Protective effects of vitamin B₆ alone and in combination with L-cysteine and NaHS on ethanol and indomethacin-induced gastric lesions in mice

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Abstract

Objective(s): This study was undertaken to investigate the protective effects of vitamin B₆ cofactor for cystathionine-γ lyase and cystathionine-β synthase (producers of H₂S), alone and in combination with L-cysteine, H₂S precursor, on indomethacin, and ethanol-induced gastric lesions in male NMRI mice.

Materials and Methods: Fasted male NMRI mice were randomly assigned into 12 groups (7 in each). The gastroprotective activity of vitamin B₆ alone and in combination with L-cysteine and sodium hydrosulfate (NaHS) was evaluated against ethanol-, and indomethacin-induced gastric lesions. The animals were received vehicle, vitamin B₆, L-cysteine, L-cysteine + vitamin B₆, NaHS or NaHS + B₆ before the induction of gastric lesions by ethanol (50%, 0.5 ml/25 g of body weight, orally) or indomethacin (40 mg/kg, orally). One and five hours after the administration of ethanol and indomethacin, respectively, the animals were sacrificed using anesthetics. The stomachs were removed, rinsed with normal saline and assessed for gastric wall mucus changes.

Results: Pretreatment with L-cysteine, sodium hydrosulfate, and vitamin B₆ significantly decreased the total area of gastric lesions (P<0.01). The mucus production in L-cysteine-, sodium hydrosulfate-, and vitamin B₆-treated animals were significantly higher than in control rats (P<0.05). The gastroprotective activity of L-cysteine and sodium hydrosulfate in combination with vitamin B₆ were higher than when administered alone (P<0.05).

Conclusion: The result of this survey showed that the protective activity of L-cysteine and sodium hydrosulfate enhances in the presence of vitamin B₆.

Introduction

Hydrogen sulfide (H₂S) is a colorless gas with a strong odor, produced in different environments, but it is also found in mammalian tissues, where it is generated during cysteine metabolism (1). It is formed in mammalian cells by the activity of two pyridoxal phosphate-dependent enzymes including cystathionine-γ lyase (CSE) and cystathionine-β synthase (CBS), which convert L-cysteine to sulfide sulfide (2).

Beneficial effect of H₂S, its precursors and donors have been reported on different experimentally gastric ulcer in rat and mouse (3-5). Recently, H₂S is introduced as a rescue molecule for mucosal defense and repair (6). The underlying gastro-protective mechanisms of hydrogen sulfide were attributed to maintenance and elevation of gastric mucosal blood flow (3, 7), stimulation of bicarbonate secretion (8), reduction of pro-inflammatory cytokine expression/release (5), increase of prostaglandin synthesis (9), decrease of reactive oxygen metabolite production (10) and enhancement of tissue repair (11).

To our knowledge, there is no study about the effect of vitamin B₆ as an essential cofactor for endogenous production of H₂S, on indomethacin-, and ethanol-induced gastric mucosal lesions in mice. Therefore, this study aimed to evaluate the effect of vitamin B₆ alone and in combination with L-cysteine and sodium hydrosulfate on two models (indomethacin and ethanol) of experimentally-induced gastric ulcer in mice.

Materials and Methods

Chemicals

Ethanol was purchased from Merck (Germany). L-cysteine and sodium hydrosulfate were purchased...
from Sigma (USA). Indomethacin and vitamin B6 were purchased from Tolid Daru (Iran). L-cysteine and indomethacin were dissolved in distilled water and prepared freshly.

**Animals**
NMRI male mice (25 to 30 g) were supplied from the animal house of Ahvaz Jundi Shapur University of Medical Sciences, Ahvaz, Iran. Animals were fed on conventional diets and tap water. They were maintained under standard conditions of humidity, temperature (22±2°C) and light/dark cycle (12 hr: 12 hr). All experiments were carried out in accordance with ethics committee of Ahvaz Jundishapur University of Medical Sciences (RDC-9202). Animals were fasted 24 hr before the experiments. Animals were randomly divided into 12 groups (7 in each).

**Animal grouping and procedures**
In the first set of experiments, to evaluate the protective effect of L-cysteine, sodium hydrosulfate and vitamin B6 alone and in combination on ethanol-induced gastric lesions, six groups of mice were recruited. They were positive control, L-cysteine, vitamin B6, L-cysteine+vitamin B6, NaHS, and NaHS+vitamin B6 treated groups. The positive control rats were given normal saline (0.1 ml, IP) 30 min before the induction of gastric lesions by ethanol (50%, 0.5 ml/25g of body weight, orally) (12); L-cysteine-treated animals received a single dose of L-cysteine (100 mg/kg, IP) 60 min before the induction of gastric lesion by ethanol (5); vitamin B6-treated animals administered a single dose of vitamin B6 (10 mg/kg, IP) (13) 30 min before the induction of gastric lesion by ethanol; L-cysteine+vitamin B6 treated rats received a single intraperitonel administration of L-cysteine (100 mg/kg, IP) and vitamin B6 (10 mg/kg, IP) 60 min and 30 min, respectively before the induction of gastric lesion by ethanol; animals in the fifth group given a single intraperitoneally administration of NaHS (100 mg/kg, IP) and vitamin B6 (10 mg/kg, IP) 60 min and 30 min, respectively before the induction of gastric lesion by indomethacin; animals in the fifth group received a single administration of NaHS (80 µg/kg, IP) 30 min prior to intervention and rats in the sixth groups administered NaHS (80 µg/kg, IP) + vitamin B6 (10 mg/kg, IP) 30 min before the induction of mucosal lesions by indomethacin. Five hours after the administration of indomethacin, animals were sacrificed under a high dose of diethyl ether. Calculating the total area of gastric lesions and determining the gastric wall mucus were carried out similar to the first protocol.

**Determination of gastric wall mucus**
To measure the gastric wall mucus, Perera et al method was used (16). Briefly, the stomachs were washed by normal saline, and then the gastric wall mucus was scraped, and homogenized in 1 ml of distilled water. Difference between the weight of obtained homogenate and the original 1 ml of water considered as the weight of mucus (mg).

**Histological evaluation**
For histological evaluation, stomachs from control and treated animals were fixed in 10% formalin, dehydrated in grade ethanol, and embedded in paraffin. Thereafter, sections of tissue were cut at 5 µm using a microtome, stained with hematoxylin and eosin, and assessed under an Olympus microscope (IX50).

**Statistical analysis**
Data are shown as mean±SEM. Statistical analysis was performed by one-way ANOVA and followed by post hoc Tukey’s test. Significance was set at a P<0.05 level.

**Results**
**Effect of L-cysteine, NaHS and vitamin B6 alone and in combination on gastric mucosa lesions induced by ethanol and indomethacin**
Histological examination showed gastric lesions such as multiple erosions, exfoliation and necrosis of superficial cells, hemorrhages in the mucosal layer, and severe alterations in the architecture of glandular parts of the gastric mucosa one hour after ethanol (50%, 0.5 ml/25 gr of body weight) administration and five
Table 1. Effect of L-cysteine (100 mg/kg), vitamin B<sub>6</sub> (10 mg/kg, IP), NaHS (80 µg/kg), L-cysteine+vitamin B<sub>6</sub> and NaHS+vitamin B<sub>6</sub> on gastric mucus content in ethanol and indomethacin-induced gastric lesions in NMRI male mice.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mucus (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline+ethanol</td>
<td>32±1</td>
</tr>
<tr>
<td>L-cysteine+ethanol</td>
<td>28±1.5*</td>
</tr>
<tr>
<td>Vitamin B&lt;sub&gt;6&lt;/sub&gt;+ethanol</td>
<td>26±1.6*</td>
</tr>
<tr>
<td>NaHS+ethanol</td>
<td>26±3.0*</td>
</tr>
<tr>
<td>L-cysteine+vitamin B&lt;sub&gt;6&lt;/sub&gt;+ethanol</td>
<td>32±2.1**</td>
</tr>
<tr>
<td>NaHS+vitamin B&lt;sub&gt;6&lt;/sub&gt;+ethanol</td>
<td>32±2.3**</td>
</tr>
<tr>
<td>Saline+indomethacin</td>
<td>24±1.5</td>
</tr>
<tr>
<td>L-cysteine+indomethacin</td>
<td>29±1.8*</td>
</tr>
<tr>
<td>Vitamin B&lt;sub&gt;6&lt;/sub&gt;+indomethacin</td>
<td>31±2.5*</td>
</tr>
<tr>
<td>NaHS+indomethacin</td>
<td>30±1.9*</td>
</tr>
<tr>
<td>L-cysteine+vitamin B&lt;sub&gt;6&lt;/sub&gt;+indomethacin</td>
<td>36±3.1**</td>
</tr>
<tr>
<td>NaHS+ vitamin B&lt;sub&gt;6&lt;/sub&gt;+indomethacin</td>
<td>37±2.1**</td>
</tr>
</tbody>
</table>

(All of the sections stained with hematoxylin and eosin; ×100 magnification).

As shown in Figure 2, a single administration of ethanol (50%, 0.5 ml/25 g of body weight) produced gastric mucosal lesions in fasted male NMRI mice. Figure 2 also shows that pretreatment with L-cysteine, vitamin B<sub>6</sub>, NaHS and L-cysteine+vitamin B<sub>6</sub> protected the gastric mucosa against ethanol-induced gastric lesions. The total area of gastric lesions in pretreated groups, L-cysteine, vitamin B<sub>6</sub>, and NaHS, was significantly lower than in control rats (P<0.01, P<0.001 and P<0.001, respectively). The protective activity of vitamin B<sub>6</sub> was pronounced when co-administered with L-cysteine and NaHS. The total area of mucosal lesions in animals receiving a combination of L-cysteine+vitamin B<sub>6</sub> and NaHS+vitamin B<sub>6</sub> was significantly lower than in L-cysteine-, and NaHS-treated rats (P<0.05). The gastro-protective activity of vitamin B<sub>6</sub> in combination with L-cysteine was higher than when administered alone (P<0.05) (Fig. 4).

**Effect of L-cysteine and vitamin B<sub>6</sub> alone and in combination pretreatment on gastric wall mucus**

The mucus content in L-cysteine-, NaHS-, and vitamin B<sub>6</sub>-treated animals was significantly higher than in control rats (P<0.05). The mucus content in L-cysteine and NaHS in combination with vitamin B<sub>6</sub> was higher than when administered alone (P<0.05) (Table 1).

**Discussion**

The findings of the present study showed that: (1) the administration of L-cysteine, NaHS and vitamin B<sub>6</sub> reduced the total area of the acute gastric mucosal lesions induced by ethanol and indomethacin; (2) the protective activity of L-cysteine and NaHS on ethanol-, and indomethacin-induced gastric lesions significantly increased in the presence of vitamin B<sub>6</sub>; and (3) the contents of gastric wall mucus in the control groups (animals received ethanol or indomethacin) were lower than in L-cysteine-, vitamin B<sub>6</sub>-, NaHS-, and L-cysteine+vitamin B<sub>6</sub>-treated rats.

H<sub>2</sub>S is produced in mammalian cells via both enzymatic and nonenzymatic pathways, although the nonenzymatic pathway only accounts for a small portion of H<sub>2</sub>S production (17). Among enzymes involved in H<sub>2</sub>S production, CBS and CSE have been investigated extensively, both using pyridoxal 5'-phosphate (vitamin B<sub>6</sub>) as a cofactor. Vitamin B<sub>6</sub> is a water-soluble molecule that is involved in a wide range of metabolic, physiological and developmental functions.
processes. It itself is an enzyme cofactor required for more than 140 biochemical reactions (18).

Moreover, the vitamin is a potent antioxidant, rivaling carotenoids or tocopherols in its ability to quench reactive oxygen species (19). Many studies have shown the neuroprotective activity of vitamin B6 (20). In vitro studies have shown the anti-tumor and anti-inflammatory effect of vitamin B6 (21, 22). Furthermore, it has been demonstrated that dietary vitamin B6 inhibits nitric oxide (NO) production in response to LPS administration (21).

Nonsteroidal anti-inflammatory drugs cause gastrointestinal damage through blocking prostaglandin (PGs) synthesis [inhibition of the cyclooxygenase (COX) enzymes] and also through inhibiting a number of autacoids [NO and H2S] acting in concert with prostaglandins in maintaining the gastric mucosal barrier (23). PGs have been demonstrated to stimulate mucus and bicarbonate secretion as well as mucosal blood flow, and induce angiogenesis (24). All these factors contribute to accelerated ulcer healing.

The results of the present study showed that L-cysteine, NaHS and vitamin B6 increase mucus secretion. Therefore, it can be concluded that this protective activity could largely be due to potentiation of mucosal barrier through increasing gastric wall mucus secretion. This effect in concert with other protective effect including up-regulating the gene expression of cyclooxygenase-2 in mucosal tissue of the rat stomach as shown by our previous report (14) and also by Wallace et al report that showed endogenous and exogenous hydrogen sulfide through the up-regulation of COX-2, promotes resolution of colitis in rats (9), and explains the protective activity of vitamin B6 against indomethacin-induced gastric lesions.

Our recent report also showed that NaHS [a H2S donor] and L-cysteine [a H2S precursor] decrease the acid output in response to gastric distention (14). Therefore, these results suggest that one of the possible mechanisms of the protective activity of L-cysteine and vitamin B6 against indomethacin and ethanol could be largely mediated by their antacid effects. Abdallah et al showed that indomethacin causes a remarkably significant increase in ulcer index, gastric juice free and total acidity (25). Taken together, these findings show that the excitatory effect of indomethacin on acid output as shown by Abdallah et al report was prevented by the anti-secretory effect of H2S as shown by previous work.

It has been indicated that the endogenous production of H2S increases in various models of tissue injury (26, 27). Wallace et al demonstrated that the expression of CSE and CBS profoundly increases after the induction of gastric ulcer, and that the administration of propargylglycine (PAG) prevents

![Figure 2](image_url)

**Figure 2.** A graphic representation of the ulcer index following ethanol administration among various treatment groups. C-E (control): rats were received ethanol (0.5/25 g of body weight, orally); L-cys: animals were given L-cysteine (100 mg/kg) 60 min prior to ethanol administration; B6: animals received vitamin B6 (10 mg/kg, IP) 30 min prior to ulcer induction by ethanol; L-cys+B6: animals received L-cysteine 60 min prior to intervention and vitamin B6 30 min prior to ulcer inducing by ethanol. **P<0.01, ***P<0.001 versus the control group and *P<0.05 versus the L-cysteine-, and vitamin B6-treated groups.*

![Figure 3](image_url)

**Figure 3.** Histological evaluation of gastric mucosa. Representative gastric sections were obtained 5 hr after indomethacin administration. C-I: Control group indicate severe disruption to the upper half of mucosal thickness and necrotic lesions penetrating deeply into mucosa; L-Cys, B6, NaHS; L-Cys+B6, NaHS+B6: animals pretreated with L-cysteine (100 mg/kg, IP), vitamin B6 (10 mg/kg, IP), NaHS (80 µg/kg, IP), L-cysteine (100 mg/kg, IP)+vitamin B6 and NaHS (80 µg/kg, IP)+vitamin B6, demonstrate moderate to mild disruption of the surface epithelium. All of the sections stained with hematoxylin and eosin; ×100 magnification.
gastric ulcer healing by L-cysteine (a H₂S precursor) treatment (4). Several studies have demonstrated that PAG exacerbates gastric mucosal lesions induced by acetylsalicylic acid (3). Taken together, these findings imply that under damage conditions, the body limits injury by increasing the production of protective autacoids such as H₂S. Also, it can be concluded that potentiation of the involved tissue by exogenous H₂S precursor, by L-cysteine pretreatment, or by increasing the activation of involved enzymes, CSE and CBS, to increase the endogenous production/release of H₂S by pretreatment of essential enzyme cofactor, vitamin B₆ or by a combination of them significantly protected the gastric mucosal tissue against indomethacin and ethanol as shown by the present results.

Ethanol induces severe mucosal ulcers in the stomach mainly by activation of the inflammatory reaction (28, 29). The mucosal lesions resulted from ethanol is characterized by epithelial cellular loss, mucosal edema, and sub-epithelial hemorrhage (12, 30). As shown in Figure 1, pretreatment with L-cysteine, vitamin B₆ and NaHS protected the gastric mucosa against ethanol-induced mucosal lesions. Recently, we have shown that L-cysteine and NaHS protected the gastric mucosa against ischemia-reperfusion injury in rat through down-regulating the mRNA expression and plasma level of pro-inflammatory cytokines, IL-1β and TNF-α (5). These findings together show that L-cysteine, NaHS and vitamin B₆ by increasing H₂S production/release, and it in turn through a decrease in pro-inflammatory cytokines protect the gastric mucosa against ethanol. The interesting finding of the present study was the gastro-protective activity of pyridoxine (vitamin B₆) against ethanol and indomethacin-induced mucosal lesions. As shown in Figures 1 and 2, the protective activity of both L-cysteine and NaHS increase when co-administered with vitamin B₆. These results clearly show that H₂S production increases in the presence of enzyme cofactor. In the present study, we did not measure the tissue levels of hydrogen sulfide but a higher gastroprotective effect of L-cysteine and NaHS after the administration of vitamin B₆ represents an increase in the production of hydrogen sulfide.

Conclusion

The result of this survey for the first time showed the gastroprotective effect of vitamin B₆ on ethanol and indomethacin-induced gastric lesions in rats. The findings of this study demonstrated that: a) Pretreatment with B₆ decreased the total area of acute gastric mucosal lesions induced by ethanol and indomethacin. b) The protective activity of L-cysteine enhances in the presence of vitamin B₆ cofactor for natural enzymatic pathways for endogenous production of H₂S. c) The gastric wall mucus production in B₆ and L-cysteine pretreated rats was higher than in the control.

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Conflict of interest

All authors declare that they have no conflicts of interest.

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