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Selenoprotein P in seminal fluid is a novel biomarker of sperm quality

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ABSTRACT

Hepatically-derived selenoprotein P (SePP) transports selenium (Se) via blood to other tissues including the testes. Male *Sepp*-knockout mice are infertile. SePP-mediated Se transport to Sertoli cells is needed for supporting biosynthesis of the selenoenzyme glutathione peroxidase-4 (GPX4) in spermatozoa. GPX4 becomes a structural component of sperm midpiece during sperm maturation, and its expression correlates to semen quality. We tested whether SePP is also present in seminal plasma, potentially correlating to fertility parameters. Semen quality was assessed by sperm density, morphology and motility. SePP was measured by an immunoluminometric assay, and trace elements were determined by X-ray fluorescence spectroscopy. SePP levels were considerably lower in seminal plasma as compared to serum (0.4 ± 0.1 mg/l vs. 3.5 ± 1.0 mg/l); Se concentrations showed a similar but less pronounced difference (48.9 ± 20.7 μg/l vs. 106.7 ± 17.3 μg/l). Se and Zn correlated positively in seminal fluid but not in serum. Seminal plasma SePP concentrations were independent of serum SePP concentrations, but correlated positively to sperm density and fraction of vital sperm. SePP concentrations in seminal plasma of vasectomized men were similar to controls indicating that accessory sex glands are a testes-independent source of SePP. This notion was corroborated by histochemical analyses localizing SePP in epithelial cells of seminal vesicles. We conclude that SePP is not only involved in Se transport to testes supporting GPX4 biosynthesis but it also becomes secreted into seminal plasma, likely important to protect sperm during storage, genital tract passage and final journey.

1. Introduction

Selenium (Se) is an essential micronutrient required for the biosynthesis of 25 human gene products containing selenocysteine in their primary sequences [1]. Se is needed for normal spermatogenesis [2] and a preferential supply of testes with the trace element is established in mammals [3,4]. Testes express a number of selenoproteins including phospholipid hydroperoxide–glutathione peroxidase (PH-GPX, GPX4) which is an essential enzyme [5]. Testicular GPX4 expression is stringently regulated by gonadotropins [6]. It undergoes a functional metamorphosis during spermatogenesis from an active selenoenzyme into a cross-linked structural component of mature spermatozoa stabilizing the midpiece region [7]. The nuclear form of GPX4 undergoes a similar cross-linking reaction and is involved in sperm DNA condensation [8]. Accordingly, single nucleotide polymorphisms (SNP) in *GPX4* are associated with male fertility [9]. GPX4 activity can be re-constituted from sperm and correlates to sperm viability, morphological integrity and forward motility [10]. Low levels of immunoreactive GPX4 in sperm are associated with poor male fertility [11]. These findings concur to transgenic mouse models, as male infertility resulted from the genetic inactivation of mitochondrial *Gpx4* or from spermatocyte-specific ablation of *Gpx4* [12,13]. The importance of GPX4 genotype and sperm GPX4 concentrations for male fertility is thus firmly established.

Selenoprotein P (SePP) is a second selenoprotein implicated in male fertility [14,15]. Male mice with genetic *Sepp* inactivation (*Sepp*-ko) are infertile [16,17]. Sperm cells of *Sepp*-ko mice display structural defects including a characteristic hairpin-like kink at the midpiece-principal piece junction indicating Se deficiency and insufficient Gpx4 expression [18,19]. Expression of a human SePP transgene in liver of *Sepp*-ko mice restores sperm structure and male fertility [19]. Accordingly, SePP is considered to serve a Se transport function from liver to testes supporting testicular GPX4 biosynthesis and spermatogenesis. This notion has been

Abbreviations: GPX, glutathione peroxidase; Se, selenium; SePP, selenoprotein P.

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corroborated by X-ray fluorescence microscopy of trace element concentrations during spermatogenesis in transgenic mice [20]. *Sepp* mRNA is expressed in Leydig cells potentially affecting androgen biosynthesis [21]. The low density lipoprotein receptor-related protein 8 (apolipoprotein E receptor 2, ApoER2) has been identified as a testicular Sepp-receptor implicated in Sepp uptake and Se-dependent spermatogenesis [22]. ApoER2 is expressed in Sertoli cells thus ideally located to supply the growing spermatids with Se [22]. A biochemical sequence of transport events can be deduced; Se supply of testes originates in liver where dietary Se is converted into SePP. Secreted SePP travels the circulation and becomes taken up by Sertoli cells via ApoER2 providing growing spermatids with Se needed for GPX4 biosynthesis.

However, also sperm-free seminal plasma contains considerable Se levels [23]. In humans, concentrations are 2–3-times lower than in serum, whereas in bulls, seminal Se can exceed serum Se concentrations by one order of magnitude [24]. Seminal Se increases in response to Se supplementation while sperm Se content remains constant indicating different regulatory mechanisms [25]. GPX4 accounts for the major fraction of Se in testes and mature sperm [15], but its presence and activity in seminal plasma is marginal [26]. Since SePP controls transport of Se to Sertoli cells, we hypothesized that SePP is found in seminal plasma as a convenient biomarker of testicular Se supply. This hypothesis proved only partially correct. SePP was indeed identified in seminal plasma and correlated to sperm quality. Surprisingly, SePP concentrations were normal in seminal plasma of vasectomized men indicating that SePP was not derived from testes. These findings suggest a second role for SePP in male fertility, unrelated to its transport function.

2. Materials and methods

2.1. Human serum, seminal plasma and tissue samples

Human serum and seminal plasma samples were obtained from men visiting the Department of Urology, University Hospital Charité, for diagnosis of fertility, and from healthy volunteers. Semen analyses were carried out using established standard criteria at the Department of Urology at Charité Berlin according to WHO guidelines (4th edition, 1999). Semen samples were evaluated with respect to sperm density, motility, morphology, and vitality as well as density of round cells and leukocytes. The analyses were conducted according to the Declaration of Helsinki and had been approved by the local ethics committee.

2.2. Western blot analyses

SePP expression was analyzed by Western Blot analysis using SePP-specific sheep antisera as described [27,28]. Briefly, aliquots of human serum (0.05 μl) and seminal plasma (0.5 μl) were separated by 10% SDS–PAGE and blotted onto Protran nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Membranes were stained with Ponceau Red in order to verify uniform loading and complete protein transfer. Unspecific binding was blocked by incubation in 5% BSA. Primary antibodies were applied over night at 6 μg/ml followed by donkey-anti-sheep antibodies (45 min, 1:5000, Serva, Germany). Development of signals was achieved with the ECL system (Amersham, Braunschweig, Germany).

2.3. Protein precipitation analyses

Protein precipitations were performed using trichloric acid (TCA). Briefly, one volume of TCA (50%) was added to four volumes of sample and incubated at 4 °C for 20 min. After centrifugation at 15,000 rpm supernatants were removed and pellets were washed twice with ice-cold acetone (−20 °C). Pellets were dried at room temperature and then solubilized in four volumes of HNO_3 (30%).

2.4. Quantification of SePP and analysis of trace element concentrations

SePP-quantification was performed in duplicates using an immunoluminometric assay as described [27]. Stability of SePP in seminal plasma was determined with respect to incubation time and freeze–thaw cycles using fresh samples from healthy donors. Trace elements were measured using total-reflection X-ray fluorescence (TXRF) spectroscopy via a benchtop TXRF device (Picofox S2, Bruker-nano, Berlin, Germany). Samples were diluted 1:2 using HPLC-H_2O. Gallium was added as internal standard. Seronorm (Level T2) standard serum (SERO AS, Billingstad, Norway) was used for quality control, and intra- and inter-assay variations were <15% for samples containing >10 μg Se/l.

2.5. Immunohistochemical analysis

Routinely sampled and paraffin-embedded tissue samples of seminal vesicles were immunostained with a monoclonal mouse anti-SePP antibody (MAB0761, dilution 1:1400, Abnova GmbH, Heidelberg, Germany). Antigen retrieval was achieved by boiling in citrate buffer at pH 8.0 for 15 min. Endogenous peroxidases were blocked by incubation in a 1% solution of hydrogen peroxide for 15 min. Antibody incubation and further processing was done by Ventana immunostainer Bench Mark XT system (Ventana Medical Systems Inc., USA) using routine immunohistochemistry protocols.

2.6. Statistical analysis

Data sets were verified for normal distribution using the Kolmogorov–Smirnov test. One-way ANOVA was used for variance analyses, Bonferroni's post hoc test for detecting significant differences using GraphPad Prism 4 software. Data are expressed as mean + SD. Statistical significance was defined as $p < 0.05$ (∗), $p < 0.01$ (∗∗) or $p < 0.001$ (∗∗∗). Associations between semen parameters were analyzed by Spearman's rank-order correlation.

3. Results

3.1. Identification and stability of SePP in seminal plasma

SePP isoforms in human blood and seminal plasma showed a similar migration pattern, however some additional faster migrating bands were present in seminal samples (Fig. 1A). Seminal plasma SePP proved stable over a time period of up to 24 h at 4 °C. At room temperature, some loss was observed after 7 h (Fig. 1B). Seminal plasma SePP was unaffected by up to four freeze–thaw cycles (Fig. 1C). These results indicate that SePP represents a relatively stable component of human seminal plasma.

3.2. Comparison of Se and SePP concentrations in serum and seminal plasma

SePP concentrations were quantified in matched serum and seminal plasma samples of control and vasectomised men. SePP concentrations were almost an order of magnitude lower in seminal plasma than in the corresponding serum samples (Fig. 2A). SePP and Se concentrations in seminal plasma of men who had undergone vasectomy prior to sampling were comparable to control samples (Fig. 2A and B). When samples were treated with TCA to precipitate selenoproteins from small selenocompounds,

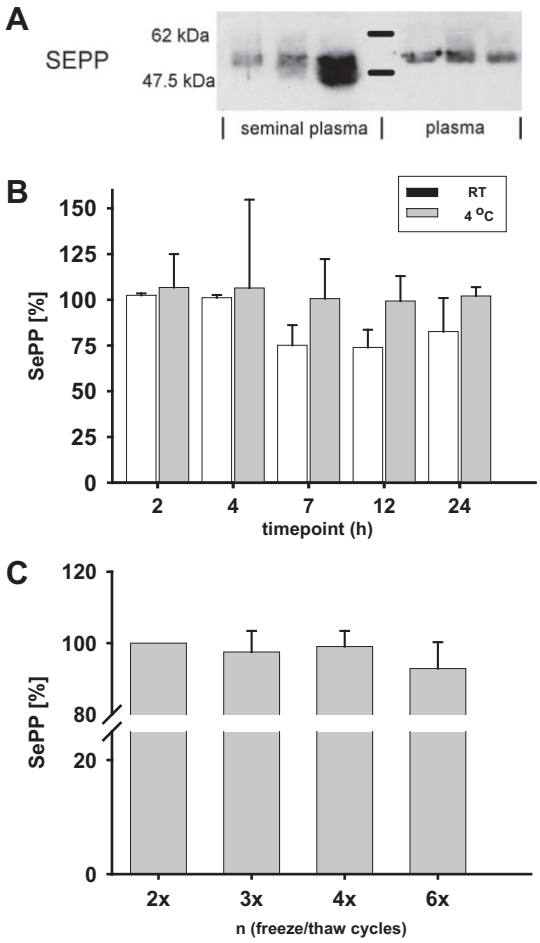

Fig. 1. Characterization of SePP in seminal plasma. (A) Serum and seminal plasma samples from healthy men showed a similar immunoreactive band pattern. Seminal plasma samples contained additional smaller SePP fragments not present in serum. (B) Stability of SePP in seminal plasma was tested at room temperature (RT) and 4 °C. SePP proved stable at 4 °C, but was partially lost by incubation at RT for incubation periods >4 h ($n = 4$). (C) Stability of SePP in seminal plasma was assessed as a function of freezing and thawing. SePP was stable for four consecutive freeze–thaw cycles ($n = 4$ independent samples).

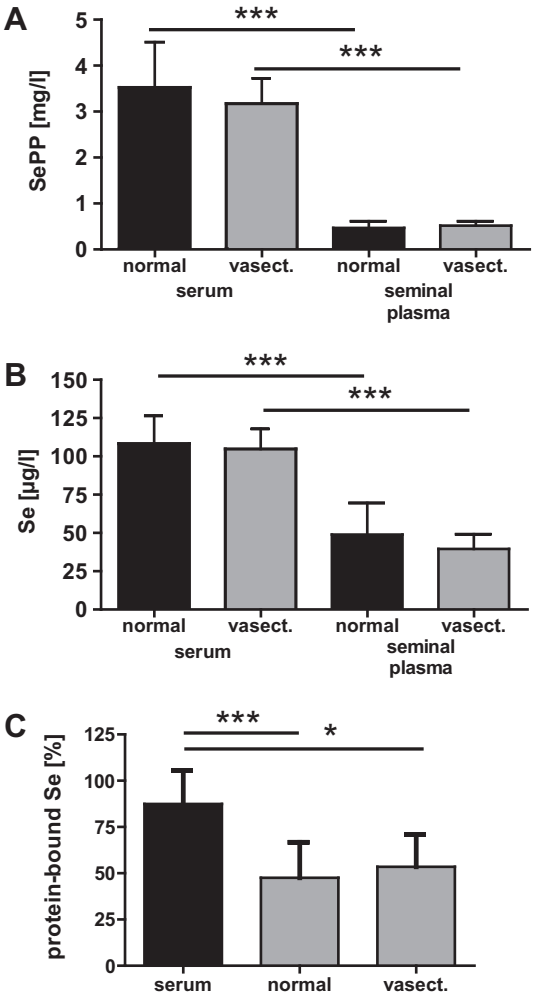

Fig. 2. Comparison of Se and SePP in human serum and seminal plasma. (A) Serum and seminal plasma samples were analyzed from normal ($n = 18$) and vasectomized ($n = 4$) men. SePP concentrations were on average seven times higher in serum as compared to seminal plasma samples. (B) In comparison, Se concentrations were only twice as high in serum as compared to seminal plasma. (C) Samples were treated with TCA and centrifuged to separate selenoproteins from small selenocompounds. In serum ($n = 12$), 90% of Se was associated with TCA-precipitated proteins. In seminal plasma of healthy ($n = 8$) and vasectomized ($n = 4$) men, TCA treatment precipitated only around 50% of Se. Bars indicate mean (+ SD).

around 90% of total Se was precipitated from serum. This is in agreement with former studies in humans [29] and mice [30]. In contrast, seminal plasma yielded two fractions with equally high Se concentrations indicating a relatively high abundance of small selenocompounds (Fig. 2C). The pattern of Se-containing molecules thus differs considerably between serum and seminal plasma samples.

3.3. SePP immunoreactivity in seminal vesicles

The findings above indicate that SePP is expressed within the male reproductive tract and becomes secreted into the seminal fluid. Two endocytic receptors have been described to bind SePP and mediate Se-uptake, i.e., ApoER2 (LRP8) and megalin (LRP2). ApoER2 is expressed in testes located at the basolateral membrane of Sertoli cells [22], whereas megalin is strongly expressed in seminal vesicles [31]. From our analyses, we excluded the testes as the major source of seminal plasma SePP and rather favored the seminal vesicles. An immunohistochemical analysis supports this notion as positive SePP immunostaining is detected on epithelial cells, specifically in budding vesicles derived thereof, implying active secretion by seminal vesicles (Fig. 3). A potential route of megalin-mediated SePP uptake from the circulation into seminal vesicles as necessary prerequisite for Se accumulation needed for de novo SePP biosynthesis and secretion can thus be envisaged.

3.4. Associations of selenium, zinc and SePP concentrations in serum and seminal plasma

Trace elements in matched serum and seminal plasma samples were measured in order to characterize their relation to SePP in these matrices. Zn concentrations were 143.6 ± 95.6 mg/l in seminal fluid and 1.6 ± 0.3 mg/l in serum samples. In comparison, Se concentrations were only 0.11 ± 0.02 mg/l in serum and 0.05 ± 0.02 mg/l in seminal plasma samples. There was a strong correlation of Se and Zn in seminal plasma (Fig. 4A) but not in serum (not shown). Se and SePP concentrations correlate in human serum over a large concentration range until SePP expression becomes saturated [32]. In seminal plasma, a similar positive correlation between Se and SePP is observed (Fig. 4B). In contrast, there was no significant correlation of SePP concentrations between serum and seminal plasma samples. These findings indicate that the Se status in both compartments is actively controlled and that

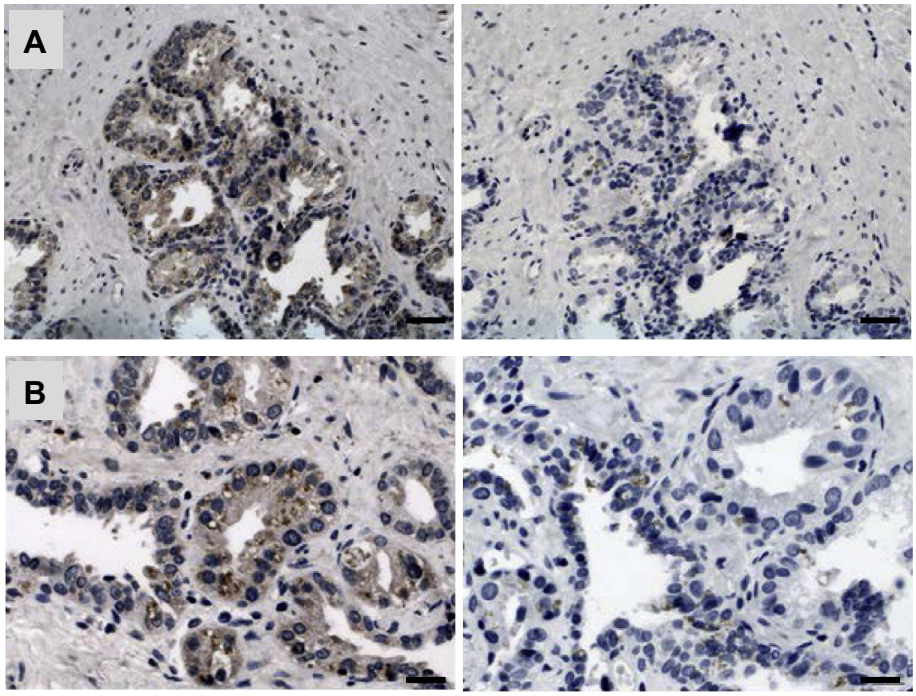

Fig. 3. SePP immunoreactivity in seminal vesicles. Seminal vesicle tissue specimen were analyzed for SePP expression by a SePP-specific antiserum. (A) Dark brownish SePP expression was evident in the stained sections (left) but not in control sections (right). (B) Immunoreactive SePP localized to secretory epithelial cells facing the seminal vesicle lumen. The pale granular signals in the control section (right) are due to unspecific staining of the typical lipofuscin pigment of seminal vesicles. The bar indicates 50 μm (top) and 20 μm (bottom), respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

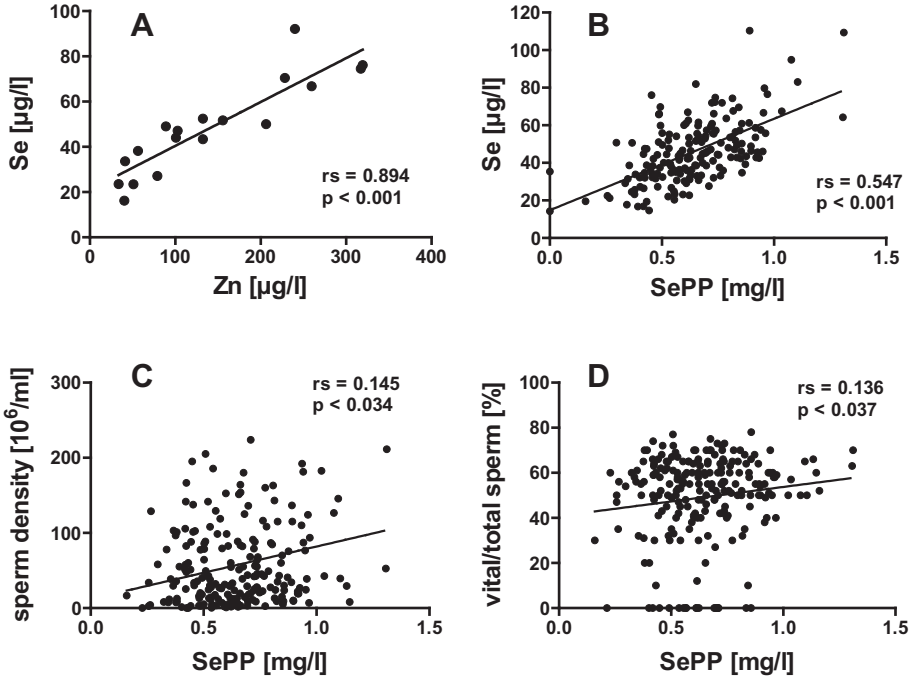

Fig. 4. Associations of seminal plasma Se, Zn and SePP with sperm parameters. (A) Se and Zn concentrations and (B) Se and SePP concentrations correlate positively in seminal plasma of healthy men. (C) Sperm density and (D) the fraction of vital sperm positively correlate to seminal plasma SePP concentrations. Significance and Spearman correlation coefficients are indicated.

SePP is not diffusing, filtered or passively transported from serum into seminal plasma.

3.5. Associations of seminal SePP with fertility parameters

Routine semen analyses were performed in samples from adult men ($n = 244$) visiting the Urology Department for diagnostics of fertility. SePP was quantified in the respective seminal plasma samples. SePP concentrations showed a positive correlation to sperm density (Fig. 4C) and to the fraction of vital sperm (Fig. 4D). Total sperm concentrations, sperm motility or morphological alterations (sperm with cytoplasmic droplets, round-headed sperm, head, midpiece or flagellum structure anomalies) were not significantly associated with seminal plasma SePP

concentrations. These results imply that SePP positively interacts with sperm during storage and on their journey supporting their vitality.

4. Discussion

The relation of GPX4 with male fertility presents as an exciting and puzzling story of hypotheses and errors, until it was established as a protein with moonlighting activities first protecting sperm as an active selenoenzyme before becoming a structural component in mature sperm [7,15]. However, two notions argue against the idea that GPX4 represents the major factor linking nutritional Se supply to male fertility. Firstly, there is a privileged supply of Se to testes as part of a hierarchy among the different tissues whereby essential organs are preferred in times of deficiency [33,34]. And secondly, there is also a hierarchical order of selenoprotein biosynthesis prioritizing essential enzymes like GPX4 [35,36]. Both mechanisms ensure that sperm maturation does not stringently depend on the daily Se intake; otherwise sperm quality and fertility of men would differ strongly between populations with sufficient or poor Se status, e.g., between Americans (ample Se intake) versus most Asian, European or African men (marginal Se supply). This interpretation is in line with the data from experimental animals and humans alike indicating that under normal conditions, testes Se is unrelated to Se intake [25]. A second Se-dependent pathway can thus be hypothesized relating Se with male fertility, translating supplemental Se into improved fertility in strongly Se-deficient men [37].

At present, SePP is mainly considered as the endogenous Se transporter supplying privileged tissues with the essential trace element. Functional insights were provided by the analyses of *Sepp*-ko mice as they presented with male-specific infertility [16,17] which was rescued by expression of a human SePP transgene in liver [19]. The identification of ApoER2 as the SePP-receptor of Sertoli cells seemed to close the chapter on the physiological relevance of SePP for male fertility [22]. A picture emerged in which dietary Se is converted by hepatocytes into tissue-available SePP and then taken up via testicular ApoER2 supporting GPX4 biosynthesis during spermatogenesis. The nature and origin of Se in seminal plasma remained an unresolved issue.

This study points to a second role of SePP in male fertility as it is found in seminal plasma contributing significantly to its Se concentration. Importantly, seminal SePP was unrelated to serum SePP concentrations suggesting that it is an actively added constituent. Seminal plasma SePP correlated positively with sperm density and percentage of vital sperm, but not with total sperm count, sperm with altered morphology or sperm motility. This pattern is profoundly different to findings related to testes GPX4, which correlates to motility, count and structural integrity of sperm [10]. Seminal plasma SePP thus fulfills an unrelated function as compared to serum SePP and sperm GPX4. This interpretation is corroborated by normal SePP concentrations in seminal plasma of vasectomized men indicating an origin in accessory sex glands. The anatomical sequence along with the course of molecular events suggest that seminal plasma SePP is not involved in GPX4 biosynthesis, chromatin condensation or flagellum formation, but rather in protection of sperm and seminal fluid from damage in view of its powerful antioxidative and scavenging activities against phospholipid hydroperoxides and peroxynitrite-mediated oxidation [38,39].

Seminal plasma SePP may hereby increase the fraction of vital sperm being ejaculated in face of the high levels of reactive oxygen species from sperm internal metabolism and external sources [40]. Alternatively, it might preserve the structural integrity of the accessory sex glands [41] or protect seminal plasma proteins involved in transport of spermatozoa and elimination of damaged sperm [42]. Such positive protection has been observed in respective clinical trials involving Se and/or *N*-acetyl cysteine [43] or other antioxidants [44]. Further studies will be needed to test the precise contribution of SePP for sperm protection and whether seminal SePP concentrations correlate to fertility success being positively influenced by respective supplementation attempts. Quantitative analysis of seminal plasma SePP might help to identify subfertile men in need of Se or antioxidant treatment in order to improve their sperm quality and fertility odds. The observed stability of SePP in seminal plasma supports its suitability as a novel and suitable biomarker in such attempts.

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