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Ameliorating and protective effects *mesalazine* on ethanol-induced gastric ulcers in experimental rats

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ABSTRACT

Gastric ulcer is a frequent gastrointestinal tract (GIT) disorder that affects about 10% of the world population. Drug candidates that can provide high efficacy and low toxicity are needed value for the prevention and treatment of gastric ulcers. The present study aimed to assess the protective effect of mesalazine against ethanol-induced gastric mucosal injury in rats. The rats were divided into five groups, normal, ethanolic, standard (recipient ranitidine 50 mg/kg), experimental groups 1, 2 (receive mesalazine at doses of 50 and 100, respectively). The protective effect of mesalazine was evaluated by the ulcer index, histological examinations, measurement of oxidative stress parameters, antioxidant systems, and some gastric mucosal protection factors.

Pre-treatment of rats with doses of 50 and 100 mg/kg Mesalazine (5-ASA) in experimental groups 1 and 2 increased the pH of gastric juice and reduced the gastric ulcer index compared to the ethanolic group. Also, the results indicated that mesalazine, reduced the tissue reactive oxygen species (ROS), malondialdehyde (MDA), and protein carbonyl (PCO) levels, serum nitric oxide (NO), and increased the level of tissue NO and glutathione (GSH) and activity of Catalase (CAT), Based on these results, it can be concluded that mesalazine strengthens the antioxidant defense system of gastric mucosal cells during oxidative damage caused by ethanol.

1. Introduction

Gastric ulcer is a frequent gastrointestinal tract (GIT) disorder that affects about 10% of the world population (Adefisayo et al., 2017). A gastric ulcer occurs due to an imbalance between the constructive and destructive mechanisms of the gastric system. Constructive factors include mucin and peptide secretions, prostaglandin secretion, and blood flow, while harmful factors comprise gastric acid, pepsin secretion, and Helicobacter pylori. The gastric ulcer can occur in all layers of the stomach. Injury to any layer causes disturbance in normal physiologic functioning, leading to the excessive release of gastric acid, reactive oxygen radicals, nitric oxide synthase, and lipid peroxidation. Nonsteroidal anti-inflammatory drugs (NSAIDs), alcohol intake, bacterial infection, stress, and refluxed bile salts can be responsible for gastric ulceration. Ethanol is mostly used to induce gastric ulcers in animal models. Ethanol ingestion causes gastric cell necrosis and vascular injury, and consequently, ulceration. These effects are attributed to the generation of hydroperoxy free radicals and superoxide anions, which are produced as a result of ethanol metabolism in the body (Aziz et al., 2019).

Several medications, such as antibiotics, antacids, proton-pump inhibitors, and histamine H_2-receptor antagonists (Ranitidine (RAN)), are readily available to treat ulcers. However, these agents face major problems due to their limited efficacy against gastrohelcosis and severe side effects, for instance, gynecomastia, hypoacidity, impotence, osteoporotic bone fracture, hypergastrinemia, and cardiovascular disease risks. Thus, new drug candidates who could provide high efficacy and low toxicity are needed value for the prevention and treatment of Gastric ulcer (Zhou et al., 2020).

Mesalazine or 5-aminosalicylic acid (5-ASA, (5-amino-2-hydroxybenzoic acid)) is an anti-inflammatory drug used to treat inflammatory bowel disease. It is well tolerated by most patients and can induce mucosal healing, specifically in ulcerative colitis (Moura et al., 2016; Ramadan et al., 2018). 5-ASA is a highly effective antioxidant, free radical scavenger, and metal chelator against reactive oxygen species (ROS). In addition, its inhibitory effects against free radicals have already been confirmed in studies (Conner and Grisham, 1996). Recently, the in vitro effect of 5-ASA on amyloid fibril formation and defibrillation was investigated (Faramarzian et al., 2020). On the other hand, 5-ASA has anti-cancer effects, and it is believed that this ability is

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due to the removal of molecules that cause oxidative damage in the mucosa.

5-ASA is the active moiety of sulfasalazine, which is metabolized to sulfapyridine and 5-ASA. Most patients with adverse effects from sulfasalazine will tolerate 5-ASA (Moura et al., 2016; Ramadan et al., 2018). Sulfasalazine is used to treat ulcerative colitis. There are reports of its therapeutic use against ethanol-induced gastric damage in rats. The findings suggest that sulfasalazine may be useful in treating gastric lesions (Cho et al., 1987). But so far, there have been no reports of pre-treatment effects of 5-ASA on ethanol-induced gastric ulcer. Therefore, the present study aimed to assess the protective effect of 5-ASA against ethanol-induced gastric mucosal injury in rats. The protective effect of 5-ASA was evaluated by the ulcer index, histological examinations, measurement of oxidative stress parameters, antioxidant systems, and some gastric mucosal protection factors.

2. Materials and methods

2.1. Chemicals and drugs

Mesalazine (5-ASA) was provided by an industrial company, "chemidarou" (Tehran, Iran). Ranitidine (RAN) was purchased from Kharazmi Pharmaceutical Company (Khorramabad, Iran). 5, 5́-dithiobisnitro benzoic acid (DTNB), 2́, 7́-dichlorofluorescein diacetate (DCFH-DA), 2, 4-dinitrophenylhydrazine (DNPH), and guanidine hydrochloride from Sigma-Aldrich (St. Louis, MO, USA) were prepared. Ethanol, hydrogen peroxide (H_2O_2), 5-sulfosalicylic acid ($C_7H_6O_6S.2H_2O$), trichloroacetic acid (TCA), thiobarbituric acid (TBA), 1-naphtylethylenediamine and Sulfanilamide were obtained from Merck Co (Germany). All other chemicals used were analytical grade.

2.2. Animals

In this experimental study, 30 adult male Wistar rats with a weight range of 180–220 ± 20 g were used. These rats were purchased from the animal sciences department of Kermanshah University of Medical Sciences. They were kept for three days in the animal house of the Faculty of Veterinary Medicine of Lorestan University in standard temperature and light conditions. All procedures performed in this study involving animals were following the ethical standards and the international regulations of the usage and welfare of laboratory animals and were approved by the clinical ethics committee of the Faculty of Veterinary Medicine of Lorestan University with (Ethical code: LU. ECRA. 2020.32).

2.3. Ethanol-induced gastric mucosal injury

In this study, rats were weighed on the first day and kept under 12 h at the light and 12 h at the darkness, along with enough water and food to adapt to environmental conditions. After adaptation, on the second day, rats were randomly grouped into five groups, except the normal group, remained fasting for 24 h (without food). During these 24 h, the rats were given enough water with free access to prevent further loss of body water. On the third day, all rats were weighed first, then, according to the following grouping, all groups, except the first group, received the drugs with gavage nidel (Li et al., 2013).

The rats were divided into five groups, as follows:

- First group: normal group (normal healthy rats that do not receive extract, drug or ethanol during the research);
- Second group: ethanolic group (a group that receives only 1 ml of ethanol per rat);
- Third group: standard group (recipient RAN 50 mg/kg + 1 ml ethanol per rat);
- Fourth group: experimental group 1 (receives 5-ASA at a dose of 50 mg/kg + 1 ml ethanol per rat);
- The fifth group is experimental group 2 (receives 5-ASA at a dose of 100 mg/kg + 1 ml ethanol per rat).

During the gavage of the drugs to the third to fifth groups, to create the same stress conditions caused by the gavage, the first and second groups were given 1 ml of gavage distilled water.

In this study, 1 h after drug gavage, to induce gastric ulcer, 1 ml of 96% ethanol per gavage was administered except for rats of the first group (normal group). One h after the ulcer induction, animals were anesthetized by diethyl ether-saturated cotton ball in a Desiccator for 2–5 min and euthanized by cervical dislocation. Blood samples were collected and centrifuged (3000 g/10 min), where clear serum was separated and stored at −20 °C until analysis. In parallel, animal stomachs were rapidly taken away, opened along the greater curvature, where their contents were collected for volume and pH determination. After that, gastric tissue specimens were rinsed gently with a physiology saline solution to remove any blood clots and then examined macroscopically to calculate gastric ulcer index (Mousa et al., 2019). In the next step, the stomach was cut into two equal parts lengthwise, one part was transferred to a sterile tissue container containing 10% formalin for histological studies, and the other part was transferred to a sterile tissue container to evaluate of oxidative stress markers and antioxidant parameters and frozen at −20 °C.

2.4. Gastric ulcer index (UI) and percentage inhibition (%I)

The Ulcer index (UI) in terms of square millimeters (mm^2) and ulcer inhibition percentage (%I) were determined according to the method suggested by Takagi and Okabe (1968) with a slight change. In this method, the surface of the injured area was first measured with a ruler, and the degree of the ulcer's degree was determined based on the severity of the ulcer using Table 1. The UI and %I were calculated using the following formulas (Bhattamisra et al., 2019):

$$\textbf{Ulcer index (UI)} = \frac{\text{Total degrees of Ulcers in each group}}{\text{Number of animals in each group}}$$

$$\textbf{Inhibition of ulceration}(\%\textbf{I}) = \frac{\text{Ethanolic group UI} - \text{Pretreatment group UI}}{\text{Ethanolic group UI}}$$

2.5. Measurement of gastric juice volume and pH

The method described by Dashputre and Naikwade (2011) measured the volume and pH of gastric juice. Gastric juice of each rat for 10 min at 2000 g was centrifuged. Then the pellet was removed, and the volume of gastric juice was measured, and in the following to 1 ml of supernatant, 1 ml of distilled water was added, and its pH was measured by a pH meter (Hanna pH meter model 211/USA).

2.6. Histological analysis

Stomach samples were fixed in 10% buffered formalin for>48 h. The samples were dehydrated in graded alcohol and embedded in paraffin wax, and the sections were cut into a thickness of 5 μm. Subsequently, the samples were stained with hematoxylin and eosin (H&E) for histological analysis. The pathological changes in the gastric tissues were

Table 1
Gastric ulcer scoring system based on the severity of the ulcer.

Ulcer score	Gastric Lesions
0	**No lesion**
1	**Mucosal edema and petechiae**
2	**One to five small lesions (1–2 mm),**
3	**More than five small lesions or one intermediate lesion (3–4 mm)**
4	**Two to more intermediate lesions or one gross lesion (>4 mm)**
5	**Perforated ulcers**

observed under a light microscope (Chen et al., 2019).

2.7. Biochemical analysis (stomach homogenate preparation)

To measure biochemical parameters, stomach tissue samples were homogenized (Jambi and Khattab, 2019). Homogenates of stomach tissue samples (10% (w/v)) were prepared with ice-cold 100 mM PBS buffer (pH 7.4). The homogenates were centrifuged at 12,000 g for 20 min (4 °C). The supernatant was collected for further experiments.

2.8. Measurement of reactive oxygen species (ROS)

The rate of ROS formation in the reaction mixture was measured by following the oxidation of 2, 7-dichlorofluorescein diacetate (DCFH-DA) to a highly fluorescent 2, 7- dichlorofluorescein (DCF) compound, according to the published method with a slight change (Bahramikia et al., 2009). Each sample consisted of 1.7 ml of phosphate buffer solution (50 mM, pH = 7.4), 0.2 ml of gastric homogenate, and 100 μl of DCFH-DA solution (10 μM). The samples were incubated in a hot water bath at 37 °C temperature for 15 min. ROS concentrations were measured by DCF formation using a Cary-Eclipse fluorescence spectrophotometer (Agilent/USA) with excitation wavelength and emission at 488 and 521 nm.

2.9. Determination of malondialdehyde (MDA)

As a marker of lipid peroxidation, the MDA level was measured by the double heating method (Draper and Hadley, 1990). In short, to 0.5 ml of each homogenized sample of gastric tissue, 2.5 ml of trichloroacetic acid (TCA, 10%) was added and placed in a boiling water bath for 15 min. After cooling the test tubes at room temperature and 4 °C, the samples were centrifuged at 3000 g for 10 min, and then 0.3 ml of supernatant per tube to new tubes containing 0.3 ml were transferred from TBA solution (0.67%). In the next step, each tube was placed in a boiling water bath (95 °C) for 20 min. Then, after cooling to room temperature and at 4 °C, the samples' absorption was measured at 532 nm against the Blank solution. MDA absorption was calculated based on the molecular absorption coefficient of MDA-TBA complex ($\varepsilon = 1/56 \times 10^5\ cm^{-1}.M^{-1}$) and was expressed based on nmol/mg Protein.

2.10. Determination of protein carbonyl (PCO)

PCOs were measured using the method of Reznick and Packer (1994). 1 ml of DNPH (10 mM in HCl) was added to the reaction mixture (2 mg protein). The samples were incubated at room temperature for 1 h and were Vortexed every 15 min. Then, 1 ml of trichloroacetic acid (TCA) (10%, w/v) was added to each reaction mixture and centrifuged at 3000 g for 10 min. In the next step, the protein precipitate was washed three times with 2 ml of ethanol/ethyl acetate solution (v/v, 1:1) and dissolved in 1 ml of guanidine hydrochloride solution (6 M, pH = 2.3). After 10 min of incubation of the samples at room temperature, the absorption of them at a wavelength of 370 nm was read against the blank solution, and the PCO content was calculated based on the molar extinction coefficient of DNPH ($\varepsilon = 2.2 \times 10^4\ cm^{-1}.M^{-1}$) (Bahramikia et al., 2009). The data were expressed as nmol/mg protein.

2.11. Determination of total nitric oxide (NO) levels in serum and tissue

The NO level in the rats' serum and gastric tissue was determined by measuring the accumulation of its stable degradation products, namely nitrite and nitrate. The serum and tissue nitrite levels were determined using the Griess reagent, according to Hortelano et al. (1995). Griess reagent is a mixture (1: 1) of sulfanilamide (SA) 1% in phosphoric acid 5% and 1- naphthyl ethylene diamine (NED) 0.1%, which in the presence of nitrite forms a red diazo complex. Color intensity was measured at 540 nm, and the results were expressed as μmol/L using the standard $NaNO_2$ curve (Bahramikia and Yazdanparast, 2012).

2.12. Assessment of catalase (CAT) activity

CAT activity was measured by the Aebi (1984) method with a slight modification. 100 μl of stomach tissue homogenate supernatant to the cuvette containing 1900 μl of phosphate buffer (50 mM, pH = 7) was added. Then the reaction by adding 1 ml of hydrogen peroxide (H_2O_2) was beginning. The sample absorption was read at 15-s intervals and Within 60 s, and the rate of H_2O_2 decomposition was measured by a UV/VIS spectrometer (T80+/England) at 240 nm. The activity of the CAT enzyme was calculated based on the following formula.

$$\textbf{Activity of enzyme} = \frac{\Delta A \times \text{total volume}}{\varepsilon \times \Delta t \times \text{enzyme volume} \times \text{mg Pr}}$$

ΔA = Absorption difference ؛ Δt = time difference ؛ Molar absorption coefficient (($\epsilon\ H_2O_2 = 43.6\ cm^{-1}\ M.^{-1}$

2.13. Determination of glutathione (GSH)

The GSH level was measured by Jollow et al. (1974) with a slight change. To the 0.5 ml of gastric tissue homogenate, 1 ml of sulfosalicylic acid (4%) was added and placed at 4 °C for 1 h. After this time, the samples were centrifuged for 15 min at 3000 g at 4 °C. To 1 ml of the supernatant of each sample, 0.1 ml of DTNB (4 mg/ml) and 0.9 ml of phosphate buffer (0.1 M, PH = 7.4) were added. The samples' absorption was read after the formation of yellow color at a wavelength of 412 nm. Reduced glutathione was expressed as μg/mg of protein.

2.14. Determination of protein

The protein contents of homogenate were determined, according to Bradford (1976) using bovine serum albumin (BSA) as standard. This method is based on the interaction of the Coomassie Blue G250 dye with proteins. At the pH of the reaction, the interaction between proteins of high molecular weight and the dye causes a shift in the dye to the anionic form, which absorbs strongly at 595 nm. For the measurement of protein, 5 μl of the sample was added to each well. Then, 195 μl Bradford's solution was added to each well. After 5 min, a reading was taken at a wavelength of 595 nm.

2.15. Statistical analysis

The results were expressed as the mean ± S.D. Student's t-test detected statistical differences between the two groups. When it was analyzed more than two groups, it was used one-way analysis of variance (ANOVA), followed by the Dunnett post-hoc test. Values of $P < 0.05$ were considered statistically significant. The data were analyzed using GraphPad Prism 8.0 software (San Diego, CA, USA).

3. Results

3.1. Effect of 5-ASA pre-treatment on gastric ulcer assessment (ulcer index and percentage inhibition of ulceration, gastric juice volume, and pH)

According to Table 2, the pH of gastric juice in the stomachs of rats in the ethanolic group decreased significantly compared to the pH of gastric juice in the stomachs of normal group rats. This (pH = 2.6) is one of the reasons for getting the highest number of related to ulcer index in this group (ethanolic). On the other hand, rats of the standard group, which pre-treated with RAN as a standard drug, had a significant increase in gastric juice pH, indicating a high effect of the drug in reducing acidity (secretion of acid) caused by ethanol gavage. In addition, the ulcer index of this group is low, and the ulcer inhibition percentage by this drug is observed at the highest level.

Table 2
The effects of different doses of 5-ASA and RAN drug on gastric ulcer caused by ethanol.

Groups	Gastric juice volume (mL)	Gastric juice pH	Ulcer index (UI)	Inhibition of ulceration (%I)
Normal	1.61 ± 0.40	6.5 ± 0.08	0	0
Ethanolic (%96)	2.62 ± 0.82	2.6 ± 0.04	4 ± 0.00	0
RAN (50 mg/Kg) + Ethanol	1.58 ± 0.46 [a]	8.5 ± 0.65[b]	1.16 ± 0.43 [a]	70
5-ASA (50 mg/Kg) + Ethanol	2.10 ± 0.70 [b]	4.8 ± 0.17 [b]	1.83 ± 0.70 [a]	54
5-ASA (100 mg/Kg) + Ethanol	1.80 ± 0.30 [a]	5.5 ± 0.10 [a]	1.5 ± 0.43 [a]	62

The results were expressed as mean ± S.D. for 6 rats in each group. (Mesalazine = 5-ASA; Ranitidine = RAN) [a] Significant difference with $P < 0.01$ compared to ethanolic group, [b] Significant difference with $P < 0.05$ compared to ethanolic group.

Pre-treatment with doses of 50 and 100 mg/kg 5-ASA in experimental groups 1 and 2 increased the pH of gastric juice in rats of these two groups compared to the ethanolic group. The gastric ulcer index in these two groups indicates the effect of this drug in preventing ulceration.

3.2. Macroscopic and histological findings

3.2.1. Macroscopic evaluation

Acute gastric lesions were induced by intra-gastric administration of ethanol. As shown in Fig. 1, a significant increased area of gastric ulcer formation was observed in the ethanol-stimulated rats compared with that in normal rats. Pre-treatment with 5-ASA or RAN effectively prevented the severe gastric mucosal damage caused by ethanol.

3.2.2. Histological studies

Findings observations of microscopic lesions are as follows:

- In normal group: Structure of normal mucosal tissue, free of edema, hyperemia or hemorrhage, and inflammatory cell infiltration was observed (Fig. 2A).
- In the ethanolic group: extensive destruction and necrosis of mucosal tissue, severe hemorrhage, edema, and infiltration of leukocyte cells were observed (Fig. 2B).
- In RAN 50 mg/kg recipient group: Mild degeneration and necrosis at the apex of mucosa tissue, very small amounts of edema, hemorrhage, and infiltration of inflammatory cells were observed (Fig. 2C).
- In 5-ASA 50 mg/kg recipient group: necrosis Up to one-third of the anterior mucosa, low to moderate amounts of hyperemia or

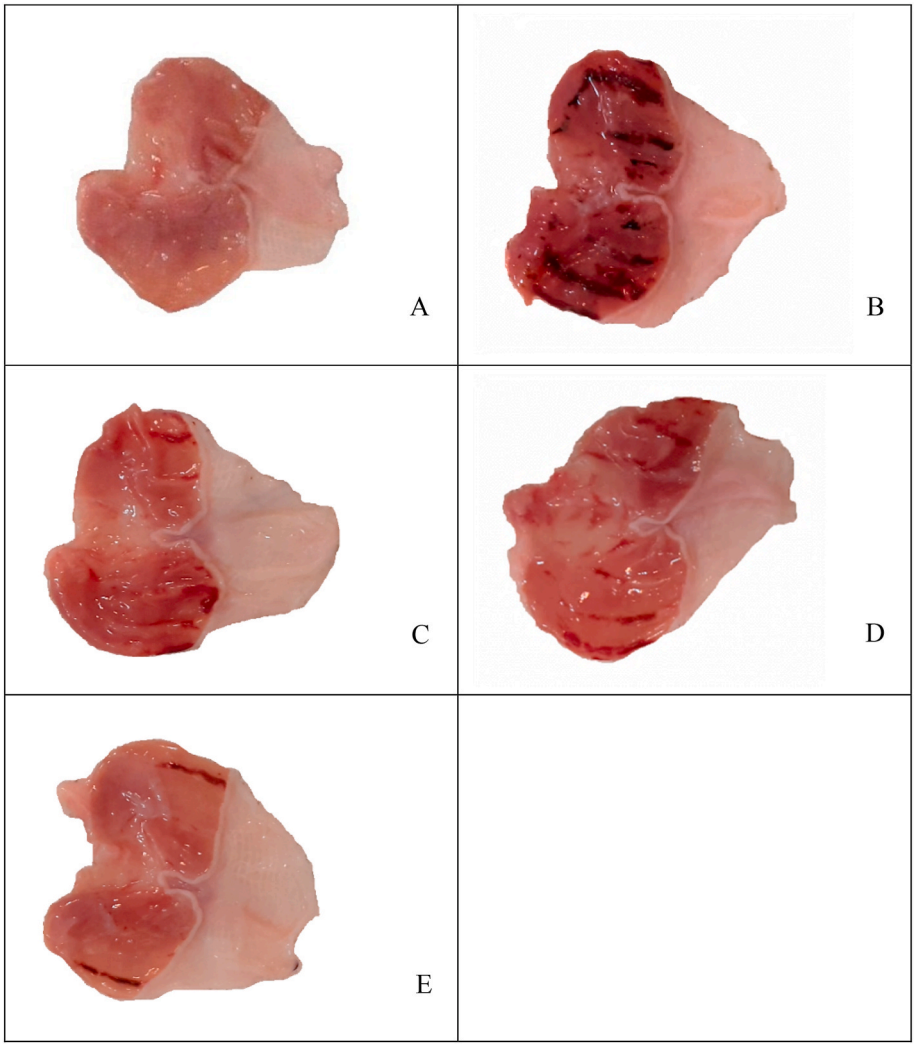

Fig. 1. Macroscopic view of the stomach tissue in rats of normal group (A), ethanolic group (B), RAN 50 mg/kg recipient group (C), 5-ASA 50 mg/kg recipient group (D), 5-ASA 100 mg/kg recipient group (E).

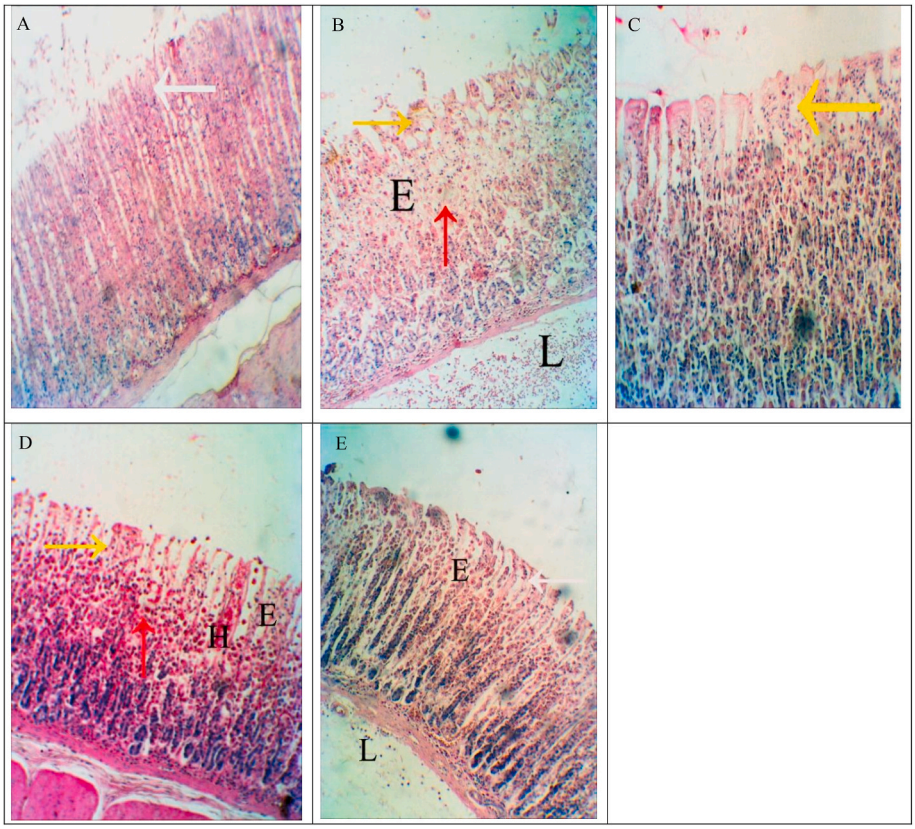

Fig. 2. Microscopic view of the stomach tissue in rats of normal group (A), ethanolic group (B), RAN 50 mg/kg recipient group (C), 5-ASA 50 mg/kg recipient group (D), 5-ASA 100 mg/kg recipient group (E). Yellow arrow: Mild degeneration and necrosis of the mucosa at the apex of the mucosa; Red arrow: necrosis and destruction of mucosa in the depth of the mucosa; White arrow: Healthy appearance or with minimal changes in the coating structure and mucosal layer; (E: edema, L: infiltration of inflammatory cells, H: Hemorrhage).

hemorrhage, edema, and low infiltration of inflammatory cells was seen (Fig. 2D).

- In 5-ASA 100 mg/kg recipient group: destruction and Mild necrosis at the apex of mucosal tissue, very small edema, hemorrhage, and infiltration of inflammatory cells were seen (Fig. 2E).

3.3. Effect of 5-ASA pre-treatment on gastric tissue oxidative stress markers

3.3.1. Level of ROS

According to Fig. 3, the induction of gastric ulcer by ethanol in the ethanolic group caused a significant increase in the ROS rate of the tissue of this group compared to the normal group. DCF fluorescence intensity in the standard group (RAN 50 mg/kg recipient group) was significantly reduced compared to the ethanolic group, indicating a small amount of ROS in the tissue of this group. On the other hand, pre-treatment with

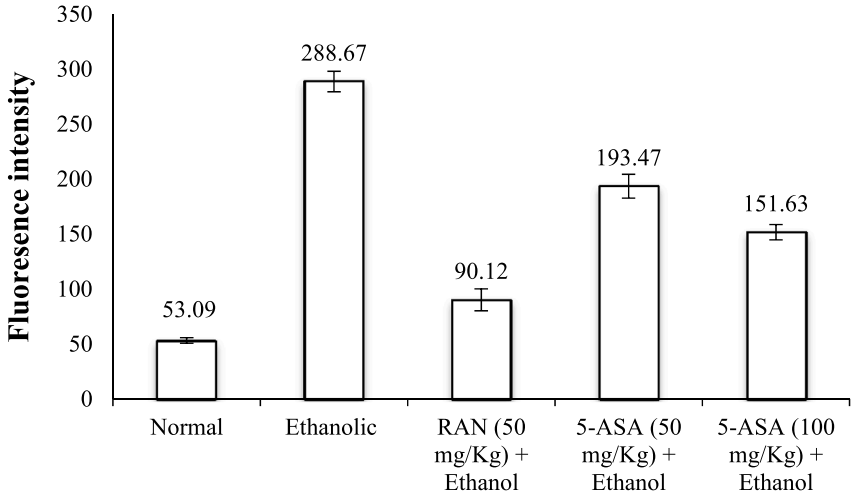

Fig. 3. The antioxidant effect of 5-ASA and RAN drugs on changes in gastric tissue ROS level in the studied groups. The results were expressed as Mean ± standard deviation for 6 rats in each group. (Mesalazine = 5-ASA; Ranitidine = RAN).

doses of 50 and 100 mg/kg of 5-ASA also significantly reduced tissue ROS levels in experimental groups 1 and 2, indicating the good antioxidant capacity of this drug.

3.3.2. CAT enzyme activity

Changes in the activity of the CAT enzyme in this study are shown in Fig. 4. The induction of gastric ulcers by ethanol significantly reduced the activity of this enzyme in the ethanolic group compared to the normal group. On the other hand, the administration of RAN in the standard group significantly increased the activity of the CAT enzyme compared to the ethanolic group. Also, administration of 50 and 100 mg/kg doses of 5-ASA significantly increased CAT enzyme activity in experimental groups 1 and 2, which was statistically significant.

3.3.3. Level of serum NO

NO is one of the reactive nitrogen species that acts as an important radical molecule in the intracellular signaling pathway and has a protective role in vascular dilation. However, if excessive is Increased, this radical can be destructive, so given the importance of this molecule, its amount was measured in the serum of the groups being tested.

As shown in Fig. 5, serum NO levels in the ethanolic group increased significantly compared to the normal group. However, the administration of RAN in the standard group significantly reduced the serum NO level of this group compared to the ethanolic group. Also, administration of 50 and 100 mg/kg doses of 5-ASA depending on the dose reduced serum NO levels in experimental groups 1 and 2.

3.3.4. Level of tissue NO

Blood flow to the stomach tissue is supplied through vessels inside the tissue. Given the role of NO in vasodilation, if its rate in the tissue is considered a sign of a decrease or increase in blood flow in the tissue, the result of this test is understandable, which can be cited according to the articles presented in this field.

According to Fig. 6, the induction of gastric ulcer by ethanol in the ethanolic group caused a significant decrease in the NO level of this group compared to the normal group. On the other hand, the administration of RAN in the standard group increased the level of NO in this group, which significantly increased. Also, administration of 50 and 100 mg/kg doses of 5-ASA in experimental groups 1 and 2 increased the amount of NO to be dose-dependent manner, which was statistically significant.

3.3.5. Level of MDA

As shown in Table 3, the amount of MDA in the ethanolic group was significantly increased from the normal group. The MDA level in the standard group (RAN 50 mg/kg dose recipient group) was significantly reduced. The level of MDA in rats pre-treated with doses of 50 and 100 mg/kg of 5-ASA was dose-dependently reduced, compared to the ethanolic group.

3.3.6. Level of PCO

PCO as an indicator of protein oxidation is one of the important parameters indicating oxidative damage, so this test was performed to evaluate the antioxidant effect of drugs. As shown in Table 3, the administration of RAN in the standard group significantly inhibited the formation of PCO. Also, 5-ASA administration in experimental groups 1 and 2 significantly and dose-dependently prevented the formation of PCO.

3.3.7. Level of tissue GSH

As shown in Table 3, the amount of GSH in the ethanolic group rats decreased significantly compared to the normal group. On the other hand, the administration of RAN in the standard group significantly increased the non-enzymatic antioxidant in this group. Also, administration of 50 and 100 mg/kg doses of 5-ASA in experimental groups 1 and 2 significantly increased the GSH level of these two groups compared to the ethanolic group. The increase in GSH level at a dose of 100 mg/kg 5-ASA was equivalent to the standard group (RAN 50 mg/kg recipient group).

4. Discussion

Numerous studies using ethanol-induced gastric ulcer models have been extensively performed in animals to investigate the protective effect of plants (Birdane et al., 2007) and drugs. The effect of ethanol on ulcer formation is that its high concentration can directly destroy gastric mucosa tissue and cause acute mucosa inflammation, mucosal hyperemia, edema, hemorrhage, erosion, and ulceration the gastric mucosa. Ethanol can cause gastric mucosa damage by enhancing gastric mucosa damage factors, weakening mucosa protection factors, and overloading calcium in cells. In the meantime, because there are alcohol dehydrogenase and xanthine oxidase in the stomach, they are absorbed by the stomach. Alcohol dehydrogenase can catalyze ethanol into acetaldehyde, and xanthine oxidase can metabolically catalyze ethanol into free

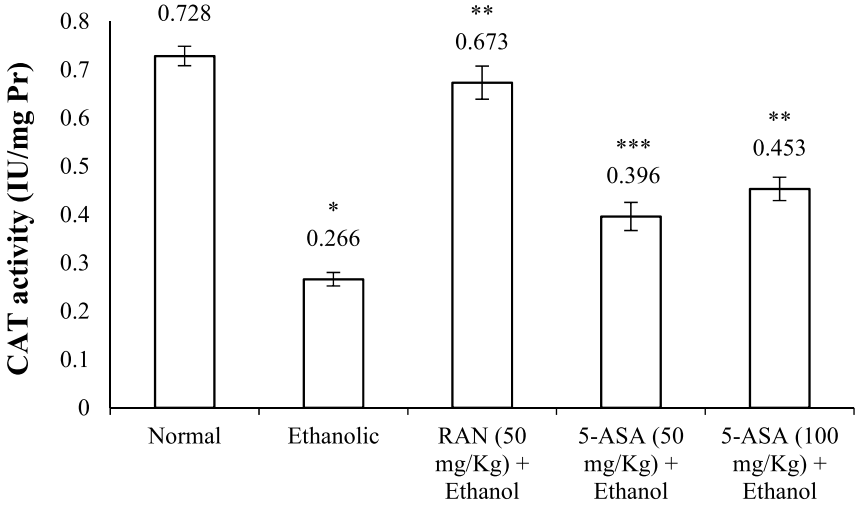

Fig. 4. Changes in the activity of the CAT enzyme in the gastric tissue of rats in the understudy groups. The results are shown based on the mean ± standard deviation of the data for 6 rats in each group. * Significant difference with $P < 0.05$ compared to the normal group, ** Significant difference with $P < 0.05$ compared with the ethanolic group, *** Significant difference with $P < 0.02$ compared to the ethanolic group.

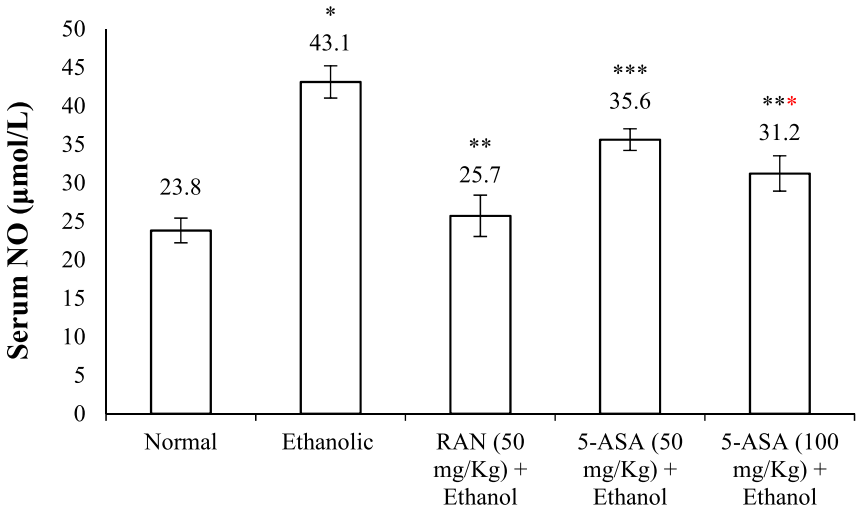

Fig. 5. Changes in NO levels in serum rats in the understudy groups. . The results are shown based on the mean ± standard deviation of the data for 6 rats in each group. * Significant difference with $P < 0.001$ compared to normal group, ** Significant difference with $P < 0.05$ compared to ethanolic group, *** Significant difference with $P < 0.001$ compared to ethanolic group.

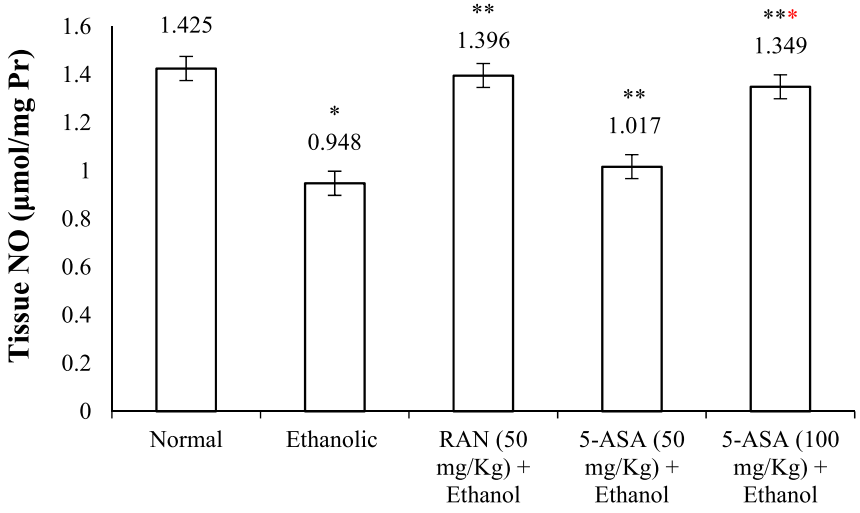

Fig. 6. Changes in NO levels in the gastric tissue of rats in the understudy groups. The results are shown based on the mean ± standard deviation of the data for 6 rats in each group. * Significant difference with $P < 0.001$ compared to the normal group, ** Significant difference with $P < 0.001$ compared to the ethanolic group, *** Significant difference with $P < 0.05$ compared to the ethanolic group.

radicals. Free radicals have a very important role in the process of alcoholic tissue injury. Free radicals obviously by enhancing mucosal cell lipid peroxidation reaction and reducing in mucus production and subsequently increase in the production of ROS result in damage capillary endothelial cells (Chen et al., 2017).

On the other hand, evidence suggests that excessive production of free radicals from ethanol gavage is directly related to the infiltration and activation of neutrophils. It may be one of the sources of free radicals, infiltration, and accumulation of neutrophils in the gastric mucosa, which leads to damage to cellular components such as lipids and proteins (Mshelia et al., 2017). These events are likely to be consistent with the increase in ROS in the ethanolic group of the present study. On the other hand, hypochlorous acid (HOCL) as one of the reactive oxygen species, which reflects the role of neutrophils in gastrointestinal diseases, causes protein oxidation and the formation of carbonyl groups in proteins, which is called PCO are called. Therefore, PCO, as a diagnostic marker of oxidative stress, is important in many diseases. Its detection and reduction play a decisive role in the recovery of these diseases (Dalle-Donne et al., 2003; Elliott and Wallace, 1998). In the present study, pre-treatment of the studied groups with 5-ASA and RAN prevented the increase of ROS level and PCO formation, which could be due to the amplification of their antioxidant defense system of gastric cells.

Lipid peroxidation is an important cellular injury mechanism, and many studies have shown that ethanol can stimulate lipid peroxidation in cell membranes via ROS (Cederbaum, 2001). Ethanol-induced lipid peroxidation in gastric epithelial cells accelerates gastric ulcer by destroying membrane integrity and increasing cell permeability. MDA, the end product of the peroxidation of unsaturated fatty acids in cell membranes, is commonly used as a reliable indicator in the process of lipid peroxidation in tissues. Therefore, the determination of MDA levels can be used to estimate ethanol-induced gastric tissue damage. In the present study, ethanol gavage significantly increased the production of MDA in gastric tissue, which this result can be confirmed by increasing the concentration of MDA in gastric tissue by ethanol administration in

Table 3
Effect of 5-ASA on ethanol-induced changes in gastric tissue oxidative stress markers: MDA level, inhibition of PCO formation, GSH level in rats.

Groups	MDA (nmol/mg Pr)	PCO (nmol/mg Pr)	GSH (μg/mg Pr)
Normal	0.27 ± 0.010	–	5.65 ± 0.08
Ethanol (%96)	0.55 ± 0.022 [a]	14.98 ± 0.4	4.22 ± 0.02 [f]
RAN (50 mg/kg) + Ethanol	0.41 ± 0.017 [b]	12.99 ± 0.2 [d]	5.01 ± 0.04 [g]
5-ASA (50 mg/kg) + Ethanol	0.53 ± 0.020 [b]	14.65 ± 0.2 [e]	4.44 ± 0.04 [h]
5-ASA (100 mg/kg) + Ethanol	0.43 ± 0.013 [c]	13.28 ± 0.41 [e]	5.01 ± 0.03 [h]

The results were expressed as mean ± S.D. for 6 rats in each group. (Mesalazine = 5-ASA; Ranitidine = RAN).
[a] Significant difference with $P < 0.02$ compared to normal group.
[b] Significant difference with $P < 0.05$ compared to ethanol group.
[c] Significant difference with $P < 0.02$ compared to ethanol group.
[d] Significant difference with $P < 0.001$ compared to ethanol group.
[e] Significant difference with $P < 0.01$ compared to the ethanolic group.
[f] Significant difference with $P < 0.001$ compared to the normal group.
[g] Significant difference with $P < 0.05$ compared to an ethanolic group.
[h] Significant difference with $P < 0.001$ compared to the ethanolic group.

other studies (Yang et al., 2017; Rocha et al., 2011; Antonisamy et al., 2015).

On the other hand, one of the dangerous species resulting from the enzyme myeloperoxidase (MPO) in neutrophils is HOCL, which is obtained from the oxidation of chloride ions with H_2O_2. Therefore, the antioxidant defense elements of gastric cells have taken action at this time, and by destroying H_2O_2, they are trying to counter this process. CAT, as an important antioxidant enzyme, converts H_2O_2 to water and oxygen. At the same time, GSH, with its having sulfhydryl groups (SH) in its structure, can act as a scavenger for ROS and thereby prevent Lipid peroxidation. GSH can also act as a cofactor for the enzyme glutathione peroxidase (GPx) and neutralize H_2O_2. It has been shown that under ethanol-induced gastric mucosal damage, the CAT activity and GSH content in gastric tissue reduces. This reduction leads to an increase in ROS accumulation and, consequently, an increase in lipid peroxidation and PCO formation (Antonisamy et al., 2014; Das and Vasudevan, 2007). Similar results can be seen in the study of Amaral et al. (2013), which shown the ethanol-induced mucosal damage increase the production and accumulation of ROS and MDA by increasing the activity of the MPO enzyme, which is associated with decreased activity and CAT and GSH levels in gastric tissue. Liu et al. (2016) reported that ethanol administration by increasing MDA and PCO could damage gastric tissue.

5-ASA, as a drug used in this study, leads to reduced ROS, MDA, and PCO and increased CAT and GSH. As mentioned earlier, one of the sources of ROS is an increase in the activity of the MPO enzyme in ethanol-induced damage (Amirshahrokhi and Khalili, 2016), which increases the damage to lipids and proteins by increasing MDA and PCO in gastric tissue. Numerous studies have shown that 5-ASA works by different mechanisms, including free radical scavenging, restricting the migration of macrophages and neutrophils to damaged areas, and inhibiting lipid peroxidation (Brogden and Sorkin, 1989). However, excessive ROS increase neutrophils-induced leads to defects in the antioxidant defense system, increased oxidative stress, and mucosal damage. Therefore, 5-ASA by increasing the expression of antioxidant enzymes through activating Nuclear factor erythroid 2-related factor 2 (NRF2), moves toward the decomposition of oxidants Resulting from neutrophils (Kang et al., 2017). In this regard, the study of Kiremit-Korkut et al. (2004) showed that 5-ASA increased the amount of GSH; Oxygen-derived free radicals scavenge and reduced MPO and MDA activity by reducing the accumulation of neutrophils.

In this study, RAN was used as a standard drug. This drug inhibits gastric acid secretion by blocking histamine H_2-receptors and has antioxidant properties (Ahmadi et al., 2011); therefore, it has a relatively good effect to 5-ASA. This effect can be seen in biochemical results and macroscopic studies and increasing the pH of gastric juice, which has led to a decrease in the ulcer index in this study. There is evidence that gastric juice does not normally affect gastric mucosa. Still, after gastric mucosal injury, the stomach is stimulated. It secretes a large amount of stomach acid, which increases the volume of gastric juice and reduces the pH of gastric juice. These events lead to worsening gastric mucosal damage and, thus, exacerbate gastric injury (Liu et al., 2019). In this study, 5-ASA may effectively reduce gastric juice volume and increase the pH of gastric juice in rats that receive alcohol, and thereby protects the gastric tissues and prevents ethanol-induced gastric injuries.

NO, as a gaseous free radical, plays a role in a variety of physiological functions, some of which have destructive effects in pathophysiological conditions. Due to the increased production of NO by inducible nitric oxide synthase (iNOS), and anion superoxide ($O_2^{\bullet-}$) resulting from ethanol metabolism or produced by neutrophils (Tamura et al., 2013; Kwiecien et al., 2002), NO is prone to react with $O_2^{\bullet-}$, which their reaction leads to the formation of proxy nitrite ($ONOO^-$). $ONOO^-$ is a species with cellular toxicity that can oxidize various cellular components such as proteins, lipids, and DNA, leading to disruption of important cellular processes, disruption of cellular signaling pathways, and induction of cell death through both apoptosis and necrosis. On the other hand, previous studies have shown that ethanol consumption is associated with increased expression of iNOS, which leads to an increase in NO levels. Given that in the present study, NO production in ethanolic group serums in response to gastric ulcer ethanol-induced increased, therefore, this could be due to the increased induction of iNOS expression, which in this context can be noted in the study of Yu et al. (2014). Li et al. (2015) also reported that ethanol gavage significantly increased serum NO levels and iNOS expression in the ethanolic group compared to the normal group.

On the other hand, NO as a vasodilator factor that has a short half-life, by regulating vascular and nutrient blood flow, it maintains the epithelial integrity of the stomach and mucus barrier and, most importantly, plays an important role in angiogenesis, tissue regeneration, and may cause ulcer healing (Moawad et al., 2019). As a result, its reduction in the gastric tissue of ethanolic group rats in our study and oxidative damage Likely decreased endothelial nitric oxide synthase (eNOS) expression as a protective factor, as well as the effect of direct-ethanol damage on epithelial cells and sub-mucosal endothelial vessels. This event leads to the rupture of blood capillaries and reduced blood flow in these vessels, and in support of this, we can mention the study of Zhang et al. (2020) (Elliott and Wallace, 1998). Numerous studies have also confirmed that as a result of ethanol gavage, NO levels in the gastric tissue of rats in the ethanolic group decrease (Sidahmed et al., 2013; Arab et al., 2015, 2019; Rouhollahi et al., 2014).

5. Conclusion

In this study, 5-ASA, by reducing tissue ROS, MDA, and PCO levels and increasing factors such as CAT, and GSH, strengthen the antioxidant defense system of gastric mucosal cells during oxidative damage caused by ethanol. In addition, the compound reduced serum NO and increased tissue NO levels. Although the mechanism of action of exact 5-ASA is unknown, however, probably 5-ASA by reducing iNOS expression and increasing eNOS expression leads to a change in the nitric oxide system. In addition, there is evidence of radical NO scavenging and inhibition of its production by lowering the expression of iNOS by 5-ASA, which confirms its effect (Moura et al., 2016; Couto et al., 2010; Kennedy et al., 1999).

Author agreements

We the undersigned declare that all authors have seen and approved the final version of the manuscript being submitted. They warrant that the article is the authors' original work, hasn't received prior

publication and isn't under consideration for publication elsewhere.

CRediT authorship contribution statement

Mohammad Beiranvand: Conceptualization, Validation, Writing - review & editing, Supervision, Investigation, Resources, Project administration, Funding acquisition. **Seifollah Bahramikia:** Methodology, Software, Data curation, Writing - original draft, Visualization, Software, Validation.

Declaration of competing interest

The authors declare that there are no conflicts to declare.

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