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Failure of the Expression of Phospholipid Hydroperoxide Glutathione Peroxidase in the Spermatozoa of Human Infertile Males[1]

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ABSTRACT

Phospholipid hydroperoxide glutathione peroxidase (PHGPx) was intensely expressed in mitochondria in the midpiece of human spermatozoa by immunostaining with anti-PHGPx monoclonal antibodies. The PHGPx not only reduced phospholipid hydroperoxide but also scavenged hydrogen peroxide in human spermatozoa. We found a dramatic decrease in the level of expression of PHGPx in the spermatozoa of some infertile males by immunoblotting with anti-PHGPx monoclonal antibodies. These individuals accounted for about 10% of the group of 73 infertile males that we examined. All seven patients with PHGPx-defective spermatozoa were classified as suffering from oligoasthenozoospermia, a defect in which both the number and the motility of spermatozoa are significantly below normal. Males with PHGPx-defective spermatozoa accounted for 26% of the 27 infertile males with oligoasthenozoospermia. No defects in expression of PHGPx in spermatozoa were observed in 31 fertile volunteers. After a 3-h incubation, the relative number of motile spermatozoa with low-level expression of PHGPx was significantly lower than that of spermatozoa with normal expression of PHGPx. The PHGPx-defective spermatozoa failed to incorporate rhodamine 123, revealing a loss of mitochondrial membrane potential. Ultrastructual analysis of mitochondria by electron microscopy demonstrated that the morphology of mitochondria in PHGPx-defective spermatozoa was abnormal. The results suggest that failure of the expression of mitochondrial PHGPx in spermatozoa might be one of the causes of oligoasthenozoospermia in infertile men.

apoptosis, sperm, sperm maturation, sperm motility and transport, spermatogenesis, stress, testes

INTRODUCTION

A frequent cause of male infertility is defective sperm function, which accounts for close to a quarter of couples at infertility clinics [1–3]. However, identification of the causes of male infertility is quite difficult. Male infertility can be found in men with apparently normal seminal fluid. Various conditions that have not always been fully characterized can be classified as generalized *oligoasthenozoospermia.* Considerable efforts are now focused on the identification of ultrastructural and/or molecular defects in the spermatozoa or the seminal plasma in the hope of developing new solutions to various types of male infertility.

The susceptibility of human spermatozoa to oxidative stress was first suggested as a cause of male infertility by Jones et al. [4]. Human spermatozoa contain high concentrations of polyunsaturated fatty acids (PUFA), in particular, docosahexaenoic acid that has six double bonds per molecule [5, 6]. These PUFA render spermatozoa vulnerable to peroxidative damage by oxygen radicals. The generation of reactive oxygen species (ROS) and the peroxidation of sperm lipids can have negative effects on motility [7], midpiece abnormalities [8], and sperm-oocyte fusion, and each of these properties is required for fertilization in vertebrates [9]. Mitochondria are a major physiological source of ROS that can be generated during mitochondrial respiration that results in the production of ATP [10]. Abnormalities of mitochondria, such as those associated with mitochondrial diseases, result in loss of sperm motility and male infertility [11].

Spermatozoa are protected by various antioxidants and antioxidant enzymes in the seminal plasma and in the spermatozoon itself. Cytosolic glutathione peroxidase (cGPx) [12], Cu,Zn superoxide dismutase (Cu,Zn-SOD) [13], and Mn-SOD [14] have been found in human spermatozoa. The presence of catalase remains controversial [15]. The SODs reduce superoxide to hydrogen peroxide, and cGPx can reduce H_2O_2 to H_2O in spermatozoa. No correlation between human infertility and the expression of Cu,Zn-SOD and cGPx in spermatozoa has been found [12, 16]. Knockout mice that lacked Cu,Zn-SOD or cGPx were found to be fertile [17, 18]. The possible involvement of Mn-SOD in infertility remains to be determined as elimination of Mn-SOD in knockout mice was lethal [19, 20].

Phospholipid hydroperoxide glutathione peroxidase (PHGPx) is the only known intracellular antioxidant enzyme that can directly reduce lipid hydroperoxides in membranes. Phospholipid hydroperoxide glutathione peroxidase is expressed at high levels in rat and mouse testes [21–23]. Expression of PHGPx in testes is induced after puberty [22], and an extraordinarily high rate of transcription of the gene for PHGPx was detected in the layer of late sper-

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matocytes and round spermatids in mice [23] and rats [24]. In rat epididymal spermatozoa, PHGPx is present in sperm heads and midpiece [25–27]. The abundance of PHGPx in spermatozoa suggests that this unique antioxidant enzyme might be involved in the development of spermatozoa. Phospholipid hydroperoxide glutathione peroxidase is one of the major selenoproteins in spermatids, and selenium deficiency results in impairment of both the production and motility of spermatozoa. Furthermore, extensive peroxidation of lipid in spermatozoa has been reported in oligoasthenozoospermic males [28]. Phospholipid hydroperoxide glutathione peroxidase in mitochondria plays a major role in suppressing the effects of ROS that are produced by the mitochondrial respiratory chain [29, 30] and in preventing apoptotic cell death [31]. The characteristic features of male infertility are the limited production and reduced motility of spermatozoa.

To our knowledge, no information is available about the expression and function of PHGPx in the testis and spermatozoa of fertile and infertile men. In this study, we examined the expression of PHGPx in the human testis and in spermatozoa from fertile and infertile men. We found a significant decrease in the level of expression of mitochondrial PHGPx in the spermatozoa of some infertile men with oligoasthenozoospermia.

MATERIALS AND METHODS

Reagents

Antibodies against human Mn-SOD and human voltage-dependent anion channel (VDAC; Porin 31HL) were purchased from Stressgen Biotechnologies Corp. (La Jolla, CA) and Calbiochem Novabiochem Corp. (La Jolla, CA), respectively. Monoclonal antibodies (mAbs) against subunit IV of cytochrome c oxidase and rhodamine 123 (Rh123) were obtained from Molecular Probes, Inc. (Leiden, The Netherlands).

Collection of Semen and Measurement of Changes in Sperm Motility

Seventy-three samples of semen were obtained from men who had sought help for infertility at the Department of Urology, School of Medicine, Tokyo Medical and Dental University. Semen samples from 31 fertile volunteers, who had produced children in the most recent 2-yr period, were also analyzed. Informed consent was obtained from all individuals. Semen was collected after masturbation that followed 3 days of sexual abstinence. Concentrations of spermatozoa and percentage of motile spermatozoa in semen were analyzed at room temperature immediately after complete liquefaction (2 h). After complete liquefaction, spermatozoa were incubated in seminal plasma at room temperature, and the motility of spermatozoa in the seminal plasma was measured at intervals of 1 h. Motility was determined with the CellSoft Automated Semen Analyzer (version 3.51c; Cryo Resources, Ltd., New York, NY). At least 200 spermatozoa were examined for each analysis of motility.

Production of PHGPx-Specific mAbs

We generated and purified a glutathione-*S*-transferase (GST)-PHGPx fusion protein using GSH-affinity column as described previously [32]. We immunized Balb/c mice with the purified fusion protein combined with adjuvant twice at 2-wk intervals. Spleen cells were isolated and fused with P3-X63-Ag.653 cells as described previously [33]. Hybridomas were cultured in synthetic medium (GIT medium; Nihon Pharmaceutical Co., Tokyo, Japan). Hybridoma supernatants were screened for binding to PHGPx that was bound to the plate by immunoblotting and by immunoprecipitation analysis. Hybridoma cells were cloned twice by limiting dilution, and three clones (6F10, 7D2, and 8B8) were established for immunoblotting and two clones (3H10 and 3F7) were established for immunoprecipitation. The various IgG mAbs were purified by ammonium sulfate precipitation and subsequent affinity chromatography on a column of protein G-coupled Sepharose (Pharmacia Biotech Inc., Uppsala, Sweden).

Immunoblotting Analysis, Immunostaining, and Immunohistochemical Analysis

The proteins in spermatozoa (2×10^6 cells) that had been separated by centrifugation from the semen of healthy volunteers and infertile men were separated by SDS-PAGE on 15.0% acrylamide gels and transferred to polyvinylidene difluoride (PVDF) membrane filters (Millipore Co., Bedford, MA) in 25 mM Tris, 192 mM glycine, 10% (w/v) methanol at 4°C in a protein-transfer system (Bio-Rad Laboratories, Inc., Melville, NY), operated at 50 V for 150 min as described previously [34]. Each PVDF membrane with blotted proteins was blocked by incubation with 3% (w/v) skimmed milk in 10 mM Tris-HCl, pH 7.4, that contained 150 mM NaCl and 0.1% Tween-20 (TBS-T) for 1 h. The PVDF membrane was then incubated separately with anti-PHGPx mAb (6F10), anti-human Mn-SOD mAb, and anti-VDAC mAb that had been diluted with TBS-T to an appropriate concentration for 2 h. Then each PVDF membrane was incubated for 1 h with horseradish peroxidase-conjugated goat antibodies against mouse IgG (Zymed, South San Francisco, CA). The binding of antibodies to the antigen on the PVDF membrane was detected with an enhanced chemiluminescence Western blotting analysis system (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Immunostaining with anti-PHGPx mAb (6F10) was undertaken to determine the distribution of PHGPx in human spermatozoa. Aliquots of 1×10^7 cells of spermatozoa, separated from semen, were permeabilized by treatment with cold methanol for 5 min, washed three times with PBS, and fixed on coverslips, coated with 0.1% (w/v) poly-L-lysine at 25°C for 30 min. Fixed cells were washed with PBS and blocked with PBS that contained 2% BSA at 25°C for 30 min. The cells were then incubated with 2 μg/ml mouse mAb against cytochrome c oxidase subunit IV monoclonal antibodies or anti-PHGPx mAb (6F10) in PBS plus 2% BSA at 25°C for 2 h. Then the cells were washed with PBS and incubated with Cy3-conjugated goat anti-mouse IgG (Amersham) that had been diluted to 10 μg/ml with PBS plus 2% BSA at 25°C for 1 h. The fluorescence of Cy3 in spermatozoa was monitored and photographed with an Axiovert 135M inverted microscope (Carl Zeiss, Oberkocken, Germany) equipped with a Planapochromat 63× objective.

Immunohistochemical staining with anti-PHGPx mAb was performed as described previously [35]. In brief, human testes were resected surgically from patients with a diagnosis of seminoma at Kitasato University Hospital. All tissues were fixed in 10% formalin and processed for embedding in paraffin wax. Immunohistochemical staining was performed with a combination of microwave-oven

heating and the standard streptavidin-biotin-peroxidase complex methods (LSAB kit; Dako, Copenhagen, Denmark). Counterstaining of nuclei was achieved with 0.3% methyl green solution. The mAbs used were the 6F10, 8B8, and 7D2 mAbs as described above. To confirm the specificity of binding of mAbs, normal mouse serum, as a negative control, was applied instead of each primary antibody. No staining was detected with the negative control.

In Situ Hybridization with a cRNA Probe for Human PHGPx

For RNA in situ hybridization (ISH), a cDNA for human PHGPx (220 base pairs from the second initiation codon to nucleotide 527 with a *Hin*dIII site) was cloned into p-Bluescript [36]. The digoxigenin (DIG)-labeled cRNA probe was prepared with a DIG RNA Labeling Kit (Boehringer Mannheim GmbH, Germany) as described previously [37]. The plasmid that included the cDNA for human PHGPx was either linearized with *Eco*RI and transcribed with T3 RNA polymerase to generate a 0.33-kilobase antisense probe, or it was linearized with *Xho*I and transcribed with T7 RNA polymerase to generate a sense probe. The nucleotide sequence of the inserted cDNA was determined with a DNA sequencer (ALF Red DNA sequencer; Amersham Pharmacia Biotech). Testicular tissue was fixed in 4% paraformaldehyde for 24 h and embedded in paraffin. Five-micrometer-thick sections were cut and placed on slides that had been coated with silane (Muto Pure Chemicals Co. Ltd., Kyoto, Japan). The technique for ISH was based on that described by Oka et al. [37] with minor modifications. The hybridization mixture contained 50% deionized formamide, 10 mM Tris-HCl (pH 7.6), 200 μg/ml yeast tRNA, 1× Denhardts solution, 10% dextran sulfate, 10% NaCl, 0.25% SDS, 1 mM EDTA (pH 8.0), and approximately 2 μg/ml of the DIG-labeled cRNA probe. A 25-μl aliquot of this mixture was applied to each section and covered with parafilm. Hybridization was allowed to proceed in a humid chamber for 16 h at 50°C. Subsequent immunohistochemical staining was performed with 500-fold diluted polyclonal anti-DIG Fab antibody (Boehringer-Mannheim) and a mixture of 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (Boehringer-Mannheim). In addition, stages of spermatogenesis in human testes were determined on semiserial sections of testis that had been stained with hematoxylin and eosin. The specificity of the immunostaining reaction was confirmed by hybridization with a hybridization mixture without any DIG-labeled probe and by hybridization with the sense probe.

Activity of PHGPx and cGPx in Human Spermatozoa, in Other Cells, and in Seminal Plasma

Approximately 2×10^6 sperm cells from the semen of each healthy fertile volunteer, 3×10^7 human endothelial cells (ECV304), and 3×10^7 rat basophil leukemia cells (RBL2H3) were washed twice with ice-cold PBS, collected, and sonicated in 1 ml of PBS that had been supplemented with 5 mg/ml leupeptin and 17 mg/ml PMSF. Each sonicate was centrifuged for 20 min at 20 000 × *g* and 4°C. Supernatants and seminal plasma were used for assays of the reduction of phospholipid hydroperoxide and H_2O_2 by PHGPx and cGPx. The reducing activity for phospholipid hydroperoxide, namely, PHGPx activity, was measured using phosphatidylcholine hydroperoxide (PCOOH) as described previously [38]. The reduction of hydrogen peroxide by glutathione peroxidase was monitored by following the decrease in absorbance at 340 nm of NADPH in the presence of 2 mM GSH and 0.25 mM H_2O_2. To examine the ratio of PHGPx activity to cGPx activity, the protein in the total homogenate was separately immunoprecipitated with anti-PHGPx mAb (3H7) and anti-cGPx antibodies bound to protein A-Sepharose for 2 h. After centrifugation, the residual activity of each enzyme in the homogenate was determined as described above. The activities of PHGPx and cGPx were calculated from the residual and total activities of each homogenate.

Measurement of Mitochondrial Membrane Potential

Changes in the mitochondrial membrane potential of spermatozoa were examined by monitoring the fluorescence of Rh123 as described previously [30]. Semen was incubated for 7 h after the ejaculation, and then 2×10^5 sperm cells were stained with Rh123 (1 μg/ml) for 20 min and washed with PBS. The stained sperm cells were dropped onto glass slides. Fluorescence due to Rh123 in spermatozoa was monitored and photographed with the Axiovert 135M inverted microscope equipped with the Planapochromat 63× objective.

Electron Microscopy

Motile spermatozoa were collected by a 60-min swim up in human tubal fluid (Gibco BRL, Rockville, MD). Samples were prepared from three fertile volunteers and three patients with low-level expression of PHGPx. The suspension with spermatozoa was centrifuged at 200 × *g* for 20 min. The supernatant was discarded, and the pellet was fixed in a solution of 2.5% glutaraldehyde in 0.1 M phosphate buffer. After two rinses in 0.1 M phosphate buffer, the sample was postfixed in a solution of 1% OsO_4 in 0.1 M phosphate buffer, dehydrated through a graded series of ethanols, and embedded in Epon-Araldite. Ultrathin sections were stained in 5% aqueous uranyl acetate and lead citrate and examined with a transmission electron microscope (TEM; Hitachi H-7100, Hitatinaka, Japan) operated at 80 kV. We examined 20 longitudinal profiles of sperm heads and the mitochondria in 20 longitudinal sections of midpieces.

Statistical Analysis

The data were analyzed by a Mann-Whitney *U*-test with StatView software, version 4.01 (Abacus Concepts, Palo Alto, CA).

RESULTS

Expression of PHGPx in Human Testis and Spermatozoa

We examined the expression of PHGPx in human testis and spermatozoa using several monoclonal antibodies against PHGPx (five clones: 6F10, 8B8, and 7D2 for immunoblotting analysis; 3F7 and 3H10 for immunoprecipitation). As shown in Figure 1A, mAbs 6F10, 7D2, and 8B8 specifically detected a 20-kDa PHGPx in human spermatozoa. Furthermore, mAbs 3F7 and 3H10 specifically immunoprecipitated ^{75}Se-labeled PHGPx in extracts of human ECV304 endothelial cells (data not shown). The localization of PHGPx and its mRNA in human testis was determined by immunohistochemical staining (Fig. 1, B and C) and by ISH, respectively (Fig. 1, D–F). Immunohistochemical analysis with anti PHGPx mAb (7D2 in Fig. 1, B and C) revealed that PHGPx was abundantly distributed in late

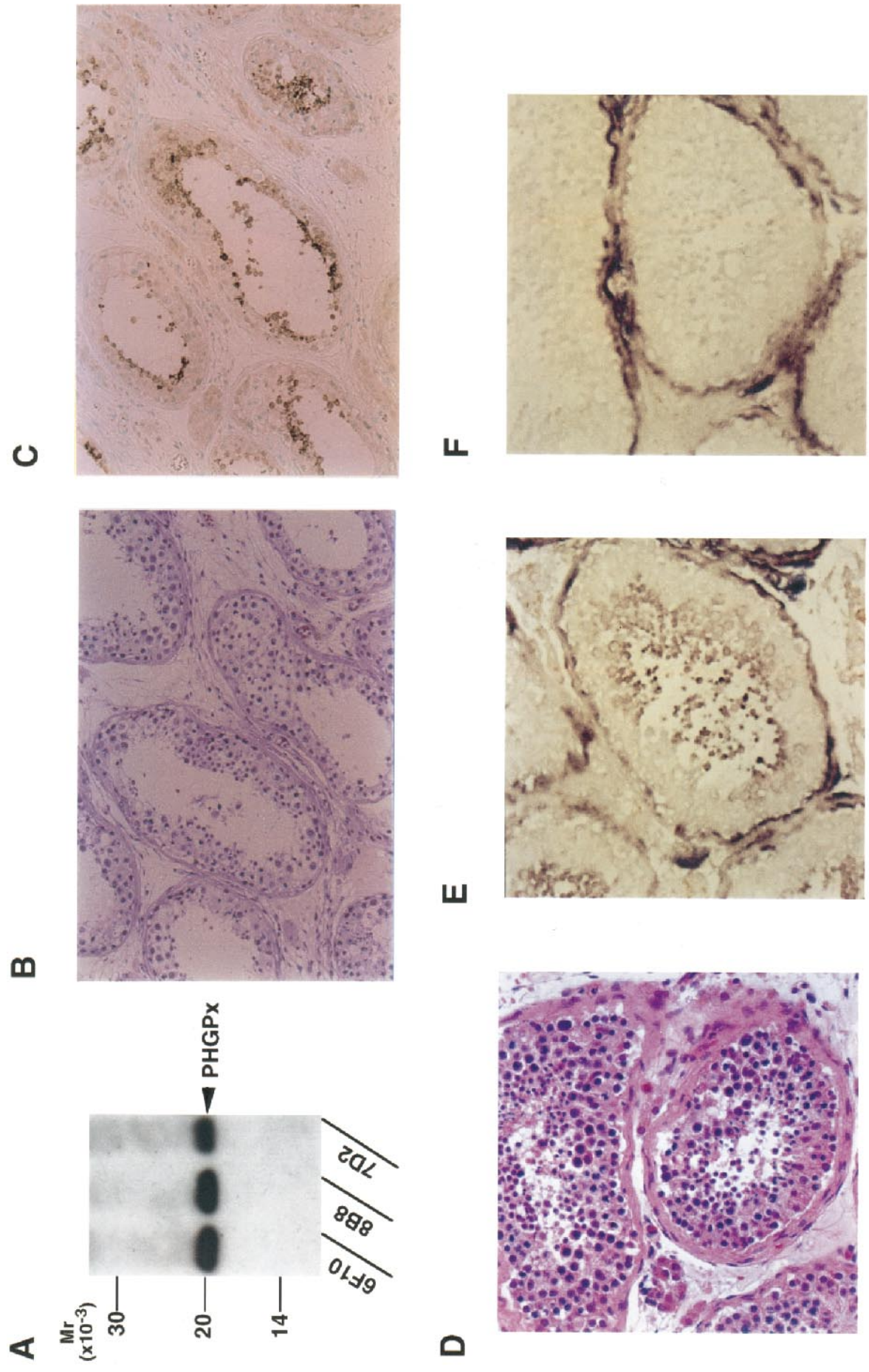

FIG. 1. Expression of mRNA for PHGPx and of PHGPx itself in human testis and spermatozoa. **A**) Immunoblotting analysis with anti-PHGPx mAbs (6F10, 8B8, and 7D2) of proteins in human spermatozoa. **B**) Hematoxylin and eosin stain; magnification 160-fold. **C**) Immunohistochemical staining with anti-PHGPx mAb (7D2) of human testis. **D–F**) In situ hybridization showing localization of PHGPx mRNA in human testis. The three micrographs show adjacent sections of an identical region. **D**) Hematoxylin and eosin stain; magnification 160-fold. **E**) In situ hybridization using the DIG-labeled antisense probe for PHGPx mRNA. **F**) In situ hybridization using the DIG-labeled sense probe (negative control).

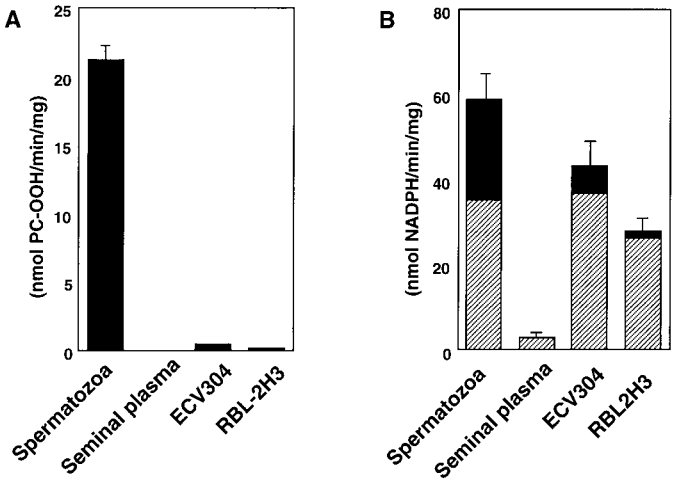

FIG. 2. Reduction of phospholipid hydroperoxide and H_2O_2 by PHGPx and cGPx in spermatozoa and other cells. Total activity was measured in lysates of spermatozoa, in lysates of cultured cells, and in seminal plasma, as described in the text. Proteins in samples were immunoprecipitated separately with anti-PHGPx and anti-cGPx antibodies, and residual activities in lysates were measured. **A**) Reduction of phospholipid hydroperoxide. **B**) Reduction of H_2O_2. Black areas indicate activity due to PHGPx. Shaded areas indicate activity due to cGPx. Column show means ± SD of results from three samples.

spermatocytes and spermatids. In situ hybridization indicated that the distribution of mRNA for PHGPx was almost the same as that of PHGPx, as determined by immunohistochemical staining (Fig. 1, D–F). Staining with the antisense probe for PHGPx was mainly observed in late spermatocytes and spermatids (Fig. 1E), and the sense probe gave negative results (Fig. 1F). Few signals were detected in spermatogonia and Sertoli cells. These results indicate that PHGPx is induced in late spermatocytes during spermatogenesis.

Activities of PHGPx and cGPx in Human Spermatozoa

The reduction of phospholipid hydroperoxide and H_2O_2 were determined in human spermatozoa and two lines of cultured cells (Fig. 2). The activity of PHGPx was determined after precipitation with cGPx-specific polyclonal antibodies, while the activity of cGPx was determined after the immunoprecipitation of PHGPx by mAb 3H7. The activity of PHGPx in human spermatozoa to reduce phosphatidylcholine hydroperoxide (PCOOH) was 20 times higher than that of PHGPx in human endothelial cell line (ECV304) and the rat basophil leukemia cell line (RBL2H3; Fig. 2A). No PHGPx activity was detected in human seminal plasma. The ability of cGPx in human spermatozoa to reduce H_2O_2 was almost the same as that in ECV304 cells and RBL2H3 cells (Fig. 2B). The reduction of H_2O_2 was predominantly due to cGPx in ECV304 cells and RBL2H3 cells. The reduction by PHGPx of H_2O_2 (23.5 ± 5.2 nmol NADPH/min/mg) in human spermatozoa was significantly higher than that in ECV304 cells (6.1 ± 3.5 nmol NADPH/min/mg) and RBL2H3 cells (1.7 ± 1.5 nmol NADPH/min/mg). Although PHGPx appeared to account for 15% of the total reduction of H_2O_2 in ECV304 cells, about 40% of the reduction of H_2O_2 appeared to be due to PHGPx in human spermatozoa. These results show that

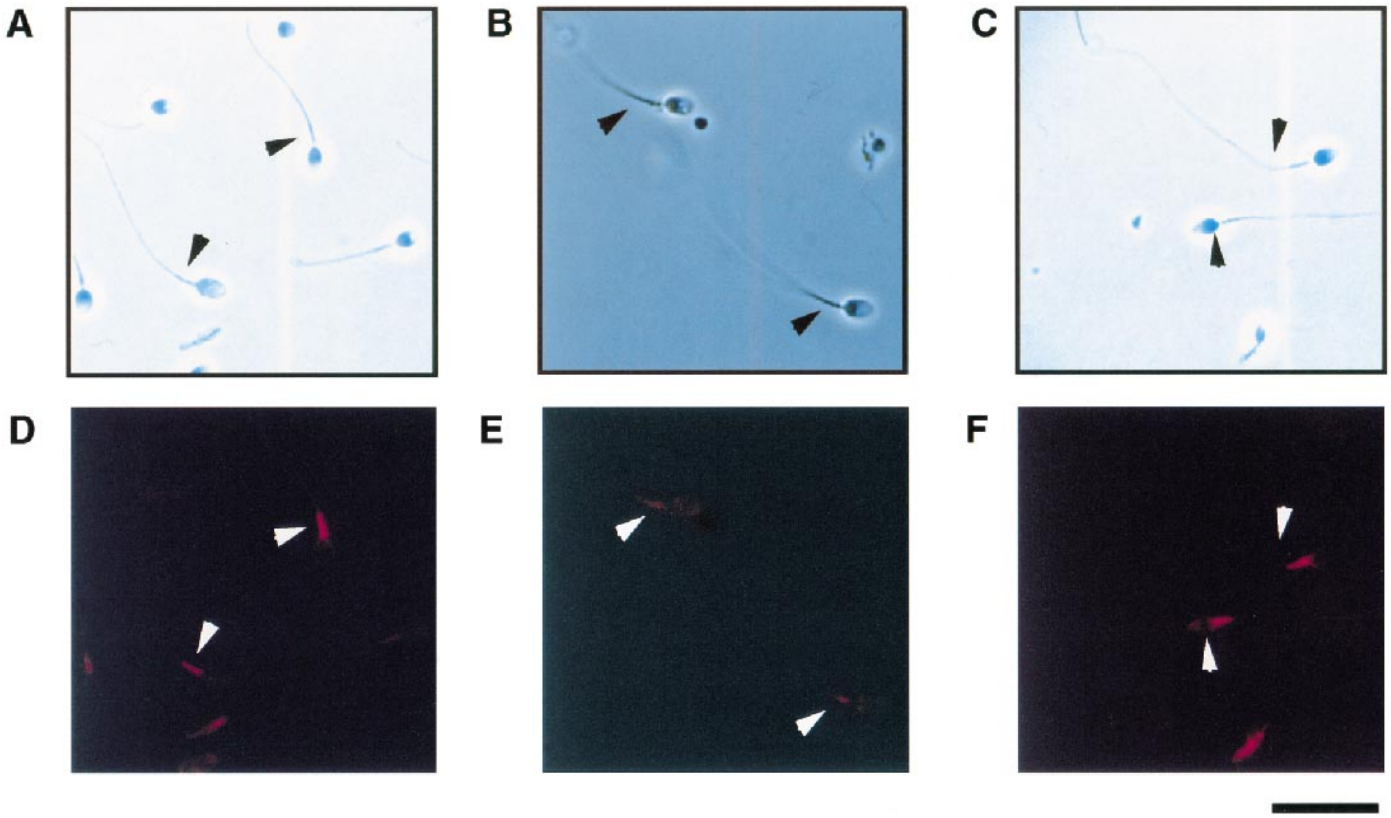

FIG. 3. Immunostaining with anti-PHGPx of spermatozoa from fertile and infertile males. Normal fertile spermatozoa (**A**, **C**, **D**, and **F**) and PHGPx-defective infertile spermatozoa (**B** and **E**) were stained with Cy3-conjugated anti-PHGPx mAbs (6F10; **A**, **B**, **D**, and **E**), and Cy3-conjugated anti-cytochrome c oxidase mAbs, as a mitochondrion-specific probe (**C** and **F**). Confocal images (**A**, **B**, and **C**) and fluorescence images (**D**, **E**, and **F**), bar = 10 μm.

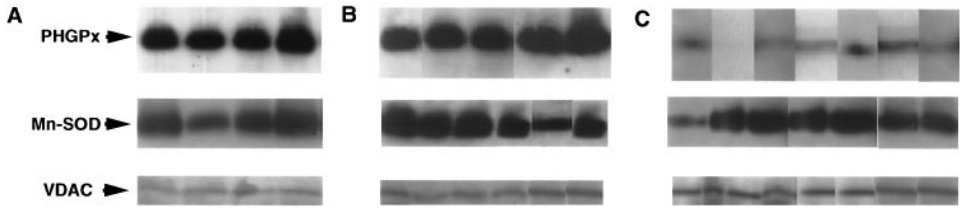

FIG. 4. Reduced expression of PHGPx in spermatozoa from infertile males, as revealed by immunoblotting analysis. Lysates of human spermatozoa (2×10^6 cells) from ejaculates were subjected to SDS-PAGE and then proteins were blotted on PVDF membranes. Immunoblotting was performed with antibodies against PHGPx, Mn-SOD, and VDAC, respectively, as described in the text. **A**) Spermatozoa from normal fertile volunteers. **B**) Normal levels of PHGPx in the spermatozoa of some infertile male. **C**) Low levels of PHGPx in spermatozoa from infertile males, with normal levels of Mn-SOD and VDAC.

PHGPx is a major antioxidant enzyme that reduces hydroperoxides in human spermatozoa.

Failure of PHGPx Expression in Spermatozoa from Infertile Men

Immunoblotting analysis revealed large amounts of PHGPx in human spermatozoa, as shown in Fig. 1A. Localization of PHGPx in spermatozoa was revealed by immunostaining as shown in Fig. 3. Strong PHGPx-specific fluorescence was observed in the midpiece of spermatozoa from a fertile volunteer (Fig. 3, A and D). The profile of fluorescence due to PHGPx was identical to that of the mitochondrial marker protein cytochrome c oxidase, indicating that PHGPx was localized in the mitochondria of spermatozoa (Fig. 3, C and F). By contrast, only faint fluorescence was detected in the midpiece of spermatozoa from infertile male, indicating a defect in the expression of PHGPx (Fig. 3, B and E). No abnormalities in morphology were detected in the midpiece, head, and tail of PHGPx-defective spermatozoa by confocal microscopy (Fig. 3, A and B).

We examined the expression of PHGPx in spermatozoa from 73 infertile patients and 31 fertile volunteers by immunoblotting analysis (Fig. 4). We found a dramatic decrease in the expression of PHGPx in spermatozoa from 7 of the 73 infertile men (Fig. 4C). The amounts of PHGPx were from 20% to 10% of those in the spermatozoa of fertile volunteers. No defect in the expression of PHGPx in spermatozoa was found in the case of the 31 normal fertile volunteers (Fig. 4A). Expression of PHGPx in the spermatozoa from the other 66 infertile men was very similar to that in the spermatozoa from the fertile volunteers (Fig. 4B). No decreases in expression of other mitochondrial proteins, such as Mn-SOD and VDAC, were detected in spermatozoa with low-level expression of PHGPx (Fig. 4, A–C). These results demonstrate that the significant decrease in the level of PHGPx in the spermatozoa of some infertile men was not due to a decrease in the number of mitochondria in the spermatozoa.

Impairment of Generation and Motility of Spermatozoa in Infertile Men with PHGPx-Defective Spermatozoa

The total numbers of spermatozoa in the ejaculates of fertile volunteers were significantly higher than in those of infertile males (Table 1). Moreover, the total numbers of spermatozoa in the semen from PHGPx-defective infertile men were very low compared to those in the semen of infertile men with normal levels of PHGPx. We examined the motility of PHGPx-defective spermatozoa with an automated analyzer after liquefaction (Fig. 5). More than 50% of spermatozoa of fertile volunteers were motile, and no reduction in the motility of sperm was observed for 5 h. By contrast, fewer than 50% of spermatozoa were motile in the case of the PHGPx-defective spermatozoa, and motility decreased in a time-dependent manner (Fig. 5). There was a statistically significant difference ($P < 0.01$) in motility between PHGPx-defective spermatozoa and normal spermatozoa during a 3-h period (Table 1). In the case of spermatozoa from infertile men, the decrease in motility of PHGPx-defective spermatozoa was also significantly greater than that of spermatozoa with normal levels of expression of PHGPx.

Classification of Fertile Volunteers and Infertile Patients According to the Criteria of the World Health Organization

Fertile men, infertile men, and infertile with PHGPx-defective spermatozoa were classified according to the criteria of the World Health Organization [39] (Table 2). Asthenozoospermia is diagnosed when motile spermatozoa account for fewer than 50% of the total spermatozoa. In oligozoospermia, the concentration of spermatozoa is below 2×10^6 cells/ml. When both the motility and the concentration of spermatozoa are below normal, the diagnosis is oligoasthenozoospermia. Among the 31 fertile men, 23 had normozoospermia, 7 had asthenozoospermia, and 1 had oligozoospermia; none had oligoasthenozoospermia. Among the 73 infertile men that we examined, 18 had normozoos-

TABLE 1. Comparison of numbers of total spermatozoa and of rates of reduction in motility during a 3-h incubation between normal and PHGPx-defective spermatozoa.

	Healthy fertile volunteers (n = 27)	Infertile males (n = 44)	
		Normal PHGPx (n = 38)	PHGPx-defective (n = 6)
Total cells per ejaculate ($\times 10^6$)	$136.7 \pm 71.4^{A*}$	$90.7 \pm 82.5^{B*}$	$17.6 \pm 15.5^{A*B*}$
Motility (%; relative to time 0) after a 3-h incubation	$96 \pm 4.5^{A*}$	$70 \pm 29^{B\dagger}$	$49 \pm 26^{A*B\dagger}$

* $P < 0.01$.
† $P < 0.05$.
A Data for healthy volunteers and PHGPx-defective infertile males are compared.
B Data for infertile males with normal levels of PHGPx in spermatozoa and PHGPx-defective spermatozoa are compared.

TABLE 2. Classification of fertile and infertile males by the criteria proposed by the World Health Organization.

	Fertile volunteers (n = 31)	Infertile patients (n = 73)	Infertile patients with PHGPx-defective spermatozoa (n = 7)
Normozoospermia*	23	18	0
Oligozoospermia	1	7	0
Asthenozoospermia	7	23	0
Oligoasthenozoospermia	0	25	7

* See text for details of criteria.

permia, 7 had oligozoospermia, 23 had asthenozoospermia, and 25 had oligoasthenozoospermia. All of the seven subjects with PHGPx-defective spermatozoa belonged to the group with oligoasthenozoospermia. Thus, infertile men with PHGPx-defective spermatozoa accounted for about 10% of the total number of infertile men and for 26% of the infertile men with oligoasthenozoospermia.

Ultrastructure and Function of Mitochondria in PHGPx-Defective Spermatozoa

We examined the membrane potential in mitochondria of spermatozoa using Rh123, which is selectively taken up by mitochondria at a rate that depends on the mitochondrial membrane potential. After incubation of spermatozoa for 5 h in semen, we separated spermatozoa from seminal plasma, stained them with Rh123 for 20 min, and monitored the fluorescence of Rh123 in the spermatozoa with a fluorescence microscope. Fluorescence was clearly observed in the midpiece of fertile spermatozoa (Fig. 6C). By contrast, PHGPx-defective spermatozoa failed to incorporate Rh123, and fluorescence due to Rh123 was undetectable (Fig. 6D).

The ultrastructure of mitochondria was assessed by transmission electron microscopy (Fig. 7). The mitochondria in the midpiece of PHGPx-defective spermatozoa had extremely variable and abnormal morphology as compared to that of normal spermatozoa. Characteristic findings were mitochondria with an increased relative area of the matrix; thickening of membranes, in particular of outer membranes; and swelling, with loss of cristae. These changes were observed in all the spermatozoa examined from two PHGPx-defective individuals and in 60% of the spermatozoa of another PHGPx-defective individual.

DISCUSSION

Human spermatozoa in ejaculated semen contain large amounts of PHGPx, which is one of the major antioxidant enzymes that scavenges phospholipid hydroperoxides and H_2O_2 in human spermatozoa. We found that the reducing activity against phospholipid hydroperoxide in human spermatozoa was of the same order as that in rat epididymal spermatozoa [26]. In most somatic cells, H_2O_2 is reduced primarily by cGPx. However, the reduction by PHGPx of H_2O_2 was similar to that by cGPx in normal human spermatozoa. Immunostaining revealed that PHGPx was mainly distributed in the midpiece of human spermatozoa, confirming previous studies of rat epididymal spermatozoa by Godeas et al. [25] and Ursini et al. [27].

In this study we found that infertile males with PHGPx-defective spermatozoa accounted for about 10% of the total number of infertile males examined and for 35% of infertile males with oligoasthenozoospermia. No abnormal expression of PHGPx was detected in spermatozoa from 31 fertile males. All of the infertile males with PHGPx-defective spermatozoa were grouped in the class with oligoasthenozoospermia. These observations suggest that insufficient expression of PHGPx in spermatozoa might lead to serious impairment of fertilization. Aitken et al. [40] indicated that half of the spermatozoa from oligozoospermic men were defective, with loss of motility and elevated levels of reactive oxygen species. Zalata et al. [28] reported a decrease in relative levels of PUFAs, including docosahexaenoic acid, in the phospholipids of spermatozoa in case of the oligoasthenozoospermia. The defects associated with spermatozoa in oligoasthenozoospermic patients might be due, at least in part, to a significant decrease in the level of mitochondrial PHGPx, a major antioxidant enzyme in the spermatozoa.

A prominent feature of PHGPx-defective spermatozoa was loss of motility. Moreover, the motility of these spermatozoa decreased significantly during a 5-h incubation in semen. This loss of motility might be one of the causes of infertility in these patients. Aitken et al. [40] and Sharma and Agarwal [1] suggested that loss of motility might be

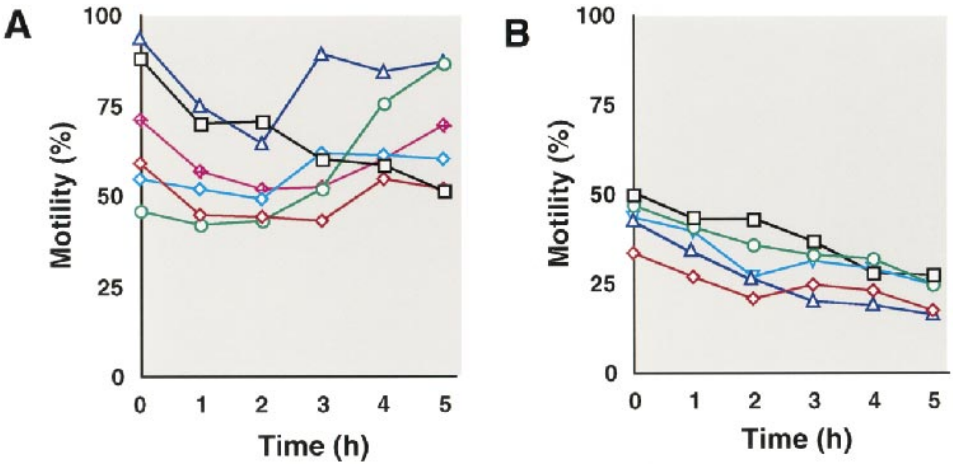

FIG. 5. Impaired motility in semen of PHGPx-defective spermatozoa from infertile males. Spermatozoa from normal fertile volunteers (**A**) and PHGPx-defective spermatozoa from infertile males (**B**) were incubated in semen at room temperature after complete liquefaction. Motility was measured as described in the text. At least 200 cells were examined in each case at each indicated time. Individual symbols refer to individual samples.

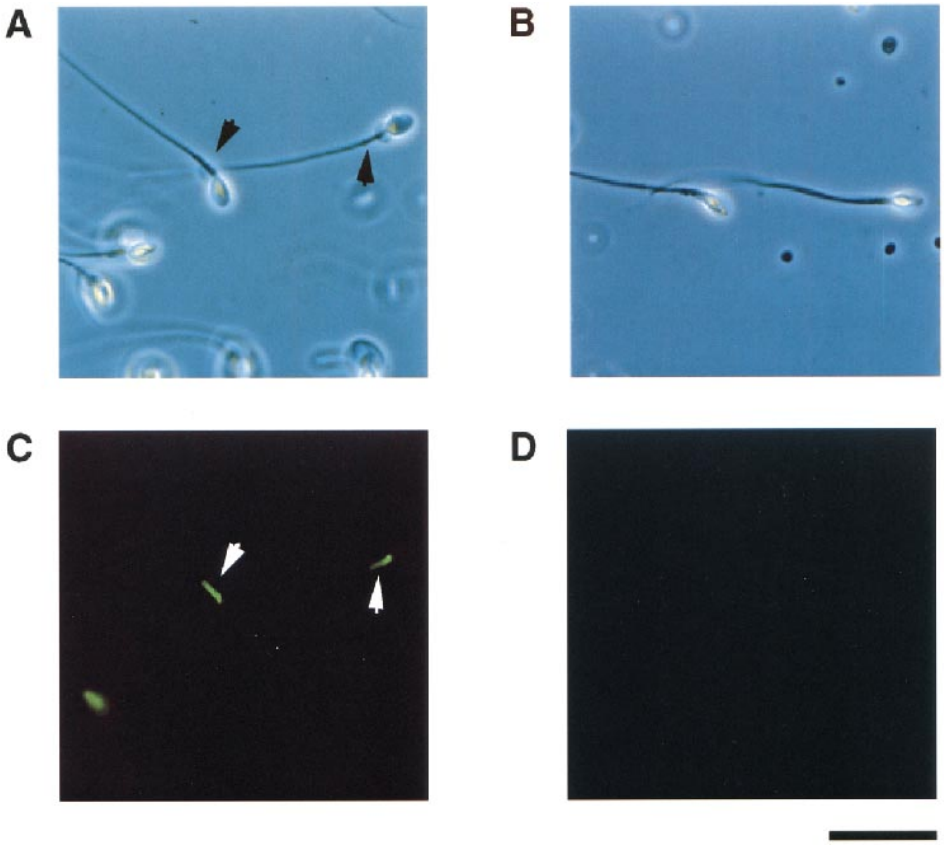

FIG. 6. The mitochondrial membrane potential in normal and PHGPx-defective spermatozoa. Five hours after ejaculation, normal fertile spermatozoa (**A** and **C**) and PHGPx-defective spermatozoa from an infertile male (**B** and **D**) were stained with 1 μg/ml Rh123 for 20 min. Fluorescence due to Rh123 was photographed under a fluorescence microscope (**C** and **D**). Confocal images (**A** and **B**). Bar = 10 μm.

induced by ROS generated by the spermatozoon itself or by leukocytes in the semen. In our population of males with PHGPx-defective spermatozoa, loss of spermatozoal motility was not due to leukocytes, because no accumulation of leukocytes was apparent in their semen. In PHGPx-defective spermatozoa, the activity of PHGPx (0.07 ± 0.02 nmol PCOOH/min/10^6 cells) was lower than the activity (0.28 ± 0.05 nmol PCOOH/min/10^6 cells) in fertile spermatozoa. We reported previously that mitochondrial PHGPx suppresses the peroxidation of lipids and protects mitochondrial functions in cells treated with inhibitors of the respiratory chain [30]. Decreases in PHGPx activity should result in the production of ROS and the subsequent dysfunction of mitochondria in the spermatozoa of infertile males because Mn-SOD is expressed at normal levels in these mitochondria. Mitochondrial dysfunction could be a direct cause of the impairment of the functions of spermatozoa. Inhibitors of respiratory enzymes, such as KCN, and uncouplers significantly depress the motility of spermatozoa (data not shown). Weinberg et al. [41] showed that nitric oxide, which inhibits the ATP-generating ability of enzymes in the electron-transport system, inhibits the motility of human spermatozoa. Folgero et al. [11] showed that sperm motility was apparently reduced in patients with mitochondrial disease caused by the reduced activities of complexes I and IV. We observed a decrease in the membrane potential of mitochondria and disruption of mitochondrial morphology in PHGPx-defective spermatozoa (Figs. 6 and

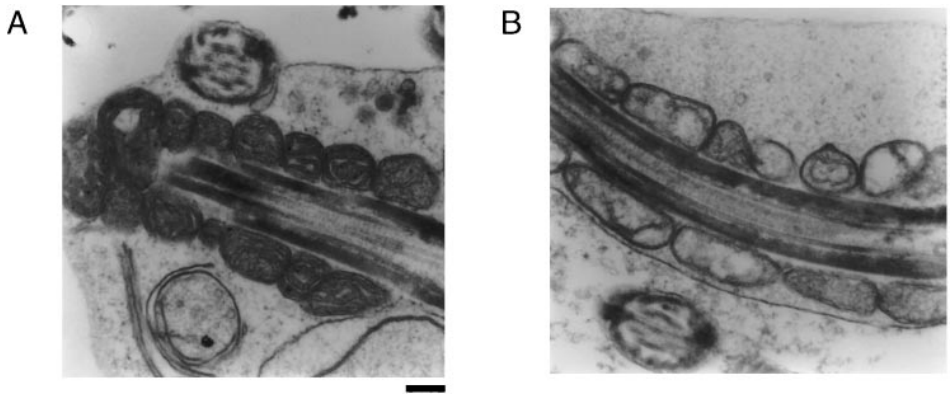

FIG. 7. Ultrastructure of mitochondria in midpiece of a normal (**A**) and a PHGPx-defective (**B**) spermatozoon. Bar = 13 nm.

7). Thus, it appears that a defect in the expression of PHGPx in the mitochondria of spermatozoa might be closely linked to loss of motility as a result of the disruption of mitochondrial activity.

Another major change associated with reduced expression of PHGPx was a marked decrease in the number of spermatozoa in the ejaculate. The defect in the generation of spermatozoa might have resulted from some abnormality in spermatogenesis in these infertile males. Spermatogenesis is a complex process, and little information is available about the regulation of spermatogenesis at the molecular level. Recent studies with knockout mice suggest that apoptosis might be closely linked to the control of spermatogenesis. The first wave of spermatogenesis, during which spermatogonia develop to spermatocytes, is normally accompanied by extensive apoptosis of germ cells [42]. Apoptosis might be involved at various stages of spermatogenesis and might regulate both development and quality control during the production of spermatozoa. Knockout mice that lack *Bcl-w* [43], which is an anti-apoptotic member of the *Bcl-2* family, are blocked at the later stages of spermatogenesis, with enhanced degradation of germ cells and caused male infertility. Mutation of genes for other proteins in the Bcl-2 family, such as *Bax* [44], *bcl-2* [45], and *bcl-xL* [46], leads to the accumulation of premeiotic germ cells and disruption of the differentiation of germ cells. Knockout of Apaf-1, which initiates an apoptotic signaling pathway, also causes male infertility [47]. It seems likely that the low-level generation of spermatozoa in some infertile males might reflect a defect in mitochondrial PHGPx because we recently found that PHGPx in mitochondria serves as an anti-apoptotic factor via prevention of the generation of hydroperoxides in mitochondria [31]. Mitochondrial PHGPx blocks apoptosis that is induced by the mitochondrial death pathway such as glucose deprivation, staurosporine, etoposide, and UV irradiation. Maiorino et al. [24] reported that PHGPx activity and the expression of mRNA for PHGPx in the rat testis was diminished by the administration of ethane dimethanesulfonate (EDS) as a Leydig cell-specific toxin. Moreover, Woolveridge et al. [48] reported recently that EDS accelerated the apoptosis of germ cells during the formation of rat pachytene spermatocytes and spermatids. We demonstrated the presence of PHGPx mRNA and of PHGPx itself in late spermatocytes and round spermatids by ISH*III* and by immunohistochemical staining in human testis. In rat and mouse testes, the expression of mitochondrial PHGPx is induced in late spermatocytes and round spermatids [22–24]. These results suggest the involvement of PHGPx in spermatogenesis and they also suggest that failed induction of expression of mitochondrial PHGPx as an anti-apoptotic factor in late spermatocytes might have a major effect on spermatogenesis.

An essential role for selenium in male fertility in rodents has been demonstrated. When selenium deficiency is severe, both numbers and motility of spermatozoa are reduced [49, 50]. Selenium-deficient spermatozoa have mitochondria with abnormal morphology, and the midpiece of spermatozoa is also abnormal. Under normal conditions, selenium is present at high levels in the mitochondrial capsule. Recently, Ursini et al. showed that in the rat, a major mitochondrial capsule selenoprotein is PHGPx [27]. They postulated that PHGPx might be a major structural protein in mature spermatozoa and that the distorted morphology of the midpiece of selenium-deficient spermatozoa might result from the absence of PHGPx as a major constituent of the mitochondrial capsule. In the present study, we observed no changes in the morphology of the midpiece and tail in human spermatozoa even when the level of expression of PHGPx was low (Figs. 3 and 6). The limited expression of PHGPx in spermatozoa from seven infertile males in the present study was not due to selenium deficiency because the level of expression of PHGPx in blood leukocytes of these infertile males was the same as that of fertile males (data not shown). Thus, it is likely that selenium deficiency affects the morphology of the midpiece of spermatozoa in a manner that is independent of a low level of PHGPx.

There have been, to our knowledge, no previous detailed measurements of the expression of antioxidant enzymes, including PHGPx, in the spermatozoa of infertile men. Our observations suggest an important role in male fertility for PHGPx in spermatozoa. Determination of the etiologies of male infertility is important for the development of effective therapies. The close correlation between PHGPx deficiency and severe male infertility suggests that PHGPx might be an important target in efforts to clarify the molecular mechanisms of male infertility.

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