Functional role of sperm surface glutathione *S*-transferases and extracellular glutathione in the haploid spermatozoa under oxidative stress

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Received 22 January 2003; accepted 22 January 2003

First published online 12 February 2003

Edited by Barry Halliwell

Abstract On the sperm surface, glutathione *S*-transferases (GSTs) exist as oocyte binding proteins but their detoxification function in this unique cell type is not known. Using H_2O_2- and 4-hydroxynonenal-induced sperm dysfunction models, this study demonstrates that the sperm surface GSTs are able to use extracellular reduced glutathione to inhibit the loss of functional competence of goat spermatozoa; however, in the presence of GST inhibitors, they are unable to do so. In the context of susceptibility of spermatozoa to oxidative stress, this finding that strategically located sperm surface GSTs are important for maintaining the functional competence of sperm is relevant to studies on male infertility.

Key words: Glutathione *S*-transferase; Oxidative stress; Sperm

1. Introduction

Glutathione *S*-transferases (GSTs) are a family of cytosolic or microsomal enzymes that catalyze a number of reduced glutathione (GSH)-dependent reactions [1]. Studies from this laboratory have demonstrated the presence of immunoreactive and enzymatically active GSTs on goat sperm surface that serve as oocyte binding proteins [2–5]. Although it has been shown that sperm surface GSTs are enzymatically active, the precise mechanism by which they protect spermatozoa is not clear. In comparison to somatic cells, spermatozoa are extremely susceptible to damage by reactive oxygen species (ROS) as well as ROS-generated products [6–12] due to the preponderance of oxidation-prone unsaturated fatty acids on their plasma membrane [13]. Therefore, plasma membrane-located defensive enzymes are more useful to sperm in comparison to intracellular defensive enzymes as these would have less access to surface events. To demonstrate the role of sperm surface GSTs in the protection of the cell, we used a model of H_2O_2-induced stress on goat epididymal spermatozoa, as H_2O_2 is most effective in causing membrane damage [14,15]. To our knowledge, this is the first report that demonstrates the capability of sperm GSTs to use extracellular GSH to provide protection to sperm in terms of maintaining motility, viability, mitochondrial status, oocyte binding capacity and fertilizing capabilities during exposure to H_2O_2 or to products of lipid peroxidation.

2. Materials and methods

2.1. Animals

Testes with epididymides and female reproductive tracts with ovaries of goat (*Capra hircus*) were obtained from the local slaughterhouse at New Delhi, India, as described previously [5]. Goat semen was collected from animal house facilities of the Indian Veterinary Research Institute at Izzatnagar, India.

2.2. Cell treatments

Based on pilot experiments, 1 mM H_2O_2 was selected as the optimum dose to study functional changes in sperm. To measure the actual quantity of exogenous GSH required by sperm to effect detoxification during H_2O_2 treatment, 1 mM GSH was added to 10^7 sperm in 1 ml medium in the presence of 1 mM H_2O_2 and after 1 h of incubation at 37°C, glutathione levels were estimated. In a different set of experiments, to study the effect of H_2O_2-treated sperm supernatants containing products of oxidative stress on fresh spermatozoa, supernatants were collected from cells exposed to H_2O_2 and were treated for removal of H_2O_2 (catalase treatment) and products of lipid peroxidation (treatment with *N*-ethylmaleimide). Subsequently, fresh sperm were incubated in these supernatants with or without the removal of H_2O_2 or products of oxidative stress. Following these incubations, mitochondrial potential was measured. Sperm were also treated with different doses of 4-hydroxynonenal (HNE) with or without GSH supplementation and viability was determined from which LD_{50} and LD_{75} were calculated.

2.3. Preparation of gametes and extracellular fluids and studies on viability, motility and in vitro fertilization

Sperm preparation, capacitation, motility, viability, in vitro fertilization, oocyte recovery and maturation and collection of seminiferous tubular fluid from goat testis were carried out as described previously [2,3,5]. For seminal fluid, goat ejaculates were used, for epididymal fluid, goat caudae were punctured and the fluid collected. Female reproductive tract flushings were collected from oviducts [16] by tying both ends with nylon threads and oviductal fluid with cells were scraped off with a glass slide from the infundibulum end to the isthmus with an incision. After collection of all the above fluids, cells were removed by centrifugation at $1300\times g$ for 15 min followed by further centrifugation at $10\,000\times g$ for 1 h to remove all particulate matter.

2.4. Preparation of plasma membranes and purification of GSTs

Plasma membranes were prepared from Percoll-purified sperm as described earlier [5]. GSTs were purified from detergent (0.1% NP-40)

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Abbreviations: GST, glutathione *S*-transferase; GSH, reduced glutathione; $\Delta\psi_m$, mitochondrial membrane potential; ROS, reactive oxygen species; HNE, 4-hydroxynonenal; PnA, *cis*-parinaric acid; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazole carbocyanide iodide

0014-5793/03/$22.00 © 2003 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)00103-0

extracts using glutathione Sepharose™ 4B affinity beads as described in previous reports [17,18].

2.5. Lipid peroxidation, mitochondrial changes, ATP and GSH measurements

The oxidative fluorescence decay of *cis*-parinaric acid (PnA) was used to monitor the lipid peroxidation process in spermatozoa in response to H_2O_2 addition [19]. After 30 min of incubation with PnA (10 μM) at 37°C, sperm were washed and treated with different concentrations of H_2O_2. Fluorescence signal was followed at excitation and emission wavelengths of 324 nm and 413 nm respectively. Mitochondrial membrane potential ($\Delta\psi_m$) was estimated using 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazole carbocyanide iodide (JC-1) as a probe as described earlier [20]. ATP was measured by a bioluminescence assay [21] using an ATP determination kit (Molecular Probes, Eugene, OR, USA). Readings of experimental samples and standard curves were taken in a luminometer (Lumicount, Packard, Meriden, CT, USA).

2.6. Statistical analysis

An unpaired two-tailed Student's *t*-test using T-EASE software (Version 2.0; Institute for Scientific Information®, Philadelphia, PA, USA) and analysis of variance were used for statistical analyses.

3. Results

3.1. GSH is unable to prevent the loss of sperm viability, motility and fertilizing capacity induced by H_2O_2 if GST activity is inhibited

After a series of pilot experiments, 1 mM H_2O_2 was chosen as the optimal dose to bring about necessary changes in sperm required to carry out mechanistic studies on the function of sperm surface GSTs. 1 mM H_2O_2 caused a time-dependent loss of goat sperm motility, viability (Fig. 1A,B) and the ability of the spermatozoa to bind and fertilize oocytes (Fig. 1C). Addition of 1 mM GSH in the medium containing sperm during H_2O_2 treatment could reduce this loss of viability and motility and the decreased ability of sperm to fertilize after H_2O_2 exposure (Fig. 1A–C). This suggested that GSH was protecting the sperm either by its own non-enzymatic capabilities [22] or by using sperm surface enzymes. The fact that GSTs were primarily responsible for protecting the sperm from H_2O_2-induced effects became evident when in the presence of *S*-hexyl GSH, a GSH site binding inhibitor for GSTs, addition of exogenous GSH failed to inhibit loss of motility and viability (Fig. 1B,C). The ability of GSH to use an intracellular GSH-linked detoxification enzyme was very low as extracellular GSH is unable to enter live cells.

The above observations that sperm GSTs were able to use exogenous GSH were substantiated by experiments that showed a reduction in the amount of exogenous GSH added in sperm culture supernatants during exposure to H_2O_2. In the presence of *S*-hexyl GSH the reduction in total glutathione levels achieved by H_2O_2 was reversed to control levels (Table 1) showing that catalytically active GSTs were required for the sperm to be able to use the exogenous GSH. The relationship between the inability of the sperm to use exogenous GSH in the presence of a GST inhibitor and the loss of functional competence in this group shows that utilization of exogenous GSH by sperm is necessary to maintain its functioning capabilities.

3.2. Inhibition of sperm GSTs during H_2O_2 treatment leads to the loss of sperm mitochondrial membrane potential

To determine the possible early cellular changes upstream of functional alterations, we looked at mitochondrial function

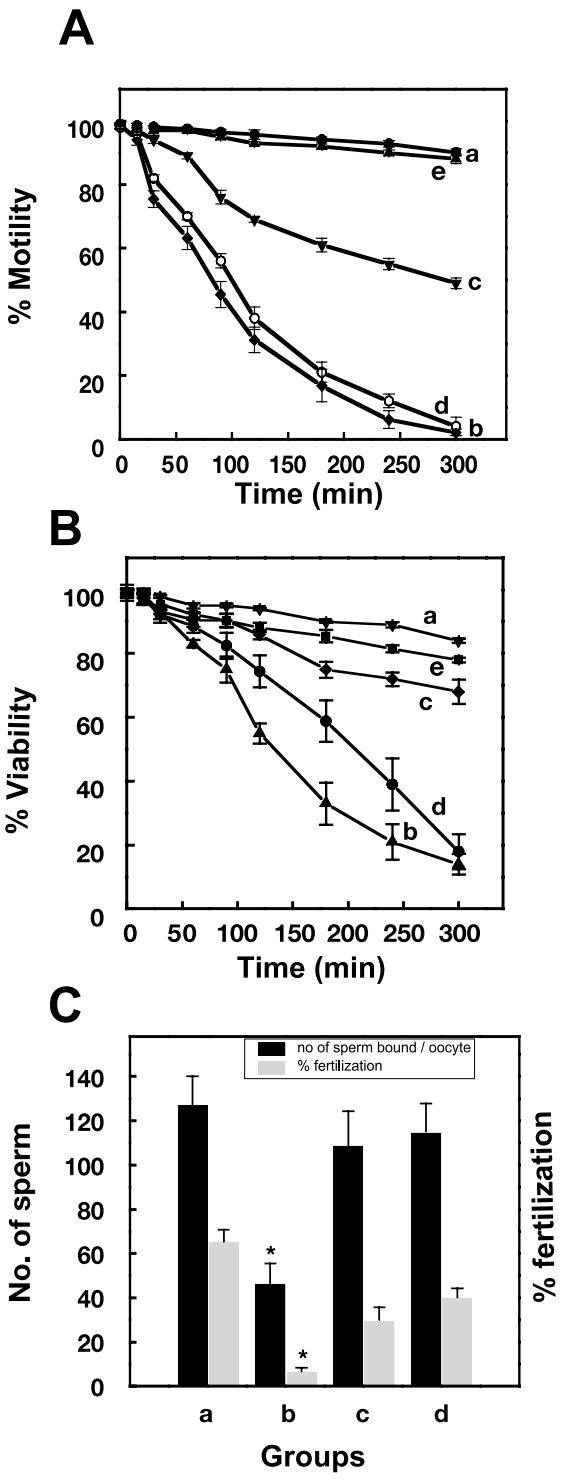

Fig. 1. Effect of H_2O_2 on the functional competence of spermatozoa and salvaging effect of exogenous GSH. A,B: Percent of motile and viable sperm respectively after treatment with (a) buffer; (b) 1 mM H_2O_2; (c) 1 mM H_2O_2+1 mM GSH; (d) 1 mM H_2O_2+1 mM GSH+200 μM *S*-hexyl GSH; (e) 1 mM H_2O_2+10 μg/ml PM-GSTs+1 mM GSH. C: In vitro oocyte binding and fertilization index of spermatozoa, which were preincubated (2 h) with (a) buffer; (b) 1 mM H_2O_2; (c) 1 mM H_2O_2+1 mM GSH; (d) 1 mM H_2O_2+10 μg/ml PM-GSTs+1 mM GSH. The asterisk represents level of significant variance in comparison to (a). $^*P < 0.05$. Data represent mean ± S.E.M.; $n = 6$.

Table 1
Glutathione levels in sperm culture supernatants, after induction of oxidative stress with 1 mM H_2O_2 in the presence of 1 mM GSH, with or without *S*-hexyl GSH ($n=4$)

Group	Total glutathione (mM) (mean ± S.E.M.)
GSH	0.81 ± 0.12
GSH+H_2O_2	0.36 ± 0.09*
GSH+H_2O_2+*S*-hexyl GSH (200 μM)	0.86 ± 0.11
GSH+*S*-hexyl GSH (200 μM)	0.89 ± 0.15

*$P < 0.05$ in comparison to the GSH group.

as motility is tightly linked to cellular energy generation. To establish if the loss of $\Delta\psi_m$ is directly related to motility, we used 1 mM H_2O_2 to bring about motility changes in sperm and assessed the $\Delta\psi_m$ in motile and immotile populations separated by the swim-up method. Immotile spermatozoa showed significantly less $\Delta\psi_m$ in comparison to the motile ones (readings at 590/530 nm, motile: 3.98 ± 0.3; immotile, 0.958 ± 0.1; $n=3$). After exposure to H_2O_2, changes in the mitochondria were reflected by a significant decrease in sperm $\Delta\psi_m$ within 60 min of exposure (Fig. 2A,B) and a loss of ATP (control, 30 ± 5 pmol/10^4 sperm; $n=6$; 1 mM H_2O_2, 14 ± 5 pmol/10^4 sperm; $n=6$). The number of sperm with low $\Delta\psi_m$ increased with the time of exposure to H_2O_2 (Fig. 2A). The presence of two GST inhibitors, ethacrynic acid (electrophile binding site inhibitor) or *S*-hexyl GSH (GSH binding site inhibitor) [23], during exposure to H_2O_2 induced a larger loss of $\Delta\psi_m$ than what was achieved with H_2O_2 alone (Fig. 2B). The presence of GSH, however, was able to prevent the fall in $\Delta\psi_m$ (Fig. 2B). Iodoacetate, aminotriazole and mercaptosuccinic acid used as inhibitors of selenium-independent GSH peroxidase, catalase and selenium-dependent GSH peroxidase respectively could not induce a greater loss of $\Delta\psi_m$ as compared to the GST inhibitors (Fig. 2B).

3.3. Sperm GST inhibition during exposure to products of lipid peroxidation leads to the loss of $\Delta\psi_m$ and viability

As H_2O_2 is known to oxidize lipids that are involved in sperm damage [24–26], the lipid peroxidation status of the sperm was checked with fluorescent PnA and an increase in peroxidation of membrane lipids signified by the loss of fluorescence was observed (fluorescence intensity units: control, 425 ± 30; $n=3$; experimental, 300 ± 35; $n=3$). To establish if products of lipid peroxidation did affect sperm, healthy motile sperm were incubated with the supernatants of H_2O_2-treated sperm and a decrease of $\Delta\psi_m$ of the spermatozoa was recorded (Fig. 3A). Catalase treatment of supernatants to remove residual H_2O_2 did not inhibit $\Delta\psi_m$ loss. When the products of lipid peroxidation in these supernatants were removed by incubation with glutathione peroxidase and GSH followed by subsequent removal of the remaining GSH with *N*-ethylmaleimide, there was a partial recovery in the decrease of $\Delta\psi_m$ (Fig. 3A). This experiment suggested that the byproducts of lipid peroxidation generated by H_2O_2 treatment were partially responsible for the loss of $\Delta\psi_m$. The question therefore was how effective the sperm GSTs were to protect sperm in the event of excess generation of products of lipid peroxidation. We used one well known major byproduct of H_2O_2-induced lipid peroxidation, HNE, to treat spermatozoa [27] and found a significant decrease in $\Delta\psi_m$ values and viability (Fig. 3B, Table 2).

HNE was toxic to the spermatozoa with an LD_{50} value of 45.31 μM (Table 2). The cytotoxic activity of HNE could be inhibited by the addition of 1 mM GSH but addition of GST inhibitors and HNE together in the presence of GSH in the medium became too lethal to the spermatozoa (LD_{50}, 17.11 μM) (Table 2). This suggested that exogenous GSH was able to salvage the cytotoxic effects of HNE when acting in concert with GSTs.

4. Discussion

In humans, several observations from fertility clinics show that glutathione is present in the seminal plasma and the level of this non-enzymatic antioxidant is significantly less in the semen of infertile subjects as compared to normal subjects [28]. Moreover, sperm motility improves in vitro with GSH supplementation [29]. In addition to the above reports, recent

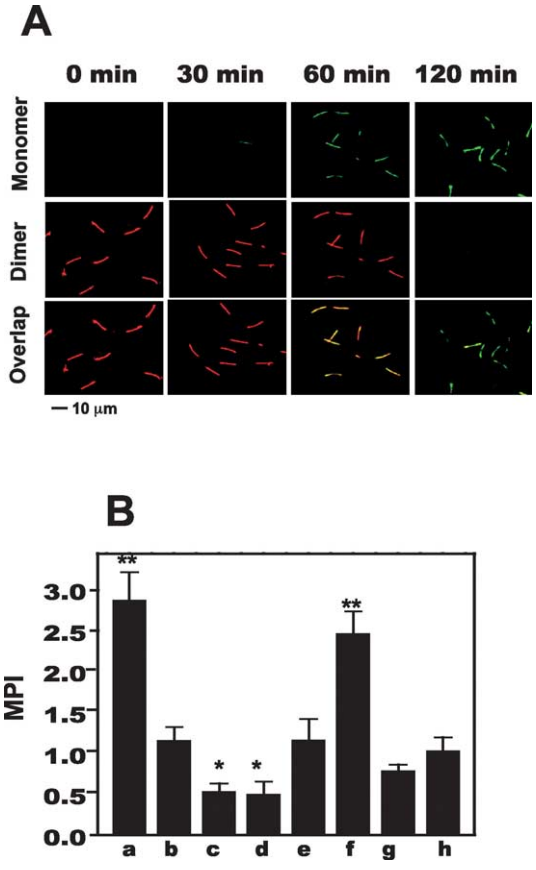

Fig. 2. Mitochondrial potential changes in spermatozoa in response to different treatments. A: Visualization of sperm mitochondria at different time points after treatment with 1 mM H_2O_2 showing localization of J-aggregates and J-monomers. Monomer: J-monomer distribution in sperm mitochondria at different time points after exposure to H_2O_2; Dimer: J-aggregate distribution in sperm mitochondria at different time points after exposure to H_2O_2; Overlap: J-monomer distribution overlapped with J-aggregate staining at different time points after exposure to H_2O_2. B: The $\Delta\psi_m$ (MPI) of spermatozoa (10^7/ml) was monitored using JC-1 after 1 h of incubation with (a) buffer; (b) 1 mM H_2O_2; (c) 1 mM H_2O_2+ethacrynic acid (100 μM); (d) 1 mM H_2O_2+*S*-hexyl GSH (200 μM); (e) 1 mM H_2O_2+mercaptosuccinic acid (200 μM); (f) 1 mM H_2O_2+GSH (1 mM); (g) 1 mM H_2O_2+iodoacetic acid (200 μM); and (h) 1 mM H_2O_2+aminotriazole (200 μM). The asterisks represent level of significant variance in comparison to the H_2O_2-treated group (b), *$P < 0.05$, **$P < 0.01$. Data represent mean ± S.E.M.; $n=6$.

studies show that the GST M1 null genotype predisposes sperm to increased oxidative damage in patients with varicocele [30] and is related to disorders of spermatogenesis [31]. These clinical observations clearly indicate that GSTs and GSH are very important for sperm function, but how they protect spermatozoa is not clear. Findings from this study support the above clinical observations by providing an insight into situations where these molecules become important.

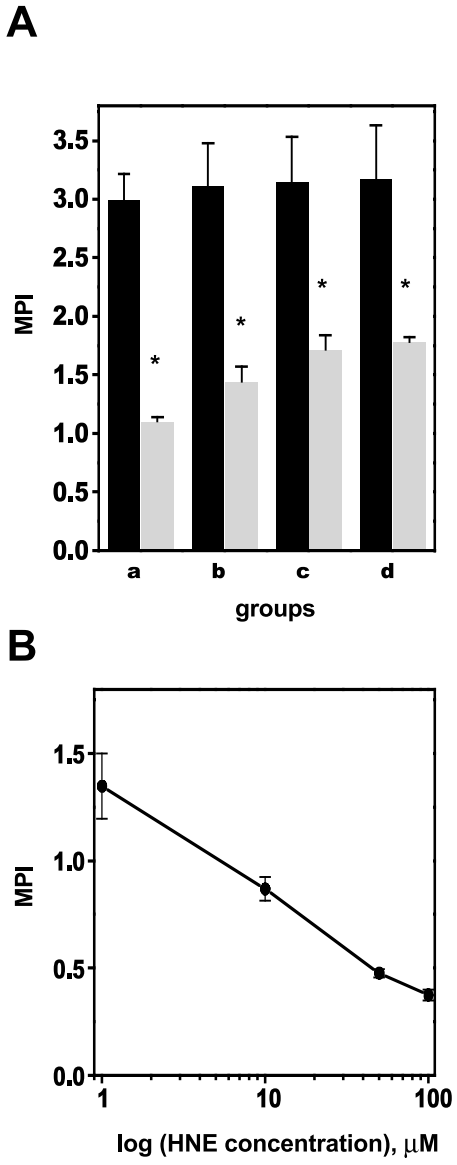

Fig. 3. Effect of products of lipid peroxidation on sperm mitochondrial potential. A: The $\Delta\psi_m$ (MPI) of viable and motile sperm (10^7/ml) was assessed using JC-1, after incubation with 2 h supernatant of 1 mM H_2O_2-treated (gray bars) or untreated sperm (black bars). The supernatant treatments were as follows: (a) untreated; (b) treated with catalase (100 U); (c) incubated with glutathione peroxidase (200 U)+1 mM GSH; (d) incubated with catalase (100 U)+glutathione peroxidase (200 U)+1 mM GSH for 20 min before incubation with fresh sperm. In groups (c) and (d), remaining GSH in the supernatants, after treatment, was inactivated with *N*-ethylmaleimide prior to incubation with fresh sperm. The asterisks represent level of significant variance compared to treated group a ($P < 0.05$). B: $\Delta\psi_m$ of spermatozoa, monitored using JC-1 fluorescent dye, after treatment with increasing concentrations of HNE for 2 h at 37°C. Data represent mean ± S.E.M.; $n = 6$.

Table 2
Effect of exogenous GSH and *S*-hexyl GSH on HNE-induced toxicity in sperm during in vitro incubations

Group	*r*	LD_{50} (μM)	LD_{75} (μM)
Control (HNE)	0.92	45.31	273.8
HNE+GSH	0.88	62.57	350.6
HNE+GSH+200 μM *S*-hexyl GSH	0.95	17.11	116.6

Data are representative of four repeats.

Experimental evidence shows that spermatozoa use exogenous GSH through the catalytic activity of surface GSTs to maintain functional competence in terms of maintaining motility, viability, mitochondrial status, oocyte binding capacity and fertilizing capabilities during exposure to H_2O_2 or to products of lipid peroxidation. The relative contribution of cytosolic GSTs, if any, using intracellular GSH would be very much less as 85% of the total GST activity on sperm is localized on the plasma membrane (total activity, 199.7 ± 20; plasma membrane, 169.7 ± 15; cytosolic, 26.2 ± 3.2; mitochondrial, 2.2 ± 0.4; nuclear, 1.02 ± 0.1; all activities expressed in nmol/min/10^8 cells; $n = 4$). In the context of spermatozoa, use of exogenous GSH is very important, as the sperm has to travel through various reproductive tract fluids to reach the oocyte and the possibilities of exposure to ROS are high because of the presence of immune cells and dead sperm in the reproductive tract [9,32]. Our studies show that GSH is present in extracellular fluids in the male and female reproductive tract (GSH content in seminiferous tubular fluid, 0.9907 ± 0.08 mM; epididymal fluid, 0.2062 ± 0.03 mM; seminal fluid, 0.7585 ± 0.08 mM; oviductal fluid, 0.1088 ± 0.03 mM; $n = 4$) and therefore, it is possible that spermatozoa use these fluids as a source for GSH whenever stress situations occur.

The capability of the spermatozoon to utilize extracellular GSH gives a survival advantage to it, as it has very meager cytoplasm – the primary storehouse of defensive enzymes. Exposure to ROS in the reproductive tract with increased lipid peroxidation would be very detrimental to these cells if GSTs did not play an active role on the sperm surface. This is because spermatozoa are extremely susceptible to damage by ROS as well as ROS-generated products [6–12] due to the preponderance of oxidation-prone unsaturated fatty acids on their plasma membrane [13]. Arguably therefore, plasma membrane-located defensive enzymes would be more useful to sperm in comparison to intracellular defensive enzymes as these would have less access to surface events. This study clearly demonstrates the functional importance of sperm GSTs in the maintenance of sperm functional competence and provides an insight into possible defects in the functioning of the GST–GSH system in sperm that has the potential to lead to impaired fertility.

Acknowledgements: Financial support from the Department of Biotechnology, Government of India, the National Institute of Immunology, New Delhi and CONRAD, Virginia, USA is appreciated.

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