

# Nonprotein Thiols and Disulfides in Rat Epididymal Spermatozoa and Epididymal Fluid: Role of $\gamma$ -Glutamyl-Transpeptidase in Sperm Maturation

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**ABSTRACT:** Sperm thiol oxidation during sperm maturation is important for sperm component stabilization, the acquisition of sperm motility, and fertilizing ability. A correct degree of oxidation is required, since spermatozoa are very susceptible to oxidative damage. The pathways involved in physiologic sperm thiol oxidation in the epididymis are not completely understood. The nonprotein thiol glutathione (GSH), in addition to playing a major role as an antioxidant and in eliminating toxic compounds, has been implicated in prooxidation processes in various cells, via  $\gamma$ -glutamyl-transpeptidase ( $\gamma$ -GT)-dependent catabolism. Little information is available on the dynamics of nonprotein thiols (NPSHs) and disulfides (NPSSNPs) in spermatozoa and epididymal fluid (EF) during sperm passage in the epididymis. It is not clear whether NPSHs and NPSSNPs are involved in sperm protein thiol (PSH) oxidation or whether GSH catabolism in the epididymis can serve as a pathway for sperm PSH oxidation. In the present study, we used the thiol fluorescence labeling agent monobromobimane to analyze NPSHs and nonprotein

disulfides (NPSSRs) (R, nonprotein or protein) in spermatozoa and EF in the rat caput and cauda epididymis. NPSH levels are shown to be significantly higher in the caput than in the cauda (spermatozoa and fluid). GSH in the caput lumen is subject to high  $\gamma$ -GT activity. A marked loss of sperm GSH and a shift to an oxidized state (resulting in a significantly higher concentration of glutathione disulfides [GSSRs] than GSH) occur during the passage of spermatozoa from the caput to the cauda epididymis. Caput EF and extracellular NPSSNPs induce sperm thiol oxidation. The results suggest that epididymal NPSH/NPSSNP participates in sperm PSH oxidation and that some reactions of GSH in the  $\gamma$ -GT pathway (in the epididymis) provide oxidizing power, leading to physiologic sperm thiol oxidation.

**Key words:** Glutathione/glutathione disulfides, cysteine/cysteine disulfides, nonprotein mixed disulfides, nonprotein-protein mixed disulfides, sperm oxidative pathways.

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Mammalian spermatozoa acquire the ability to fertilize eggs during epididymal maturation while passing from the caput to the cauda epididymis. During maturation, most sperm structures such as the chromatin and tail components become stabilized (Yanagimachi, 1994). Stabilization is achieved mainly through the oxidation of thiol groups (SH) to disulfides (SS) during sperm epididymal maturation (Bedford and Calvin, 1974; Shalgi et al, 1989). An adequate condensation and stability of the chromatin is essential to protect the genome from physical and chemical agents until the spermatozoon reaches the egg. Sperm thiol oxidation is important for the induction of progressive sperm motility (Cornwall et al, 1986) as well as for capacitation, acrosome reaction, egg attachment, and fertilization (Yanagimachi et al, 1983; Sa-

leh and Agarwal, 2002). It is also an important aspect in the protection of spermatozoa during freeze-drying (Kaneke et al, 2003).

The pathways involved in the physiologic sperm thiol oxidation in the epididymis have not been clarified. Factors within the sperm cell itself and/or in the epididymis may control sperm protein thiol (PSH) oxidation. A correct degree of oxidation is required, since spermatozoa are very susceptible to oxidative damage. Excessive activity of oxidative pathways (via reactive oxygen species and lipid peroxidation) leads to damaged spermatozoa and infertility (Maiorino and Ursini, 2002; Saleh and Agarwal, 2002; Baker and Aitken, 2004). Glutathione (GSH), which serves as a major cellular antioxidant (Kosower and Kosower, 1978; Sies, 1999), is considered important in maintaining this redox equilibrium in the mammalian testis and for protecting spermatozoa against oxidative stress. GSH and GSH-related enzymes have been studied in the testes, epididymal tissue, and spermatozoa of various species (Li, 1975; Agrawal and Vanha-Perttula, 1988a; Alvarez and Storey, 1989; Bauche et al, 1994; Lan

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et al, 1998; Storey et al, 1998; Tramer et al, 1998; Ursini et al, 1999; Lee et al, 2000). GSH-related enzymes have also been studied in epididymal cell cultures (Montiel et al, 2003) and in the aging rat epididymal duct following GSH depletion (Zubkova and Robaire, 2004). In the studies on GSH and GSH-related enzymes, the focus has mostly been on epididymal and sperm GSH function as an antioxidant.

In addition to a major role as an antioxidant and in eliminating toxic compounds, GSH has been implicated in prooxidation processes in various cells, via  $\gamma$ -glutamyl-transpeptidase ( $\gamma$ -GT)-dependent catabolism. GSH catabolism can lead to oxidative modifications of cellular PSHs (Filomeni et al, 2002; Paolicchi et al, 2002). Modulating effects of GSH catabolism have been observed on components of signal transduction pathways, such as those involving cell surface receptors and transcription factors (Accaoui et al, 2000; Filomeni et al, 2002; Paolicchi et al, 2002).

We have previously analyzed sperm PSHs and disulfides (Shalgi et al, 1989; Seligman et al, 1997), based on the use of the fluorescent thiol labeling agent monobromobimane (mBBr) (Kosower and Kosower, 1987). Labeling by mBBr can be carried out in the intact sperm before fractionation, thus avoiding possible thiol modification and loss during subsequent cell fractionation and analysis. These procedures allowed the morphologic evaluation and quantitative determination of SH and SS (after the reduction of SS by dithiothreitol [DTT]) in whole spermatozoa and subcellular fractions and the analysis of PSH status by electrophoretic separation of labeled sperm proteins (Kosower and Kosower, 1987).

Little information is available on the dynamics of GSH and glutathione disulfide (GSSG) and other nonprotein thiols (NPSHs) and disulfides in spermatozoa and epididymal fluid (EF) during sperm passage in the epididymis. It is not clear whether the NPSHs and disulfides (NPSSNs) are involved in sperm PSH oxidation or whether GSH catabolism in the epididymis can serve as a pathway for sperm PSH oxidation. Identification and quantitative analysis of mBBr-labeled NPSHs at the picomole levels can be carried out by high-performance liquid chromatographic (HPLC) methods (Fahey and Newton, 1987). In the present study, we used mBBr to analyze NPSHs and NPSSRs (R, nonprotein or protein) in the spermatozoa and EF of the rat caput and cauda epididymis and examined whether small thiols and disulfides such as GSH/glutathione disulfides (GSSRs), cysteine (CSH), and cysteine disulfides (CSSRs) are involved in sperm PSH oxidation during epididymal maturation. Our results suggest that epididymal NPSH/NPSSNP participates in sperm PSH oxidation and that  $\gamma$ -GT, which initiates the degradation of extracellular GSH, plays a role in the processes leading to sperm PSH oxidation.

## Materials and Methods

### Animals and Reagents

Mature albino Wistar-derived rat males, 4–8 months old, were used for this study. The study was carried out in accordance with the rules established by the Animal Care and Use Committee at Tel-Aviv University. All reagents were obtained from Sigma Chemical Company (St Louis, Mo), apart from mBBr, which was obtained from Molecular Probes Inc (Eugene, Ore).

### Isolation of Spermatozoa and Fluid and Labeling With mBBr

*a) Labeling of Sample Thiols*—Epididymal lumen content (fluid plus spermatozoa) was collected from the caput and cauda by established procedures. Briefly, the outer surface of the epididymis was cleaned, and the cauda and caput segments obtained from the same animal were each sliced into 135 mM NaCl/10 mM Tris-HCl buffer, pH 7.4 (TBS), containing 2.5 mM mBBr, in the presence and absence of 0.5 mM of the  $\gamma$ -GT inhibitor acivicin, and then agitated for 5 minutes to allow the release of spermatozoa and the fluid contained within the lumen of the epididymis. The spermatozoa and fluid were collected in a test tube and kept in the dark for another 10 minutes, and a sperm count was carried out.

To determine the molar concentrations of NPSH in the EF, the epididymal caudal content was quantitatively obtained by a micropipette. A needle connected to a syringe containing paraffin oil was inserted into the upper segment of the vas deferens. The cauda epididymal content (fluid plus spermatozoa) was displaced by the paraffin oil in the syringe and was collected by a calibrated micropipette through a minute incision in the epididymis at a location where the epididymal duct was relatively wide. The micropipette content was diluted with TBS containing 0.5 mM acivicin and 2.5 mM mBBr for thiol analysis. An aliquot of the whole sample was centrifuged, and the fluid fraction was separated from the spermatozoa. The concentration of NPSH in the whole sample was calculated on the basis of the dilution of the micropipette sample in the buffer. The concentration of NPSH in the fluid fraction was estimated on the basis of a fluid volume of 45% in the whole sample (Levine and Marsh, 1971). For the sperm fraction, the NPSH was based on the sperm number per milliliter of whole sample and on a packed sperm volume of 55%.

*b) Labeling of Sample Disulfides*—Whole samples (fluid plus spermatozoa), obtained from the cauda epididymis by the micropipette method, and washed caput and cauda spermatozoa obtained by slicing the epididymis were analyzed for NPSSR in relation to NPSH levels. Whole samples (fluid plus spermatozoa), EF, and washed spermatozoa were divided into 2 parts. Aliquots were incubated with 2.5 mM N-ethylmaleimide (NEM) for 30 minutes at 37°C (to block the reactive thiols, allowing the determination of disulfides) or without NEM (for the determination of thiols). All samples were incubated in the presence of 0.5 mM acivicin. Excess NEM was removed by extraction with benzene. To reduce disulfides, samples were incubated with 1 mM DTT for 10 minutes at 37°C and then labeled in the dark with 3 mM mBBr. Under these conditions, the NPSSRs include

NPSSRs (symmetrical and unsymmetrical, eg, GSSG, CSSC, GSSC) and nonprotein-protein mixed disulfides (NPSSPs).

#### Analysis of Nonprotein Fractions by HPLC

The mBBr-labeled samples were treated with 5% trichloroacetic acid (TCA) to precipitate proteins, and the samples were centrifuged. The supernatants containing the mBBr-labeled NPSHs and the labeled reduced NPSSs were freed of the unreacted mBBr and the bis-bimane derivative of DTT by extraction with  $\text{CH}_2\text{Cl}_2$ . Analysis of thiols by HPLC was carried out as previously described (Fahey and Newton, 1987). HPLC allows the identification and quantitation of mBBr-labeled NPSHs such as GSH and CSH at the picomole level. For the disulfides, the levels are presented as half-disulfide equivalents.

#### Evaluation of Sperm Thiols by Microscopy and Analysis of Sperm PSHs by Electrophoresis

Labeled spermatozoa were examined with a Leitz fluorescence microscope equipped with epifluorescence optics and filter A (for ultraviolet [UV] excitation range of 340–380 nm and emission above 450 nm). Photography was carried out using identical exposure times. For analysis of PSHs, labeled spermatozoa were solubilized in 5% sodium dodecyl sulfate (SDS) and 3% 2-mercaptoethanol at 37°C for 10 minutes in the presence of 1 mM EDTA and 0.5 mM phenylmethyl sulfonyl fluoride. Samples were sonicated for 10 seconds to liquefy the DNA and analyzed by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Following electrophoresis, gels were photographed using UV illumination to detect the fluorescent protein bands and then stained with Coomassie blue and photographed again.

## Results

#### NPSH in Whole Epididymal Content (Fluid Plus Spermatozoa)

Analyses of NPSHs in rat epididymal lumen content (fluid plus spermatozoa), obtained by slicing the caput and cauda epididymis, were carried out on protein-free extracts, as described in “Materials and Methods.” In initial experiments, the epididymal lumen content was diluted with TBS alone before being labeled with mBBr. The main NPSH identified was CSH, along with low levels of GSH and traces of other thiols (including CoA, ovoidiol-A, ovoidiol-C,  $\gamma$ -glutamylcysteine, cysteinylglycine, and several others that were not identified). In the caput, the values, expressed as pmol/ $10^6$  spermatozoa/mL, were  $1750 \pm 190$  for CSH and  $40 \pm 9$  for GSH (mean  $\pm$  SEM;  $n = 3$  experiments) (Figure 1A). In the cauda, the values were  $230 \pm 50$  for CSH and  $31 \pm 9$  for GSH (mean  $\pm$  SEM;  $n = 5$  experiments) (Figure 1B). Recovery of CSH and GSH was assessed by the addition of 6  $\mu\text{M}$  CSH or GSH to some aliquots before mBBr labeling. We found that most of the added CSH was recovered (approximately 80%). In contrast, most of the GSH was lost (recovery of added GSH was about 5%–15%, with about 60% re-

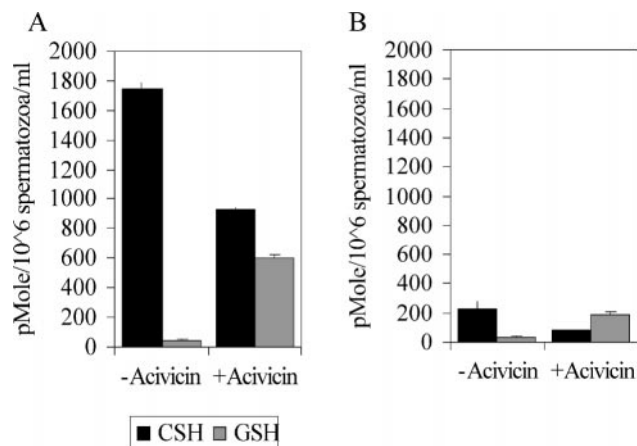


Figure 1. Cysteine (CSH) and glutathione (GSH) levels in the caput and cauda lumen (fluid plus spermatozoa). The caput and cauda epididymis segments were sliced, and their content was released into 135 mM NaCl/10 mM Tris-HCl buffer, pH 7.4 (TBS) in the absence of acivicin, (–Acivicin) or in the presence of acivicin; (+Acivicin). The samples were incubated with monobromobimane (mBBr), deproteinized, and analyzed by high-pressure liquid chromatography (HPLC), as described in “Materials and Methods.” Values for CSH and GSH are expressed as pmol/ $10^6$  spermatozoa/mL (to signify that the values reflect the nonprotein thiols [NPSHs] in the epididymal lumen, including spermatozoa and fluid). The black bars represent CSH values. The gray bars represent GSH values. (A) CSH and GSH in caput lumen. (B) CSH and GSH in cauda lumen.

covered as CSH). The results indicated that under these conditions, the apparent higher content of CSH (than that of GSH) was due to the conversion of GSH to CSH, via cleavage by  $\gamma$ -GT, followed by a dipeptidase cleavage (Kozak and Tate, 1982; Agrawal and Vanha-Perttula, 1988a). To achieve an efficient recovery of GSH when it was added to the epididymal lumen samples, 0.5 mM of the  $\gamma$ -GT inhibitor acivicin was added during sample preparation (85%–100% of GSH recovered). HPLC analysis of caput and cauda lumen content obtained in the presence of acivicin showed lower CSH levels and higher GSH levels than those obtained in the absence of the inhibitor. In the caput, CSH and GSH levels were  $940 \pm 90$  and  $610 \pm 90$  pmol/ $10^6$  spermatozoa/mL, respectively ( $n = 3$ ) (Figure 1A); and in the cauda, CSH and GSH levels were  $82 \pm 2$  and  $198 \pm 19$ , respectively ( $n = 5$ ) (Figure 1B). The combined levels of CSH and GSH obtained in the presence of acivicin were similar to those obtained in the absence of acivicin (Figure 1), indicating that the GSH was indeed converted to CSH in the absence of the  $\gamma$ -GT inhibitor. On the basis of these results, all subsequent sample preparations were carried out in the presence of 0.5 mM acivicin.

#### Concentrations of NPSH/NPSSR in the Cauda EF and in the Spermatozoa

To determine the molar concentrations of NPSH/NPSSR in the epididymal lumen, we used a calibrated micropipette (see “Materials and Methods”). Only the cauda ep-

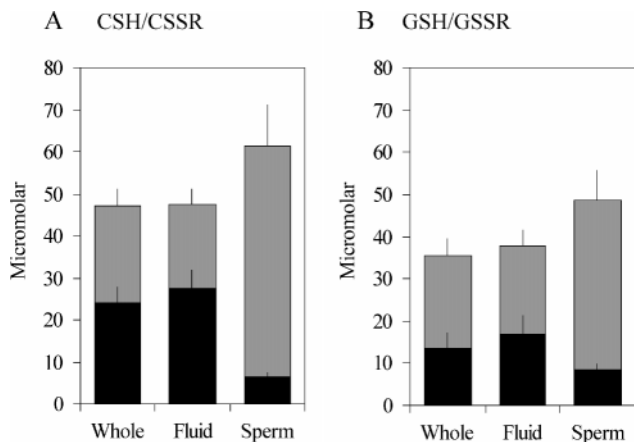


Figure 2. Cysteine/Cysteine disulfide (CSH/CSSR) and glutathione/glutathione disulfide (GSH/GSSR) levels in cauda lumen content. The lumen content was collected by a calibrated micropipette directly from the cauda epididymal duct into 135 mM NaCl/10 mM Tris-HCl buffer, pH 7.4 (TBS), containing acivicin. Whole samples, epididymal fluid, and washed spermatozoa were divided into 2 parts. One part, used for nonprotein thiols (NPSHs), was labeled with monobromobimane (mBBR). The other part, used for nonprotein disulfide (NPSSR) analysis, was first treated with N-ethylmaleimide (NEM) to block reactive thiols; disulfides were then reduced by dithiothreitol (DTT) before labeling with mBBR, as described in "Materials and Methods." Values for CSH and GSH are expressed in  $\mu\text{M}$ . **(A)** CSH/CSSR levels. **(B)** GSH/GSSR; whole lumen content (whole), epididymal fluid (fluid), and spermatozoa (sperm). The black bars represent NPSH values (CSH or GSH). The gray bars represent NPSSR values (CSSR or GSSR).

ididymis was used in this procedure, since it was adequate for obtaining fluid and spermatozoa from the cauda epididymis but was not useful for obtaining caput epididymal content without injury to the wall of the narrow caput epididymal tube. Aliquots were used for sperm counting and for the determination of NPSHs and NPSSRs, as described in "Materials and Methods." To assess the recovery of NPSSRs, 2  $\mu\text{M}$  GSSG was added to aliquots of samples before incubation with NEM. The recovery of added GSSG was approximately 80%, indicating that the procedure employed was adequate for the analysis of NPSSRs in these samples. The level of CSH in the whole samples and in the EF was  $24 \pm 4 \mu\text{M}$  and  $28 \pm 4 \mu\text{M}$ , respectively ( $n = 4$ ). Significantly less CSH was found in the spermatozoa ( $6.3 \pm 1.3 \mu\text{M}$ ,  $n = 7$ ) (Figure 2A). The results indicate that more CSH is present in the EF than in the spermatozoa. Similar results were obtained for GSH. The GSH concentrations in whole samples and in EF were  $13.5 \pm 3.5 \mu\text{M}$  and  $16.9 \pm 3.4 \mu\text{M}$ , respectively ( $n = 4$ ). Washed spermatozoa contained less GSH than the fluid ( $8.6 \pm 1.6 \mu\text{M}$ ,  $n = 5$ ). In the whole samples and in the fluid fractions, about half of the total of CSH plus CSH equivalents in disulfides (CSSR, including CSSC, nonsymmetrical small disulfides, and mixed CSS proteins) was present as CSH (Figure 2A). Similarly, about half of the total GSH plus GSH equivalents in disulfides (GSSR, including GSSG, nonsymmetrical small

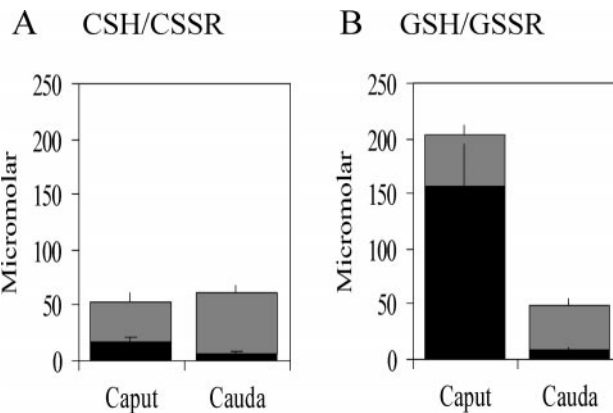


Figure 3. Cysteine/Cysteine disulfide (CSH/CSSR) and glutathione/glutathione disulfide (GSH/GSSR) levels in spermatozoa obtained from the caput and cauda epididymis. Spermatozoa were washed and suspended in 135 mM NaCl/10 mM Tris-HCl buffer, pH 7.4 (TBS), containing 0.5 mM acivicin; aliquots were used for nonprotein thiols (NPSHs) and nonprotein disulfide (NPSSR) analysis, as described in "Materials and Methods." Values for CSH and GSH are expressed in  $\mu\text{M}$ . The black bars represent NPSH values (CSH or GSH). The gray bars represent NPSSR values (CSSR or GSSR).

disulfides, and mixed GSS proteins) in the fluid was present as GSH (Figure 2B). In contrast, the spermatozoa contained little CSH and GSH but large amounts of the disulfides (Figure 2A and B).

#### Concentrations of NPSH/NPSSR in the Cauda and Caput Spermatozoa

The levels of CSH/CSSR, GSH/GSSR in caput and cauda spermatozoa are shown in Figure 3. The caput spermatozoa contained a high level of GSH when compared to that in the cauda spermatozoa ( $156 \pm 39 \mu\text{M}$  in the caput and  $8.6 \pm 1.6 \mu\text{M}$  in the cauda spermatozoa [ $n = 5$ ]). The level of GSSR was  $48 \pm 7 \mu\text{M}$  in the caput spermatozoa, with similar levels in the cauda sperm. CSH content of the caput spermatozoa was significantly lower than that of GSH; in the cauda spermatozoa, the low CSH level was similar to that of the GSH level. The level of total CSH plus CSSR in the cauda cells was similar to that in the caput cells. There was a sharp drop in the sperm GSH level as the spermatozoa passed from the caput to the cauda (Figure 3B). In addition, as shown in Figure 3, the cauda spermatozoa contained appreciable amounts of NPSSRs relative to GSH and CSH.

#### In Vitro Effects of EF, NPSHs, and Disulfides on Sperm Thiol Status

We have previously shown that in the rat, mBBR-labeled caput spermatozoa exhibit high fluorescence, indicative of high thiol levels, whereas cauda spermatozoa contain low thiol levels, and mBBR-labeled cauda sperm heads are almost totally dark (Shalgi et al, 1989). To examine the effects of EF on sperm thiol status, unwashed caput sper-

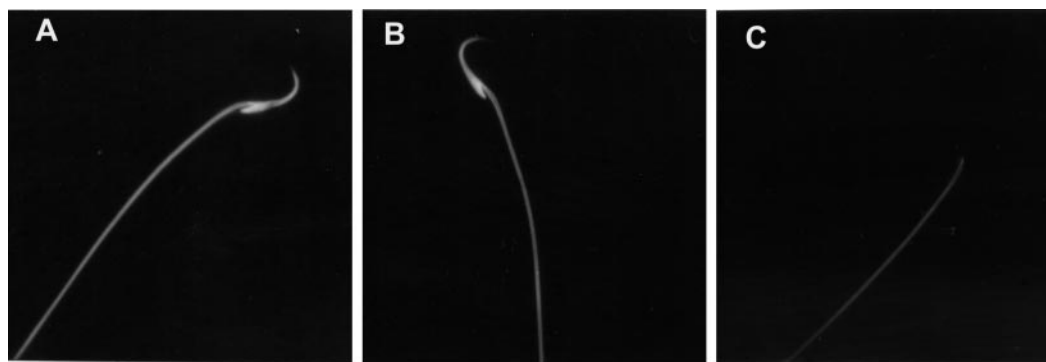


Figure 4. Fluorescence micrographs of caput spermatozoa labeled with monobromobimane (mBBr). (A) Control (nonincubated) spermatozoa, washed and labeled at time 0. (B, C) Spermatozoa labeled following 3 days of incubation at 4°C. (B) Washed spermatozoa, resuspended in 135 mM NaCl/10 mM Tris-HCl buffer, pH 7.4 (TBS). (C) Unwashed spermatozoa, incubated in the presence of epididymal fluid (EF).

matozoa (ie, samples that retained the caput EF) and washed sperm samples (freed of EF), were incubated for 3 days at 4°C and labeled with mBBr, and the thiol status of spermatozoa was assessed by fluorescence microscopy. As can be seen in Figure 4, caput spermatozoa that were washed and incubated in TBS exhibited high fluorescence in heads and tails, similar to the fluorescence intensity shown by spermatozoa that were labeled without incubation (Figure 4A and B). In contrast, a diminution in fluorescence was observed in unwashed caput spermatozoa kept for 3 days in the presence of EF. Diminution in fluorescence was especially noted in sperm heads, which were almost totally dark, with some diminution also observed in sperm tails (Figure 4C). Cauda sperm exhibited

low fluorescence when kept in either EF or TBS, especially in sperm heads, similar to the low fluorescence shown by nonincubated spermatozoa (data not shown). Thus, the heads of caput spermatozoa that were kept in the caput EF resembled cauda sperm heads.

To assess sperm tail PSH levels, mBBr-labeled spermatozoa were solubilized, and the proteins were analyzed by SDS-PAGE, as described previously (Shalgi et al, 1989). As can be seen in Figure 5, unwashed caput spermatozoa that were kept in vitro with the caput EF demonstrated a decrease in the fluorescence of various sperm protein bands when compared to the control, washed caput spermatozoa kept in buffer (Figure 5, lanes 1 and 2). No significant changes were observed in the PSH content of unwashed cauda spermatozoa kept in vitro with the cauda EF (Figure 5, lanes 3 and 4).

To examine the effects of small thiols and disulfides on sperm thiol status, washed and unwashed caput and cauda spermatozoa were treated with or without GSH, GSSG, CSH, and CSSC. As shown in the Table, the washed caput

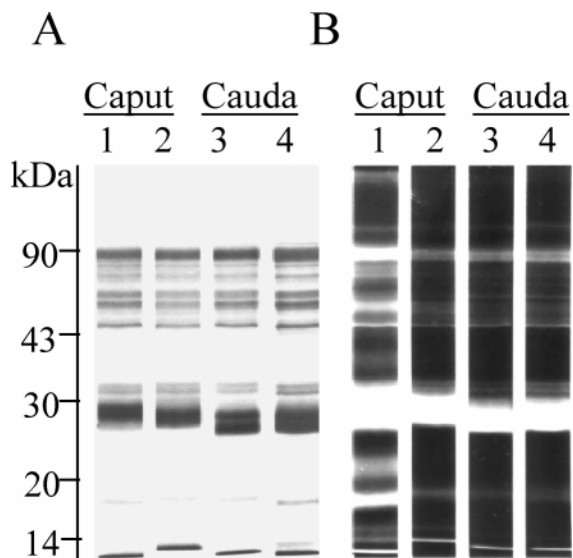


Figure 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of caput (1,2) and cauda (3,4) spermatozoa kept for 3 days at 4°C and then labeled with monobromobimane (mBBr). 1,3, washed spermatozoa, kept in 135 mM NaCl/10 mM Tris-HCl buffer, pH 7.4 (TBS); 2,4, unwashed spermatozoa kept in epididymal fluid. (A) Coomassie blue stain. (B) Fluorescence illumination. Approximate molecular masses are indicated from the migration of known protein standards.

Effects of extracellular nonprotein thiols and disulfides on thiol status in epididymal sperm heads\*

| Sperma-<br>tozoa | EF† | Relative Levels of<br>mBBr-Fluorescence of Sperm Heads |         |     |      |      |
|------------------|-----|--|---------|-----|------|------|
|                  |     | Un-<br>treated   | Treated |     |      |      |
|                  |     |  | GSH     | CSH | GSSG | CSSC |
| Caput            | —   | 10   | 10      | 10  | 1    | 1    |
| Caput            | +   | 1  | 3       | 5   | 1    | 1    |
| Cauda            | —   | 1  | 10      | 10  | 1    | 1    |
| Cauda            | +   | 1  | 1       | 1   | 1    | 1    |

\* GSH indicates glutathione; CSH, cysteine; GSSG, glutathione disulfide; CSSC, cysteine disulfide; and mBBr, monobromobimane.

† Washed (—epididymal fluid [EF]) or unwashed (+EF) caput and cauda sperm suspensions were kept at 4°C for 3 days without or with 1 mM GSH, CSH, GSSG, and CSSC; spermatozoa were then washed and labeled with mBBr. The fluorescence intensity of the sperm heads, evaluated by microscopy, was graded from 1 (very low intensity) to 10 (very high intensity).

spermatozoa (incubated in the absence of EF) exhibited a high fluorescence intensity when either untreated or treated with GSH or CSH (Figure 4B; Table). Treatment of washed caput spermatozoa with the disulfides GSSG and CSSC led to a striking diminution in head fluorescence, with some diminution observed in tail fluorescence. Unwashed caput spermatozoa (incubated in the presence of EF) exhibited a low fluorescence when compared to washed spermatozoa. This effect was especially noted in sperm heads, which became dark and resembled cauda sperm heads (Figure 4C; Table). Treatment of the unwashed caput spermatozoa with GSH or CSH led to the appearance of some fluorescence, mainly noted in the sperm heads. Spermatozoa incubated with CSH exhibited somewhat a higher fluorescence intensity than those incubated with GSH. Heads of washed cauda spermatozoa, which were almost totally dark, exhibited a high fluorescence intensity when treated with GSH or CSH (Table), with a slight increase in fluorescence of the sperm tails. Treatment of unwashed cauda spermatozoa with GSH and CSH had no effect on these spermatozoa, with heads remaining dark. The results presented indicate that EF has an oxidizing effect on sperm thiols (most easily observed in sperm heads of caput spermatozoa kept in caput EF). In addition, the results demonstrate that extracellular NPSHs and NPSSRs can interact with spermatozoa and alter the thiol status of caput and cauda spermatozoa. Thus, GSSG and CSSC lead to the oxidation of sperm thiols, especially noted in the caput sperm heads. Conversely, the thiols in the buffer (GSH, CSH) lead to an increase in sperm thiols, noted in the cauda sperm heads.

## Discussion

Oxidation-reduction (redox) reactions are important to the regulation of metabolic functions of the cell and in the protection of cellular components against oxidative damage (Sies, 1991). In the case of spermatozoa, a shift of the redox status of PSHs to disulfides occurs as the spermatozoa undergo maturation during their passage through the epididymis (Maiorino and Ursini, 2002). The epididymal region in which this change occurs differs among species (Kosower et al, 1992). In the rat, spermatozoa isolated from the caput epididymis contain high levels of thiols, while cauda epididymis spermatozoa contain mostly disulfides (Shalgi et al, 1989). An accurate degree of thiol oxidation is important for acquisition of sperm motility and fertilizing ability (Cornwall et al, 1986; Seligman et al, 1991, 1992). In addition to participation in structural changes, sperm PSH oxidation promotes tyrosine phosphorylation, which is important for normal sperm function (Aitken et al, 1995; Baker and Aitken, 2004; Seligman et al, 2004).

The mechanisms and factors responsible for the physiologic oxidation of sperm PSHs to disulfides during epididymal sperm maturation are not completely understood. Several pathways leading to sperm PSH oxidation have been considered. The selenoprotein phospholipid hydroperoxide GSH peroxidase (PHGPx) uses PSHs as substrates for the reduction of hydroperoxides when GSH levels are low (Flohé et al, 2002). PHGPx is present in testicular spermatids and is assumed to protect them from oxidative damage (Roveri et al, 1992; Godeas et al, 1997; Flohé et al, 2002). In the late stages of sperm maturation in the epididymis, when sperm GSH is low, PHGPx oxidizes PSHs. At this stage, it is involved in the stabilization of the mitochondrial capsule, resulting in mature spermatozoa, when PHGPx is present as oxidatively cross-linked, enzymatically inactive, insoluble protein (Ursini et al, 1999; Flohé et al, 2002; Maiorino and Ursini, 2002). PHGPx may also play a role in sperm chromatin condensation (Godeas et al, 1997; Maiorino and Ursini, 2002).

NADPH oxidase, present in the male genital tract, has been proposed to be an important oxidant source responsible for the production of reactive oxygen species (ROS), leading to PSH oxidation. Spermatozoa have been considered a source for NADPH oxidase, in addition to the oxidase activity in leukocytes present in the male genital tract (Griveau and Le Lannou, 1997; Maiorino and Ursini, 2002; Baker and Aitken, 2004). However, mammalian spermatozoa may not possess significant NADPH oxidase activity, as indicated by recent studies (Richer and Ford, 2001; Baker et al, 2004). Thus, ROS may mainly be generated by sources external to spermatozoa. Electron leakage from the mitochondria and nitric oxide (NO) have been proposed as additional oxidant sources (Maiorino and Ursini, 2002). The ROS generated would serve dual roles. Depending on the nature, on the location in the male genital tract, and on the level of ROS, the outcome would be a physiologic or pathologic oxidation of sperm components (Griveau and Le Lannou, 1997; Maiorino and Ursini, 2002; Baker and Aitken, 2004).

The results presented suggest that GSH catabolism via the  $\gamma$ -GT pathway is an oxidant source for sperm PSH oxidation.  $\gamma$ -GT is a ubiquitous enzyme present in many tissues, including the testis, epididymis, and seminal vesicle. The enzyme is associated with the apical surface of the epididymal epithelium and is also present within the epididymal luminal fluid in vesicles or in solubilized form (Agrawal and Vanha-Perttula, 1988a; Lan et al, 1998).  $\gamma$ -GT acts to cleave the gamma bond between glutamate and CSH in GSH to yield cysteinylglycine (Cys-Gly) and glutamate (Kozak and Tate, 1982; Lan et al, 1998). Cys-Gly is then cleaved by dipeptidase (Kozak and Tate, 1982).  $\gamma$ -GT has been shown to be important in the development of male reproductive organs and their functions (Agrawal and Vanha-Perttula, 1988b; Lee et al,

2000) and is assumed to play a role in protecting spermatozoa from oxidative stress (Lan et al, 1998). However, ROS are produced as a by-product of  $\gamma$ -GT-catalyzed GSH cleavage (Dominici et al, 1999; Filomeni et al, 2002; Paolicchi et al, 2002), and thus,  $\gamma$ -GT activity can serve as an oxidant source.

We found that the total GSH and CSH level in the caput epididymis lumen (fluid and spermatozoa) is significantly higher than that in the cauda epididymis. We also found that without  $\gamma$ -GT inhibition, most NPSH is present as CSH, with very little GSH present. In contrast, in the presence of a  $\gamma$ -GT inhibitor, the GSH level is significantly higher and is especially notable in the caput (Figure 1). The results point to  $\gamma$ -GT activity, especially significant in the caput. The high level of CSH observed in the samples, when these are processed in the absence of the  $\gamma$ -GT inhibitor, is thus a result of GSH cleavage. Our results are consistent with other published observations showing higher levels of GSH in the caput epididymis than in the cauda (Agrawal and Vanha-Perttula, 1988a; Zubkova and Robaire, 2004). In addition, amino acid analysis of EF has shown the presence of CSH with significantly higher levels present in the caput than in the cauda (Hinton, 1990).

Analysis of NPSH/NPSSR levels in the spermatozoa themselves showed significant differences in GSH/GSSR concentrations between caput and cauda spermatozoa. Caput spermatozoa contain GSH in concentrations about 3 times higher than the GSSR level. Cauda spermatozoa contain about 10 times less GSH than caput spermatozoa, whereas GSSR in cauda spermatozoa is at a level similar to that of caput spermatozoa (Figure 3). These results indicate a loss of intracellular GSH during the passage of spermatozoa through the epididymis and a shift to the oxidized state. Our results are consistent with previous observations, showing that caput epididymal spermatozoa have more GSH than cauda spermatozoa or ejaculated spermatozoa (Agrawal and Vanha-Perttula, 1988a) and that spermatozoa also contain an appreciable amount of GSSG (Storey et al, 1998). The results may also explain the lack of GSH under certain assay conditions, such as sperm collected from an excised epididymis in the absence of  $\gamma$ -GT inhibition (Bauche et al, 1994).

It has been suggested that GSH is transported to the lumen from epithelial cells and sperm cells (Agrawal and Vanha-Perttula, 1988a). There are no actual data regarding the efflux of GSH from sperm cells in addition to its being exported from the epididymal epithelial cells. GSH synthesized within the sperm cells (from  $\gamma$ -glutamylcysteine) and/or converted from GSSR (by exchange reactions) may be exported into the lumen. On reaching the cauda, rat spermatozoa contain mostly disulfides (NPSSRs). Low levels of GSH and CSH are present in the cauda spermatozoa (with the major part present as GSSR and CSSR), whereas

the cauda EF contains about 50% GSH (of total GSH/GSSR), with similar values for CSH/CSSR (Figure 2). These results indicate that in the cauda epididymis, the intrasperm milieu is at a more oxidized state than the surrounding fluid. The sperm cell is thus unique among other cells, since under physiologic conditions, in most tissues, the intracellular GSH level is higher than the GSSR level, and extracellular GSH levels are very low. These results are consistent with the notion that during the passage of spermatozoa in the epididymis, the activities of the reactions leading to sperm thiol oxidation and stabilization of sperm components diminish. Thus, GSH cleavage would occur mostly in the caput epididymis.  $\gamma$ -GT is known to be much more active in the caput epididymis than in the distal epididymal regions (Kozak and Tate, 1982; Agrawal and Vanha-Perttula, 1988b; Lan et al, 1998). Dipeptidase is also more active in the caput than in the cauda epididymis (Kozak and Tate, 1982). As a result, GSH in the cauda EF is not subjected to extensive cleavage, does not serve as a source for ROS, and may then act as an antioxidant, allowing the maintenance of reducing power in the EF in the distal parts of the epididymis. This reducing power may play a role in protecting the mature spermatozoa from excess oxidative damage to membrane components (Storey et al, 1998; Saleh and Agarwal, 2002; Baker and Aitken, 2004).

The results presented thus suggest the involvement of epididymal GSH and  $\gamma$ -GT in sperm PSH oxidation during sperm maturation. It is of interest to note that following testosterone withdrawal by castration, the level of cauda sperm thiols increases, indicating that testosterone withdrawal leads to inhibition of sperm thiol oxidation (Seligman et al, 1997). Since  $\gamma$ -GT is a testosterone-dependent enzyme (Hatier et al, 1994), testosterone withdrawal could influence PSH levels via effects on GSH catabolism.

The proposed steps involving  $\gamma$ -GT, GSH, CSH, GSSG, CSSC, and GSSC are summarized in the following scheme (Figure 6): GSH, present in the caput EF and/or exported from the epididymal epithelial cells, is cleaved by the very active caput epididymis  $\gamma$ -GT. The product Cys-Gly is further cleaved by dipeptidase to free CSH and glycine (Kozak and Tate, 1982; Lan et al, 1998). GSH and CSH are oxidized by the thiol oxidase present in the EF (Chang and Morton, 1975; Chang and Zirkin, 1978). Thiol oxidation may also be achieved via nonenzymatic chemical reactions catalyzed by metal ions (Held and Biaglow, 1994; Dominici et al, 1999; Paolicchi et al, 2002). Metal ions such as copper and iron are present in the EF (Gaur et al, 2000). CSH has been shown to autoxidize significantly faster than GSH at a pH greater than 7.0 in the presence of catalytic amounts of copper (Held and Biaglow, 1994). This autoxidation would be expected to be similarly fast with Cys-Gly. Such autoxidation has

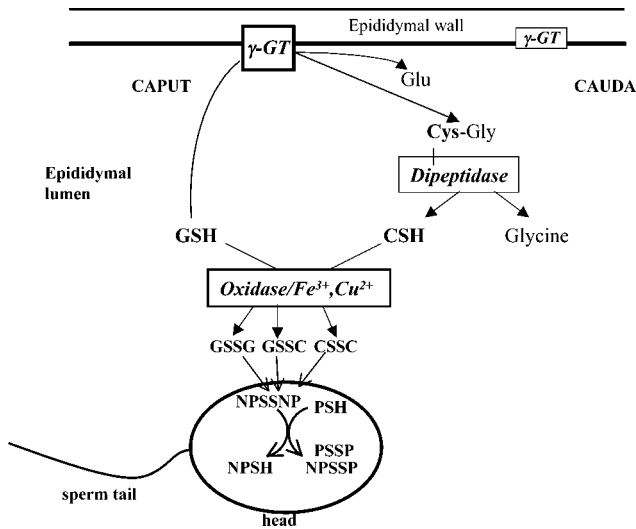


Figure 6. Scheme presenting the proposed steps that involve  $\gamma$ -glutamyl-transpeptidase ( $\gamma$ -GT), glutathione (GSH), cysteine (CSH), and glutathione and cysteine disulfides (GSSG, GSSC, and CSSC) in sperm thiol oxidation during sperm maturation in the epididymis.  $\gamma$ -GT (—, high activity; —, low activity); Glu, glutamate; Cys-Gly, cysteine-glycine; Oxidase/ $Fe^{3+}$ ,  $Cu^{2+}$ , oxidase and/or metal ions; PSH, protein thiols; PSSPs, protein disulfides; and NPSSPs, nonprotein-protein mixed disulfides.

been shown to produce hydrogen peroxide (Held and Biaglow, 1994; Dominici et al, 1999; Paolicchi et al, 2002). Thus, the initial cleavage of GSH by  $\gamma$ -GT to Cys-Gly and CSH in the EF may generate a rapidly autooxidizing environment, giving the observed NPSSNP (ie, GSSG, CSSC, GSSC). Additional studies are required to clarify the role played by enzymatic vs nonenzymatic reactions in NPSH oxidation. The disulfides (formed by enzymatic or nonenzymatic reactions) then participate in sperm PSH oxidation (as shown in the Table). This conclusion is strengthened by the finding that sperm thiols are oxidized when spermatozoa are exposed to caput EF (Figures 4 and 5; Table). The NPSSNP may enter the spermatozoa or interact with surface thiols, initiating further thiol-disulfide interchange reactions within the cell. The NPSSNP-induced sperm PSH oxidation may also involve cross-talk between GSH/GSSG and factors such as thioredoxin (Casagrande et al, 2002), sperm-specific forms of which have been identified in the sperm tail (Jiménez et al, 2002). Possible regulation of sperm thioredoxin by GSH/GSSG requires further study.

In conclusion, the results presented, along with the published data, point to GSH catabolism via  $\gamma$ -GT as a source for sperm thiol oxidation, in addition to other pathways thought to be involved (NADPH oxidase, NO, and PHGPx). The quantitative parts played by each remain to be studied.

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