

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.

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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, 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

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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 intraperitoneally 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 administration of NaHS (80 µg/kg, IP) 30 min prior to intervention and the sixth group of animals were received NaHS (80 µg/kg, IP)(14)+vitamin B₆ (10 mg/kg, IP) 30 min before the induction of mucosal lesions by ethanol. One hour after the administration of ethanol, animals were sacrificed under a high dose of diethyl ether, and their stomachs were rapidly removed, opened along the greater curvature, rinsed with physiologic saline and pinned out on ice-cooled saline. Immediately after taking photo of the stomachs for measurement of the surface area of gastric lesions by Image J software, the gastric wall mucus was scrapped for determining the gastric wall mucus.

In the second set of experiments, the gastro-protective activity of L-cysteine, NaHS and vitamin B₆ alone and in combination on indomethacin-induced gastric lesions were tested on six groups of mice. They were control-, L-cysteine-, vitamin B₆-, L-cysteine+vitamin B₆-, NaHS-, and NaHS+vitaminB₆-

treated groups. The control rats were given normal saline (0.1 ml) 30 min before the induction of gastric lesion by indomethacin (40 mg/kg, orally)(15). L-cysteine-treated animals received a single dose of L-cysteine (100 mg/kg, IP) 60 min before the induction of gastric lesion by indomethacin. Vitamin B₆-treated animals administered a single dose of vitamin B₆ (10 mg/kg, IP) 30 min before the induction of gastric lesion by indomethacin, L-cysteine+vitamin B₆-treated group given a single intraperitoneally 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 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 B₆ (10 mg/kg, IP), NaHS (80 µg/kg), L-cysteine+vitamin B₆ and NaHS+vitamin B₆ on gastric mucus content in ethanol and indomethacin-induced gastric lesions in NMRI male mice.

Groups	Mucus (mg)
Saline+ethanol	18±1
L-cysteine+ethanol	25±1.5*
Vitamin B ₆ +ethanol	26±1.6*
NaHS+ethanol	26.3±0.8*
L-cysteine+vitamin B ₆ +ethanol	31±2.1** ^α
NaHS+vitamin B ₆ +ethanol	32±2.3** ^β
Saline+indomethacin	24±1.5
L-cysteine+indomethacin	29±1.8*
Vitamin B ₆ +indomethacin	31±2.5*
NaHS+indomethacin	30±1.8*
L-cysteine+vitamin B ₆ +indomethacin	36±3.1** ^α
NaHS+ vitamin B ₆ +indomethacin	37±2.1** ^β

hours after indomethacin (40 mg/kg, orally) treatment in the corresponding control groups. Pretreatment with L-cysteine, vitamin B₆, NaHS, L-cysteine+vitamin B₆, NaHS+Vitamin B₆ attenuated the gastric lesions induced by ethanol and indomethacin (Figure 1 and 3).

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 B₆ and NaHS protected the gastric mucosa against ethanol-induced gastric lesions. The total area of gastric lesions in pretreated groups, L-cysteine, vitamin B₆ and NaHS, was significantly lower than in control rats ($P<0.01$, $P<0.001$ and $P<0.001$, respectively). The protective activity of combination of L-cysteine and NaHS with vitamin B₆ were higher than when administered them alone ($P<0.05$).

As shown in Figure 4, a single administration of indomethacin at 40 mg/kg (orally) produced gastric mucosal lesions in male fasted NMRI mice. Pretreatment with L-cysteine, vitamin B₆ and NaHS protected the gastric mucosa against indomethacin - induced gastric lesions.

The total area of gastric lesions in pretreated groups, L-cysteine, vitamin B₆ and NaHS, was signifi-

cantly lower than in control rats ($P<0.01$, $P<0.001$ and $P<0.001$, respectively). The protective effect of vitamin B₆ was pronounced when co-administered with L-cysteine and NaHS. The total area of mucosal lesions in animals received a combination of L-cysteine+vitamin B₆ and NaHS+vitamin B₆ was significantly lower than in L-cysteine-, and NaHS-treated rats ($P<0.05$). The gastro-protective activity of vitamin B₆ in combination with L-cysteine was higher than when administered it alone ($P<0.05$) (Figure 4).

Effect of L-cysteine and vitamin B₆ alone and in combination pretreatment on gastric wall mucus

The mucus content in L-cysteine-, NaHS-, and vitamin B₆-treated animals was significantly higher than in control rats ($P<0.05$). The muco-tropic effect of L-cysteine and NaHS in combination with vitamin B₆ was higher than when administered them alone ($P<0.05$) (Table 1).

Discussion

The findings of the present study showed that: (1) the administration of L-cysteine, NaHS and vitamin B₆ 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 B₆; and (3) the contents of gastric wall mucus in the control groups (animals received ethanol or indomethacin) were lower than in L-cysteine-, vitamin B₆-, NaHS-, and L-cysteine+vitamin B₆-treated rats.

H₂S is produced in mammalian cells via both enzymatic and nonenzymatic pathways, although the nonenzymatic pathway only accounts for a small portion of H₂S production (17). Among enzymes involved in H₂S production, CBS and CSE have been investigated extensively, both using pyridoxal 5'-phosphate (vitamin B₆) as a cofactor. Vitamin B₆ is a water-soluble molecule that is involved in a wide range of metabolic, physiological and developmental

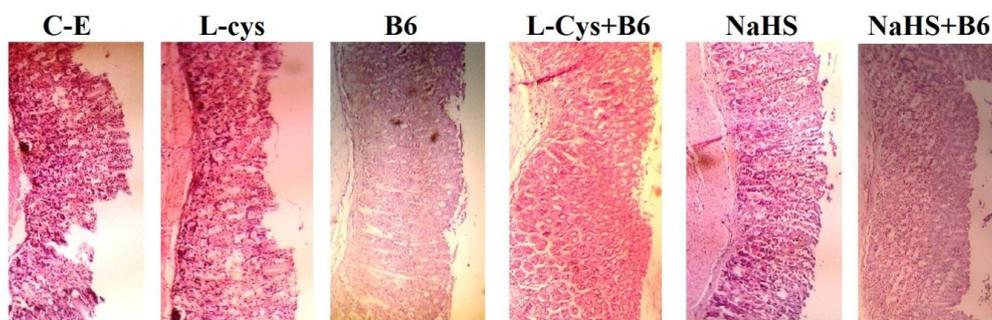


Figure 1. Histological evaluation of gastric mucosa. Representative gastric sections were obtained 1 hr after ethanol administration. C-E: Control group indicate severe disruption to the upper half of mucosal thickness and necrotic lesions penetrating deeply into mucosa; L-Cys, B6, NaHS, L-Cys+B6, NaHS+B6: 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 disruption of the surface epithelium. All of the sections stained with hematoxylin and eosin; ×100 magnification

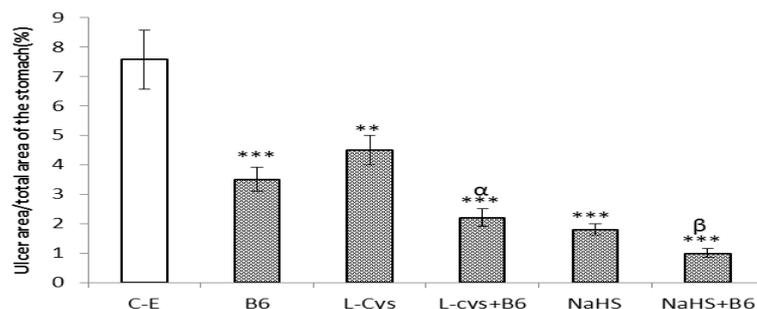


Figure 2. A graphic representation of the ulcer index following ethanol administration among various treatment groups. C-E (control): rats were received ethanol (0.5/25 g of body weight, orally); L-cys: animals were given L-cysteine (100 mg/kg) 60 min prior to ethanol administration; B6: animals received 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. $\beta P < 0.05$ as compared with NaHS-treated animals

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

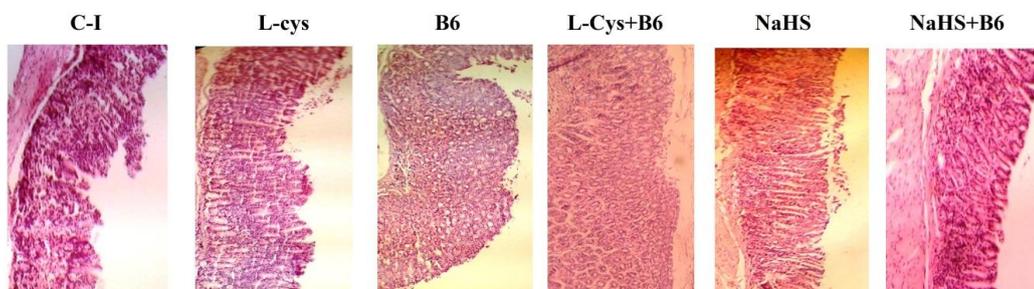


Figure 3. Histological evaluation of gastric mucosa. Representative gastric sections were obtained 5 hr after indomethacin administration. C-I: Control group indicate severe disruption to the upper half of mucosal thickness and necrotic lesions penetrating deeply into mucosa; L-Cys, B6, NaHS, L-Cys+B6, 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

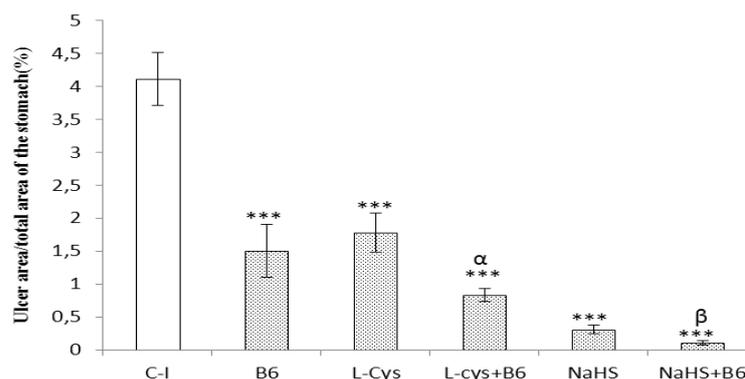


Figure 4. A graphic representation of the ulcer index following indomethacin administration among various treatment groups. C-I: control (animals were received indomethacin at 40 mg/kg, orally); L-cys: animals were given L-cysteine (100 mg/kg, IP) 60 min prior to intervention; B6: animals received vitamin B₆ (10 mg/kg) 30 min prior to intervention; L-cys+B6: animals received L-cysteine (100 mg/kg, IP) 60 min prior to intervention and vitamin B₆ (10 mg/kg, IP) 30 min prior to intervention. ***P*<0.01, ****P*<0.001 versus the control group and ^α*P*<0.05 versus the L-cysteine-, and vitamin B₆-treated groups. ^β*P*<0.05 as compared with NaHS-treated animals

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-1 β 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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