (E) On D3 and (F) on D5, the cytoplasm of several granulosa-lutein cells was positive (black arrows). GPx2 means glutathione peroxidase 1; D0 means without mating, D1, D3, and D5 is the first, third, and fifth day of pregnancy.

formed clusters also under the cytoplasmic membrane (CPM). We detected GPx1 near nuclei and under the CPM in the blastocyst (Fig. 1E). In blastomers undergoing mitosis, GPx1 was situated on the periphery of the cell. In the degenerated blastocyst (Fig. 1F), GPx1 formed clusters or was localized homogeneously in the cytoplasm.

GPx2 was not observed in any of the O/PE (Fig. 2). In the 2-cell embryo using as negative control, we did not observe GPx2, too.

Immunohistochemistry

GPx1 was observed in the ovary, oviduct, and uterus on all days. In the ovary, the follicular fluid, blood plasma, capillary wall, and cytoplasm of oocytes and germinal epithelium were positive on all investigated days, but granulosa-lutein cells showed differences. On D0, the germinal epithelium cytoplasm and the oocyte cytoplasm were positive. We did not found positivity of granulosa-lutein cells on D0 (Fig. 3A), but on D1, GPx1 was detected in the cytoplasm of few granulosa-lutein cells and in the

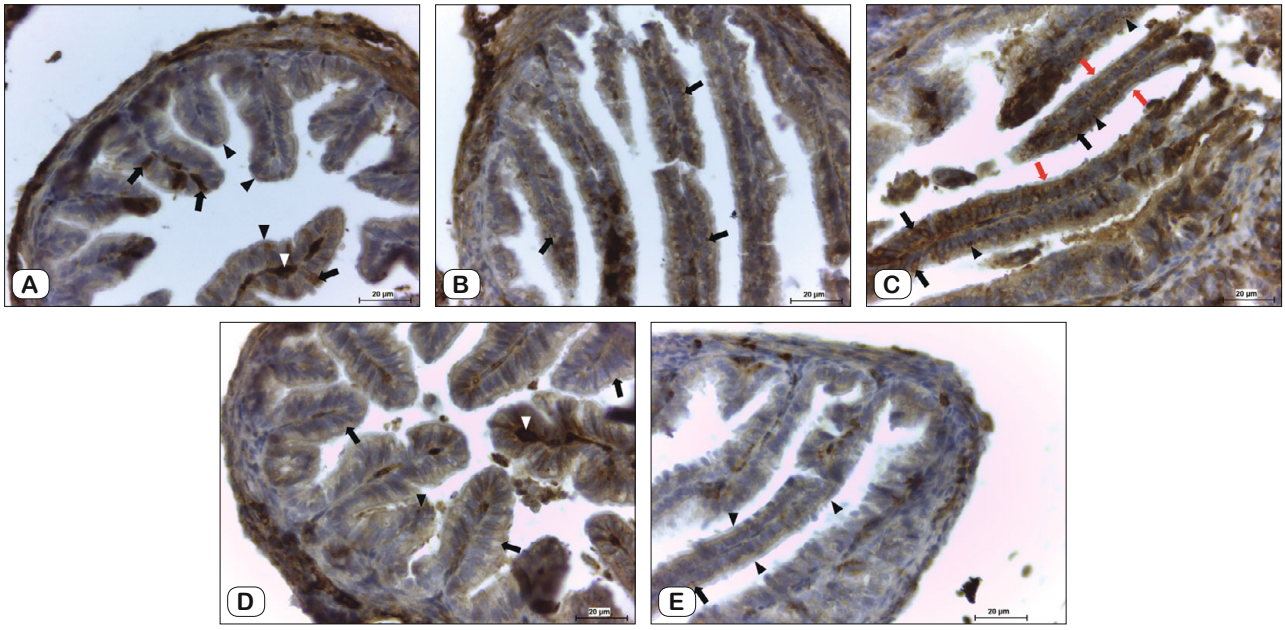

Fig. 7. A presence of GPx2 in the mouse oviduct using an immunohistochemistry. (A) On D0, the cytoplasmic positivity of few epithelial cells was observed (black arrows). Positive was also the cytoplasm of fibroblasts (white arrowhead). Kinocilia were negative (black arrowheads). (B) On D0 in the oviduct, positivity also showed granules in the cytoplasm of epithelial cells (black arrows). (C) On D1, positive were the cytoplasm of epithelial cells (black arrows) and granules (black arrowheads). Kinocilia were negative (red arrows). (D) shows negative epithelial cells without positive cytoplasmic granules (black arrows), small amount of positive cytoplasmic granules (black arrowhead), and the positive cytoplasm of fibroblasts (white arrowhead) on D3. (E) On D5, only few positive cytoplasmic granules were observed in epithelial cells (black arrows). Kinocilia were negative (black arrowheads). GPx2 means glutathione peroxidase 1; D0 means without mating, D1, D3, and D5 is the first, third, and fifth day of pregnancy.

germinal epithelium (Fig. 3B). On D3, the cytoplasm of germinal epithelium shown positivity. Granulosa-lutein cells with positive cytoplasm formed small groups (Fig. 3C). Moreover, in the granulosa-lutein cells cytoplasm, positive granules were detected. The cytoplasmic positivity of granulosa-lutein cells was showed also on D5 (Fig. 3D).

In the oviduct on D0, the cytoplasm and nuclei of some epithelial cells were positive for GPx1. The serous cells cytoplasm showed GPx1 positivity (Fig. 4A). Positivity showed fibroblasts in the stroma and capillary walls. D1 was similar as D0 (Fig. 4B). On D3, the epithelium showed the cytoplasmic and nuclear positivity. Moreover, positive granules were found in the epithelial cytoplasm (Fig. 4C). D5 showed similar positivity pattern, but positive granules were missing (Fig. 4D). Kinocilia were negative on all investigated days.

On D0 in the uterus, the GPx1 positivity of luminal epithelial cells cytoplasm was observed. In uterine glands, cytoplasmic positivity was detected just locally in few epithelial cells, but this cell type was mostly negative. Cytoplasmic positivity showed endothelial cells, fibroblasts, and mesothelial cells in the perimetrium on all investigated days. On D1, the cell type positivity pattern was similar, but many columnar epithelial cells lining the lumen showed cytoplasmic positivity. Positive cytoplasm of several epithelial cells of uterine glands and fibroblasts was observed (Fig. 5A). On D3, luminal epithelial cells were mostly negative, only few cells showed positive cytoplasm. The epithelium of uterine glands was mostly negative (Fig. 5B). On D5, only few luminal epithelial cells were positive. Several epithelial cells of uterine glands showed positive cytoplasm (Fig. 5C).

GPx2 was detected in all organs on all days. In the ovary, GPx2 was observed in the blood plasma, follicular fluid, and in the cytoplasm of germinal epithelium and follicular cells in follicles. The oocyte cytoplasm was negative on all investigated days. On D0, GPx2 was detected in the corpus luteum, specifically in the granulosa-lutein cells cytoplasm, but these cells of several corpora lutea were negative (Fig. 6A). Positive granulosa-lutein cells were located separately or formed small groups. The follicular cells cytoplasm was positive, but the oocyte cytoplasm was negative (Fig. 6B). The positivity patterns on D1 (Fig. 6C, 6D), D3 (Fig. 6E), and D5 (Fig. 6F) after mating were similar as previous, we did not detect any significant differences.

In the oviduct, GPx2 positivity was detected in the blood plasma and in the fibroblast cytoplasm. Kinocilia were negative on all investigated days. On D0, GPx2 was found in the cytoplasm of few epithelial cells, most of them were negative. The cytoplasmic positivity was also observed in fibroblasts (Fig. 7A). Moreover, in the cytoplasm of several epithelial cells were observed positive granules (Fig. 7B). On D1, the number of cytoplasmic positive epithelial cells and the amount of positive granules were similar (Fig. 7C), but D3 and D5 showed differences. On D3, we detected decreased number of positive granules in the epithelium (Fig. 7D), and on D5 we observed only few epithelial cells with positive granules (Fig. 7E).

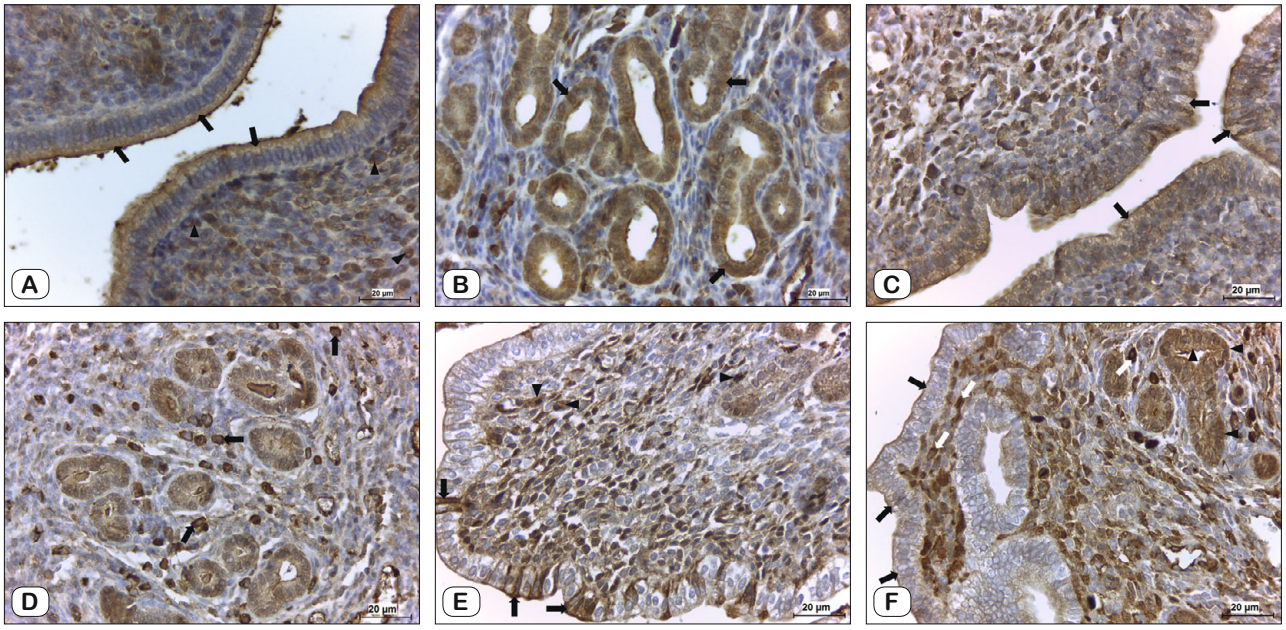

Fig. 8. A presence of GPx2 in the mouse uterus using an immunohistochemistry. (A) On D0, apical parts of the cytoplasm of epithelial cells lining the lumen were positive (black arrows). The positivity also showed the cytoplasm of fibroblasts (black arrowheads). (B) On D0, positive was also the cytoplasm of epithelial cells of uterine glands (black arrows). (C) On D1, the epithelium lining the uterine lumen was mostly negative (black arrows). (D) On D3, the cytoplasmic positivity showed fibroblasts (black arrows). Positive was also the secretion of uterine glands (white asterisk). (E) On D5, only small number of epithelial cells lining the lumen had positive cytoplasm (black arrows). (F) The epithelium lining the lumen was mostly negative (black arrows). The cytoplasmic positivity showed epithelial cells of uterine glands (black arrowheads) and fibroblasts (white arrows). GPx2 means glutathione peroxidase 1; D0 means without mating, D1, D3, and D5 is the first, third, and fifth day of pregnancy.

Concerned the uterus, GPx2 was found in many cell types on all investigated days. On D0, the positivity showed apical parts of the luminal epithelial cells cytoplasm and the cytoplasm of several fibroblasts (Fig. 8A). Positive was the epithelial cells cytoplasm in uterine glands (Fig. 8B) and their secretion. On D1 (Fig. 8C) and D3, the cytoplasmic positivity of the luminal epithelium started to disappear. On D3, the positivity showed the fibroblast cytoplasm, and the uterine glands secretion (Fig. 8D). On D5, only few luminal epithelial cells showed positive cytoplasm, but this epithelium was mainly negative. Positive cytoplasm had the uterine glands epithelium and fibroblasts (Fig. 8E, 8F). The other positivity patterns were similar on each day, blood plasma was positive and smooth muscle cells were negative.

Western blot analysis

Using Western blot, we detected GPx1 in the ovary and uterine horns. In the ovary, GPx1 was observed as 25 kDa band only on D0 in two animals. Other monitored days were negative. GPx1 was not detected in the oviduct, but in the uterus were detected two bands of different molecular weights, 17 kDa and 25 kDa, on D1 and D3. Only three animals showed GPx1 bands on D1, but on D3, GPx1 bands were detected in all animals (Fig. 9).

GPx2 was detected in all investigated organs. In the ovary, 25 kDa bands were observed only in two animals on D1. In the uterus, 25 kDa bands were detected in one animal on D0 and in one animal on D1. Concerning the uterus, only one 25 kDa band was observed from D1 to D5. On D1, GPx2 was detected in four animals, on D3 in three animals, and on D5 in five animals (Fig. 10).

Discussion

In our research, we detected GPx1, but not GPx2 in the mouse O/PE using IFA. Using IHC, GPx1 and 2 were presented in many cell types in ovaries, oviducts, and uterine horns. Employing WB, we detected the presence of both enzymes in several female genital organs.

For correct course of a preimplantation period of pregnancy, an oocyte maturation is important. It consists of two steps, a cytoplasmic and nuclear maturation. For the cytoplasmic maturation is characteristic organelle organization and a protein and mRNA accumulation, and transcription factors relevant for the development of the preimplantation embryo (34, 35). Moreover, mitochondria and endoplasmic reticulum (ER) organization occur. This supports a fertilization, pronuclei formation, and an early embryo development (36). During the nuclear maturation, a chromosome segregation occurs (34, 35). The cytoplasmic maturation is critical and leads to the correct embryogenesis in pigs. On the other hand, if there was no proper cytoplasmic maturation, the embryo development would not be adequate, even if the nuclear maturation would be correct (37).

In our research, we detected GPx1 in the cytoplasm of ovulated oocytes using IFA. GPx1 formed clusters mostly in the perinuclear space. We support results of El Mouatassim et al (1999), who found GPx1 in both oocytes and embryos (38). Lee et al (2016) found, that

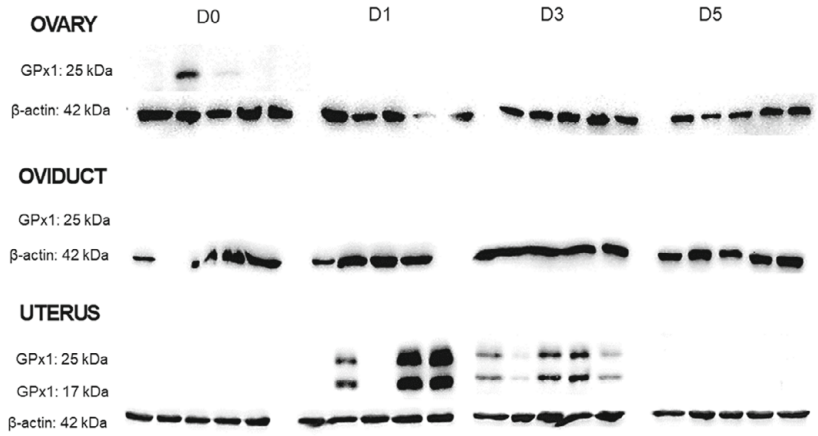

Fig. 9. A presence of GPx1 in the mouse ovary, oviduct, and uterus. 25 kDa band was detected in the ovary on D0 in two animals, whereas the oviduct was negative on all investigated days. In the uterus on D1, two bands of 17 kDa and 25 kDa, were observed in three animals, but on D3, two bands of different molecular weights were found in five animals. GPx1 is glutathione peroxidase 1; D0 means animals without mating, D1, D3, and D5 means corresponding day of pregnancy.

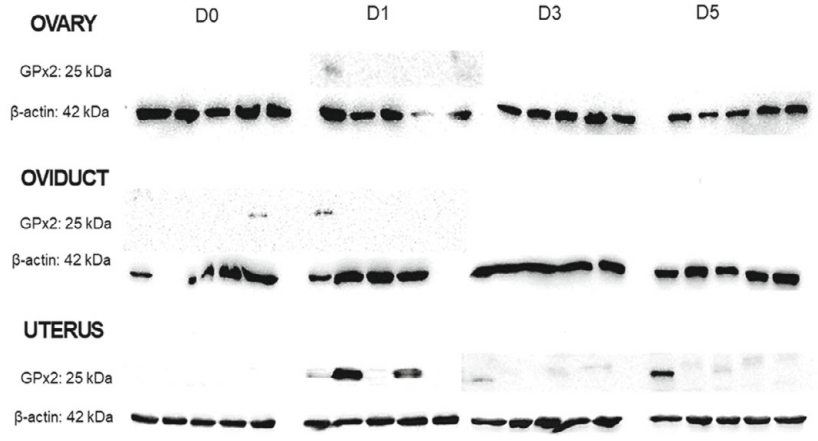

Fig. 10. A presence of GPx2 in the mouse ovary, oviduct, and uterus. 25 kDa band was detected in the ovary on D1 in two animals. In the oviduct, one 25 kDa band was detected on D0 in one animal and one band on D1 in one animal. In the uterus, one 25 kDa band was observed on D1, D3, and D5 in four, three, and five animals, respectively. GPx2 is glutathione peroxidase 2; D0 means animals without mating, D1, D3, and D5 means corresponding day of pregnancy.

in matured oocytes an amount of GSH and GPx1 expression were increased. Because of higher levels of these antioxidants, amounts of ROS decreased (39). This may suggest the incorrect nuclear maturation and the development of mouse oocytes with a defected β-oxidation (40). Li et al (2012) found, that modified levels of GSH in the cell cause disorders in the embryo development, oocyte aging after an ovulation or defective male pronuclei formation (41). Besides that, the GPx1 presence may be related to the protection of cells against ROS arising during the mitochondria metabolism (42), because GPx1 is presented in mitochondria and the cell cytoplasm (10). Based on this, GPx1 clusters we found in the oocyte cytoplasm could be GPx1 in mitochondria. The correct preimplantation embryo development relates with a distribution of mitochondria, which should be granulated and uniform during the oocyte maturation (43, 44).

After the fertilization we detected a cluster formation of GPx1 mostly in the perinuclear space of zygotes and the 2-cell embryos, but from 4-cell embryos GPx1 formed clusters also under the CPM. We suggest, that a different GPx1 distribution between 2- and the 4-cell embryos is related to the activation of the embryonic genome, which replaces a maternal genome. The maternal genome plays its role until 2-cell embryos, the embryonic genome is activated at the stage of the 2-cell embryo (45). At the stage of the 4-cell embryo, a protein synthesis pattern modifies until the 8-cell embryo in mice (46). Because in bovine 4-cell embryos and blastocysts higher levels of ROS can increase a general methylation (47), this indicates the need of a protection from the OS. The presence of GPx1 was observed from the 8-cell embryo (48) until the blastocyst on D8 (20, 49), but in our study, we observed GPx1 in the blastocysts cytoplasm already on D5. Moreover, García-Martínez et al (2020) investigated, that the blastocyst quality rises together with the higher expression of GPx1 (49). In blastomers which undergoing mitosis, GPx1 was localized mostly on the periphery of cells. This indicates, that a spindle region should be free of bigger elements localized in the cytoplasm, such as mitochondria (50). During mitosis in the *Drosophila* embryo, ER is situated mostly in the peri-spindle region (51). Because of this, some of localization options for GPx1 are mitochondria and ER.

In our study, we did not find GPx2 in ovulated O/PE. Baek et al (2011) investigated, that mRNA of GPx2 was detected at the embryonic day (ED) from 7.5 to 18.5 in embryos and in extraembryonic tissues. In extraembryonic tissues, the GPx2 mRNA expression was more abundant than in embryos. In the same study, after comparing mRNA levels of GPx2 and GPx1, they detected lower amounts of GPx2 mRNA in embryos, but higher levels in extraembryonic tissues than GPx1 (28). Because GPx2 and GPx1 are very similar (23), this suggests, that both enzymes may complement each other in the defense against OS in embryos and extraembryonic tissues (28). This may be one of reasons, why we did not detect GPx2 in preimplantation embryos from D1 to D5, but GPx1 was presented. The other reason may be a significance of GPx2 during the organogenesis (28). In mice,

the intestine development is ongoing from ED6.5 to ED18.5 (52). Moreover, GPx2 is the main GPx in the GIT (25). According to these facts, we suggest, that GPx2 is not very significant during the embryo preimplantation development, but it is important in the later stage, during the GIT development.

In females, GPx1 plays role during a follicular maturation and growing and with a follicular microenvironment maintenance (53). Moreover, GPx activity was increased in a follicular fluid in fertilized follicles compared to follicles without undergoing fertilization (54). This may be the reason, why we detected positivity in follicular fluid. It suggests the importance of GPx1 for correct microenvironment in mouse follicles. The GPx1 expression was found in bovine corpus luteum, small, large healthy and atretic follicles. The largest amount of GPx1 mRNA was detected in large healthy follicles, the smallest amount was found in small healthy and atretic follicles. GPx1 is important in the dominance and follicle growing (17) to provide follicle physiological development. It is known, that cells of large follicles have more cytochrome P450 enzymes (55) for progesterone and estradiol-17β synthesis. During this process, high amounts of H_2O_2 and $^{\bullet}O_2^-$ are formed by cytochrome P450 (56). This indicates, that GPx1 may be involved in ROS scavenging to defend oocytes against the extensive OS (57). Moreover, we detected GPx1 in granulosa-lutein cells from D1 to D5, but not on D0. This may indicate, that activity of corpus luteum is low on D0 without excessive production of OS, which should be compensated by higher GPx1 production. It seems, that after mating, the steroid hormones production in corpus luteum is higher with higher ROS formation and subsequent higher antioxidant enzymes requirements for cells protection. In aging female mice, apoptosis in granulosa-lutein cells and follicles is more abundant in case of higher ROS formation (58-60).

As was described, GPx1 is the enzyme specific for epithelial cells and increased production of an intracellular H_2O_2 (61). This may be the reason, why we detected GPx1 in oviductal epithelial cells, endometrial epithelial cells, and the epithelium of uterine glands.

After fertilization, the preimplantation embryo passes through the oviduct few days until it reaches the uterus, where the implantation occurs (62). Until the implantation, the embryo is nourished by substances produced by oviductal and uterine epithelial cells (63). These oviductal and uterine fluids include proteins, lipids, phospholipids, and other nutrients (64, 65). Moreover, the oviduct contains the glycoprotein oviductin, which plays role in the embryo development, sperm capacitation, and oocyte binding with sperm cells (66). This might explain, why we detected positive granules of GPx1 in the oviductal epithelial cells cytoplasm on D3. On D3, the mouse embryo is present in passes through the oviduct and may be protected against ROS by secreted GPx1 into the lumen.

GPx1 and GPx2 were detected from the isthmus to the ampulla of the bovine oviduct. Specifically, GPx1 was observed in all cell types and oviductal parts. The highest GPx1 expression was found in the ampulla (24) and infundibulum. The smallest expression was observed in the isthmus (67).

Santos et al (2016) observed the GPx1 presence in the canine endometrium using IHC (68). GPx1 was found in the cytoplasm of epithelial cells and uterine glands, and in the stroma, but not in cell nuclei. Similarly, we detected GPx1 in the endometrial epithelium cytoplasm on D0 and D1, but these cells were mostly negative on D3 and D5. As was described, GPx1 is important in the growth factor mediated proliferation, what may be affected by estrogens (69). Besides that, GPx1 plays role also in the epithelial cells survival and regeneration (70). One can assume, that the GPx1 presence in mouse uterine epithelial cells during preimplantation period of pregnancy may relate to these findings. Concerning GPx1 and GPx2, both were found in the uterus. GPx1 was the most abundant and GPx2 had less quantity (67).

We detected GPx1 in all investigated organs throughout the whole preimplantation period. As was discovered, GPx1 is important for fibroblasts, because fibroblasts from GPx1 knockout mice showed senescent-like cells morphology and DNA synthesis reduction (71). Moreover, we found GPx1 in endothelial cells. This may be due to significant role of GPx1 in vascular system, because mice without GPx1 showed the endothelial progenitor cell dysfunction and defective neovascularization (72). In fibroblasts and endothelial cells, GPx2 may play the similar role as GPx1.

Concerning GPx2, we confirmed GPx2 negative oocyte cytoplasm using IHC. As was mentioned above, this may be related to the GIT formation after the embryo implantation. Like in our experiment, GPx2 expression in granulosa-lutein cells was previously detected in the bovine ovary (17). The GPx2 presence in granulosa-lutein cells may indicate the need to protect these cells against ROS during the steroid hormone synthesis like GPx1, because both enzymes are very similar, as was described above.

Previously, GPx2 was found in epithelial cells, glands, and in the connective tissue with blood vessels in oviduct (24). Our results were similar, we detected GPx2 in the cytoplasm of oviductal epithelial cells. Moreover, we also observed positive cytoplasmic granules. The largest number of granules was on D0 and D1, but the number decreased on D3, and only few granules were found on D5. This suggests, that GPx2 may be secreted into the oviductal lumen, where it may protect passing embryos or sperms against OS, thus GPx2 may have the similar function as GPx1.

The presence of GPx2 in the uterus was similar to GPx1. GPx2 was detected in the luminal epithelium cytoplasm on D0 and D1, but this epithelium was mostly negative on D3 and D5. Previously, the lower GPx2 expression was detected in the mouse endometrium on D8, but GPx1 expression was increased (73).

GPx1 was detected by WB in granulosa-lutein cells of large follicles, but not in small follicles (17). The molecular weight of GPx1 is 22 kDa, as was described previously (74). We detected two GPx1 bands of different molecular weights (17 and 25 kDa) in the uterus on D1 and D3. This may be caused by post-translational modifications of the protein or may be related with the GPx1 presence in sperms or in the seminal fluid. Concerning GPx2, one 25 kDa band was detected in the uterus from D1 to D5, which may be related with the increased activity of this organ after mating. GPx2 has molecular weight 22 kDa (75). No detection of GPx1 or 2 in some organs on certain days may be caused by small amount of the protein in the specimen, hence this method could not detect it. Moreover, mouse ovaries and oviducts are very small, hence we may not have had enough material for enzymes detection.

In our study, we investigated mouse ovulated O/PE for GPx1 and GPx2 presence employing IFA. GPx1 was observed in all samples, specifically in the cytoplasm and under the cytoplasmic membrane, whereas GPx2 was not detected. Using IHC, we detected both enzymes in many cell types in all examined organs. There were several differences between investigated days, for example in corpus luteum or epithelial cells of the oviduct and uterus. WB showed the biggest difference in the uterus. Whereas two GPx1 bands of different molecular weight were observed, GPx2 showed only one band. These findings may indicate, that GPx1 in oocytes and embryos plays the significant role during the preimplantation period of the pregnancy as protectors from the OS to maintain the correct course of the pregnancy, because during the embryo development, cell metabolism is more active, and levels of ROS are elevated. Conversely, GPx2 as a GIT enzyme may have no mission during preimplantation processes in oocytes and embryos, but is possibly important after the implantation, when the GIT is developing. Concerning genital organs, both enzymes may protect cells from oxidation during steroid hormone synthesis in the ovary or may defend oocytes and embryos against OS during their passage through the oviduct and uterus.

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