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Male Fertility Is Linked to the Selenoprotein Phospholipid Hydroperoxide Glutathione Peroxidase[1]

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ABSTRACT

The selenoprotein phospholipid hydroperoxide glutathione peroxidase (PHGPx) accounts for almost the entire selenium content of mammalian testis. PHGPx is abundantly expressed in spermatids as active peroxidase but is transformed to an oxidatively inactivated protein in mature sperm, where it is a major constituent of the mitochondrial capsule in the midpiece. Male infertility in selenium-deficient animals, which is characterized by impaired sperm motility and morphological midpiece alterations, is considered to result from insufficient PHGPx content. We studied the relationship between sperm PHGPx, measured as rescued activity, and human fertility. Sperm specimens from 75 infertile men and 37 controls were analyzed for fertility-related parameters according to World Health Organization criteria. The PHGPx protein content was estimated after reductive solubilization of the spermatozoa by measuring the rescued PHGPx activity. Rescued PHGPx activity of infertile men ranged significantly below that of controls (93.2 ± 60.1 units/mg sperm protein vs. 187.5 ± 55.3 units/mg) and was particularly low in oligoasthenozoospermic specimens (61.93 ± 45.42 units/mg; $P < 0.001$ compared with controls and asthenozoospermic samples). Rescued PHGPx activity was correlated positively with viability, morphological integrity, and most profoundly forward motility ($r = 0.35$, 0.44, and 0.45, respectively). In isolated motile samples, motility decreased faster with decreasing PHGPx content. In humans, PHGPx appears to be indispensable for structural integrity of spermatozoa and to codetermine sperm motility and viability. Because the content of PHGPx, irrespective of the cause of alteration, is correlated with fertility-related parameters, PHGPx can be considered a predictive measure for fertilization capacity.

fertilization, gamete biology, male sexual function, sperm, spermatogenesis

INTRODUCTION

Phospholipid hydroperoxide glutathione peroxidase (PHGPx) is a selenoprotein [1] belonging to the family of glutathione peroxidases [2, 3]. PHGPx has long been considered a major antioxidant that, in cooperation with vitamin E, protects biomembranes [1, 4] and downregulates leukotriene biosynthesis [5], interleukin 1-induced signal transduction [6], and apoptosis [7, 8].

The particular relevance of PHGPx to male fertility was first suggested by gonadotropin-dependent abundance in rat testis [9] and further corroborated by in situ hybridization studies demonstrating maximal specific expression starting in round spermatids [10–12]. In testicular tissue, the PHGPx gene is expressed in three different ways. By alternate use of initiation codons [13], either a cytosolic protein is generated or the enzyme is targeted to the mitochondria by means of a N-terminal signal peptide [14]. Alternate splicing of the pre mRNA targets part of the PHGPx to the nucleus [15]. Taken together, these expression products account for most of the selenium content in testis and sperm of rodents. However, PHGPx activity is extremely low in mature rat sperm [16]. PHGPx protein devoid of enzymatic activity was identified as the major constituent of the keratin-like material that embeds the helix of mitochondria in the midpiece of spermatozoa [16]. From this material, PHGPx activity can be recovered upon solubilization by drastic reductive treatment [16].

In livestock and experimental rodents, selenium deficiency leads to alterations of spermatogenesis (reviewed in [17]). Moderate to severe selenium deficiency is characterized by impaired sperm motility and morphological alterations of the midpiece architecture, often resulting in disconnections of heads and tails. In extreme selenium deficiency, spermatogenesis is completely abrogated [18]. The discovery of the dual role of PHGPx, as an active peroxidase in spermatogenic cells and as a structural protein in spermatozoa, suggested new approaches to unravel the selenium dependency of male fertility. In early spermatogenesis, PHGPx as an active peroxidase may protect the rapidly dividing cells against oxidative injury or may even trigger specific differentiation processes. In late sperm maturation, facilitated by a redox switch that is accompanied by an almost complete loss of glutathione (GSH) [19, 20], the GSH peroxidase uses protein thiols as an alternative donor substrate and thereby becomes cross-linked with itself and other proteins to build up the mitochondrial capsule.

A first attempt to verify the inferred relevance of PHGPx to male fertility in humans has recently been reported by Imai et al. [21]. In that study, tiny amounts of active PHGPx that remain detectable in mature sperm were quantified and were correlated with fertility-related parameters. In the present investigation, we focused on the bulk of PHGPx in spermatozoa, i.e., the inactivated form contained in the capsule material, to assess its impact on sperm morphology and function.

MATERIALS AND METHODS

Patients

Seventy-five patients (mean ± SEM age = 33 ± 5 yr) who consulted the Center for Andrology of the University of Padova for infertility were enrolled in the study. The inclusion criteria were duration of infertility of

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TABLE 1. Seminal parameters and rescued PHGPx specific activity (mean ± SD) in spermatozoa.

Participants	n	Spermatozoa ($\times 10^6$ cells/ml)	Normal morphology (%)	Motility types (a + b) (%)	Viability (%)	PHGPx activity (mU/mg)
Controls	37	109.5 ± 111.3	57.5 ± 13.7	51.0 ± 12.1	81.2 ± 8.9	187.5 ± 55.3
Proven fathers	28	98.0 ± 77.7	58.2 ± 14.6	49.4 ± 12.0	81.8 ± 8.4	200.6 ± 54.5
Normozoospermic	9	145.2 ± 183.0	55.3 ± 10.7	57.1 ± 11.0	79.6 ± 10.5	146.7 ± 35.9
Infertile patients	75	45.8 ± 50.1*	46.0 ± 13.3**	26.0 ± 13.1**	74.2 ± 12.7**	93.2 ± 60.1**
Varicocele	31	53.1 ± 61.0	49.1 ± 11.2	29.4 ± 10.4	77.6 ± 7.6	103.9 ± 63.1**
Idiopathic	25	53.3 ± 42.4	44.8 ± 13.2	27.1 ± 12.6	73.8 ± 12.0	103.2 ± 61.5**
Cryptorchidism	12	30.5 ± 39.4	46.1 ± 15.7	27.0 ± 15.2	76.1 ± 9.5	76.3 ± 46.3**
Post-mumps orchitis	5	12.6 ± 11.2	33.2 ± 15.9	7.0 ± 9.5	59.2 ± 22.7	45.9 ± 30.3**
Testicular trauma	2	15.0 ± 19.8	44.0 ± 14.1	160 ± 22.6	52.5 ± 33.2	22.7 ± 2.91**

*$P < 0.01$.
**$P < 0.001$ vs. controls.

at least 2 yr, absence of prior or concomitant serious illnesses, and no consumption of medications during the 3 mo prior to the study. Absence of seminal infections was evaluated by sperm culture, and the presence of sperm antibodies was excluded by a Sperm-Mar test (Ortho Diagnostic System, Milan, Italy). Thirty-one patients were affected by left varicocele, 12 had undergone monolateral orchidopexy at an age of 2–9 yr, 5 had a history of post-mumps orchitis, and 2 had experienced testicular trauma. Pathogenesis of infertility remained unknown in the other 25 patients.

Thirty-seven healthy donors (age = 34 ± 3 yr) were chosen as controls. Among these, 28 were fertile proven fathers and the other 9 were normozoospermic men whose fertility remained unproven. Nine of the fertile proven fathers were asthenozoospermic, according to the criteria listed below.

The Ethical Committee of the Medical Faculty of the University of Padova approved the study, and informed consent was obtained from each participant.

Semen Processing

Semen samples were obtained by masturbation after 3–5 days of sexual abstinence. After liquefaction, seminal volume and pH, sperm concentration, motility, morphology, and viability (by exclusion of red eosin) were evaluated according to World Health Organization (WHO) guidelines [22]. Sperm motility of the native samples was determined by the same person microscopically at 400-fold magnification after 30 min. Motility was assigned to the following categories: rapidly motile (type a), moderately motile (type b), and immotile (type c). Spermatozoa were considered normal if there were no defects of the head (length, 4.0–5.5 μm; breadth, 2.5–3.3 μm; shape, oval; length:breadth, 1.5–1.75; acrosome easily distinguishable), the neck, midpiece, tail, or center part. For each sample, at least five microscopic fields were assessed to classify 200 Papanicolau-stained spermatozoa. The inclusion criteria for classification as asthenozoospermic or oligoasthenozoospermic were motility (types a and b) of <50% or motility of <50% plus count of $<20 \times 10^6$ sperm/ml.

Seminal volume and pH were in the normal range both in control and infertile participants (seminal pH between 7.6 and 8.2; semen volume between 2.0 and 4.3 ml).

In Vitro Aging of Spermatozoa

For this experiment, sperm samples containing a broad range of rescued PHGPx specific activity were selected. These samples were obtained from ejaculates. Motile spermatozoa were then isolated by the swim-up technique, as previously described [23], and the 100% motile forms (types a and b) were incubated at 36.8°C in 21 mM Hepes buffer, pH 7.4, for 24 h in an oscillating water bath. Motility was determined again after 12 and 24 h of incubation.

After the experiment, samples were also reevaluated for rescued PHGPx specific activity, and there was no change. Semen analysis revealed that the donors were asthenozoospermic, in agreement with the observation that this subgroup exhibits the widest range of PHGPx activity.

Measurement of Rescued PHGPx Activity

Reactivation of insoluble PHGPx and assessment of rescued PHGPx activity was essentially performed as previously described [24]. Ejaculates were diluted with PBS and centrifuged at $600 \times g$. The pellet, which contained spermatozoa, was washed with PBS and stored at −20°C for up to 1 wk. Pellets were resuspended at 4°C to reach a protein concentration of approximately 0.5 mg/ml and were dissolved in 0.1 M Tris-HCl, 6 M guanidine-HCl, pepsatin A (0.5 μg/ml), leupeptin (0.7 μg/ml), and 0.1M 2-mercaptoethanol, pH 7.5. Before activity measurement, mercaptoethanol and guanidine-HCl were removed by passing the sample twice through an NAP-5 column (Pharmacia, Upsala, Sweden) equilibrated with test buffer (0.1 M Tris-HCl, pH 7.5, containing 3 mM GSH, 5 mM EDTA, and 0.1 % (v/v) Triton X-100). PHGPx activity was then measured spectrophotometrically at room temperature as previously described [24] with 0.04–0.08 mg protein of the eluted samples. Phosphatidylcholine hydroperoxide (40 μM) was used as a specific PHGPx substrate. Activity is given in milliunits (mU), defined as nmoles of the hydroperoxide consumed per minute.

Statistical Analysis

Group differences were assessed by the Student *t*-test, and correlations were evaluated by linear regression. The results are given as mean ± SD. *P* values of <0.05 and <0.01 were regarded as significant and highly significant, respectively.

RESULTS

As previously reported for rat spermatozoa [16], active PHGPx is present in extremely small amounts in human sperm (<5 mU/mg) when compared with the PHGPx protein in the mitochondrial capsule, which can only be assessed by activity measurement after a reductive rescuing procedure. In sperm samples of healthy human volunteers, the rescued PHGPx activity averages near 200 mU/mg protein. With the amount of sperm protein applied to the test according to the standard procedure used [24], PHGPx activity persisting in mature spermatozoa was within the limits of experimental error and was therefore ignored.

Table 1 summarizes seminal parameters and rescued PHGPx activity in sperm of infertile participants and controls. In the infertile group, rescued PHGPx activity of 93.2 ± 60.1 mU/mg was markedly ($P < 0.001$) lower than that in the control group (187.5 ± 55.3 mU/mg). The low PHGPx content in infertile group was associated with lower sperm count ($P < 0.01$), a higher percentage of morphological alterations ($P < 0.001$), and impaired sperm motility ($P < 0.001$). The pathogenesis underlying the fertility problem did not appear to affect the general trend. Despite small sample sizes for some etiological subgroups, the PHGPx content was much lower than the value in healthy controls ($P < 0.001$).

Impaired sperm motility was inversely correlated with PHGPx content when all samples including controls were considered (Fig. 1, $r = 0.45$). When the participants were classified into normozoospermic, asthenozoospermic, and oligoasthenozoospermic, low PHGPx content was significantly associated with both diagnostic subgroups and particularly pronounced in oligoasthenozoospermic patients

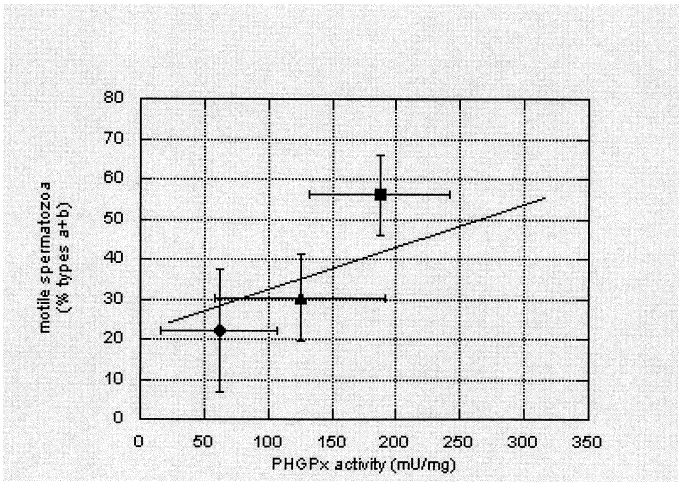

FIG. 1. PHGPx is related to the percentage of motile spermatozoa. The mean ± SD value of rescued PHGPx activity is plotted vs. mean ± SD percentage of motile spermatozoa in normozoospermic (■), asthenozoospermic (▲), and oligoasthenozoospermic (♦) men. The groups were significantly different based on the combination of the two parameters: $P < 0.001$ for normozoospermic vs. asthenozoospermic and oligoasthenozoospermic; $P < 0.05$ for asthenozoospermic vs. oligoasthenozoospermic. The regression line was calculated from all data (n = 112; $r = 0.45$).

(Fig. 1). PHGPx content in spermatozoa was also associated with a progressive loss of motility with time. Motile asthenozoospermic spermatozoa isolated by the swim-up technique [23] only moderately lost motility after 12 h and 24 h of incubation at 36.8°C when their PHGPx content was nearly normal, whereas the percentage of motile spermatozoa left over after 12 and 24 h fell to <30% or zero, respectively, if the PHGPx content was at the lower limit of the range (<60 mU/mg) (Fig. 2).

Morphological integrity similarly declined with decreasing PHGPx content ($r = 0.44$). Again, PHGPx activity and percentage of normal spermatozoa was less decreased in

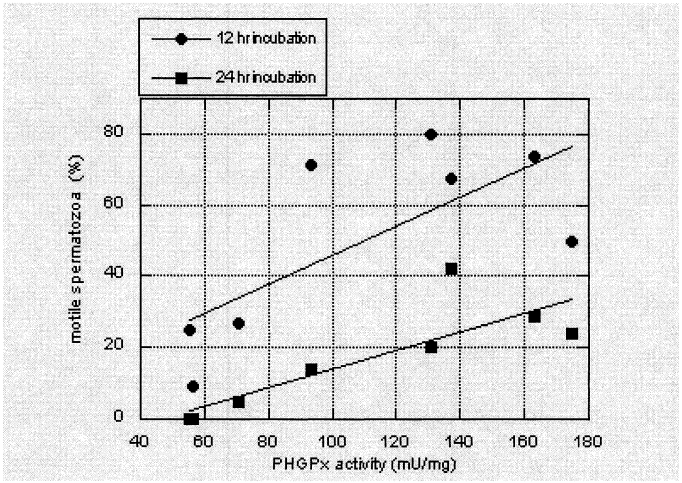

FIG. 2. Motility of in vitro incubated spermatozoa decreases as a function of rescued PHGPx activity. Motile spermatozoa were isolated by the swim-up tecnique and incubated for 12 and 24 h at 36.8°C. Motility (which was 100% at the beginning of experiment for all samples) was reassessed and plotted against the rescued PHGPx specific activity of each sperm sample. Rescued PHGPx activity was evaluated at the beginning and the end of experiment and was unchanged. Two samples with identical activity (the lowest value in the figure) completely lost motility after 24 h and thus overlap at the 0% motility point at 24 h ($r = 0.72$ and 0.84 for 12 and 24 h of incubation, respectively).

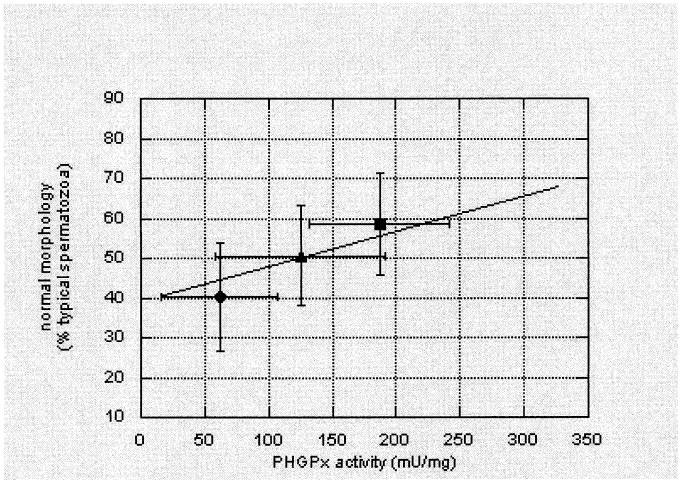

FIG. 3. PHGPx is related to normal morphology of spermatozoa. The mean ± SD rescued PHGPx activity is plotted against mean ± SD percentage of typical forms in normozoospermic (■), asthenozoospermic (▲), and oligoasthenozoospermic (♦) men. The groups were significantly different based on the combination of the two parameters: $P < 0.05$ for normozoospermic vs. asthenozoospermic; $P < 0.001$ for normozoospermic vs. oligoasthenozoospermic; $P < 0.005$ for asthenozoospermic vs. oligoasthenozoospermic. The regression line was calculated from all data (n = 112; $r = 0.45$).

asthenozoospermic samples ($P < 0.05$) than in oligoasthenozoospermic samples ($P < 0.001$) (Fig. 3). Altered neck morphology was prominent in the infertile population but did not differ significantly between the diagnostic subgroups (data not shown).

Viability was correlated with PHGPx content, but not as clearly. The positive correlation ($r = 0.34$) was primarily determined by highly scattered but mostly low values of viability in samples of extremely low PHGPx content. Thus, there is obviously not a simple relationship between viability and PHGPx content. Below 90 mU/mg, the percentage of viable spermatozoa dropped markedly. Accordingly, comparisons with controls were significant ($P < 0.01$) only for the oligoasthenozoospermic group (Fig. 4).

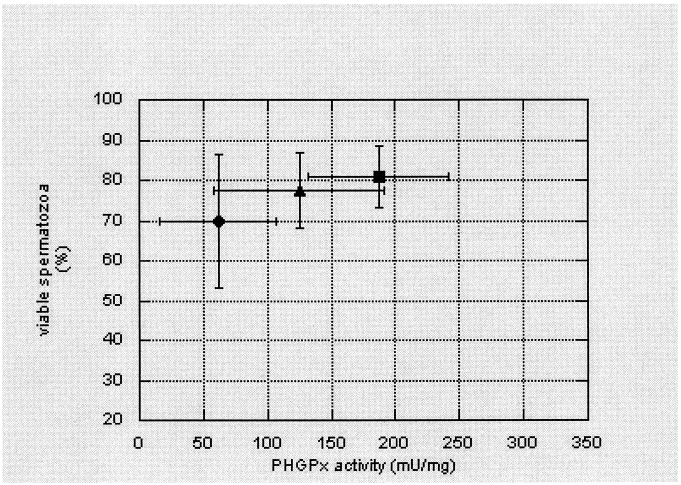

FIG. 4. PHGPx is related to viability of spermatozoa. The mean ± SD rescued PHGPx activity is plotted against mean ± SD percentage of viable spermatozoa in normozoospermic (■), asthenozoospermic (▲), and oligoasthenozoospermic (♦) men. The normozoospermic group was significantly different based on the combination of the two parameters from the oligoasthenozoospermic group ($P < 0.01$).

DISCUSSION

As in rodents [16], the vast majority of PHGPx in human spermatozoa were enzymatically inactive without prior reductive solubilization. High contents of enzymatically inactive PHGPx associated with marginal levels or absence of soluble active PHGPx have also been observed in sperm of bulls, pigs, horses, and dogs (unpublished data). In contrast, low PHGPx activity and no evidence of activatable PHGPx protein was obtained with sperm of nonmammalian vertebrates such as birds and fishes (unpublished data). Thus, only in mammalian sperm does PHGPx adopt the peculiar function of a structural protein. As such, it appears essential to build up the mitochondrial capsule, again a peculiarity of mammalian spermatozoa. PHGPx is the mitochondrial capsule selenoprotein (MCS), a term that was erroneously attributed to a sperm-mitochondria-associated cysteine-rich protein (SMCP) until cloning and sequencing of the SMCP revealed that it lacks selenium [25]. Accordingly, the sperm alterations, such as impaired motility, fuzzy appearance of the midpiece, and loss of tails, observed in selenium deficiency of livestock and experimental rodents that had previously been attributed to inadequate MCS content [18] likely resulted from impaired PHGPx biosynthesis. The correlation of PHGPx content of spermatozoa with sperm motility and structural integrity demonstrated here for clinical samples agrees with the presumed pivotal role of the selenoprotein in guaranteeing correct midpiece architecture.

The impact of PHGPx on sperm viability is less easily explained. An antioxidant function, widely discussed to be mandatory to protect sperm against abundant oxidants [7, 21, 26], cannot reasonably be attributed to PHGPx in mature spermatozoa. The overwhelming proportion of sperm in the capsule are inactive [16], the nuclear variant is cross-linked to protamine [15], and the residual active enzyme [21] cannot work as an antioxidant system because it lacks the major reducing substrate. An almost complete loss of GSH during final sperm maturation has been amply documented [19, 20], and exposed protein SH groups, which may be considered alternative substrates [15, 27, 28], are largely oxidized in epididymal spermatozoa [20]. An antioxidant role for PHGPx can, however, be envisioned for spermatogenic cells, and because of low levels of the active peroxidase during final stages of spermatogenesis, oxidative injuries might accumulate and lead to delayed impairment of viability.

Further, the widely discussed antiaptoptotic effect of PHGPx [6–8, 29], relevant to sperm viability, is involved. Spermatozoa may be considered cells ''sentenced to death'' because of extensive ubiquitination of their mitochondrial surface [30]. This proapoptotic signal is, however, shielded by the mitochondrial capsule and physiologically is unmasked after fertilization in the oocyte, where it possibly triggers proteasomal destruction of male mitochondria [31]. Low PHGPx activity during spermatogenesis might enhance apoptotic programming, leading to decreased viability in mature sperm. More importantly, the ubiquitin link to the mitochondrial surface would no longer be hidden in severely disturbed mitochondrial capsules because of inadequate PHGPx content, and the apoptotic process might be initiated prematurely in the spermatozoon.

In contrast to these phenomena, PHGPx as the catalyst and raw material for the formation of the mitochondrial capsule cannot possibly explain the association between low PHGPx content and oligospermia. Instead, we postulate a stimulatory effect of active PHGPx in early germ cells on the proliferative activity of the germ epithelium. This dual role of PHGPx in spermatogenesis is also supported by inverse genetics in mice [28]. Testicular tissue hemizygous for PHGPx displayed the expected defects of the mitochondrial capsule and a completely disorganized germ epithelium with very few advanced spermatogenic cells. How the peroxidatively active PHGPx regulates early spermatogenesis remains unknown.

The underlying causes of PHGPx deficiency in male infertility were not specifically addressed in this investigation. Selenium deficiency as a potential cause is not easily assessed because testicular selenium is not closely correlated with alimentary supply or selenium levels in easily accessible tissue. Testis, like the thyroid and the brain, tends to retain normal selenium levels even under conditions of moderate selenium deficiency [3]. PHGPx ranks high in the hierarchy of selenoproteins, which means it is still kept at normal levels when selenoproteins such as GPx-1 decline markedly because of selenium deficiency [3, 32]. Thus, sporadic shortages of selenium, as could be envisaged in a southern European population, would not be the most likely condition leading to inadequate PHGPx content of sperm. In support of this suggestion, Imai et al. [21] reported that the soluble PHGPx in infertile sperm was not correlated at all with levels in blood WBCs. Genetic defects may be suspected but also do not appear to be the prominent cause. A still ongoing screen of the PHGPx genes of our patients so far yielded a point mutation resulting in a guanine to adenine exchange of still unverified functional relevance in one case and single base polymorphisms of questionable importance (unpublished). Testicular PHGPx biosynthesis may be affected by any disturbance of the complex machinery of selenoprotein biosynthesis, which is regulated in a tissue-specific manner by largely unknown mechanisms [3]. Testosterone-dependent differentiation into spermatids is associated with PHGPx expression [10]. Thus, any disturbance of spermatogenesis should ultimately result in both low sperm count and insufficient sperm PHGPx content. This hypothesis is consistent with the low PHGPx content observed in 50 of our patients displaying infertility of plausible etiologies unrelated to selenium deficiency.

The multiple causes of impaired biosynthesis of PHGPx and its pivotal function in mature spermatozoa suggest that PHGPx is a global marker of the fertilization capacity of sperm. The reductive rescuing procedure [24], as used in this investigation, is at present considered the most reliable way to assess the total PHGPx content in sperm, although a less tedious analytical approach would be desirable. Further investigations are needed to validate the prognostic value of PHGPx assessment. However, the correlations of PHGPx content with established prognostic parameters appear to justify the effort.

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