Gastroprotective effects of hydrogen sulfide, carbon monoxide and nitric oxide on an experimental ulcer model in rats

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Abstract

Background/Aim: Gastric mucosal injury induced by several agents such as ethanol, stress or nonsteroidal anti-inflammatory drugs (NSAIDs) is a common severe disorder. Hydrogen sulfide (H2S), carbon monoxide (CO) and nitric oxide (NO) are gaseous autacoids that are endogenously produced in mammalian tissues. Recently, several studies confirmed that H2S, CO and NO play a role in gastroprotection. Our work aimed to evaluate and compared the gastroprotective effects of H2S, CO and NO on ethanol-, indomethacin- and stress-induced rat ulcer models.

Methods: The effects of NaHS (5 mg/kg), CORM-2 (5 mg/kg) and L-arginine (100 mg/kg) were investigated on gastric ulcer models induced by ethanol (1 ml 96% i.g.), stress (cold+immobility) and indomethacin (40 mg/kg i.g.). The ulcer index, gastric mucus secretion, free and total acidity, and levels of TNF-α, PGE2, MDA GSH, COX-1, COX-2 were measured.

Results: NaHS and CORM-2 decreased the increased TNF-α and MDA levels in ethanol-induced ulcer. L-arginine reduced mucin secretion, TNF-α and GSH levels in stress-induced gastric ulcer.

Conclusion: The present study showed that H2S and CO may have gastroprotective activity against ethanol-induced ulcers and NO may be gastroprotective against stress-induced ulcers.

Keywords: Gasotransmitters, Hydrogen sulfide, Carbon monoxide, Nitric oxide, Ulcer
Introduction
Gastric ulcer is a chronic disorder affecting an increasing number of individuals globally [1]. It develops due to an imbalance between exogenous protective and endogenous aggressive factors such as Helicobacter pylori, acid, pepsin, drugs, and mucosal defense mechanisms including mucosal blood flow, mucus production, bicarbonate, prostaglandins, nitric oxide and sulphydryl compounds [2, 3]. Several different ulcer models are experimentally used for evaluating the gastroprotective activity of potential agents [4]. The animal models of ethanol-induced gastric injury are similar to many aspects of humans and ensure a means for evaluating agents with potential anti-ulcer effects [5]. Oxidative stress, inflammation and reduction of gastric mucus and prostaglandin synthesis are exceedingly involved in ethanol-induced gastric ulcer pathogenesis [6]. Another major etiologic agent of gastrointestinal diseases is the consecutive use of nonsteroidal anti-inflammatory drugs (NSAIDs) which can cause topical injury to the mucosa and systemic effects by inhibiting cyclooxygenase-1 (COX-1), which is associated with mucosal prostaglandin production [7]. Indomethacin, the most popular representative agent among NSAIDs, leads to gastric ulcers by way of generation of reactive oxygen species (ROS), infiltration of leukocytes, inhibition of prostaglandin and induction of apoptosis [8]. The disturbance of gastric mucosal microcirculation, alterations of gastric secretion and abnormal gastric motility might play a role in stress-induced gastric mucosal lesions [9]. Various studies confirmed that stress increased gastric acid secretion [10], decreased prostaglandin synthesis [11] and enhanced lipid peroxidation [12] lead to stress-induced ulcers.

The gaseous mediators, H2S, CO and NO play a critical role in gastroprotection [13]. Recently several studies indicated that these mediators have gastroprotective effects in different ulcer models [14-16].

This research aimed to assess and compare the possible gastroprotective effects of the three important signaling molecules (H2S, CO and NO) against different rat ulcer models.

Materials and methods
Animals
Ninety-one Wistar male rats (7-8 weeks old, 250–300 g) were housed in cages in a controlled room with 12/12 light-dark cycles, a temperature of 20-22°C and a relative humidity of 65%-70%. Before the onset of experiments, the animals were kept in a single cage and deprived of food for 16 hours. Free access to water was allowed until 1 hour before the beginning of experiments. This study was performed in compliance with the guidelines for the care and use of laboratory animals approved by the Local Ethics Committee of Eskisehir Osmangazi University (548-1/2017). The rats were divided into thirteen groups (n = 6): (i) Control group (saline, i.p.), (ii) Ethanol control group (1 ml 96% i.g.), (iii) Ethanol+L-arginine (100 mg/kg, i.p.) group, (iv) Ethanol+CORM-2 (5 mg/kg, i.g. group), (v) Ethanol+NahS (5 mg/kg, i.g) group, (vi) Stress group, (vii) Stress+L-arginine (100 mg/kg, i.p.) group, (viii) Stress+CORM-2 (5 mg/kg, i.g. group), (ix) Stress+NahS (5 mg/kg, i.g) group, (x) Indomethacin group (40 mg/kg), (xi) Indomethacin+L-arginine (100 mg/kg, i.p.) group, (xii) Indomethacin+CORM-2 (5 mg/kg, i.g.) group, (xiii) Indomethacin+NahS (5 mg/kg, i.g.) group.

Drugs
Sodium hydrosulfide (NaHS), L-arginine, tricarboxylicdichlororuthenium (II) dimer (CORM-2) and indomethacin were purchased from Sigma-Aldrich (St. Louis, MO, USA). CORM-2 was dissolved in 1% DMSO. The other drugs were dissolved in saline before use. NaHS (5mg/kg, i.g) for H2S, CORM-2 (5mg/kg, i.g) for CO, and L-arginine (100 mg/kg, i.p.) for NO donor were examined on three gastric ulcer models. These agents were administered orally 1 hour before the induction of ulcers.

Ulcer models
Ethanol-induced gastric ulcer
One ml of absolute ethanol was administered by intragastric gavage. Treatment agents were administered 1 hour before ethanol administration. Two hours after ethanol administration, the rats were sacrificed by decapitation after 3% sevoflurane was given for anesthesia [17].

Stress-induced gastric ulcer
Animals restrained and immobilized in single cages were placed in a ventilated refrigerator at 4 °C for 4 h [18]. Drugs were administered to rats 1 hour before placing them in the refrigerator. After removing them from the refrigerator, the animals were sacrificed as previously described.

Indomethacin-induced ulcer
Gastric ulcer was induced by intragastric gavage of 40 mg/kg of indomethacin. Drugs were given to animals 1 hour before indomethacin administration [19]. Five hours after the administration of indomethacin, they were sacrificed as described previously.

Ulcer index of gastric mucosa
Stomachs of animals were quickly ligated at both ends and removed, and then opened along the great curvature. The ulcerated areas were measured with a magnifying glass. Each lesion (mm) was measured along its greatest length, five petechias were considered to be equivalent to a 1 mm-long ulcer [20]. The ulcer index was recorded and calculated in accordance with the method of Guth [21]. Ulcer length ≤ 1mm (including erosion foci) was scored as 1; 1 mm< ulcer length≤2 mm was scored as 2; 2 mm< ulcer length≤3 mm was scored as 3; 3 mm< ulcer length≤4 mm was scored as 4; ulcer length>4 mm was scored as 5. If ulcer width exceeded 2 mm, the score doubled. The total scores of the whole stomach constituted the ulcer index. After determining ulcer index, each stomach was separated into the corpus and fundus parts.

Corpus was divided into four parts and fundus was divided into six parts, all of which were weighed and stored at 80°C until determination of gastric mucus, TNF-α, PGE2, MDA GSH, COX-1, and COX-2 levels.

Determination of gastric acidity
For evaluating gastric acidity, the gastric content of the stomachs was collected, washed with 1 ml of saline and centrifuged. Gastric acidity was identified by titration with 0.01 N sodium hydroxide using methyl orange and phenolphthalein for indicators and represented as mEq/L [22].
Determination of gastric mucus

The corpus of the stomach was weighed and used for the determination of gastric mucus content (µg/g tissue) in accordance with the modified procedure of Corne et al. using Alcian blue [23].

Determination of COX-1, COX-2, PGE2, TNF-α, MDA and GSH

An enzyme-linked immunosorbent assay (ELISA) was performed to measure the level of COX-1, COX-2, PGE2, TNF-α, MDA and GSH expression from gastric fundus lysates using the appropriate kits from Shangai YL Biotech, China, per the manufacturer’s instructions.

Statistical analysis

SPSS 22 (SPSS Inc, Chicago, IL, USA) software was used for statistical analysis. The results were expressed as mean and the standard error of the mean (SEM). All data were examined for normality of distribution and analyzed by the Kruskal Wallis test followed by Tukey’s test. Differences among groups were considered significant if \( P < 0.05 \).

Results

As presented in Table 1, the ulcer index significantly increased in all ulcer models induced by ethanol, stress and indomethacin. Although CORM-2 and NaHS remarkably reduced ulcer index in the ethanol group, no significant change was observed with L-Arginine. L-Arginine caused increased ulceration in the indomethacin group, whereas CORM-2 and NaHS did not change significantly. The levels of mucin presented in Table 1 show that a dramatic increase was noticed in the indomethacin-induced ulcer model with the use of NaHS and mucin secretion was inhibited in the stress group by L-Arginine. The effects of all agents on free and total acidity were demonstrated in Figure 2, none of the three agents caused any change in the indomethacin group. Although CORM-2 significantly increased PGE2 in the ethanol group, only L-Arginine decreased COX-1 in the stress ulcer group, whereas the other agents did not cause a remarkable change in other groups. Figure 5 indicates that ethanol increased while L-Arginine and NaHS [1.62 (0.3) and 1.41 (0.4)] decreased COX-1 in the ethanol group, only L-Arginine decreased COX-1 in the stress ulcer group [1.68 (0.4)], and CORM-2 significantly increased COX-1 in the indomethacin group [2.29 (0.2)]. As shown in Figure 6, ethanol increased, while L-Arginine and CORM-2 notably decreased COX-2 in the ethanol group [0.25 (0.07) and 0.33 (0.03)] and increased COX-2 in indomethacin group [0.31 (0.01) and 0.36 (0.04)]. It was also noticed that COX-2 was inhibited by indomethacin.

| Treatment                | Ulcer Index | Mucin (mg/g tissue) | Free Acidity (meq/L) | Total Acidity (meq/L) |
|--------------------------|-------------|---------------------|----------------------|-----------------------|
| Control                  | 0.132 (0.013) | 2.12 (0.43)         | 0.8 (0.21)           | 1.36 (0.22)           |
| Ethanol Control          | 45.1 (1.21)** | 3.14 (0.31)         | 1.67 (0.17)          | 7.74 (0.46)**         |
| Ethanol+L-Arginine       | 39.82 (0.73) | 2.79 (0.55)         | 2.23 (0.49)          | 9.63 (0.64)**         |
| Ethanol+CORM-2           | 19.67 (1.06)** | 4.69 (0.34)       | 1.49 (0.36)          | 11.4 (0.75)**         |
| Ethanol+NaHS             | 2.57 (0.46)** | 5.63 (0.44)**      | 1.23 (0.46)          | 12.85 (0.76)**        |
| Stress Control           | 6.5 (0.26)** | 5.98 (1.12)**       | 0.95 (0.37)          | 2.03 (0.39)           |
| Stress+L-Arginine        | 2.9 (0.72)   | 1.19 (0.21)**       | 0.56 (0.18)          | 1.59 (0.40)           |
| Stress+CORM-2            | 20.9 (0.69)** | 4.51 (0.2)         | 0.68 (0.11)          | 2.74 (0.55)           |
| Stress+NaHS              | 9.2 (1.16)   | 3.49 (1.07)**       | 0.23 (0.05)          | 1.07 (0.16)**         |
| Indomethacin Control     | 21.23 (1.14)** | 3.34 (0.43)     | 1.73 (0.19)**        | 3.14 (0.56)           |
| Indomethacin+L-Arginine  | 37.1 (0.66)** | 3.14 (0.19)       | 0.73 (0.16)**        | 3.98 (0.80)           |
| Indomethacin+CORM-2      | 25.73 (0.49) | 7.17 (0.93)         | 1.89 (0.52)          | 5.59 (0.64)           |
| Indomethacin+NaHS        | 24.93 (1.09) | 12.14 (0.56)**      | 1.98 (0.34)          | 6.27 (0.63)**         |

All values are mean (SEM). Compared with control group *, \( P < 0.05 \), **, \( P < 0.01 \) compared with their own control group #, \( P < 0.05 \), ##, \( P < 0.01 \) compared with their own control group. Kruskal-Wallis post hoc Tukey's test.

Figure 1: Effects of H2S, CO and NO on TNF-α production in the rat gastric tissue of ethanol, stress and indomethacin-induced gastric ulcers.

Figure 2: Effects of H2S, CO and NO on PGE2 production in the rat gastric tissue of ethanol, stress and indomethacin-induced gastric ulcers.

Figure 3: Effects of H2S, CO and NO on MDA production in the rat gastric tissue of ethanol, stress and indomethacin-induced gastric ulcers.
Figure 4: Effects of H2S, CO and NO on GSH production in the rat gastric tissue of ethanol, stress and indomethacin-induced gastric ulcers.

All data represent the mean (SEM). C: Control. EC: Ethanol control. SC: Stress control. IC: Indomethacin control. L: L-arginine. CO: CORM-2. N: NaHS). Significance is represented as *P<0.01 compared to control group and **P<0.01 compared to their own control group. Kruskal-Wallis post hoc Tukey's test.

Figure 5: Effects of H2S, CO and NO on COX-1 levels in the rat gastric tissue of ethanol, stress and indomethacin-induced gastric ulcers.

All data represent the mean (SEM). C: Control. EC: Ethanol control. SC: Stress control. IC: Indomethacin control. L: L-arginine. CO: CORM-2. N: NaHS). Significance is represented as *P<0.01 compared to control group and **P<0.01 compared to their own control group. Kruskal-Wallis post hoc Tukey's test.

Figure 6: Effects of H2S, CO and NO on COX-2 levels in the rat gastric tissue of ethanol, stress and indomethacin-induced gastric ulcers.

All data represent the mean (SEM). C: Control. EC: Ethanol control. SC: Stress control. IC: Indomethacin control. L: L-arginine. CO: CORM-2. N: NaHS). Significance is represented as *P<0.05 compared to control group and #P<0.05, ##P<0.01 compared to their own control group. Kruskal-Wallis post hoc Tukey's test.

Discussion

We demonstrated that NaHS and CORM-2 had gastroprotective effects against ethanol-induced ulcers and L-arginine seems to have a protective effect against stress-induced ulcers. However, NaHS, CORM-2 and L-arginine had no gastroprotective effects against indomethacin-induced ulcers. The ulcer inducing mechanisms of ethanol, stress and NSAIDs differ. It is known that ethanol causes activated neutrophil infiltration. Then it leads to an increase in the production of proinflammatory and pro-oxidative enzymes and free radicals, therefore, the gastric mucosa is damaged [24]. Several studies reported that ethanol stimulates pro-inflammatory cytokines which have critical roles in the development of acute gastric ulcers [25]. Ethanol was also shown to reduce the production of NO [26] in the gastric mucosa causing the solubilization of gastric mucus constituents and the development of hemorrhagic lesions [27, 28]. On the other hand it was suggested that high doses of ethanol induce H2S synthesis in the gastric mucosa, and a decline of H2S levels to basal conditions protects the gastric tissue [29]. H2S effects are known to differ by the extent of concentration in the gastric tissue [30]. NSAIDs reduce endogenous gastric H2S synthesis which protects the gastric mucosa from injury and mediates gastric damage [31]. NaHS prevents ethanol-induced gastric injury in a dose-dependent way [32] and sulfhydryl compounds has gastroprotective activity on ethanol-induced gastric ulcers [33]. An interesting result of our study is that NaHS lead to a significant increase in the nitrite concentration in the indomethacin group. This can be explained by the effect of H2S on the release of Ca2+ via nitric oxide synthase (NOS) activation through the soluble guanylate cyclase (sGC) pathway [34].

Fast restitution and proliferation of gastric cells, maintenance of mucosal blood flow, secretion of protective mucus and bicarbonates, biosynthesis of endogenous prostaglandins (PG), sulfhydryl compounds, endothelial and epithelial nitric oxide (NO) and hydrogen sulfide (H2S) biosynthesis are among the complex mucosal defense mechanism [35]. PGE2 was reported to protect the gastric mucosa by an increase in stomach blood flow, and the secretion of mucus and bicarbonate ions (HCO3-) resulting in gastric protection, in part, also mediated by neutralization [36]. It was suggested that PGs synthesized both by COX-1 and COX-2 could participate in the gastroprotective mechanism [37]. In our study, L-arginine inhibited acidity and enhanced COX-1 levels in the stress ulcer group. Even though enhanced levels of mucin were observed by indomethacin, increased acidity and MDA, inhibited COX-2 and PGE2 led to gastric ulcers dramatically. While CORM-2 and L-arginine stimulated COX-2, NaHS, CORM-2 and L-arginine increased PGE2 levels and acidity. However, these effects were not enough to protect the indomethacin-induced gastric ulcer, therefore none of these agents had protective activity against this ulcer model.

CO has long been known as a toxic air pollutant. Although it is toxic at elevated concentrations, low concentrations of CO contributed to many physiological effects including anti-inflammatory, anti-apoptotic, anti-proliferative, antioxidant and vasodilatory effects similar to other gaseous mediators such as NO and hydrogen sulfide [38, 39]. The current mechanism of the gastroprotective effect of CO and H2S has been attributed to an increase in gastric microcirculation and the attenuation of inflammation by downregulation of pro-inflammatory factors in the gastric mucosa [40]. We also had similar results in terms of the effects of CORM-2 and NaHS on TNF-α and COX enzymes, and inhibition of MDA levels in ethanol group. Thus, this local hyperemic activity of H2S and CO seems to be a basic mechanism of gastroprotection.

NO contributes to the maintenance of gastric mucosal barrier integrity. It also has been reported to increase mucus and bicarbonate secretions, mucosal blood flow, and induce tissue repair when stomach tissue is damaged [41]. Researchers demonstrated that NO plays a biphasic role in the ulcerogenic response of the gastric mucosa and its donors were demonstrated to protect against gastric mucosa damage by several agents [42-44]. We found that NO may have protective effects against stress induced ulcer. In support of our results, some studies have shown...
the role of endogenous NO in the protection of gastric mucosa in stress ulcer models [45, 46].

Limitations
The molecular mechanisms of all agents were not clearly investigated to reveal the action in the current study. In addition, species-related alterations can cause significant differences; therefore, future studies should investigate whether the results will be similar when these procedures are performed on different animal species.

Conclusion
H2S and CO exert anti-ulcerogenic effects and can be effective in reducing the incidence of ethanol-induced gastric mucosal injury. NO may be protective against stress-induced ulcer. These gaseous mediators did not prevent NSAIDs-induced gastric ulcers, even though they stimulated mucin secretion and PGE2. Our present work may contribute to identify the possible mechanisms underlying their gastroprotective activities.

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