FEBS Letters 422 (1998) 296–300 FEBS 19800

Inhibition of sperm glutathione *S*-transferase leads to functional impairment due to membrane damage

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Received 12 December 1997

Abstract The role of glutathione *S*-transferase (GST) in the defense mechanisms of sperm is not known. We report here interference with normal motility, acrosome reaction and fertilizing ability of the goat sperm as a consequence of inhibition of GST activity. That these functional impairments were due to membrane changes was evident from the alteration in the lipid peroxidation status of these cells after GST inhibitor treatment. Increased reactive oxygen species production by the cell which occurred when GST activity was suppressed may be the mediator for membrane damage. The data argue for a role of GST in maintaining sperm membrane status.

Key words: Sperm; Glutathione *S*-transferase; Fertilization; Motility; Lipid peroxidation

1. Introduction

The genesis of the work described here lies in the synthesis of two key observations previously reported from our laboratory. The first observation pertains to the demonstration of immunoreactive glutathione *S*-transferase (GST) on goat sperm which was later identified to consist of both GST-Pi and Mu [1] located on the cell surface. The second observation was the inability of the antibodies directed against the NH_2-terminus of both GST-Pi and Mu to inhibit sperm GST activity while being able to interfere with the competence of the sperm to fertilize [2]. Evidently, there was a catalysis-independent function of sperm GSTs; however, it was thought unlikely that the cell would not take advantage of the detoxification function of GSTs, which are known to catalyze the conjugation of GSH with electrophiles [3]. We therefore investigated the contribution of sperm GST in the defense mechanisms of spermatozoa by modeling alterations in the enzyme activity by using two specific GST inhibitors. This issue is of basic interest as sperm function impairments occur due to defective defense mechanisms [4] but the role of GST in such events is not known.

Our data reported in this manuscript show that suppression of sperm GST activity leads to membrane damage, reflected in the loss of motility, inhibition of acrosome reaction and reduction of the ability of the inhibitor-treated sperm to fertilize oocytes in vitro. Enhanced lipid peroxidation which is associated with an increase in free radical production appears to be the reason for disruption of membrane integrity. Therefore, we propose that goat sperm GST forms an important part of the defense machinery of spermatozoa and is arguably at a more advantageous location on the sperm head in comparison to other known defensive enzymes situated in the midpiece.

2. Materials and methods

2.1. Reagents

Secondary antibodies were from Jackson Laboratories (West Grove, PA) and all other chemicals, unless specified, were from Sigma Chemical Co. (St. Louis, MO).

2.2. Cell preparation and antibodies

2.2.1. Preparation of sperm. Goat cauda epididymides were obtained from the slaughterhouse and sperm were collected from the cauda and subsequently Percoll-purified [5].

2.2.2. Preparation of sperm tail and head. Percoll-purified spermatozoa were subjected to sonication at 120 W output using a Branson sonifier (Danbury, CT) and separated heads and tails were collected after centrifugation on a 45–90% Percoll gradient. The percent contamination of the fractions by each other was microscopically determined.

2.2.3. Antisera. Two peptides designed from the NH_2-terminus of GST-Pi (Lys-Pro-Pro-Tyr-Thr-Ile-Val-Tyr-Phe-Pro-Val), designated PiN, and the NH_2-terminus of GST-Mu (Lys-Pro-Met-Thr-Leu-Gly-Tyr-Trp-Asp-Ile), designated MuN, were synthesized as described previously [2]. Antibodies raised against the two peptides in New Zealand white rabbits using standard methods [6] were designated anti-PiN and anti-MuN.

2.3. Inhibitor and antibody treatments

In all assays, GST inhibitor treatments of sperm suspended in isotonic buffer were given with 100 μM *S*-hexyl GSH, a GSH site binding inhibitor, or ethacrynic acid, a substrate analog inhibitor, for 15 min followed by a wash and resuspension of sperm in the relevant buffer. For competition with the inhibitors, sperm GST (GSP1, purification described in [2]) was used at a concentration of 10 μg/ml which was present along with the inhibitor during the period of treatment of sperm. Antibody treatments of the cells were given with antibody dilutions of 1:50 for 30 min at room temperature.

2.4. Functional assays

2.4.1. Motility assay. Percoll-purified sperm, incubated at 37°C for 1 h under different treatments with or without GST inhibitors in 5% CO_2 and air, were washed and the number of motile sperm per 200 sperm was counted for each experiment in identified fields in a Nikon Optiphot phase contrast microscope [7].

2.4.2. Sperm-oocyte interactions. The influence of GST inhibitors on the fertilizing capacity of goat spermatozoa was assessed by treating these cells with the GST inhibitors and subsequently washing them prior to exposure to the oocytes prepared according to Pawshe et al. [8] for 22 h at 38°C under 5% CO_2 in air. Oocytes were classified as normal fertilized (2P) when the female and male pronuclei were visible, as polyspermic (Poly) if multiple pronuclei were seen, and as

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Abbreviations: GST, glutathione *S*-transferase; ROS, reactive oxygen species; SOD, superoxide dismutase; GPx, glutathione peroxidase; CDNB, 1-chloro-2,4-dinitrobenzene; SPTL, sperm Tyrode's lactate medium; GSP1, purified goat sperm GST; GSH, glutathione; MDA, malondialdehyde; TBA, thiobarbituric acid; X-XO system, xanthine-xanthine oxidase system; HOST, hypoosmotic swelling test

PII S0014-5793(98)00032-5

Table 1
Effect of GST inhibitors on goat in vitro fertilization

Group	Total oocytes	Unfertilized (MII and 1P)	Fertilized (2P and Poly)	Oocytes fertilized (%)
1. Control (vehicle only)	52	16	36	69 ± 2.9
2. *S*-Hexyl GSH (100 μM)	34	24	10	29 ± 0.6
3. Ethacrynic acid (100 μM)	43	39	4	9 ± 1.1

Data are expressed as means ± S.E.M of three experiments. 1 vs. 2, $P < 0.001$; 1 vs. 3, $P < 0.0001$.

unfertilized when metaphase II plate (MII) or one pronucleus (1P) was present.

2.4.3. Hypoosmotic swelling test (HOST) and acrosome reaction
Percoll-purified sperm were treated with or without GST inhibitors or antibodies and subjected to HOST [9]. The percentage of swollen sperm (undamaged) vs. sperm with straight tails (damaged) was calculated from a total count of 300 sperm for each experimental group. Percoll-purified sperm (10^7) incubated with or without GST inhibitors for 15 min at 37°C were acrosome-reacted by adding 10 μg/ml of the calcium ionophore A23187 at 37°C for 10 min [10] and acrosin release was measured by the acrosin assay [11].

2.5. Biochemical analyses

2.5.1. GST assay. GST assay with live spermatozoa (10^7 or 10^6 intact cells or 10^8 separated heads or tails) using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate in GST buffer without EDTA or isotonic medium of 20 mM PBS [12] in the presence or absence of inhibitors or antisera was carried out according to Warholm et al. [13].

2.5.2. Measurement of reactive oxygen species (ROS). Percoll-purified sperm (10^7) were exposed for 30 min at 37°C to GST inhibitors and ROS were measured using luminol as the chemiluminescent substrate [14].

2.5.3. Lipid peroxidation assay. The xanthine-xanthine oxidase (X-XO) system was used to generate ROS [15], and the lipid peroxidation status of spermatozoa was monitored using the generation of malondialdehyde (MDA) [14].

2.5.4. Glutathione peroxidase (GPx) assay. The GPx assay was carried out according to Alvarez and Storey [16].

2.6. Statistical analysis
Data were analyzed using one-way analysis of variance [17]. Tadpole III software (Elsevier Biosoft, Cambridge, UK) was used for the analysis.

3. Results and discussion

This study provides evidence that sperm GSTs are important components in the defense machinery of spermatozoa. GSTs have been described mainly as cytosolic detoxification enzymes with a microsomal form described as membrane-bound [3]. Evidence for the functional involvement of goat sperm Pi and Mu GSTs in fertility-related events came from our earlier studies which demonstrated a reduction of the fertilizing ability of goat sperm when treated with antibodies directed against the NH_2-terminus of Pi and Mu GSTs. However, there was no inhibition of GST activity with these antibodies, which opened up an interesting possibility that sperm GSTs possess a non-catalytic function which is related to fertility. However, GSTs being important defensive enzymes, their presence at the strategic location of the sperm head made it more likely that the cell would use the catalytic function of this molecule. Therefore, an attempt was made to study the effects of alterations of enzyme activity of these molecules on sperm function.

Our earlier data established the ability of *S*-hexyl GSH, a GSH site binding inhibitor, and ethacrynic acid, a substrate analog inhibitor, to reduce sperm GST activity [2]. Using these two inhibitors, we obtained evidence that the reduction of enzyme activity was interfering with the ability of sperm to fertilize oocytes (Table 1). A 15 min preincubation with the inhibitors at a dose of 100 μM was chosen as this treatment was found to affect sperm function without being cytotoxic for the duration of the experiment.

The fertilizing capacity of sperm is closely related to its ability to move and to undergo acrosome reaction [4]. It was therefore of interest to check the status of these two functions after inhibitor treatment. Both inhibitors tested reduced the motility of sperm (Table 2), showing that interference with GST activity was impeding a prime function of spermatozoa. Inhibitor-treated sperm were also unable to undergo calcium ionophore-induced acrosome reaction, which

Table 2
Effect of GST inhibitors on goat sperm motility

Group	Motility (%) at two time points	
	0 h	1 h
1. Control	98 ± 0.6	90 ± 2.5
2. *S*-Hexyl GSH (100 μM)	91 ± 3.6	56 ± 2.1
3. Ethacrynic acid (100 μM)	100 ± 0.3	41 ± 2.7

Data are expressed as means ± S.E.M of triplicate assays. At 1 h: 1 vs. 2, 1 vs. 3, $P < 0.001$.

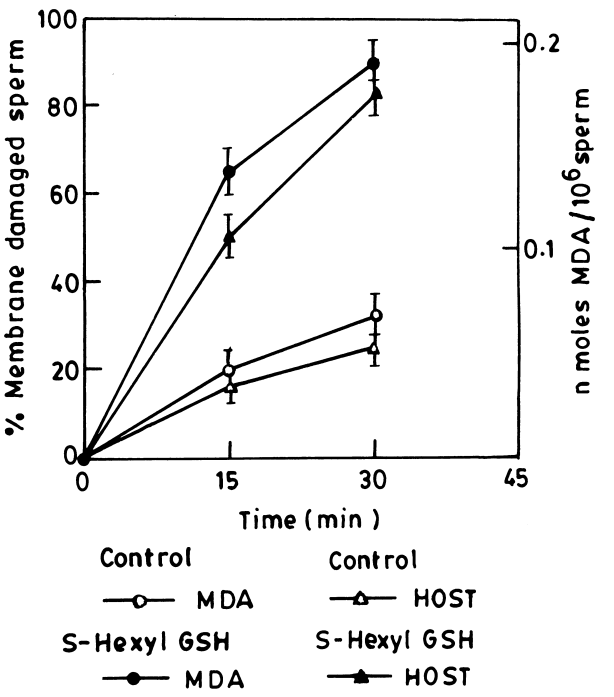

Fig. 1. Correlation between the degree of MDA accumulated and the number of membrane-damaged sperm as measured by HOST in the control and *S*-hexyl GSH (100 μM)-treated groups. Data are means ± S.E.M. of three experiments.

Table 3
Effect of GST inhibitors on the membrane of goat spermatozoa as measured by HOST

Group	Membrane-damaged (%)
1. Control	22 ± 0.17
2. *S*-Hexyl GSH (100 μM)	90 ± 0.16
3. *S*-Hexyl (100 μM)+GSP1 (10 μg/ml)	38 ± 0.07
4. Ethacrynic acid (100 μM)	53 ± 0.15
5. Anti-MuN	25 ± 0.11
6. Anti-PiN	24 ± 0.11

Data are expressed as means ± S.E.M of three experiments. 1 vs. 2, 2 vs. 3: $P < 0.001$; 1 vs. 4: $P < 0.05$.

was reflected in the reduction of acrosin release by the sperm. The values for acrosin release for inhibitor treated sperm were 4.87 ± 0.1 (*S*-hexyl GSH) and 4.52 ± 0.14 (ethacrynic acid) mU acrosin/min/10^7 sperm in comparison to control cells with acrosin release concentrations of 9.5 ± 0.4 mU acrosin/min/10^7 sperm. Therefore, two biologically significant membrane-related events were interrupted and involvement of the membrane was confirmed by HOST (Table 3) which is extensively used to test sperm function [18]. Antibodies were unable to cause membrane disruption which agrees with the fact that they could not inhibit catalytic function of sperm GST. That the membrane changes induced by inhibitors were primarily due to alterations in GST activity was confirmed when exogenous sperm GST, present to compete for the binding of the inhibitors to sperm surface GST molecules, significantly reduced the degree of membrane damage.

To identify the mechanism of membrane damage, accumulation of MDA was measured to check changes in lipid peroxidation and an increase was observed in the inhibitor-treated cells (Table 4). Due to the necessity of sperm to undergo fusion events, their membranes are rich in unsaturated fatty acids which are susceptible to attack by the lipid peroxides formed [4]. The excellent correlation between the HOST results and MDA accumulation (Fig. 1) confirms that interference with the biological function of sperm is mainly through lipid peroxide-induced membrane damage. Again, the presence of exogenous GSP1 during inhibitor treatments was able to reduce MDA accumulation indicating specificity of the GST inhibitor effects.

Lipid peroxidation-induced membrane damage is often caused by increased free radical production. Therefore, our data on the ability of superoxide dismutase (SOD) and catalase to reduce MDA accumulation consequent on inhibitor treatment (Table 4) confirmed the involvement of free radicals in the process of membrane damage caused by inhibitor treatment. There is no report available on the ability of goat sperm to produce ROS, although reports on the generation of free

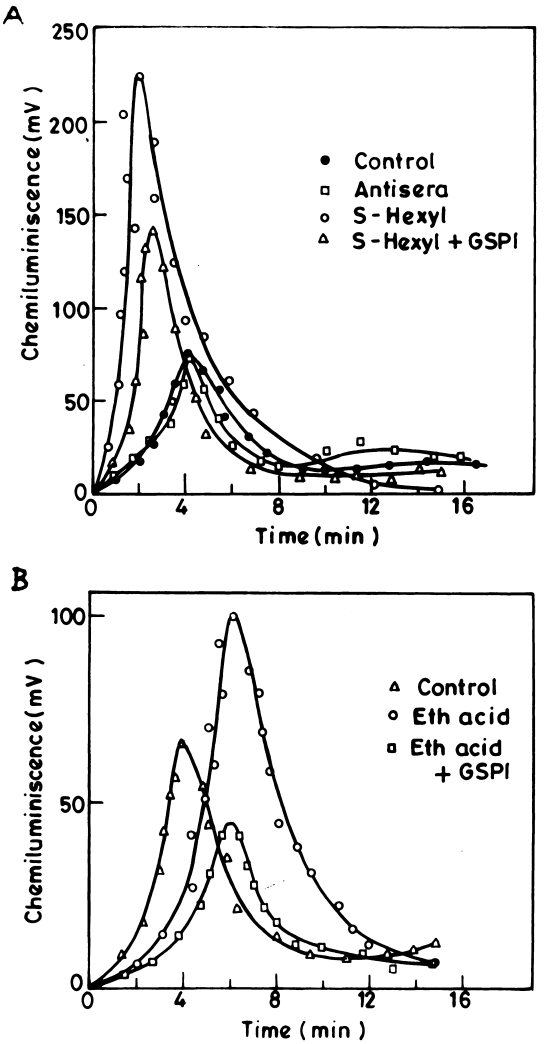

Fig. 2. ROS production in the presence or absence of GSP1 with (A) *S*-hexyl GSH (100 μM) and (B) ethacrynic acid (100 μM). Control represents ROS production by normal goat sperm without any treatment. Representative data from one experiment (out of four) are presented.

radicals by sperm of other species exist [4,19–21]. Our studies revealed that goat sperm were capable of producing ROS and this production increased in the presence of *S*-hexyl GSH and ethacrynic acid. This increase in ROS induced by the GST inhibitors could be suppressed by the presence of GSP1 during treatment (Fig. 2A,B); however, antibodies to GST were unable to cause any increase in ROS (not shown). Therefore,

Table 4
Lipid peroxidation status of goat spermatozoa on treatment with GST inhibitors

Group	MDA produced (nmol MDA/10^6 sperm)
1. Control	0.90 ± 0.17
2. *S*-Hexyl GSH (100 μM)	2.25 ± 0.16
3. *S*-Hexyl (100 μM)+GSP1 (10 μg/ml)	1.09 ± 0.07
4. *S*-Hexyl GSH (100 μM)+100 U/ml catalase	1.95 ± 0.11
5. *S*-Hexyl GSH (100 μM)+100 U/ml SOD	1.10 ± 0.08
6. Ethacrynic acid (100 μM)	2.87 ± 0.15
7. Anti-MuN	0.85 ± 0.15
8. Anti-PiN	0.80 ± 0.16

Data are expressed as means ± S.E.M of three experiments. 1 vs. 2, 2 vs. 3, 1 vs. 6: $P < 0.001$; 2 vs. 5: $P < 0.01$.

Table 5
Effect of the X-XO system on membrane damage (HOST) and lipid peroxidation in goat sperm

Group	HOST (% membrane-damaged sperm)	MDA produced (nmol/MDA/10^6 sperm)
1. X-XO system	95 ± 0.05	3.07 ± 0.05
2. X-XO+GSP1 (10 μg/ml)	62 ± 0.19	1.27 ± 0.19
3. X-XO+100 U/ml catalase	52 ± 0.10	1.80 ± 0.12
4. X-XO+100 U/ml SOD	68 ± 0.3	1.72 ± 0.19
5. Control (without X-XO)	24 ± 0.16	1.00 ± 0.15

HOST: 1 vs. 2, 1 vs. 3, 1 vs. 4: $P < 0.05$; 1 vs. 5: $P < 0.0001$. MDA produced: 1 vs. 2, 1 vs. 5: $P < 0.0001$; 1 vs. 3, 1 vs. 4: $P < 0.05$.

interference with the catalytic function of the molecule could bring about an increase in the production of ROS. ROS generation by sperm is a normal physiological event, however, an imbalance in ROS can cause oxidative stress and is frequently associated with male infertility [22]. Since it is known that ROS can induce GST activity [3], it is possible that inhibition of GST was causing disruption in the delicate balance of ROS production thereby resulting in non-physiological levels of free radicals. To ensure that the membrane changes similar to those observed with GST inhibitor treatments can be induced by oxidative stress, MDA accumulation and the number of membrane-damaged sperm were checked after exposing the sperm to ROS generated by the X-XO system. Augmentation in both end points measured was observed and both increases could be reduced in the presence of GSP1, catalase or SOD (Table 5). This clearly indicated that sperm GSTs were involved at some point of the detoxification pathway that is activated in response to free radical-induced stress. Sperm motility was reduced from 95 ± 1% at 0 h to 12 ± 0.9% at 1 h after 15 min exposure to the X-XO system. Therefore, the data described above contribute to the fact that ROS can cause membrane damage in sperm which affects critical sperm functions and are very similar to changes caused by treatment of sperm with GST inhibitors mediated through the production of ROS by the X-XO system.

GPx is a defensive enzyme catering to free radical elimination, and certain GST subunits express GPx activity [3]. Since no detectable GPx activity with GSP1 was recorded (data not shown) it can be concluded that the inhibitory effects of GST inhibitors were seen through the inhibition of GST activity only and were not due to GPx activity of GST subunits.

The extent to which the lipid peroxidation cascade will proceed depends upon the antioxidant strategies employed by the spermatozoa. The ability of these cells to manage oxidative stress is limited by their deficiency in defensive enzymes [12] as they discard the majority of their cytoplasm during maturation. It is believed that scavenger molecules like ascorbic acid, uric acid, the chain-breaking antioxidant α-tocopherol and enzymes like SOD and catalase present in human semen partly compensate for the absence of defensive enzymes on sperm [23]. The sperm membrane overlying the head is rich in unsaturated fatty acids due to its fusogenic nature and hence it is more susceptible to lipid peroxide-induced damage. Our observation that GST activity was primarily localized to the head (Table 6) provides an argument for the existence of a defensive enzyme at a very strategic location overlying the acrosome in comparison to other defensive enzymes like GPx and SOD reportedly localized in the sperm midpiece [18]. Immunoreactive sites to anti-GST-Pi or anti-GST-Mu show that the tail also expresses GSTs although the enzyme activity is low.

In summary, our studies provide evidence for the first time that GSTs on sperm are important detoxification enzymes located at the strategic position of sperm head. In support, we show disruption of normal functioning of sperm at inhibition of GST activity mediated through lipid peroxide-induced membrane damage. Thus, in combination with our earlier data on antibodies inhibiting fertilization rates without affecting catalytic activity, these experiments indicate a multifunctional role of GSTs on sperm.

Acknowledgements: Financial support from the Department of Biotechnology, Government of India, the National Institute of Immunology, New Delhi and The Population Council, New York is gratefully acknowledged.

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Table 6
Immunoreactive GST and GST activity on goat sperm head and tail

	(A) Immunoreactivity (A_{490})		(B) Enzyme activity (nmol/min/10^8)	
	Anti-MuN	Anti-PiN	CDNB (1 mM)	Ethacrynic acid (5 μM)
1. Head	0.17 ± 0.015	0.16 ± 0.012	16.25 ± 1.2	183 ± 11
2. Tail	0.06 ± 0.004	0.10 ± 0.008	2.70 ± 0.2	10 ± 1

Data are expressed as means ± S.E.M of triplicate assays. (A) Anti-MuN: 1 vs. 2: $P < 0.0001$. (B) With CDNB: 1 vs. 2: $P < 0.0001$; with ethacrynic acid: 1 vs. 2: $P < 0.0001$.

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