TÜBİTAK

Turkish Journal of Veterinary and Animal Sciences

http://journals.tubitak.gov.tr/veterinary/

Research Article

Turk J Vet Anim Sci
(2016) 40: 40-46

doi:10.3906/vet-1505-92

Postnatal expression profiles of phospholipid hydroperoxide glutathione peroxidase in spermatogenesis in mice

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Received: 30.05.2015 • **Accepted/Published Online:** 06.09.2015 • **Final Version:** 05.01.2016

Abstract: Phospholipid hydroperoxide glutathione peroxidase (PHGPx) is known to be an antioxidant enzyme that may be implicated in spermatogenesis, but its functional significance in the development of testes remains poorly understood. To demonstrate the roles of PHGPx in spermatogenesis during postnatal development, expression profiles of PHGPx mRNA and protein in testes and epididymides obtained from mice 1–9 and 27 weeks after birth were spatiotemporally analyzed. In testes, PHGPx mRNA first appeared in 2-week-old mice, while its protein was first detected in late spermatocytes and round spermatids from 3-week-old mice and levels were greatly increased in elongated spermatids and immature spermatozoa of 5-week-old mice. These increased levels were maintained until 27 weeks as well as weak expression in Leydig cells. In the epididymides, PHGPx mRNA was weakly detected in all ages examined, while PHGPx protein first appeared at 4 weeks, greatly increased after 6 weeks, and was observed strongly in luminal sperm and weakly in surrounding tubular epithelium until 27 weeks. These findings indicate that PHGPx is essential for postnatal spermatogenic activity in mice.

Key words: PHGPx, testes, epididymides, expression profile, spermiogenesis

1. Introduction

Phospholipid hydroperoxide glutathione peroxidase (PHGPx) is an antioxidant that belongs to the glutathione peroxidase (GPx) superfamily and can reduce hydrogen peroxide, alkyl peroxides, and fatty acid hydroperoxides like other GPx members, as well as hydroperoxides in lipoproteins and complex lipids (1). PHGPx is associated with regulation of mitochondrial apoptosis and protection of mitochondrial ATP generation against oxidative stress and eicosanoid production in somatic cells (2,3). PHGPx is expressed in the mitochondria, cytosol, and nuclei of all tissues; it is found in especially high levels in testes and is preferentially expressed after puberty (4,5). Transgenic mice that do not have the PHGPx protein were found to be viable, but they exhibited structural abnormalities in their sperm (6,7). Although previous studies proposed that PHGPx may be involved in sperm function in male fertility and could be considered a prognostic parameter for fertilization capacity (8,9), further studies are necessary to understand the functional significance of PHGPx in spermatogenesis.

In the present study, the expression profiles of PHGPx were spatiotemporally investigated in the testes and epididymides of postnatal mice to better understand the function of PHGPx in spermatogenesis during postnatal development.

2. Materials and methods

2.1. Animals

Male ICR mice (1–9 and 27 weeks old, n = 15 per age; Koatech, Gyeonggi, Korea) were housed in polycarbonate cages in a well-ventilated room maintained at 21 ± 2 °C and 55 ± 10% relative humidity under a 12-h light/dark cycle. Mice were fed a standard mouse chow (Samyang Ltd., Incheon, Korea) and provided with tap water ad libitum.

The left testes and epididymides were fixed with Boun's solution for immunohistochemistry, while the right organs were immediately frozen at –70 °C for RNA or protein extraction. All procedures were conducted in compliance with the Guide for Care and Use of Animals (NIH # 86-23) and approved by the Chungbuk National University Animal Care Committee (CBNUA-588-13-01).

2.2. Quantitative real-time PCR

Total RNA was isolated from mouse testes and epididymides using TRIzol Reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. Total RNA concentrations were determined based on the

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UV absorbance. Next, 2 μg of total RNA was reverse-transcribed using random primers and high capacity cDNA reverse transcription kits (Applied Biosystems, Foster City, CA, USA). Quantitative PCR reactions were performed using Power SYBR Green PCR Master Mix (Applied Biosystems). Amplification of PHGPx and β-actin was performed using a Model 7500 Real-Time PCR System (Applied Biosystems) and primers designed by TIB Mol-Bio Synthesis (Berlin, Germany). Primer sequences used in this study were as follows: PHGPx forward: 5'-TAAGAACGGCTGCGTGGT-3', PHGPx reverse: 5'-GTAGGGGCACACACTTGTAGG-3', β-actin forward: 5'-TTTCCAGCCTTCCTTCTTGGGTATG-3', β-actin reverse: 5'-CACTGTGTTGGCATAGAGGTCTTTAC-3'. Each PCR program was started with UNG (uracil-N-glycosylase) incubation at 50 °C for 2 min, followed by incubation at 95 °C for 10 min. This was followed by 40 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. Data were acquired and analyzed with the 7500 System SDS software (version 1.3.1.21, Applied Biosystems). Amplification kinetics were recorded in real-time mode as sigmoid process curves for which fluorescence was plotted against the number of amplification cycles. Beta-actin was used as an internal standard to normalize expression of the target transcripts. Data from five independent runs were analyzed using a comparative Ct method as previously described (10).

2.3. Protein extraction and western blotting

Total protein was extracted from mouse testes and epididymides. To quantify protein concentrations in tissues, western blotting was conducted as previously described (11). Anti-PHGPx polyclonal antibody was purchased from Epitomics, Inc. (Burlingame, CA, USA) and anti-GAPDH monoclonal antibody was purchased from Cell Signaling Technology (Beverly, MA, USA). Secondary horseradish peroxidase-linked antirabbit IgG antibody was purchased from Ab Frontier (Seoul, Korea). Membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:7000; Ab Frontier) for 1 h at room temperature, after which the blot was developed using the WEST-one Western Blot Detection System (iNtRON, Gyeonggi, Korea). Membranes were subsequently exposed to X-ray film from Agfa Health Care (Mortsel, Belgium) for 5 and 20 s. After digitalizing the film, ImageJ software from the National Institutes of Health (Bethesda, MD, USA) was used to quantify band intensities. Experiments were performed in each of five testes and epididymides and data were presented as the means ± standard errors.

2.4. Immunohistochemistry

Tissue sections were deparaffinized with xylene and rehydrated through an ethanol gradient. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in methanol for 15 min, after which sections were washed in PBS for 20 min 4 times. Sections were then blocked from nonspecific binding by 20 min of incubation in diluted normal serum and then incubated with the PHGPx antibody for 1 h at 37 °C. Next, sections were incubated for 30 min at room temperature with a biotinylated secondary antibody (Vectastain ABC Kit; Vector, Burlingame, CA, USA) followed by 40 min at room temperature with the peroxidase-conjugated biotin-avidin complex (Vector). Finally, the bound peroxidase was revealed by immersing the sections in diaminobenzidine (Vector). Sections were rehydrated for 15 min in deionized water to remove any precipitated Tris and dehydrated in a series of ethanol and xylene. Negative control experiments for the antibodies were performed as described above with omission of the primary antibody.

2.5. Statistical analysis

Statistical differences between groups were identified by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. $P < 0.05$ was considered to indicate significance. All data are expressed as the mean ± standard error of the mean (SE). All analyses were conducted using SPSS for Windows, version 10.0 (SPSS, Chicago, IL, USA).

3. Results

3.1. Developmental expression profiles of PHGPx in postnatal testes

The temporal expression levels of PHGPx mRNA were examined in testes of mice 1–9 and 27 weeks after birth using quantitative real-time PCR analysis (Figure 1A). PHGPx mRNA was weakly expressed in testes at 2 weeks old and began to increase at 3 weeks of age. As compared to that at 3 weeks old, significantly high expression levels were observed at 5–8 weeks, with the highest levels being observed in 9-week-old mice. These levels were maintained until 27 weeks old ($P < 0.05$). The postnatal levels of PHGPx protein were evaluated in same-aged testes by western blot analysis (Figure 1B). PHGPx immunoreactivity in testes was detected initially at 3 weeks of age, was significantly increased at 5 weeks, and was maintained at the highest level until 27 weeks as compared to that of 4 weeks ($P < 0.05$). During spermatogenesis, the developmental localization of PHGPx was examined by immunohistochemical analysis (Figure 2). No PHGPx immunostaining was detected in the seminiferous tubules of mice of 1–2 weeks old (Figures 2A and 2B). However, at 3 weeks, immunostaining was initially observed in both late spermatocytes and round spermatids (Figure 2C). With the onset of spermiogenesis, PHGPx immunoreactivity was remarkably upregulated in elongated spermatids and immature spermatozoa until 27 weeks. In addition, PHGPx was weakly detected in interstitial Leydig cells after 3 weeks (Figures 2D–2F).

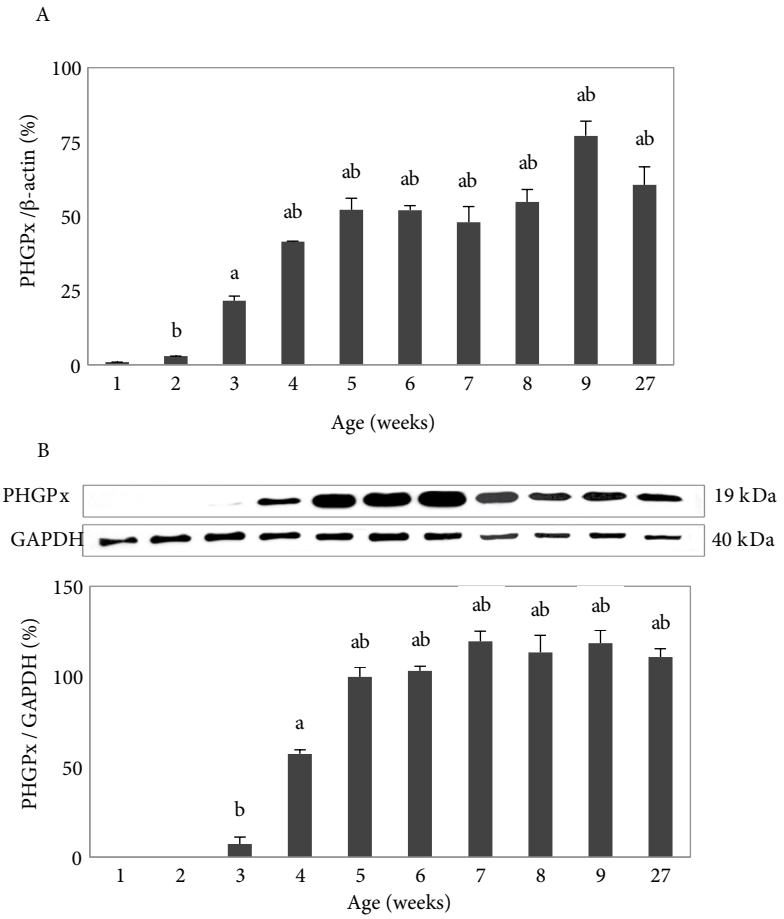

Figure 1. Temporal levels of PHGPx in postnatal testes. Quantitative real-time PCR analyses of PHGPx mRNA in mouse testes (A) after birth. Relative levels of PHGPx immunoreactivity in same-aged testes (B) detected by western blot analyses. Data represent means ± SE (n = 5). The relative ratio of PHGPx to β-actin or GAPDH level (100%) in each bar represents the average of all samples. Significant differences at $P < 0.05$: a, vs. 2 weeks old (A) or 3 weeks old (B) and b, vs. 3 weeks old (A) or 4 weeks old (B).

3.2. Developmental expression profiles of PHGPx in postnatal epididymides

In epididymides, PHGPx mRNA was weakly detected at all ages examined. PHGPx mRNA showed significantly high levels after 5 week old ($P < 0.05$), a peak level being observed at 7 weeks, and then it slightly decreased after 8 weeks (Figure 3A). However, western blot analysis indicated that epididymal PHGPx protein first weakly appeared at 4 weeks, significantly increased up to 9 weeks, and was then maintained at high levels until 27 weeks as compared to that of 5 weeks at $P < 0.05$ (Figure 3B). PHGPx protein was weakly localized in the ductus epididymis until 4 weeks (Figures 4A–4C) and slightly increased in the epididymal epithelium after 5 weeks (Figures 4D–4I). PHGPx immunoreactivity was strongly detected in intraluminal spermatozoa (Figure 4D–4I).

4. Discussion

In mice, spermatids first appear about 22 days after birth and the maximal interval between type A spermatogonia and their release as mature spermatozoa from the seminiferous tubules is about 35.5 days (12). PHGPx is localized in the mitochondria of the spermatozoa midpiece (13). Mice that do not have PHGPx produce structurally abnormal sperm and are infertile (6,7). PHGPx appears after 3 weeks of age and increases after puberty in rat and mouse testes (13,14). In the present study, although PHGPx mRNA was weakly observed at 2 weeks of age and showed peak levels at 9 weeks of age in mouse testes, PHGPx protein was first detected at 3 weeks of age, rapidly increased after 4 weeks, and was maintained at high levels until 27 weeks. PHGPx immunostaining first appeared in pachytene spermatocytes and round spermatids at 3 weeks

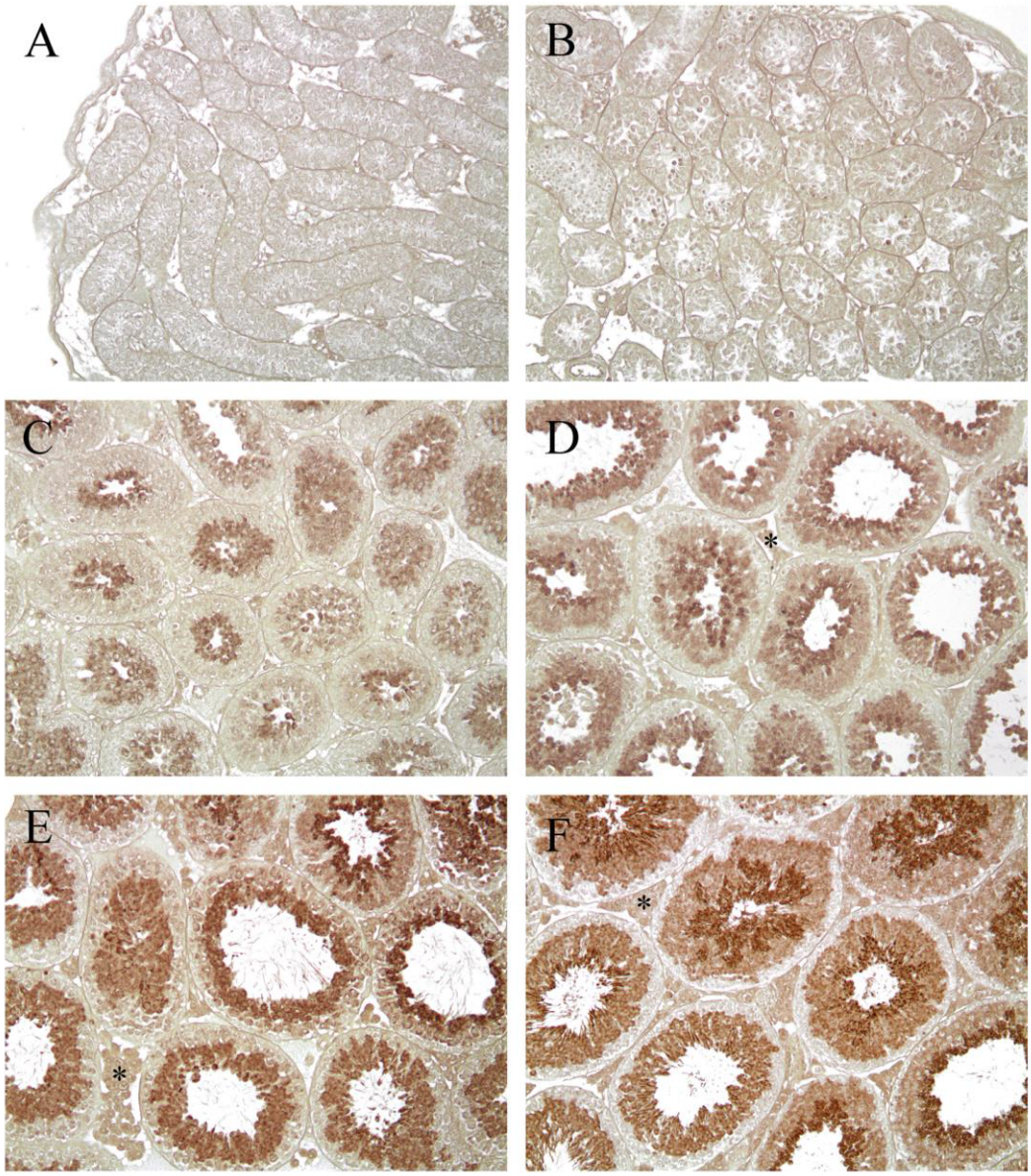

Figure 2. Developmental localization of PHGPx in postnatal testes. PHGPx immunoreactivity in the seminiferous tubules from mice of 1–5 weeks old (A–E) and 27 weeks old (F). The PHGPx signal was mainly observed in pachytene spermatocytes, round and elongated spermatids, and spermatozoa. In part, PHGPx weakly appeared in Leydig cells (asterisks) after 3 weeks. 200×.

and was strongly detected in elongated spermatids and immature spermatozoa after 5 weeks. Moreover, PHGPx immunostaining was strongly observed in sperm of the epididymis. In previous studies, PHGPx protein and mRNA were first detectable in premeiotic germ cells of 3-week-old mice (5,13). Cryptorchidism is a congenital defect of the testis in many species of mammals, which leads to infertility by overproduction of reactive oxygen species (15). We recently demonstrated that PHGPx is upregulated in degenerative spermatids, multinucleated giant cells, and Leydig cells in testes and desquamous spermatids in the epididymis after an experimental bilateral cryptorchidism in mice (16). Spermiogenesis is highly susceptible to oxidative stress because isolated spermatids have a limited capacity for both DNA repair and glutathione stock (17). Therefore, the predominance of PHGPx in spermatids is thought to reflect the necessity of shielding these cells from oxidative stress. These results

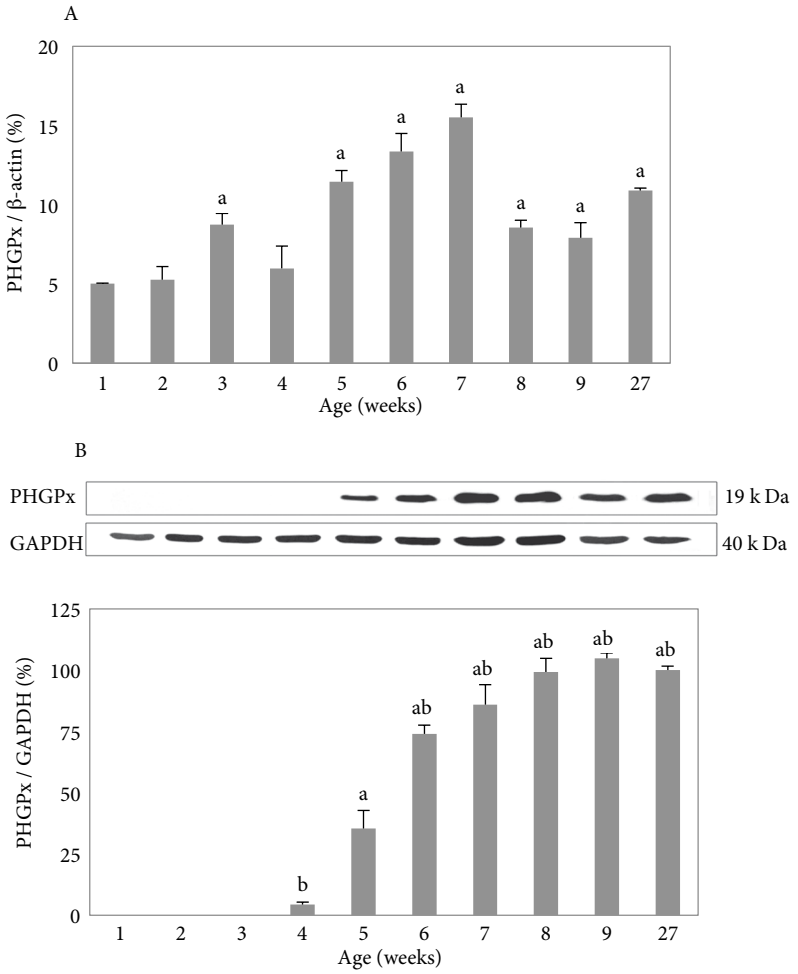

Figure 3. Temporal levels of PHGPx in postnatal epididymides. Quantitative real-time PCR analyses of PHGPx mRNA in mouse epididymides (A) after birth. Relative levels of PHGPx immunoreactivity in same-aged epididymides (B) detected by western blot analyses. Data represent means ± SE (n=5). The relative ratio of PHGPx to β-actin or GAPDH level (100%) in each bar represents the average of all samples. Significant differences at $P < 0.05$: a, vs. 1 week old (A) or 4 weeks old (B) and b, vs. 5 weeks old (B).

support the notion that PHGPx may be an essential protein for spermiogenesis and sperm capacity.

In epididymides, PHGPx mRNA showed a low level in mice of all ages examined. In contrast, PHGPx protein levels were high after 5 weeks, which is in accord with the release time of mature spermatozoa from the testes (12). Extremely abundant PHGPx protein was observed in luminal spermatozoa compared to the tubular epithelium. Conversely, epithelial PHGPx was higher in the cauda than the caput and was maintained until 27 weeks after birth. Similarly, PHGPx mRNA expression was higher in the cauda epididymis than the corpus and caput epididymis of adult rats (18). Following GPx5 deletion in the epididymal lumen, PHGPx expression increased significantly in the cauda epididymis, but not in the caput (19). These results suggest that epididymal PHGPx synthesized posttranscriptionally may primarily be associated with spermatozoa storage and maturation and partially participate in maintenance of the normal integrity in the epididymis as a local factor.

In conclusion, we found that PHGPx expression increases during the onset of puberty in both testes and

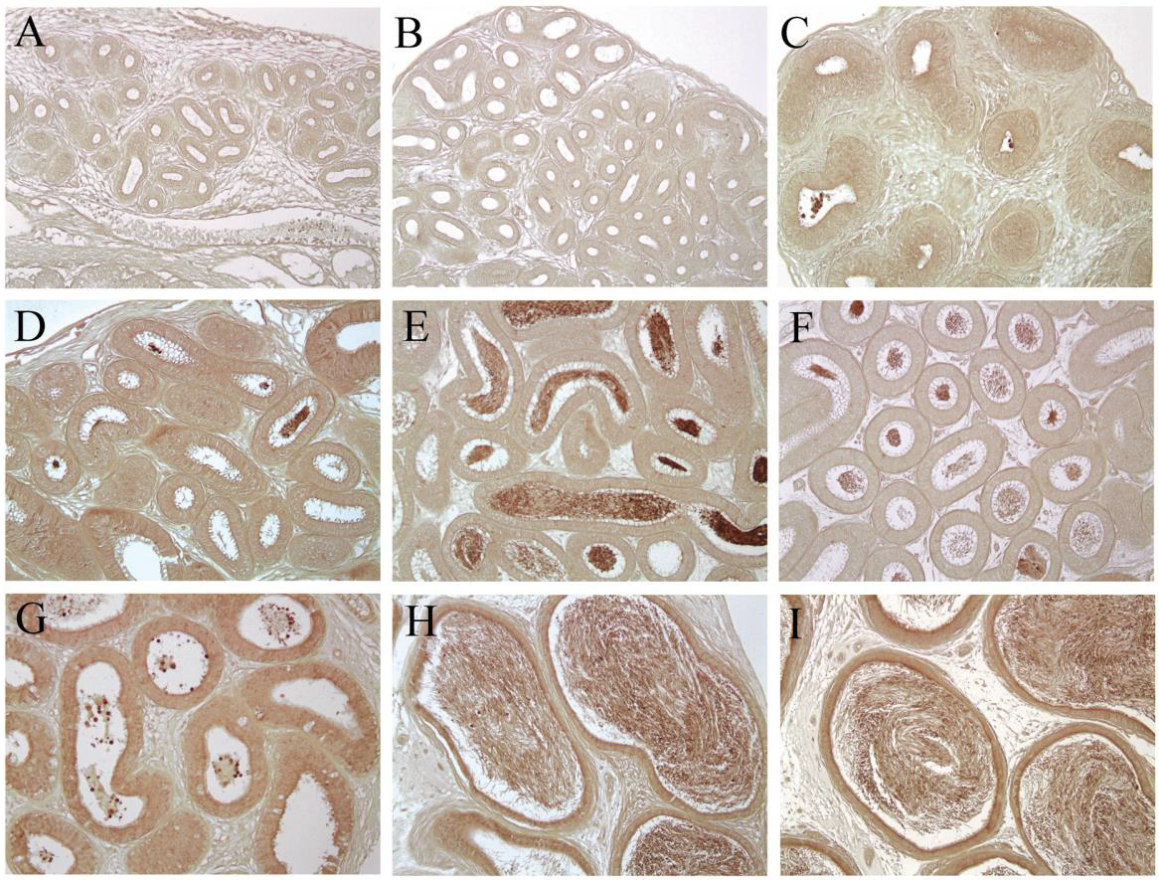

Figure 4. Developmental localization of PHGPx in postnatal epididymides. The representative PHGPx immunoreactivities in caput (D–F) and cauda (G–I) epididymides from mice of 1–3 weeks old (A–C), 5 weeks old (D and G), 7 weeks old (E and H), and 27 weeks old (F and I). 200×.

epididymides. During spermatogenesis, PHGPx was expressed stage-specifically in spermatogenic cells and detected in sperm and the epididymal epithelium during postnatal development in mice. These results indicate that PHGPx may play an important role in spermiogenesis and function as a key regulator in male fertility.

Acknowledgments

This work was supported by the Basic Science Research Program (NRF-2013R1A2A2A03016519) through the National Research Foundation of Korea (NRF), funded by the Ministry of Education, Science, and Technology.

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