

BASIC RESEARCH

## Protein and non-protein sulfhydryls and disulfides in gastric mucosa and liver after gastrototoxic chemicals and sucralfate: Possible new targets of pharmacologic agents

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### Abstract

**AIM:** To investigate the role of major non-protein and protein sulfhydryls and disulfides in chemically induced gastric hemorrhagic mucosal lesions (HML) and the mechanism of gastroprotective effect of sucralfate.

**METHODS:** Rats were given 1 mL of 75% ethanol, 25% NaCl, 0.6 mol/L HCl, 0.2 mol/L NaOH or 1% ammonia solutions intragastrically (i.g.) and sacrificed 1, 3, 6 or 12 min later. Total (reduced and oxidized) glutathione (GSH + GSSG), glutathione disulfide (GSSG), protein free sulfhydryls (PSH), protein-glutathione mixed disulfides (PSSG) and protein cystine disulfides (PSSP) were measured in gastric mucosa and liver.

**RESULTS:** Reduced glutathione (GSH) was depleted in the gastric mucosa after ethanol, HCl or NaCl exposure, while oxidized glutathione (GSSG) concentrations increased, except by HCl and NaOH exposure. Decreased levels of PSH after exposure to ethanol were observed, NaCl or NaOH while the total protein disulfides were increased. Ratios of reduced to oxidized glutathione or sulfhydryls to disulfides were decreased by all chemicals. No changes in thiol homeostasis were detected in the liver after i.g. administration of ethanol. Sucralfate increased the concentrations of GSH and PSH and prevented the ethanol-induced changes in gastric mucosal thiol concentrations.

**CONCLUSION:** Our modified methods are now suitable

for direct measurements of major protein and non-protein thiols/disulfides in the gastric mucosa or liver. A common element in the pathogenesis of chemically induced HML and in the mechanism of gastroprotective drugs seems to be the decreased ratios of reduced and oxidized glutathione as well as protein sulfhydryls and disulfides.

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**Key words:** Non-protein and protein thiol; Gastric mucosal injury; Gastroprotection; Sucralfate

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### INTRODUCTION

The biochemical basis of chemically induced acute gastroduodenal hemorrhagic mucosal lesions (HML) and protection remains unclarified. Injury to gastric mucosa and isolated gastric mucosal cells by damaging chemicals or *H. pylori* involves a complex sequence of biochemical and morphological events, e.g., activation of oxidative stress pathways, diminished prostaglandin (PG) and GSH metabolisms and early microvascular injury<sup>[1-10]</sup>.

In addition to the PG-dependent mucosal protection<sup>[11,12]</sup> increasing evidence indicates that endogenous sulfhydryls (SH) play an important role in the maintenance of gastroduodenal integrity and in the protection against chemically-induced lesions in cells, tissues and organs<sup>[13-15]</sup>. The relatively high concentration of non-protein sulfhydryls (NPSH) which is mostly reduced glutathione (GSH,  $\gamma$ -glutamyl-cysteinyl glycine) besides cysteine (CSH), coenzyme A and other thiols in the gastric mucosa also indicates their possible implications for gastroprotection<sup>[16]</sup>.

The direct organo- or cytoprotective effects of reduced GSH, N-acetylcysteine (NAC) or other SH compounds, (e.g., taurin) were demonstrated biochemically and pharmacologically *in vivo* against concentrated ethanol<sup>[2,13,16-20]</sup>, non-steroidal anti-inflammatory drugs

(NSAID)<sup>[1,21,22]</sup> and acrylonitrile<sup>[23]</sup> as well as in vitro with cultured or isolated gastric mucosal cells against ethanol<sup>[24-27]</sup> or HCl<sup>[28]</sup>.

Rapid activation and release of SH protease cathepsin B was detected after ethanol exposure in the rat stomach. Both the increase of protease activity and development of acute gastric mucosal lesions were prevented by sulfhydryl alkylators iodoacetate, iodoacetamide and N-ethylmaleimide (NEM)<sup>[29,30]</sup>. In addition, inactivation of endogenous protease inhibitors and activation of cathepsins B, L and H were demonstrated after ammonia-solution and ethanol exposures in the stomach. It has been concluded that various cysteine proteases and their endogenous inhibitors may have a role in the pathogenesis of chemically induced gastric mucosal lesion and protection<sup>[30-32]</sup>.

Other studies also indicate that changes in PSH, PSSG and PSSP are also involved in both mechanisms of tissue damage and defense<sup>[33,34]</sup>.

Various chemicals such as ethanol (50% or 75%), NaCl (25%), HCl (0.6 N), NaOH (0.2 N) or ammonia-solution (1%) provoke acute HML in minutes<sup>[3,5,11,31]</sup>. Since previous biochemical and pathophysiologic studies with endogenous SH compounds involved only ethanol and NSAID, we tested the hypothesis that changes in PSH, NPSH and disulfides (especially depletion of GSH in the rat gastric mucosa) might represent common pathogenic factors in the mechanisms of mucosal injury induced by endogenous chemicals such as HCl or ammonia (a product of *H pylori*), as well as exogenous NaOH, ethanol and hypertonic NaCl. In addition, we also investigated the possibility, whether non-protein and protein sulfhydryls might be protective mechanisms by which gastroprotective drugs such as sucralfate overcome these damaging substances.

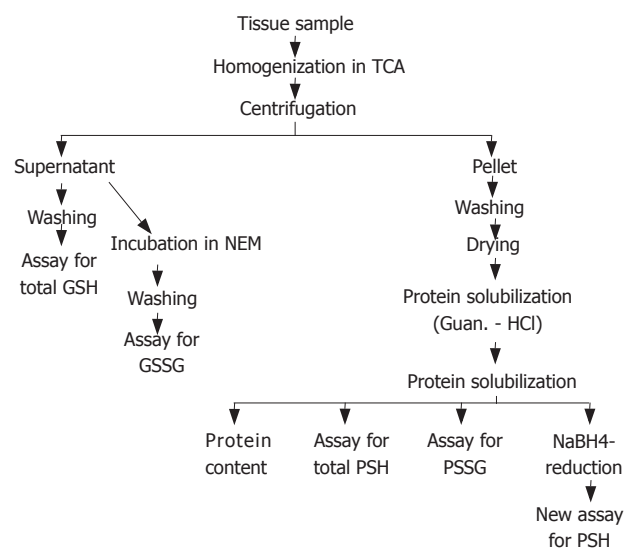
## MATERIALS AND METHODS

### Animals

Female Sprague-Dawley (S-D) rats (Taconic Farms, Germantown, NY), weighing 160-200 g were fasted for 24 h before experiment, but water was available ad libidum. Groups of rats ( $n = 4-6$ ) were given 1 mL of deionized water (in controls) or 1 mL of 75% (v/v) ethanol, 0.6 mol/L HCl, 0.2 mol/L NaOH, 25% (w/v) NaCl and 1% (v/v) ammonia water solution intragastrically (i.g.) by using gavage with a rubber stomach tube (Rusch No. 8). Rats were killed by cervical dislocation 1, 3, 6 or 12 min later. The abdomen was opened and the stomach was rapidly removed, opened along the greater curvature, rinsed in cold saline and blotted. A small piece of liver was also removed. The mucosa of the glandular stomach was scraped off with a blunt knife, weighed, then put in 5% (w/v) of ice-cold trichloroacetic acid (TCA) solution prepared with 0.01 mol/L HCl containing 5 mmol/L EDTA making a 1:20 w/v dilution. The liver samples were also rapidly weighed and put in TCA solution which was kept on ice.

### Chemicals

N-Ethylmaleimide (NEM), 5,5'-dithio-bis(nitrobenzoic acid) (DTNB), glutathione, reduced form, crystalline (GSH), glutathione, oxidized form (GSSG), L-cysteine



**Figure 1** Flow diagram of sample preparation and measurement of thiols in the rat gastric mucosa and liver.

HCl (CSH), glutathione reductase (GSSGR) (EC 1.6.4.2) type III from baker's yeast (155 units/mg protein), nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), guanidine HCl, bovine serum albumin (BSA), n-octyl alcohol, sodium borohydride (NaBH<sub>4</sub>), Trizma base, sodium phosphate dibasic and sodium hydroxide, were purchased from Sigma Chemical, (St. Louis, MO). Ammonium hydroxide (ammonia water), hydrochloric acid, ethylenediamine-tetraacetate (EDTA) disodium, ethyl ether, sodium chloride, sodium hydroxide, sodium phosphate monobasic, TCA were obtained from Fisher Scientific (Pittsburgh, PA). Ethanol was purchased from Florida Distillers (Lake Alfred, FL). Sucralfate was obtained from Marion Laboratories (Kansas City, MO).

### Preparation of samples

Figure 1 shows the flow chart of all major steps of biochemical procedures. Tissue samples were homogenized in TCA (1:20, w/v) solution for 60 s in plastic conic tubes using an ultra turrax homogenizer (Tissumizer, Tekmar Co., Cincinnati, OH) driven at 50000 r/min for 10 s three times. The homogenates were kept on ice and centrifuged at 4°C, 6000 × g for 15 min. Supernatants and pellets were separated for further preparation and biochemical assays of NPSH and protein SH fractions.

The supernatants were washed 5 times with ethyl ether (1:2, v/v) at 0°C. With this procedure triglycerides and TCA could be removed and the remaining 0.01 mol/L HCl could maintain slight acidity of solution to minimize the spontaneous oxidation of GSH. One portion of diluted (1:10-100, v/v) supernatants with 0.1 mol/L phosphate buffer saline (PBS, pH 7.4) at 0°C without further treatment was used for enzymatic and spectrophotometric measurement of the total glutathione (GSH + GSSG) levels.

Another 0.5-1.0 mL portion of the original supernatant was incubated (1:1, v/v) with 0.05 mol/L NEM in 0.1 mol/L PBS containing 5 mmol/L EDTA (pH 7.4) for 1 h at room temperature. Using this procedure the tissue

concentration of GSSG could be determined following preliminary reaction of reduced GSH with excess of NEM. The rapid and complete reaction between GSH and the SH alkylator NEM prevents the participation of reduced GSH in the enzymatic assay as well as its further spontaneous oxidation to GSSG<sup>[35,36]</sup>. After removal of non-reactive NEM by 10 extractions with cold ether (1:3, v/v) the GSSG could be measured enzymatically with GSSGR with or without further dilution.

The protein pellets were vigorously washed with 3.0 mL of ice-cold 5% (w/v) TCA containing 5 mmol/L EDTA in 0.01 mol/L HCl, centrifuged at  $4000 \times g$  for 15 min then washed 3 times again in 3.0 mL of ethyl ether. The vacuum-dried pellets were suspended for protein denaturation in 2.0 mL of 6.0 mol/L guanidine HCl plus 1.0 mL of 0.1 mol/L PBS, pH 7.4, containing 5 mmol/L EDTA with vigorous agitation for 10 min producing an almost complete dissolution of pellet. The clear supernatant (i.e., "protein solution") was separated by centrifugation at  $4000 \times g$  for 20 min for further preparation of samples or for biochemical assays.

Detection of protein disulfide groups (PSSP) was based on the reduction of disulfide bridges with 0.1 mol/L sodium borohydride (NaBH<sub>4</sub>) as reducing agent, and DTNB (Ellman's reagent) as thiol disulfide exchanger on previously denaturated protein samples<sup>[37,38]</sup>: 200  $\mu$ L of 0.1 mol/L NaBH<sub>4</sub> in 0.1 mol/L NaOH containing 0.02% Na<sub>2</sub>EDTA and a drop of octyl alcohol as an antifoaming agent was added to an 50  $\mu$ L aliquot of diluted (50%) "protein solution". The reduction was performed for 1.5 h at 37°C in a shaking water bath. The excess of sodium borohydride was removed by adding 30  $\mu$ L of 6 mol/L HCl. Since DTNB color should be developed in a mild alkaline environment, the pH of the reaction mixture was brought to about pH 8 with 400  $\mu$ L of 2.0 mol/L Tris-HCl buffer, pH 8.3, containing 0.02% EDTA.

#### **Enzymatic assays of both the total glutathione and oxidized glutathione**

This sensitive spectrophotometric measurement of SH groups was based on formation of color product resulting from reaction of DTNB and GSH<sup>[39,40]</sup>. The components, dissolved in 0.1 mol/L PBS (pH 7.4) containing 5 mmol/L EDTA were added in the following amounts: 100  $\mu$ L of original or NEM-treated supernatant (containing 0.05-0.5 mg tissue) mixed with 600  $\mu$ L of PBS (0.1 mol/L, pH 7.4), 100  $\mu$ L of 6 mmol/L Ellman's reagent (0.6  $\mu$ mol) and 100  $\mu$ L of GSSGR solution (10  $\mu$ g). The reaction was initiated with 100  $\mu$ L of 2 mmol/L NADPH (0.2  $\mu$ mol). The mixture (1.0 mL) was incubated while shaking at 37°C for exactly 6 min, and the absorbance at 412 nm was measured 10 min later against water<sup>[41]</sup> using a cuvette with 1 cm light path (standard assay for all GSH-containing disulfides).

The difference between the original and NEM-treated supernatants indicates the level of GSSG.

#### **Measurement of free protein SH**

One hundred  $\mu$ L of diluted (50%) "protein solution" was mixed with 800  $\mu$ L of PBS (0.1 mol/L, pH 7.4) containing 5 mmol/L EDTA and 100  $\mu$ L of 6 mmol/L

DTNB solution. Absorbance at 412 nm was measured spectrophotometrically 10 min later.

#### **Determination of protein-GSH mixed disulfides**

The diluted (50%) "protein solution", 100  $\mu$ L, 10  $\mu$ g GSSGR in 100  $\mu$ L PBS, 600  $\mu$ L of PBS (0.1 mol/L, pH 7.4, containing 5 mmol/L EDTA), 100  $\mu$ L of 6 mmol/L DTNB and 0.2  $\mu$ mol NADPH in 100  $\mu$ L PBS were mixed and kept at 37°C in shaking water bath for exactly 6 min. Absorption at 412 nm was read immediately afterwards.

#### **Measurement of protein cystine disulfides**

The half cystine residues of proteins were quantified using Ellman's reagent after guanidine-induced protein denaturation plus sodium borohydride reduction of cystine disulfide bonds, using untreated "protein solution" samples as control as described earlier in this paper.

#### **Assay of protein concentration**

Protein concentration was determined using the Coomassie blue (BIO-RAD) method<sup>[42]</sup> after 50% dilution of the "protein solution" sample. Absorption at 595 nm was measured with samples, blanks and known concentrations of bovine serum albumin.

#### **Investigations with sucralfate**

The experiments with gastroprotective doses of sucralfate were carried out in additional groups of fasted rats which were given 1 mL of 75% ethanol by gavage and were killed sacrificed 12 min later. Two of the groups received sucralfate at 10 or 50 mg/100g i.g. alone or (at 50 mg/100 g) 30 min before the administration of ethanol. The concentrations of all five types of non-protein and protein thiols in homogenates of mucosal scrapings of glandular stomach were measured immediately after autopsy as described above.

#### **Data analysis and statistical evaluation**

The nmol GSH/mg wet tissue and nmol GSSG/mg wet tissue were calculated from freshly prepared GSH dose response curves (5-100 ng). The dose curves of GSH and GSSG following reduction were identical. Protein SH data were expressed as nmol cysteine/mg protein corrected for the background absorbance measured in samples without protein solution. Protein disulfides were expressed as nmol cysteine (half cysteine)/mg protein after reducing disulfides with sodium borohydride. Protein-GSH mixed disulfides were expressed as nmol GSSG equivalent/mg protein calculated from a GSSG (or GSH) dose curves prepared in the standard assay.

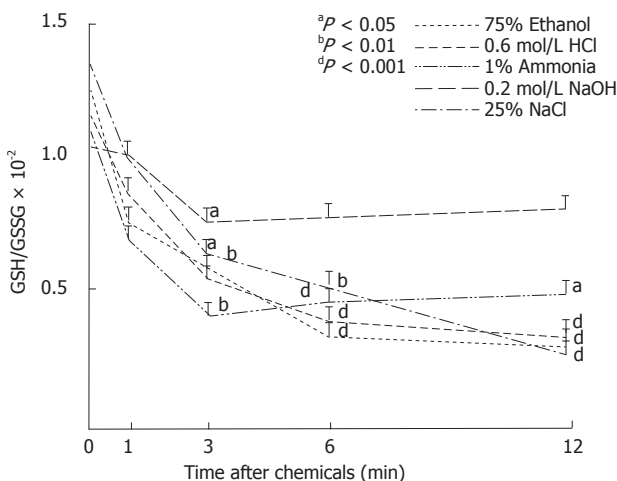
All experiments were carried out 3 times, the biochemical measurements were performed in duplicates and the results were pooled. Arithmetic means and standard errors of mean (SEM) were calculated. Statistical evaluation was performed using Mann-Whitney *U*-test, ANOVA and two-tailed Student's *t*-test for unpaired comparisons. The level of significance was  $P < 0.05$ . All experiments were carried out according to the protocol approved by the Ethics Committee.



**Table 1 Total glutathione (GSH + GSSG) in the mucosa of rat glandular stomach and liver following i.g. administration of gastrotoxic chemicals**

	0 min	1 min	3 min	6 min	12 min
Stomach					
Ethanol	2.6 ± 0.3	1.7 ± 0.5	1.6 ± 0.3	1.4 ± 0.3 <sup>a</sup>	1.2 ± 0.2 <sup>b</sup>
HCl	2.3 ± 0.7	1.5 ± 0.6	0.9 ± 0.4	0.9 ± 0.3	0.5 ± 0.2 <sup>a</sup>
NaOH	2.6 ± 0.5	2.4 ± 0.4	1.9 ± 0.6	1.9 ± 0.6	1.9 ± 0.5
NaCl	2.9 ± 0.6	2.5 ± 0.5	2.0 ± 0.2	1.8 ± 0.1	1.5 ± 0.3 <sup>a</sup>
Ammonia	3.0 ± 0.7	2.6 ± 0.5	2.3 ± 0.4	2.8 ± 0.4	2.9 ± 0.7
Liver					
Ethanol	3.8 ± 0.5	3.9 ± 1.0	4.1 ± 0.6	4.3 ± 0.8	4.2 ± 0.5

The damaging agent, 1 mL of 75% ethanol, 0.6 mol/L HCl, 0.2 mol/L NaOH, 25% NaCl or 1% ammonia-water was given by gavage to fasted S-D rats (*n* = 4-6), and the animals were killed subsequently at time-points indicated. Data are expressed as mean ± SEM of nmol GSH mg wet tissue. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01, *vs* corresponding control values (0 min).



**Figure 2** Ratios of reduced glutathione (GSH) and glutathione disulfide (GSSG) in mucosa of glandular stomach after i.g. administration of gastrotoxic chemicals in rats.

## RESULTS

The total glutathione (GSH + GSSG) concentration in the glandular stomach mucosa of control rats was 2.3-3.0 nmol GSH/mg tissue measured by the enzymatic method. In these experiments the GSH level in normal rat liver was slightly higher (3.8 + 0.5 nmol GSH/mg tissue) than in the gastric mucosa (Table 1).

Very rapid and time-dependent depletion-which reached statistical significance within 6-12 min - were found in total GSH concentrations in the gastric mucosa after i.g. administration of ethanol, HCl or hypertonic NaCl solution. In contrast, the diminished GSH levels in the gastric mucosa did not reach the level of statistical significance following 0.2 mol/L NaOH or 1% ammonia solution. Similarly, no significant alterations were found in GSH concentrations in the liver at any time after i.g. injection of ethanol (Table 1).

GSSG represents 0.7%-4.0% of total GSH pools. The concentrations of GSSG were significantly and time-dependently increased in the glandular stomach from 0.7%-1.4% to 3.2%-4.0% after ethanol, ammonia water or hypertonic NaCl treatments. However, these parameters

**Table 2 Oxidized glutathione (GSSG) in the mucosa of rat glandular stomach and liver following i.g. administration of gastrotoxic chemicals**

	0 min	1 min	3 min	6 min	12 min
Stomach					
Ethanol	20.0 ± 3.0	17.0 ± 3.5	26.0 ± 4.0	32.0 ± 5.1	39.0 ± 2.1 <sup>b</sup>
HCl	19.0 ± 6.0	18.0 ± 4.0	18.0 ± 2.8	22.0 ± 4.8	20.0 ± 4.5
NaOH	20.0 ± 2.0	25.0 ± 2.1	26.0 ± 3.7	25.0 ± 4.6	24.0 ± 3.1
NaCl	27.0 ± 1.9	25.0 ± 2.1	35.0 ± 5.7	35.7 ± 6.9	58.0 ± 8.9 <sup>b</sup>
Ammonia	28.1 ± 8.9	34.7 ± 7.1	57.0 ± 9.9	63.0 ± 10.3 <sup>a</sup>	60.0 ± 9.7 <sup>a</sup>
Liver					
Ethanol	74.0 ± 13.1	81.4 ± 15.2	64.0 ± 11.3	58.9 ± 10.8	66.6 ± 13.3

The damaging agent, 1 mL of 75% ethanol, 0.6 mol/L HCl, 0.2 mol/L NaOH, 25% NaCl or 1% ammonia-water was given by gavage to fasted S-D rats (*n* = 4-6), and the animals were killed subsequently at time-points indicated. Data are expressed as mean ± SEM of pmol GSSG in 1 mg wet tissue. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01, *vs* corresponding control values (0 min).

**Table 3 Protein SH (PSH) in the mucosa of rat glandular stomach and liver following i.g. administration of gastrotoxic chemicals**

	0 min	1 min	3 min	6 min	12 min
Stomach					
Ethanol	32.9 ± 1.2	29.6 ± 1.9	22.4 ± 1.1 <sup>b</sup>	15.9 ± 1.3 <sup>d</sup>	13.7 ± 1.0 <sup>b</sup>
HCl	34.2 ± 3.0	39.8 ± 2.1	38.7 ± 1.3	35.7 ± 3.3	31.7 ± 4.2
NaOH	26.9 ± 1.0	22.3 ± 2.1	13.0 ± 4.1 <sup>b</sup>	11.8 ± 3.5 <sup>b</sup>	14.0 ± 4.9 <sup>a</sup>
NaCl	26.7 ± 3.6	26.5 ± 2.1	23.1 ± 6.9	13.0 ± 6.7	15.1 ± 2.1 <sup>a</sup>
Ammonia	29.8 ± 2.9	22.6 ± 2.0	26.6 ± 3.9	30.9 ± 2.1	26.3 ± 4.9
Liver					
Ethanol	55.4 ± 4.8	51.2 ± 2.4	53.0 ± 1.8	54.3 ± 4.1	50.6 ± 4.5

The damaging agent, 1 mL of 75% ethanol, 0.6 mol/L HCl, 0.2 mol/L NaOH, 25% NaCl or 1% ammonia-water was given by gavage to fasted S-D rats (*n* = 4-6), and the animals were killed subsequently at time-points indicated. Data are expressed as mean ± SEM of nmol cysteine in 1 mg protein. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01, <sup>d</sup>*P* < 0.001, *vs* corresponding control values (0 min).

did not increase markedly after exposure to HCl or NaOH solutions. Ethanol did not substantially modify glutathione disulfide levels in the rat liver at any time during the 12 min period studied, and the proportion of GSSG remained below 1% (Table 2).

The moderate or marked changes in GSH and GSSG levels resulted in significant decreases of the GSH/GSSG ratios after the administration of all gastrotoxic chemicals used. However, lower ratio values were found in the gastric mucosal thiol concentrations following ethanol, HCl or NaCl than after ammonia or NaOH administration. The relatively small changes in GSH or GSSG levels in the mucosa after ammonia or NaOH exposures resulted in statistically significant decreases in the GSH/GSSG ratios (Figure 2).

The concentrations of PSH in the gastric mucosa decreased rapidly and significantly following exposure to ethanol, nevertheless, were also diminished after NaOH or hypertonic NaCl. These values remained unchanged in the liver samples following ethanol and in the gastric mucosa after exposure to ammonia or HCl solutions (Table 3).

Table 4 shows the concentrations of PSSG in protein

**Table 4 Protein-GSH mixed disulfides (PSSG) in rat glandular stomach mucosa and liver following various chemicals given i.g.**

	0 min	1 min	3 min	6 min	12 min
Stomach					
Ethanol	0.17 ± 0.04	0.28 ± 0.08	0.37 ± 0.03 <sup>b</sup>	0.35 ± 0.02 <sup>b</sup>	0.49 ± 0.04 <sup>d</sup>
HCl	0.20 ± 0.02	0.62 ± 0.11 <sup>a</sup>	0.83 ± 0.15 <sup>a</sup>	0.83 ± 0.16 <sup>a</sup>	0.84 ± 0.09 <sup>b</sup>
NaOH	0.22 ± 0.03	0.31 ± 0.10	0.36 ± 0.09	0.45 ± 0.12	0.43 ± 0.15
NaCl	0.20 ± 0.02	0.19 ± 0.04	0.17 ± 0.04	0.20 ± 0.01	0.22 ± 0.05
Ammonia	0.26 ± 0.03	0.25 ± 0.04	0.27 ± 0.04	0.36 ± 0.11	0.32 ± 0.04
Liver					
Ethanol	0.68 ± 0.03	0.86 ± 0.03	0.74 ± 0.03	0.64 ± 0.11	0.81 ± 0.10

The damaging agent, 1 mL of 75% ethanol, 0.6 mol/L HCl, 0.2 mol/L NaOH, 25% NaCl or 1% ammonia-water was given by gavage to fasted S-D rats ( $n = 4-6$ ), and the animals were killed subsequently at time-points indicated. Data are expressed as mean ± SEM of nmol GSSG in 1 mg protein. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , <sup>d</sup> $P < 0.001$ , *vs* corresponding control values (0 min).

**Table 5 Protein disulfides (PSSP) in rat glandular stomach mucosa and liver following various chemicals given i.g.**

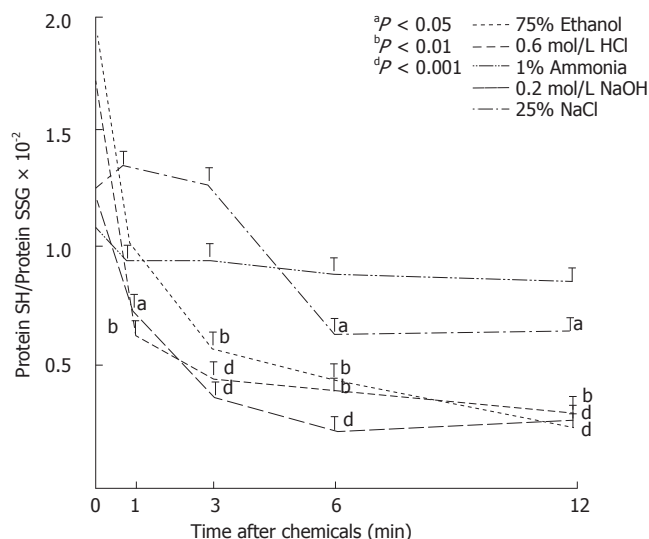
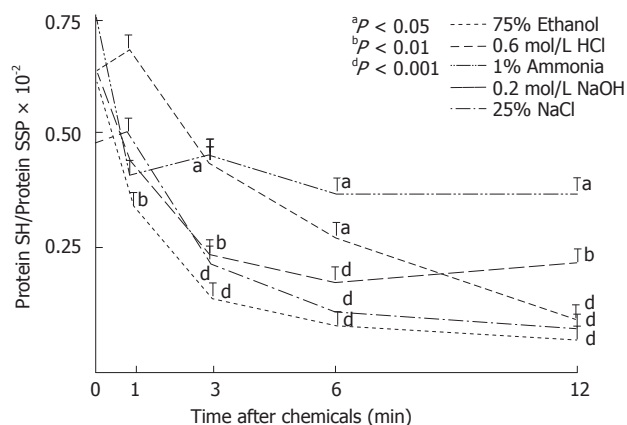
	0 min	1 min	3 min	6 min	12 min
Stomach					
Ethanol	0.55 ± 0.08	0.94 ± 0.06 <sup>b</sup>	1.36 ± 0.20 <sup>b</sup>	1.72 ± 0.05 <sup>d</sup>	1.71 ± 0.13 <sup>d</sup>
HCl	0.54 ± 0.20	0.57 ± 0.04 <sup>a</sup>	0.90 ± 0.30	1.33 ± 0.45	1.69 ± 0.09 <sup>b</sup>
NaOH	0.44 ± 0.07	0.52 ± 0.17	0.55 ± 0.04	0.66 ± 0.14	0.59 ± 0.20
NaCl	0.57 ± 0.02	0.53 ± 0.02	1.00 ± 0.12 <sup>b</sup>	1.01 ± 0.18 <sup>b</sup>	1.35 ± 0.05 <sup>d</sup>
Ammonia	0.39 ± 0.07	0.52 ± 0.17	0.56 ± 0.15	0.78 ± 0.20	0.64 ± 0.15
Liver					
Ethanol	0.89 ± 0.25	0.75 ± 0.20	0.95 ± 0.30	0.86 ± 0.17	0.69 ± 0.05

The damaging agent, 1 mL of 75% ethanol, 0.6 mol/L HCl, 0.2 mol/L NaOH, 25% NaCl or 1% ammonia-water was given by gavage to fasted S-D rats ( $n = 4-6$ ), and the animals were killed subsequently at time-points indicated. Data are expressed as mean ± SEM of nmol half cystine residue in 1 mg protein. <sup>b</sup> $P < 0.01$ , <sup>d</sup> $P < 0.001$ , *vs* corresponding control values (0 min).

samples of the gastric mucosa and liver of control and treated rats. The concentration of PSSG was higher in the liver at all the times examined than in the gastric mucosa of control rats or during the development of gastric mucosal damage. Furthermore, time-dependent and significant increments of these mixed disulfides were found in the mucosa after administration of ethanol or HCl. Since these proteins contained moderately (e.g. HCl) or markedly (e.g. ethanol, NaOH, NaCl exposures) less free SH groups than those obtained from control groups, the ratios of PSH to PSSG groups were rapidly and significantly decreased by the gastrotoxic chemicals used, except by ammonia solution (Figure 3).

The concentrations of cystine disulfides in the proteins were also significantly increased in the gastric mucosa by ethanol, HCl or NaCl measured after denaturation and borohydride reduction. No significant changes were found in the liver after ethanol or in the gastric mucosa following ammonia water or NaOH administration (Table 5).

Figure 4 shows the changes in the ratios of PSH to PSSP prepared from rat gastric mucosa. In contrast to moderate alterations by ammonia solution, the ratio of PSH to PSSP in the proteins rapidly decreased by all other

**Figure 3** Ratios of protein SH (PSH) to protein-GSH mixed disulfides (PSSG) in the gastric mucosa after i.g. administration of gastrotoxic chemicals in rats.**Figure 4** Ratios of protein SH (PSH) to protein disulfides (PSSP) in the gastric mucosa following i.g. administration of gastrotoxic chemicals in rats.

chemicals calculated from the protein thiol data in the gastric mucosa.

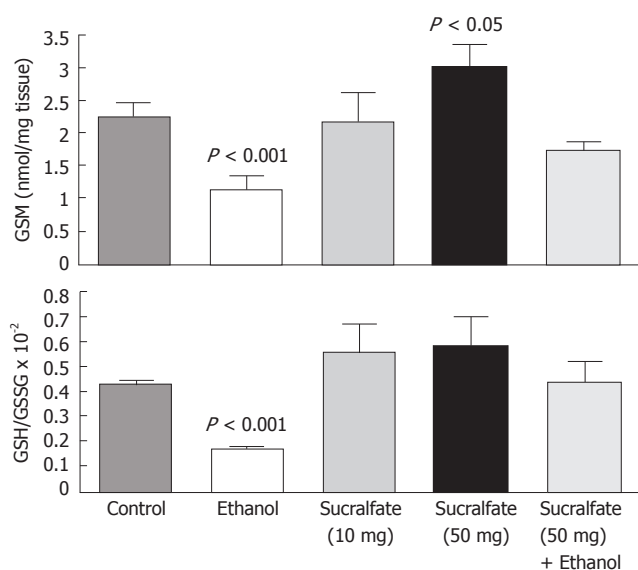
All measured alterations in PSH, NPSH and disulfides of the gastric mucosa as well as the calculated ratios are summarized in Table 6. The common elements seem to be the trend in decreased GSH and increased GSSG levels both of which reached level of statistical significance with three out of five gastrotoxic chemicals tested, very often in an overlapping manner. Similar changes were apparent with decrease in PSH and increase in PSSP, i.e., in 3/5 and 4/5 cases, respectively. Consequently, the most consistent patterns of alterations were, however, detected in the slight or marked decreases in GSH/GSSG and the PSH/PSSP ratios involving all the five tested and commonly used gastrotoxic chemicals. One should also stress that these biochemical changes preceded the full development of hemorrhagic erosions which were virtually absent at 1 min, barely detectable at 3 and 6 min, and visible at 12 min.

Our direct pharmacologic results demonstrate that the gastric mucosal levels of reduced GSH and the ratio of GSH/GSSG increased dose-dependently increased

**Table 6** Changes in the concentrations of nonprotein and protein sulfhydryls and disulfides in the rat glandular stomach in the early phase of chemically-induced mucosal injury

Agent	Total GSH	GSSG	GSH/GSSG	PSH	PSSG	PSSP	PSH/PSSG	SH/PSSP
Ethanol	DD	II	DD	DD	III	III	DD	DD
HCl	D	I	DD	D	No	II	No	DD
NaOH	DD	No	D	No	I	I	DD	DD
NaCl	No	No	D	D	No	I	DD	D
Ammonia	No	I	D	No	No	No	No	D

Total GSH: GSH + GSSG; GSSG: glutathione disulfide; PSH: protein SH; PSSG: protein-GSH mixed disulfide; PSSP: protein disulfides; I: increase; D: decrease, I or D:  $P < 0.05$ ; II or DD:  $P < 0.01$ ; III or DDD:  $P < 0.001$ ; No: no significant change in comparison with corresponding controls in the 12 min experiments.



**Figure 5** Concentration of GSH (nmol/mg tissue) and ratio of GSH/GSSG in the glandular mucosa of rat stomach after i.g. administrations of sucralfate (10 and 50 mg/100 g, b.w.), 1 mL of 75% ethanol, and sucralfate (50 mg/100 g, b.w.) plus ethanol ( $n = 5$ ). Data are expressed as means SEM.

following sucralfate administration. In addition, the ethanol-induced decrease in GSH concentration was almost completely prevented by pretreatment with gastroprotective dose (50 mg/100 g body weight) of sucralfate (Figure 5). The decrease of mucosal PSH/PSSP caused by ethanol was also abolished after pretreatment with sucralfate.

## DISCUSSION

Because of previous observations with gastric mucosal thiols<sup>[12,13]</sup> and protein sulfhydryls (including SH-proteases)<sup>[30,31]</sup> we wanted to study the early changes in tissue levels of major sulfhydryls and disulfides in the mucosa of the glandular stomach in a time-dependent manner after the administration of various gastrotoxic chemicals, i.e., before the development of HML.

Our results of the present study revealed rapid changes in SH and disulfide concentrations in the rat gastric mucosa after exposure to damaging chemicals such as ethanol, hypertonic NaCl, HCl, NaOH or ammonia solutions. The depletion of reduced GSH and the elevation of GSSG concentration were more marked after ethanol, HCl or NaCl, than following base solutions such as NaOH and

ammonia water. The ratios of reduced to oxidized glutathione were significantly decreased by all gastrotoxic chemicals. No changes in the thiol homeostasis were found in the liver.

Previous investigations demonstrated that concentrated ethanol, acid and base solutions and other chemicals may induce time-dependent severe acute mucosal lesions in the rat stomach within minutes<sup>[2,3,5,6,31]</sup>. Different biochemical events, i.e., generation of oxygen free radicals, lipid peroxidation, impaired prostaglandin metabolism, endothelial injuries have been implicated in the pathogenesis of chemically-induced acute mucosal lesions or epithelial cell damages<sup>[2,3,5,7,9,11]</sup>. Lipid peroxides and peroxiradicals in the gastric mucosa can be detoxified by GSH peroxidase *via* formation of GSSG from reduced GSH. Cytotoxins can also be eliminated by GSH conjugation catalyzed by glutathione S-transferase. Other findings revealed decreased GSH and increased GSSG in the gastric mucosa, thus increased oxidative stress might be one of the first mechanisms of the mucosal damage. The role of lipid peroxidation in the development of HML seems to be more limited than previously thought<sup>[2,3,43]</sup>.

Several researchers found a significant decrease of reduced GSH in gastric mucosa following chemicals such as NSAID and acrylonitrile<sup>[1,13,16,21-23]</sup>. Acute depletion of GSH during gastric mucosal injury may be due to: (1) direct oxidation of GSH to GSSG; (2) formation of mixed disulfides with protein SH groups or cysteine; (3) non-enzymatic conjugation with GSH<sup>[44]</sup>.

Exogenously administered SH agents such as L-cysteine, N-acetylcysteine, cystamine, cysteamine, penicillamine, GSH exert dose-dependent protection against the ethanol-induced HML in the rat stomach<sup>[1,2,13,17]</sup>. Direct cytoprotective effect of GSH was demonstrated *in vitro* against ethanol<sup>[25]</sup> or acid-induced<sup>[28]</sup> injury in cultured rat gastric mucosal cells. On the other hand, s.c. injection of SH alkylators such as iodoacetate or NEM 10 min before or after gastroprotective thiol or PG compounds eliminated the protective effect of SH drugs in ethanol model<sup>[45,46]</sup>. These results also show the importance of reduced SH groups in gastroprotection.

We detected significant increase of GSSG concentration in the glandular stomach after ethanol, HCl or hypertonic NaCl exposures, but this increment was minor following ammonia or NaOH solutions. The increased level of oxidized GSH is one of the elements in pathogenesis of mucosal injury. It is known that GSSG is rapidly reduced to GSH by NADPH-dependent GSSGR<sup>[47]</sup>. When the

rate of GSH oxidation to GSSG exceeds the capacity of GSSGR, GSSG is actively transported out of the cell. GSSG selectively destroys cysteine protease inhibitors<sup>[48]</sup>, consequently it may activate lysosomal thiol protease cathepsins B, H, L in gastric mucosal which participate in the early development of HML<sup>[29-31,45]</sup>.

Thus, our present studies revealed a rapid and significant decrease in the concentration of gastric mucosal protein SH following i.g. administration of ethanol, hypertonic NaCl or NaOH. Tissue levels of PSH remained unchanged during the development of ammonia or HCl-induced gastric lesions. Furthermore, slight or profound increases of PSSG or PSSP were detected in gastric mucosa using GSSGR enzymic method or borohydride reduction of disulfide bridges in the protein samples, respectively. But ratios of protein SH and disulfides (PSSG + PSSP) were significantly decreased by all chemicals used. No changes were found in liver protein SH concentrations after i.g. administration of ethanol. Thus, protein thiol changes are also involved in the pathogenesis of chemically-induced gastric mucosal lesions in rats. What are the possible mechanisms?

Protein SH groups play a critical role in the catalytic mechanisms of numerous enzymes including metabolic enzymes such as creatine kinase and glyceraldehyde-3-phosphate dehydrogenase. A large group of these enzymes play an important role in cell metabolism or membrane transport. PSH has been implicated in the maintenance of plasma membrane integrity and ion fluxes, of mitochondrial permeability<sup>[49]</sup>, of membrane-bound ATP-ase<sup>[50]</sup>, and of various receptors and G protein which might be one of the universal mediators of gastric mucosal protection. PSH are important for the maintenance of protein structure and functions in the gastric mucosa, e.g., their modifications via oxidation, alkylation and conjugation may change the functions of mucosal parenchymal or endothelial cells<sup>[13,51]</sup>. On the other hand, numerous thiol proteases such as calpain in the plasma membranes and cytosol, and the lysosomal cysteine protease cathepsins B, H and L may be activated during the early phase of acute gastric HML<sup>[30,32]</sup>.

The results presented here have revealed that exposure of gastric mucosa to toxic concentrations of chemicals also caused a rapid loss of total protein SH groups. During this period cysteine proteases can be activated and released<sup>[30-32]</sup>. Our group demonstrated previously that formation of reversible protein disulfides might be advantageous in the maintenance of integrity of gastric mucosa due to: (1) their possible antioxidant or reactive metabolite-eliminating effects; (2) a more stable tertiary structure integrity and functions of several structure (membrane) protein or metabolic enzymes might be more resistant against noxious influences; (3) inhibition of PSH may be associated with decreased activity of cysteine proteases.

Our results with rats given sucralfate alone revealed a significant and dose-dependent increase in both the GSH concentration and the GSH/GSSG ratio in the gastric mucosa. In addition, a gastroprotective dose of sucralfate almost completely prevented the ethanol-induced decrease of GSH concentration and ratio of GSH/PSSP. These results are also in agreement with our previous pharmacologic experiments demonstrating an SH-sensitive

step in the mechanism of gastroprotection by sucralfate<sup>[52]</sup>.

We conclude that rapid and time-dependent depletion of total GSH in the gastric mucosa precedes the development of erosions induced by ethanol, HCl or NaCl. This biochemical change was not evident after exposure to ammonia or NaOH solutions. However, GSSG concentrations in glandular mucosa increased, except after HCl or NaOH exposure. Thus, the ratios of reduced to oxidized GSH concentrations markedly decreased by all gastrototoxic chemicals studied. Considerable decreases of PSH concentration were also detected after concentrated ethanol, NaCl and NaOH solutions, while the protein disulfides were increased, resulting in diminished ratios of PSH and protein disulfides in the gastric mucosa. We also conclude that sucralfate increases the concentration of GSH in the gastric mucosa and this biochemical effect may be one of the mechanisms of gastroprotection by sucralfate and possible other similar drugs.

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