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Biological Significance of Glutathione S-Transferases in Human Sperm Cells

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Abstract

INTRODUCTION

*O*xidative damage to the sperm membranes leads to reduce ejaculate quality and has been considered as one of the causes of male subfertility or infertility.[1,2] It is well recognized that oxidative stress is induced by reactive oxygen species (ROS) and/or reactive nitrogen species (RNS). Specifically, high concentrations of ROS/RNS alter sperm membranes fluidity and integrity due to lipid peroxidation and decrease sperm motility, viability, and fertilization ability.[3] However, ROS and RNS must be maintained at appropriate levels to ensure appropriate physiological function of spermatozoa (hyperactivation, capacitation, and acrosome reaction). Since spermatozoa contain a large amount of polyunsaturated fatty acids (PUFAs) in spermal membranes which might easily be oxidized by ROS/RNS, they are particularly vulnerable to oxidative stress.[4,5] The ROS/RNS production during spermatogenesis or transit in the female genital tract might be exacerbated by environmental, infectious, and lifestyle etiologies.[6]

The appropriate balance between the generation of ROS/RNS and their neutralization is crucially important for the protection of spermatozoa against oxidative damage and is maintained by antioxidant systems. All living organisms have antioxidative defense systems to eliminate ROS/RNS. Antioxidants, which are aimed at the reduction of ROS/RNS generation, may play an important role in improving sperm quality. Superoxide dismutase, catalase, and glutathione peroxidase are in the first line of antioxidant protection; however, among other antioxidant enzymes, crucial role belongs to glutathione S-transferases (GSTs, EC 2.5.1.18).[7]

GSTs are multifunctional proteins ubiquitously expressed in most living organisms which catalyze the conjugation of glutathione (GSH, a tripeptide γ-Glu-Cys-Gly) with different toxic substrates provided with an electrophilic center. Among antioxidant defense mechanisms, GSTs play a pivotal role in detoxification of cytotoxic products, e.g., organic hydroperoxides formed during oxidative stress.[8] In response to ROS production, the GSTs can alter the levels of cellular GSH.[9] Enzymes are also involved in the regulation of different metabolic processes, signaling, and stress response.[10] There are three major families of GSTs as follows: cytosolic, mitochondrial, and microsomal (membrane-associated) GSTs. The cytosolic GSTs are the largest family. GSTs have broad substrate specificity. 1-chloro-2,4-dinitrobenzene (CDNB) is the most common substrate used to assay GSTs in the laboratory; however, not all, GSTs show catalytic activity with it.[11]

GSTM1 null genotype contributes to the increased risk of male infertility.[12] Chen *et al*.[8] have shown that spermatozoa of varicocele patients with the GSTM1 null genotype are more vulnerable to oxidative stress.[13] However, how they protect sperm cells in normozoospermic and pathospermic samples from oxidative stress is not clear.

In the present paper, to demonstrate the functional role of GSTs in sperm cells, we used a H_2O_2-induced stress on human ejaculated spermatozoa obtained from both normospermic and pathospermic patients.

SUBJECTS AND METHODS

Semen specimens were obtained from 30 men, aged 26–49 years with infertility. Exclusion criteria were as follows: azoospermia, genital infection, participants with infertility over 10 years, chronic illness and serious systemic diseases, smokers, and alcoholic men (due to their well-known high seminal ROS levels and decreased antioxidant activity which may affect GSTs). Participants currently on any medication or antioxidant supplementation were not included in the study. Specimens were also obtained from 10 male volunteers aged 25–50 years with normal semen analysis according to the WHO guidelines (2010) to serve as the fertile control. Semen specimens were collected by masturbation into a sterile plastic container after at least 3 days (3–5 days) of abstinence. After liquefaction (at 37°C with 5% CO_2 in air), semen samples were examined for spermogram parameters (volume, pH, sperm concentration, morphology, and motility according to the World Health Organization guidelines).

The present study was approved by ethics committe of Danylo Halytsky Lviv National Medical University University (protocol No 6 from March 29, 2017).

Spermatozoa were randomly assigned into one of the four experimental groups as follows: control group of cells (normospermic and pathospermic samples) that were incubated without treatment (Group 1); in the second group, spermatozoa were exposed to 1 mM of H_2O_2 alone; in the third group, sperm cells were treated with 150 μM of ethacrynic acid (EA) (electrophile-binding site inhibitor); and in the fourth group, sperm cells treated with 1 mM of H_2O_2 together with 150 μM of EA. Cells were exposed to mentioned concentration of chemicals for 5 h.

The GST activity was determined spectrophotometrically using enzyme-catalyzed condensation of GSH with CDNB as an electrophilic substrate. The product formed (2,4-dinitrophenyl-glutathione) absorbs light at 340 nm with an extinction coefficient of 9.6 mM/cm. The reaction mixture in a volume of 3 mL contained 2.5 mL of 0.1 M potassium phosphate buffer (pH 6.5), 0.2 mL of 15 mM CDNB, and 0.1 mL of 30 mM GSH, as enzyme source. The reaction was initiated by GSH. The results were expressed as μM/min/mg protein.

Peroxidative damage was estimated spectrophotometrically by the assay of thiobarbituric acid reactive substances (TBARSs) and expressed in terms of nmol of malondialdehyde (MDA) formed per mg protein. In brief, in a 3-ml reaction volume cell, homogenate supernatant was mixed with 0.15 M Tris-KCl buffer (pH 7.4) and 30% trichloroacetic and 52 mM thiobarbituric acid. The mixture was heated for 45 min at 80°C, cooled, and centrifuged for 10 min at 3000 rpm. The absorbance of the clear supernatant was measured against distilled water blank at 531.8 nm in a spectrophotometer and finally expressed as percentage change of nmol of MDA formed per mg protein.

The sperm motility was evaluated under a microscope. The sperm viability was assessed using eosin-nigrosin staining protocol (WHO, 2010). The results were expressed as the percentage of live sperm. The methods of variation statistics were used (software MS Office). The results are presented as the mean ± standard error. Means between the variables were analyzed using Student's *t*-test. Correlation between variables was performed with Pearson's correlation. Differences were considered statistically significant at $P < 0.05$ for all analyses.

RESULTS

In the first part of our study, we aimed to identify the biological role of GSTs in sperm cells under H_2O_2-induced oxidative stress. Since GST is a main determinant for adaptive response to oxidative stress, thus, we hypothesized that its pharmacologic inhibition might exacerbate the severity of

oxidative injury. Specifically, the effects of GST inhibition (using its potent inhibitor, EA) on sperm motility [Figure 1] and viability [Figure 2] were evaluated.

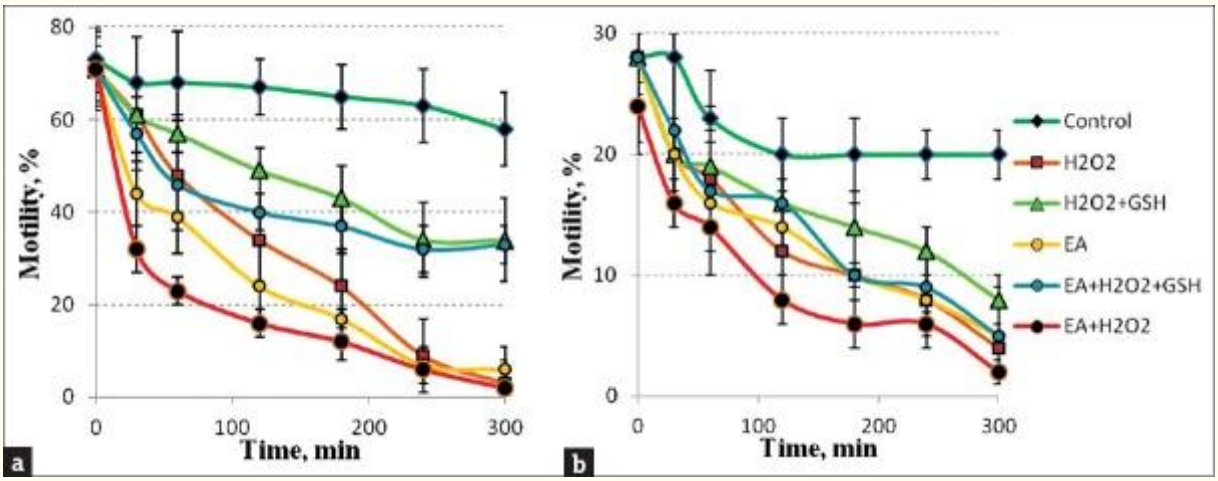

Figure 1

The effects of H_2O_2, ethacrynic acid, and glutathione on the sperm motility in normozoospermic (a) and asthenozoospermic (b) samples

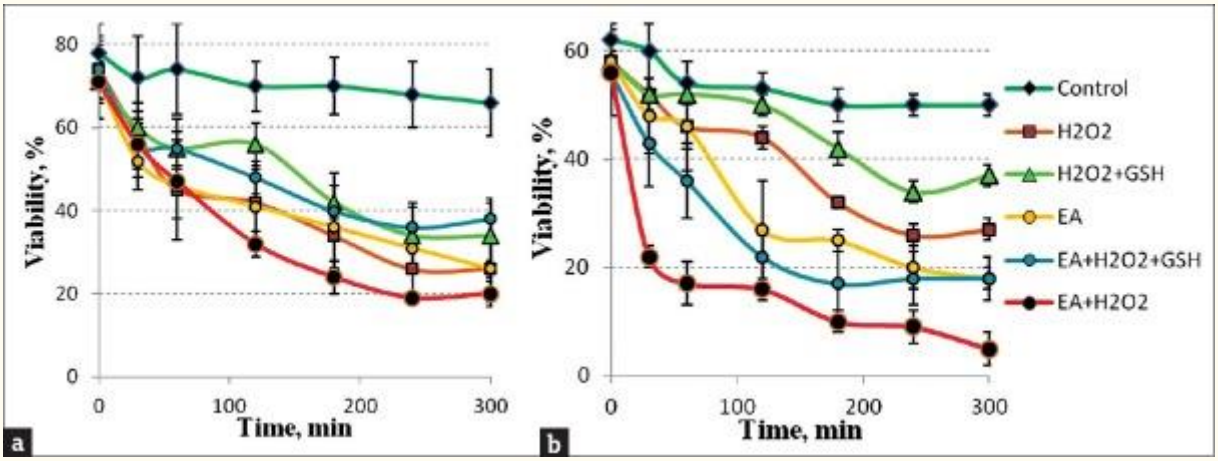

Figure 2

The effects of H_2O_2, ethacrynic acid, and glutathione on the sperm viability in normozoospermic (a) and asthenozoospermic (b) samples

1 mM H_2O_2 caused a time-dependent decrease in sperm motility and viability. Addition of 1 mM GSH in the medium containing spermatozoa during H_2O_2 treatment could reduce this loss of viability and motility. The same effect was observed when ascorbic acid used as antioxidant (data are not presented). This evidence suggested that both GSH and ascorbic acid protect the spermatozoa since they are nonenzymatic antioxidants. The fact that GSTs were primarily responsible for the protection of spermatozoa from H_2O_2-induced oxidative stress became evident when, in the presence of EA, sperm motility and viability were drastically decreased in incubation medium containing H_2O_2 in both normospermic and pathozoospermic samples. The presence of GSH in incubation medium attenuated this inhibitory effect only in normozoospermic samples [Figure 1a], but not in asthenozoospermic samples [Figure 1b]. Similar effects were observed for oligozoospermic and oligoasthenozoospermic samples (not represented in this paper).

Second, we aimed to identify the effect of GSTs inhibition on lipid peroxidation status in sperm cells under H_2O_2-induced oxidative stress. Since H_2O_2 is known to oxidize lipids in membranes, the lipid peroxidation status in sperm cells was determined by TBARS level. As can be seen

from Figure 3, the TBARS level was increased in spermatozoa incubated with H_2O_2 or EA. The most drastic increase in TBARS level was observed when sperm cells were incubated with H_2O_2 + EA.

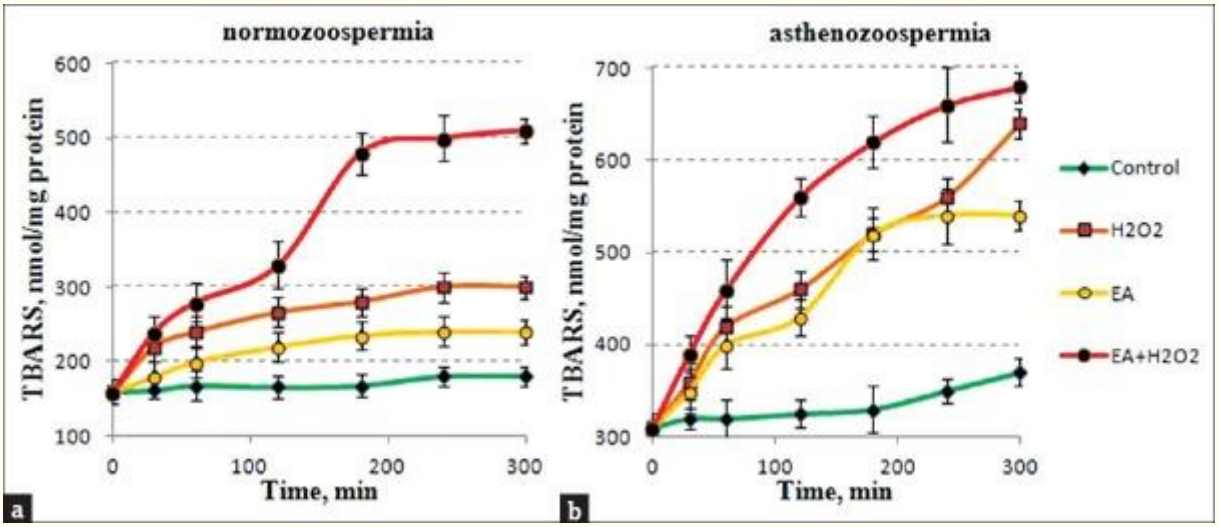

Figure 3

Kinetics profile of oxidative stress in sperm cells treated with H_2O_2, ethacrynic acid, and glutathione

The strong positive correlation between sperm motility ($r = 0.72$; $P < 0.05$)/sperm viability ($r = 0.64$; $P < 0.05$) and TBARS accumulation confirms that lipid peroxide-induced membrane damages are involved in disturbances of sperm function.

Finally, we checked the inhibition profiles of GST by EA in sperm cells obtained from normospermic and pathospermic samples. It was shown that EA in the concentration range of 0.01–10 mM suppresses GSTs activity of spermatozoa in dose-dependent manner [Figure 4]. The inhibition curves in spermatozoa obtained from asthenospermic, oligospermic, and astenooligozoospermic samples were not significantly different from normozoospermic patients. The inhibition constant and Hill coefficient are not significantly different in sperm cells between normospermic and asthenozoospermic samples [Table 1].

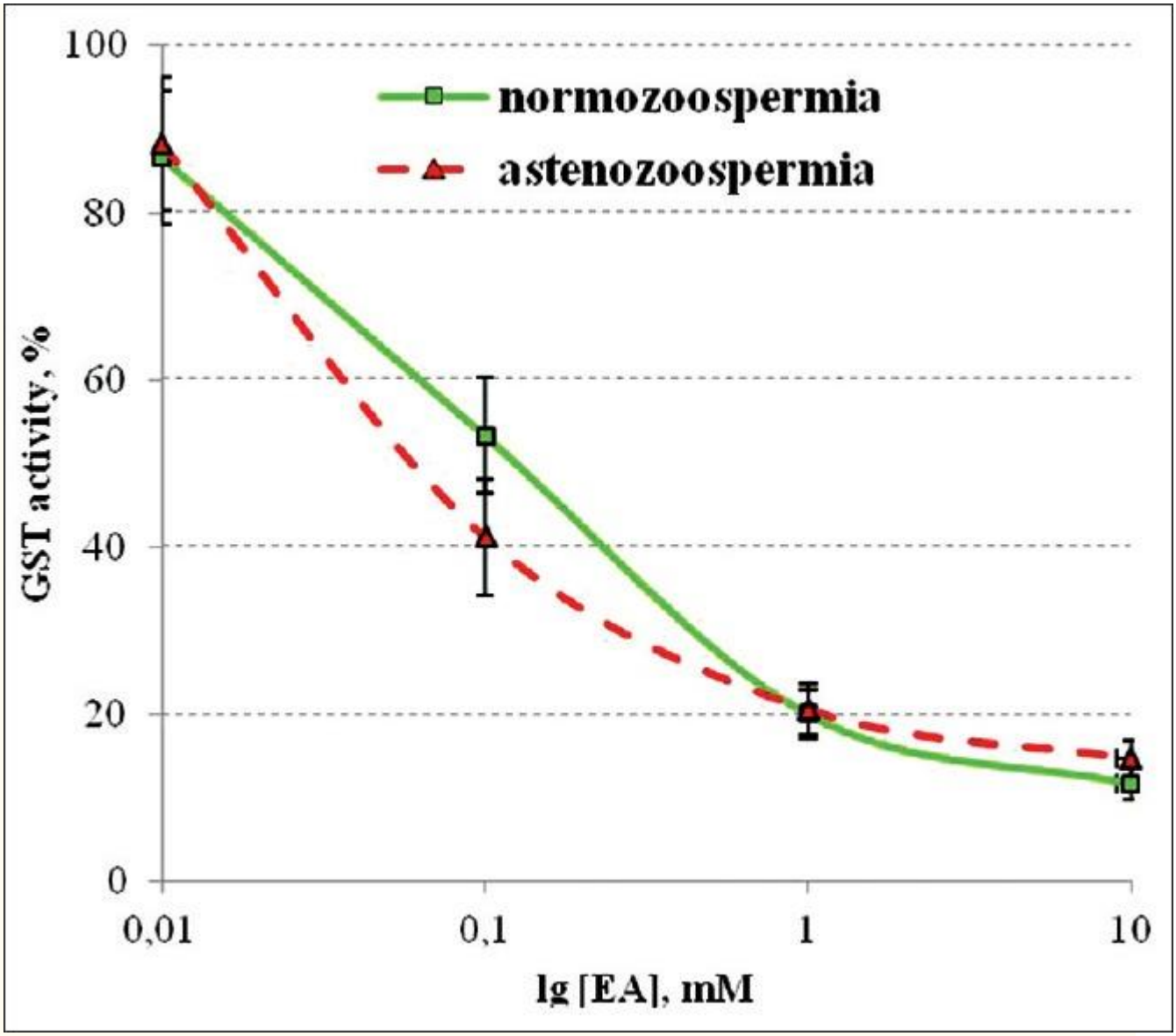

Figure 4

Inhibition of glutathione *S*-transferases activity by ethacrynic acid in spermatozoa obtained from normospermic and asthenozoospermic samples, *M*±*SE*, *n* = 6–8. Results are given as percentage of the control activity taken as 100% (enzyme activity in the absence of ethacrynic acid)

Table 1

Kinetic parameters of the glutathione S-transferase in the 150 μM ethacrynic samples obtained from normo- and pathozoospermic samples

Kinetic parameters	Fertile men (normozoospermia)	Infertile men (asthenozoospermia)
IC_{50}, μM	257.6±19.6	239.6±38.3

Kinetic parameters	Fertile men (normozoospermia)	Infertile men (asthenozoospermia)
n_H	2.03±0.06	2.17±0.19

IC_{50}=Inhibition constant, n_H=Hill's coefficient

DISCUSSION

It is known that GSTs catalyze the conjugation of GSH to a variety of electrophilic compounds and have crucial role as cell housekeepers involved in the detoxification of both endogenous and exogenous substances. Regarding the GSTs that have been studied in sperm cells, little is known about their functional role in pathozoospermic patients. It was shown that GSTM1 null genotype predisposes sperm to increased oxidative damage in patients with varicocele,[12] which clearly indicates that GSTs are crucially important for sperm functions. However, how they protect sperm cells in normozoospermic and pathospermic samples from oxidative stress is not clear.

The present study shows an association between GSTs activity and oxidative stress in spermatozoa from participants with male infertility. The most important parameters of ejaculate quality and determinants of paternity are sperm velocity and viability.[14,15] In this study, we have shown that sperm velocity and viability were reduced in EA-treated samples. The reducing sperm motility might be explained by different nonexclusive mechanisms. First, reduced sperm motility could arise from oxidative damage to the spermal membranes due to GSHs inhibition.

Second, reduced sperm quality is associated with the content of PUFA in spermal membranes. It was found that the lower proportion of PUFAs in the sperm membrane could lead to lower sperm performance.[16] Since PUFA is lower in asthenozoospermic, oligoasthenozoospermic, and oligozoospermic samples than in normozoospermic samples, it may explain a decreased sperm quality in pathospermic samples.[17]

Experimental results show that exogenous GSH can protect sperm cells through the GSTs. GSH is the most abundant nonenzymatic antioxidant in the animal cells.[18] GSH maintained sperm motility and viability during exposure to H_2O_2-induced oxidative stress. Taking into account that GSH is present in female reproductive tract; sperm cells might use this fluid as a source for GSH to protect from oxidative stress. Since sperm cells have very meager cytoplasm which is primary storehouse of defensive enzyme, the capability of sperm cells to utilize exogenous GSH gives a survival advantage to them.[19]

Pharmacological inhibition of GSTs significantly exaggerated the processes of lipid peroxidation as expressed by TBARS levels. TBARSs are end products of lipid peroxidation which reflects the amount of lipid damage due to ROS attacks.[13] We assessed the lipid peroxidation by determining the TBARS levels in spermatozoa and found their increased levels in EA-treated samples. In previous studies, higher TBARS levels in spermatozoa were negatively correlated with sperm motility and sperm concentration.[20] The increased levels of lipid peroxides in EA-treated samples revealed in this study indicate cellular damage.

In the present study, we also analyzed the inhibition profiles of GST by EA in sperm cells obtained from normospermic and pathospermic samples. The obtained value of IC_{50} for EA in sperm cells obtained from normozoospermic samples did not differ from pathospermic samples and were in micromolar range. These results are in agreement with obtained value of IC_{50} for EA in the presence of CDNB as substrate for GST of goat sperm (0.05 mM).[21]

This study demonstrates the biological significance of GSTs as detoxification agents in sperm functions. Our results sustain that the evaluation of GSTs activity in sperm cells of infertile men can

be helpful in fertility assessment and for the evaluation of protective treatment considering antioxidant strategies.

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Conflicts of interest

There are no conflicts of interest.

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