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Preliminary report

Gastroprotective effect of 2-mercaptoethane sulfonate against acute gastric mucosal damage induced by ethanol

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ABSTRACT

Gastric mucosal damage induced by ethanol is a serious medical problem. Recent evidences suggest that reactive oxygen species and inflammatory mediators play a key role in the destruction of gastric mucosa. The present study was aimed to evaluate the potential beneficial effect of MESNA (2-mercaptoethane sulfonate) against ethanol-induced gastric mucosal damage in mice. The animals were orally pretreated with vehicle or MESNA and then treated with acidified ethanol to induce gastric mucosal damage. One hour after ethanol ingestion mice were euthanized and stomach samples were collected for biochemical analysis. Macroscopic and histopathological evaluation of gastric mucosa showed that pretreatment with MESNA attenuated gastric lesions induced by ethanol. Administration of MESNA significantly increased glutathione content and superoxide dismutase and catalase activity in the gastric tissues. In addition, MESNA markedly reduced ethanol-induced lipid peroxidation, myeloperoxidase activity, tumor necrosis factor-alpha, interleukin (IL)-1β, IL-6, and monocyte chemotactic protein-1 levels. These findings suggest that the thiol-containing compound MESNA is able to decrease alcohol-induced oxidative stress and inflammation in the gastric tissue. It seems that MESNA may have a protective effect against ethanol-induced gastric mucosal damage.

1. Introduction

Gastric ulcer is the most prevalent gastrointestinal disorder that affects many people worldwide. Although the mechanism of gastric ulcer has not been completely elucidated, it is shown that the gastric mucosal injury results from an imbalance between aggressive and protective factors. The risk factors that contribute to the formation of gastric lesions include consumption of alcohol, non-steroidal anti-inflammatory drugs (NSAIDs), increased secretion of hydrochloric acid, *Helicobacter pylori*, stress, and tobacco [1]. It has been established that ethanol as an exogenous irritant damages the gastrointestinal mucosal cells and leads to gastric ulcers by different molecular mechanisms. Experimental and clinical studies showed that the reactive oxygen species (ROS) and inflammatory mediators induced by ethanol contribute to the pathogenesis of ethanol mediated gastric lesions. After entering cells, ethanol is metabolized and facilitates production of the superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl free radical ($HO^{\bullet}$). Generation of highly reactive oxygen species induces lipid peroxidation of cellular membranes and leading to cell death and epithelial damage. Furthermore, in the gastric mucosal cells, ethanol significantly reduces the levels of major antioxidants that can eliminate reactive oxygen species [2,3]. It has also been demonstrated that neutrophil infiltration into the gastric mucosa and release of proinflammatory cytokines such as TNF-α, IL-1β, IL-6 and chemokine MCP-1 are involved in the pathogenesis of gastric mucosal injury [4–7].

Recently, many studies have focused on novel approaches for prevention of gastric mucosa from alcohol-induced injury. Several compounds with antioxidant and anti-inflammatory activity have been studied for their gastroprotective effects [3,5,6,8,9]. 2-Mercaptoethane sulfonate (MESNA) is a small, synthetic, sulfhydryl-containing molecule that has the ability to scavenge reactive oxygen species through its antioxidant effects. MESNA is frequently used with cyclophosphamide and ifosfamide to reduce hemorrhagic cystitis induced by acrolein, a toxic urinary metabolite of oxazaphosphorine-alkylating agents. Sulfhydryl group present in MESNA binds to acrolein within the urinary tract and forms nontoxic stable thioether that is safely eliminated in the urine. Adverse effects associated with MESNA are few and well tolerated by most patients. Several studies have been shown that MESNA is effective in the treatment of oxidative stress induced disorders. It has been reported that MESNA prevents doxorubicin-induced plasma protein oxidation, cisplatin-induced ovarian damage and ischemia reperfusion-induced oxidant damage of the intestinal mucosa [10–12]. The present study was aimed to investigate the effectiveness and the possible mechanisms of action of MESNA in a mouse model of ethanol-induced gastric mucosal damage.

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2. Material and methods

2.1. Animals

Experiments were performed on male Swiss albino mice weighing 26–30 g. Animals were kept in our animal house under controlled conditions and allowed free access to tap water and a standard diet. Mice were fasted overnight with free access to water before the induction of gastric mucosal lesions. All animal procedures were carried out in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals (NIH publication 85–23, revised 1996).

2.2. Materials

5,5′-Dithiobis-2-nitrobenzoic acid, tetramethylbenzidine, glutathione reduced, Tris–HCl buffer, hydrogen peroxide, thiobarbituric acid, trichloroacetic acid and 1,1,3,3-tetraethoxypropane were purchased from Sigma-Aldrich. MESNA was obtained from Baxter (Germany). Ethanol was purchased from Taghtir Co. (Iran). Protease inhibitors (Complete Mini tablets) were purchased from Roche (Germany).

2.3. Experimental design

Intragastric administration of ethanol/HCl has long been used as an experimental method to induce gastric mucosal damage in animals. HCl in ethanol solution accelerates the progress of ulcerogenesis and enhances ethanol-induced gastric mucosal toxicity [13,14]. Mice were randomly allocated into four groups (8 mice each). The first group received normal saline orally and served as the control group. The second group of mice was given MESNA alone. The third group of mice was given a single dose of acidified ethanol. The fourth group was given 400 mg/kg MESNA and acidified ethanol. MESNA administered orally 1 h before application of the ulcerogenic agent. One hour after treatment with MESNA the animals received acidified ethanol (60% ethanol/0.3 M HCl, 0.1 ml/10 g body weight, orally) to induce gastric mucosal injury. The dose of MESNA used in this study (400 mg/kg) was selected based on our preliminary experiments and previous studies [15,16].

2.4. Sample collection

One hour after administration of ethanol/HCl mice were anesthetized with ketamine and xylazine and their stomachs were excised. The stomachs were opened along the greater curvature and gently washed with cold saline solution to remove the gastric contents. The gastric tissue samples were frozen in liquid nitrogen and kept at −80 °C for biochemical determinations.

2.5. Determination of gastric mucosal injury index

Macroscopic scoring of gastric mucosal damage was performed by light microscopy. The severity of gastric damage induced by ethanol/HCl was expressed as an ulcer index (UI) and calculated based on the number and diameter of the lesions [13]. $UI = \Sigma(A) + (2B) + (3C)$ (A is the number of small lesions up to 1 mm; B is the number of lesions up to 3 mm; and C is the number of linear lesions >3 mm). The mean ulcer index was calculated for each group and then statistically analyzed.

2.6. Histopatological analysis

A small specimen of each stomach was fixed in 10% formalin and embedded in paraffin, and sections were stained with hematoxylin and eosin (H&E). The histopathology examination was performed under light microscopy by an experienced pathologist according to the method as described previously [17]: (1) hemorrhage (score 0–4), (2) mucosal edema (score 0–4), (3) epithelial cell loss (score 0–3), and (4) inflammatory cell infiltration (score 0–3).

2.7. Preparation of gastric tissue homogenate

The gastric tissues were cut into small pieces and homogenized in ice-cold Tris–HCl buffer (pH 7.4, containing protease inhibitor cocktail) using a homogenizer (Heidolph, Germany). The homogenates were centrifuged at 20,000 ×*g* for 20 min in a refrigerated centrifuge at 4 °C. The supernatants were collected and stored at −80 °C until analysis.

2.8. Measurement of malondialdehyde (MDA) levels

Gastric tissue MDA level was determined using the thiobarbituric acid method as described in our previous study [4]. Briefly, 100 μl of the supernatant was added to a reaction mixture containing 100 μl of 20% (w/v) trichloroacetic acid (TCA) and 100 μl of TBA (0.1 M). The mixture was incubated in a boiling water bath for 60 min to complete the reaction. After cooling, the samples were centrifuged at 10,000 ×*g* for 5 min at 4 °C and the supernatants were separated from the pellets. The absorbance of the supernatants was measured at 532 nm using a 96-well microplate reader (BioTek, USA).

2.9. Measurement of reduced glutathione (GSH) content

To determine GSH, 50 μl of 10% trichloroacetic acid (TCA) was added to 50 μl of the supernatant to precipitate the proteins. The mixture was centrifuged at 10,000 ×*g* for 5 min at 4 °C and the supernatants were separated from the pellets. 50 μl of the resulting supernatant was added to 150 μl of DTNB (5,5′-Dithiobis-2-nitrobenzoic acid) in phosphate buffer (0.2 M, pH 7.6, 1 mM EDTA). The intensity of the yellow color formed was read at 412 nm using a microplate reader.

2.10. Measurement of superoxide dismutase (SOD) activity

The activity of superoxide dismutase in gastric supernatant samples was measured using a SOD assay kit (BioVision) according to the manufacturer's protocol.

2.11. Measurement of catalase (CAT) activity

CAT activity was assessed according to the method as described previously [18]. Briefly, 10 μl of the supernatant was added to a cuvette containing 0.5 ml of phosphate buffer (50 mM, pH 7). Reaction was started by the addition of 0.5 ml of H_2O_2 (30 mM). The rate of change in absorbance was measured at 240 nm and CAT activity was defined as the quantity of enzyme degrading 1 mmol of H_2O_2 per minute.

2.12. Measurement of myeloperoxidase (MPO) activity

The activity of MPO in gastric tissue samples was determined according to our previous study [4]. Briefly, The reaction mixture for analysis consisted of 50 μl sample, 50 μl of 15 mM tetramethylbenzidine (TMB) and 100 μl of hydrogen peroxide (25 mM) diluted in phosphate buffer (50 mM, pH 5.4). The assay was performed in a microplate and the reaction was started by adding hydrogen peroxide. The rate of change in absorbance was measured at 370 nm and MPO activity was defined as the quantity of enzyme degrading 1 μmol of hydrogen peroxide per min at 25 °C.

2.13. Measurement of pro-inflammatory cytokines

The cytokine levels of TNF-α, IL-1β, IL-6 and MCP-1 were measured by using mouse ELISA kit from eBioscience according to the manufacturer's instructions.

2.14. Statistical analysis

Data were presented as mean ± standard deviation (SD). Multiple group comparisons were analyzed statistically by one-way analysis of variance (ANOVA) with post hoc Tukey test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Macroscopic examination

Macroscopic analysis of the gastric mucosa showed that oral administration of acidified ethanol produced extensive mucosal ulcerations in the glandular portion of the stomach (Fig. 1a). Ulcers of the gastric mucosa appeared as elongated bands of hemorrhagic lesions parallel to the long axis of the stomach. Pretreatment of mice with a single dose (400 mg/kg) of MESNA reduced mucosal damage induced by ethanol. Additionally, as shown in Fig. 1c, MESNA treated group showed a significant decrease ($p = 0.002$) in the gastric ulcer index as compared to ethanol group.

3.2. Microscopic examination

Histopathological analysis of the gastric mucosa revealed that oral administration of acidified ethanol induced gastric mucosal injury, characterized by hemorrhagic damage, submucosal edema, loss of epithelial cells and inflammatory cell infiltration (Fig. 1b). Animals pretreated with MESNA showed less microscopic mucosal injury as compared with the control group treated with ethanol. As shown in Fig. 1d, mice pretreated with MESNA also showed a significant decrease ($p < 0.001$) in the total histopathological scores when compared with the ethanol group. No histopathological alteration was observed in the stomach of MESNA only-treated mice.

3.3. Effect of MESNA on MDA level and MPO activity

Oral administration of ethanol significantly increased the content of MDA and the activity of MPO in the gastric tissue in comparison to the vehicle-treated control group (Table 1). Pretreatment with MESNA remarkably reduced the formation of MDA ($p = 0.001$) and the level of MPO ($p < 0.001$) in the gastric tissue compared with the ethanol group. These data indicate that MESNA pretreatment is able to inhibit

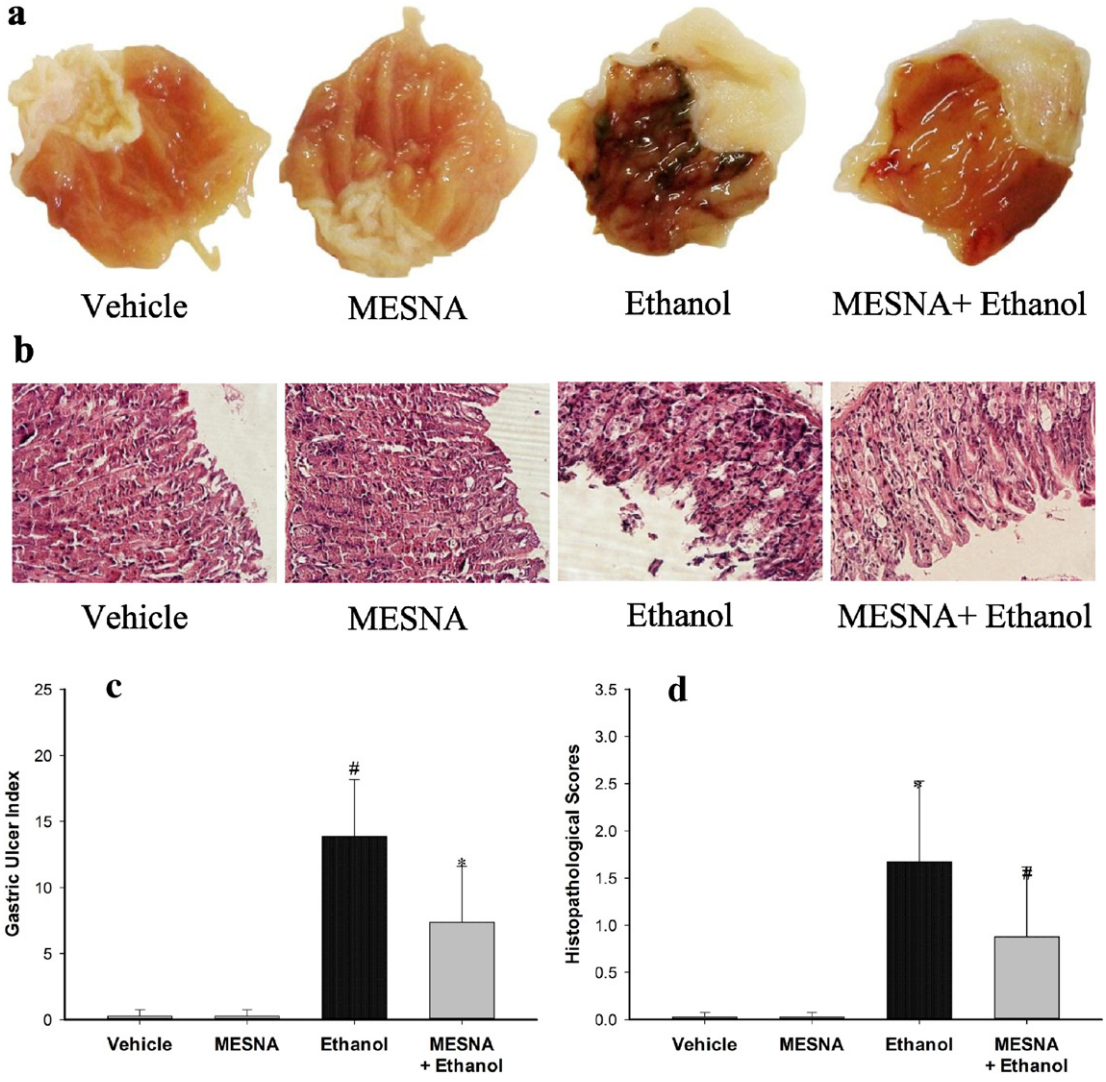

Fig. 1. Effect of MESNA and ethanol in a mouse model of ethanol-induced gastric mucosal damage. (a) Macroscopic appearance of the gastric mucosa. (b) Histological appearance of the gastric mucosa. The gastric tissue sections were analyzed by H&E staining (magnification is ×400). (c) Gastric mucosal ulcer index in each experimental group. The results are the mean ulcer score ± SD. (13.87 ± 4.2 for ethanol control group; 7.3 ± 4.25 for MESNA + ethanol group). (d) Total histopathological scores in the gastric mucosa. The results are presented as the mean of the total score ± SD. (1.66 ± 0.85 for ethanol control group; 0.87 ± 0.7 for MESNA + ethanol group). $^{\#}p < 0.001$ compared with vehicle-treated group; $^{*}p < 0.01$ compared with ethanol control group.

Table 1
Effects of MESNA on the gastric tissue levels of MDA, MPO, GSH, SOD and CAT in four study groups.

Groups	MDA (nM/mg tissue)	MPO (mU/mg tissue)	GSH (nM/mg tissue)	SOD (mU/mg tissue)	CAT (mU/mg tissue)
Vehicle	24.5 ± 2.5	18.6 ± 1.47	3.5 ± 0.25	48.4 ± 2.78	62.3 ± 19
MESNA	23.17 ± 3.26	17.8 ± 3.39	3.74 ± 0.67	47.6 ± 2.57	65.6 ± 17
Ethanol	45.3 ± 8.7#	32.3 ± 5.1#	0.3 ± 0.26#	31.6 ± 8.4#	19.5 ± 5.7#
MESNA + ethanol	30.9 ± 6.76**	19.1 ± 2.3**	3 ± 0.42**	46 ± 6.5**	47.2 ± 7.4**

Data are means ± SD.
# $p < 0.01$ compared with vehicle-treated group.
** $p < 0.01$ compared with ethanol control group.

ethanol-induced lipid peroxidation and neutrophil infiltration in the gastric mucosa.

3.4. Effect of MESNA on GSH, SOD and CAT levels

Measurement of gastric endogenous antioxidant GSH levels and SOD and CAT activity showed that ethanol administration induced significant depletion of gastric contents of GSH, SOD and CAT when compared with those in the vehicle-treated group (Table 1). Pretreatment with MESNA, significantly restored the levels of GSH ($p < 0.001$) and SOD ($p < 0.001$) and CAT ($p = 0.002$) activity in the gastric mucosa as compared with those in the ethanol group. These results demonstrate an antioxidant effect of MESNA in this experimental model.

3.5. Effect of MESNA on pro-inflammatory cytokines

The levels of proinflammatory cytokines TNF-α, IL-1β, IL-6 and chemokine MCP-1 in the gastric mucosa were considerably elevated in the ethanol treated group as compared to the vehicle-treated control group ($p < 0.001$) (Fig. 2). Pretreatment of mice with MESNA significantly decreased the production of TNF-α ($p = 0.001$), IL-1β ($p < 0.001$), IL-6 ($p < 0.001$) and MCP-1($p < 0.03$) in the gastric tissue as compared to the mice treated with ethanol. These findings show an anti-inflammatory effect of MESNA in the gastric tissue.

4. Discussion

Chronic alcohol abuse can cause gastrointestinal toxicity including gastric mucosal lesions. In the present work, we studied the gastroprotective activity of MESNA in a mouse model of ethanol-induced gastric mucosal damage. Oral administration of ethanol is commonly used as an experimental model of gastric mucosal injury in mice. We found that application of acidified ethanol caused macroscopic gastric lesions and pretreatment with MESNA significantly reduced gastric mucosal lesions induced by ethanol. The gastroprotective effect of MESNA was further confirmed by histopathological examination.

Ethanol induces its toxic effects primarily by oxidative stress and subsequent inflammatory mechanisms. Several studies have shown that ethanol administration induces oxidative stress by increasing the production of reactive oxygen species and depletion of cellular antioxidant capacity. Ethanol also induces the activity of cytochrome P450 enzymes, which contribute to the generation of oxygen radicals. It has been shown that ethanol-induced reactive oxygen species, in particular superoxide anion ($O_2^{\bullet -}$), is derived from mitochondria [2]. These highly toxic oxygen species are extremely reactive and cause damage to proteins, lipids and DNA which ultimately results in cell death. Lipid peroxidation is an important mechanism of cellular injury and many studies have demonstrated that ethanol can induce lipid peroxidation in cell membranes through reactive oxygen species [19]. Ethanol induced lipid peroxidation in gastric epithelial cells triggers the loss of membrane integrity, increase in cell permeability and leads to acceleration of gastric ulcer formation. Malondialdehyde (MDA) is an end-product of the peroxidation of polyunsaturated fatty acids within cell membranes, and commonly used as a reliable marker of lipid peroxidation process in tissues. Therefore, determination of MDA levels can be used to estimate the degree of ethanol-induced gastric damage. In the present study, we found that ethanol administration significantly increased the production of MDA in the gastric tissue. This result is in agreement with other studies, which revealed that the concentration of MDA in the gastric tissue can be elevated by ethanol administration [3,8,9]. Pretreatment with MESNA significantly prevented the ethanol-induced increase of MDA levels in the gastric tissues. This finding shows an antioxidant activity of MESNA and may indicate that MESNA reduces alcohol-induced gastric injury through the preventing of lipid peroxidation process.

The cells of the gastrointestinal mucosa have endogenous antioxidant systems which protect cells against reactive oxygen species. Antioxidant enzymes involved in the elimination of oxygen radicals include superoxide dismutase (SOD) and catalase. The enzyme SOD catalyzes the dismutation of superoxide anions ($O_2^{\bullet -}$) into oxygen (O_2) and H_2O_2. Hydrogen peroxide could be enzymatically metabolized into oxygen and water by catalase [20]. It has been shown that the tissue levels of SOD and catalase was decreased in ethanol-induced gastric damage and increased during treatment. SOD and catalase act as an important antioxidant defense in the gastric cells against ethanol induced oxidative stress [21,22]. Reduced glutathione (GSH) is a critical cellular antioxidant, and plays an important role in intracellular protection against toxic compounds. The antioxidant properties of GSH are due to the presence of the sulfhydryl (SH) group in its structure. GSH acts as a reactive oxygen species scavenger and inhibitor of lipid peroxidation and serves as a cofactor for glutathione S-transferase enzyme which removes toxic compounds by binding with GSH. It has been demonstrated that GSH is involved in a variety of cellular functions such as regulation of cell signaling and DNA repair mechanisms [23]. It has been proven that ethanol-induced gastric mucosal damage is associated with a significant decrease in GSH content of the gastric tissue. Depletion of GSH results in accumulation of reactive oxygen species, enhanced lipid peroxidation, and DNA damage [24]. In our study, acute ethanol administration significantly decreased gastric levels of GSH and SOD and catalase activity, which is in agreement with other studies [3,22]. The reduction in SOD and catalase activity might be attributed to the enzyme consumption due to neutralizing the excessive superoxide anions in the gastric tissues. The depletion of GSH content may result from its consumption by detoxifying ethanol-induced oxygen species and toxic products. MESNA pretreatment prevented the depletion of GSH and restored SOD and catalase activity in the gastric mucosa damaged by ethanol. These results suggest that the ability to increase GSH content and SOD and catalase activity is an important mechanism of action of MESNA. In other words, this finding confirms that the gastroprotective effect of MESNA in this experimental model is mediated through an antioxidant mechanism.

It has been shown that ethanol induces the infiltration of neutrophils into the gastric mucosa. Neutrophils are an important source of reactive oxygen radicals and play a critical role in the development of gastric mucosal damage. The enzyme myeloperoxidase (MPO) is a biochemical marker of neutrophil infiltration in the injured tissue. This enzyme is a

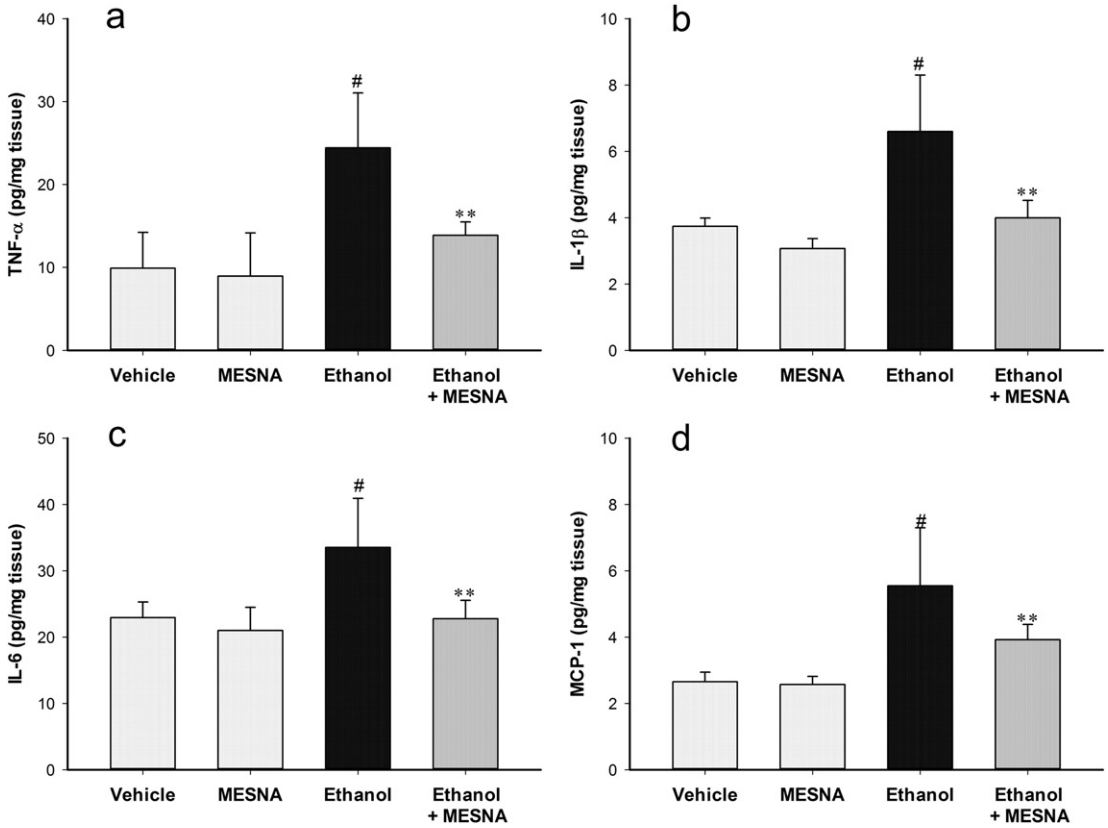

Fig. 2. Effect of MESNA on (a) TNF-α, (b) IL-1β, (c) IL-6 and (d) MCP-1 production in gastric tissue of mice. Administration of ethanol increased the levels of TNF-α, IL-1β, IL-6 and MCP-1 in gastric tissue, whereas pretreatment with MESNA (400 mg/kg) prevented the ethanol-induced increase in TNF-α, IL-1β, IL-6 and MCP-1. Results are means ± SD. The mean and SD values of ethanol control and MESNA + ethanol groups, respectively, are 24.4 ± 6.6 and 13.8 ± 1.6 in TNF-α, 6.6 ± 1.7 and 4 ± 0.5 in IL-1β, 33.5 ± 7.4 and 22.7 ± 2.7 in IL-6, 5.5 ± 1.7 and 3.9 ± 0.4 in MCP-1. $^{\#}p < 0.01$ compared with vehicle-treated group; $^{**}p < 0.01$ compared with ethanol group.

peroxidase enzyme found in the azurophilic granules of neutrophils. Following degranulation of neutrophils, MPO is released and catalyzes the oxidation of chloride ion with hydrogen peroxide (H_2O_2) to form hypochlorous acid (HOCl), which is a cytotoxic free radical [25–27]. In the present study, ethanol administration significantly increased the MPO activity, which represents infiltration of neutrophils into the gastric tissue. The increase of MPO activity in the gastric mucosal tissue may also be explained by the increase in the oxidative stress caused by ethanol. Pretreatment with MESNA prevented the increase of MPO activity in the gastric tissue of animals receiving ethanol. Other studies have also shown that MESNA is able to inhibit MPO activity in tissues [16,28]. The protective effect of MESNA against ethanol-induced gastric mucosal damage may be partially described by the ability of this drug to reduce neutrophil infiltration and MPO activity.

Ethanol-induced gastric mucosal damage was accompanied by the significant increase in the levels of proinflammatory cytokines, such as TNF-α, IL-1β, IL-6 and MCP-1. It has been shown that the production of TNF-α is one of the earliest events in ethanol-induced gastric injury. This pro-inflammatory cytokine triggers the production of other cytokines and activates infiltration of neutrophils which aggravate tissue damage through release of reactive oxygen species [5,6]. Moreover, it has been reported that ethanol-induced gastric epithelial cell apoptosis is associated with the increase of mucosal TNF-α levels [29]. IL-6 and IL-1β are pleiotropic inflammatory cytokines that express during gastric inflammation and enhance the effects of oxidative stress. It has been suggested that these inflammatory cytokines may trigger the oxidative pathways responsible for tissue damage in gastric ulcer disease [4,8]. The MCP-1 is a member of the chemokine family, and the most potent chemotactic factor for monocytes. It has been demonstrated that MCP-1 plays a key role in the initiation of mucosal inflammatory responses during the development of gastric mucosal injury. Inflammatory cells such as macrophages are the main source of MCP-1. Increased production of MCP-1 may be due to an increase in the number and activation of macrophages in injured gastric mucosa [7]. Nuclear factor-kappa B (NF-κB) is an important transcription factor that regulates inflammatory processes and immune responses. NF-κB mediates the induction of pro-inflammatory cytokines (e.g., TNF-α, IL-1B and IL-6), chemokines (e.g., MCP-1) and several other adhesion molecules. It has been reported that MESNA prevents ischemia-reperfusion-induced intestinal mucosal damage by inhibition of NF-κB activation [12]. In the present study, we showed that the oral administration of ethanol increased the levels of proinflammatory cytokines TNF-α, IL-1β, IL-6 and chemokine MCP-1 in the gastric tissue. Pretreatment with MESNA significantly induced a decrease in the levels of these proinflammatory cytokines, suggesting an anti-inflammatory effect of MESNA. It is important to note that MESNA have previously been shown to reduce TNF-α production in various experimental models, although the underlying molecular mechanisms have not been fully understood [10,30].

In conclusion, the data presented in the current study suggest that prophylactic administration of MESNA can attenuate the ethanol-induced gastric mucosal damage. The gastroprotective effect of MESNA may be primarily attributed to the decrease of lipid peroxidation process, increase the antioxidant capacity, and inhibition of inflammatory responses. Further studies are needed to determine whether MESNA could be used to protect against alcohol induced gastric damage in humans.

Conflict of interest

There is no conflict of interest.

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