Original Article

The protective effects of rosmarinic acid on ethanol-induced gastritis in male rats: antioxidant defense enhancement

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Abstract

Background and purpose: Gastritis is one of the most current gastrointestinal disorders worldwide. Alcohol consumption is one of the major factors, which provides gastritis. Rosmarinic acid (RA) is found in many plants and has powerful antioxidant and anti-inflammatory effects. In this study, the protective effect of RA was evaluated on the histopathological indices, antioxidant ability, and prostaglandin E2 (PGE2) secretion in male rats.

Experimental approach: Forty-two animals were divided into control, ethanol-induced gastritis, and RA groups, 6 each. The protective groups included RA administration before gastritis induction at 50 mg (R-G50), 100 mg (R-G100), 150 mg (R-G150), and 200 mg (R-G200) doses. Gastritis was induced by gavage of 1 mL pure ethanol in fasted animals. After 1 h of gastritis induction, the rats were sacrificed and stomach tissue was removed.

Findings/Results: Histological evaluation revealed that RA significantly attenuated gastric ulcers, leucocyte infiltration, and hyperemia. It also increased mucosal layer thickness and restored gastric glands. Furthermore, RA decreased malondialdehyde level, increased superoxide dismutase, catalase, and glutathione in the stomach tissue, and raised gastric PGE2 level.

Conclusion and implications: Our study demonstrated that rosmarinic acid has a notable effect on gastritis protection that could be due to increased antioxidant defense and PGE2 secretion, eventually maintenance of mucosal barrier integrity and gastric glands.

Keywords: Alcohol; Gastritis; Oxidative stress; PGE2; Rosmarinic acid.

INTRODUCTION

Gastritis is one of the most common digestive diseases, which develops to gastric ulcer, gastric perforation, and even gastric cancer (1). The major factors in gastritis and gastric ulcer are frequent use of non-steroidal anti-inflammatory drugs (NSAID), smoking, stress, infection, and excessive consumption of alcohol (2,3). The known pathophysiology in gastric injuries includes overproduction of reactive oxygen species (ROS), mucosal integrity loss, increased secretion of inflammatory cytokines, and necrosis (4-6). It was identified that ethanol intake produces both ROSs which leads to peroxidation of membrane lipids, reduction of antioxidant capacity (7), and

inhibits cyclooxygenase (COX) pathway (8). Alcohol increases superoxide anion and hydroxyl free radicals and decreases non-protein sulfhydryl groups such as glutathione (GSH) in the mucosal layer. Superoxide anions then react with membrane lipids and produce lipid peroxides, and malondialdehyde (MDA) is a major product of lipid peroxidation. On the other hand, superoxide dismutase (SOD) and catalase (CAT) enzymes eliminate ethanol-induced superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) to oxygen, water, and H_2O_2 and thereby decline ROS (3).

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Overgeneration of ROS, lipid peroxidation in the cell membranes, and loss of antioxidant capacity destroy the epithelial layer integrity, gastric mucus barrier, and glands and eventually damage the mucosal and submucosal cells and blood vessels endothelial and infiltration of leucocytes take place (9). Today, the main strategies for the improvement of gastric injuries are proton pump inhibitors, histamine type 2 receptor blockers, and anti-acids. However, some of these drugs have shown a lot of side effects, interaction, and recurrence (2,10). Therefore, many researches on the numerous synthetic and natural compounds are being carried out and herbal compounds are one of the most attractive agents in this regard.

Rosmarinic acid (RA) is a synthetic compound but is commonly found in plants from the Lamiaceae family including *Rosmarinus officinalis* (*R. officinalis*; rosemary), *Coleus aromaticus, Origanum vulgare L.* (oregano), and *Thymus vulgaris L.* (thyme) (10). Previously, the beneficial effects of RA on diabetes, sepsis, hepatotoxicity, and brain injuries are well-demonstrated (11-14). RA has a high power on the reduction of oxidative stress in most tissues. Several reports have revealed that RA decreased MDA as a marker of lipid peroxidation in the cell membrane and increased GSH, SOD, and glutathione peroxidase (GPx) as antioxidant defenses (12,15,16). Moreover, RA attenuated apoptosis and fibrosis in the renal cells, cardiomyocytes, and hepatic cells (17,18). Nevertheless, academic studies on the effect of RA on gastric injuries are few and limited.

Therefore, we designed this experiment to assess the protective effects of RA on ethanol-induced gastritis in male rats and its role in histological evaluations, protection of gastric glands, and enhancement of antioxidant defense.

MATERIAL AND METHODS

Reagents

RA was purchased from Sigma-Aldrich (536954-5G). MDA (ZB-MDA-96A), SOD (ZB-SOD-96A), CAT (ZB-CAT-96A), GSH (ZB-GSH-96A), and PGE2 (ZB-10504C-R9648) kits were purchased from Zellbio company, Germany.

Experimental procedures

Forty-two male Wistar rats (200 ± 20 g) were used in this study. Animals were placed in a temperature-controlled room at a 12/12-h light/dark cycle with free access to the food and tap water. All procedures were according to the Guidelines for Animal Care and Use at the Qom University of Medical Sciences (Ethics ID. IR.MUQ.REC.1399.054). Animals were randomly divided into 7 equal groups, 6 each, including control group: the fasted animals that received 1 mL (p.o.) saline by gastric gavage; 2); RA group: the fasted animals that received 200 mg/kg RA p.o. by gavage to find out possible toxic effects (19); gastritis group: the fasted animals that received a single dose of 100% ethanol (1 mL p.o. by gavage, single dose) to induce gastritis (2); R-G50, R-G100, R-G150, and R-G200 groups: the fasted animals that received single dose of 50, 100, 150, and 200 mg/kg (in 1 mL volume) RA, respectively 1 h before gastritis induction by gavage (19,20). After 1 h of ethanol gavage, the animals received 50 mg/kg of sodium thiopental (intraperitoneal, i.p.) and were sacrificed (21,22). The abdomen was exposed and the stomach removed. A segment of the stomach was placed in a 10% buffered formaldehyde solution. Another segment of the stomach was stored at -70 °C for evaluation of PGE2, MAD, SOD, CAT, and GSH levels.

Histological study

After about 24 h, the tissues were dehydrated, embedded in paraffin, sectioned with a microtome, and stained with hematoxylin and Eosin (H&E) stains. All sections were studied by Olympus light microscope (CX23 LED Microscope, Japan). The stomach tissue photos were evaluated by Image J software (National Institutes of Health, Image J 1.49f, USA).

Determination of oxidative stress indices

Briefly, 100 mg of the stomach tissue was weighted, added 1 mL phosphate buffer, and centrifuged at 3000-4000 rpm 20 min. Supernatants were then collected, allocated, and kept at -70 °C for GSH, MDA, CAT, and SOD measurements, according to the manufacturer's instructions. Antioxidant kits measured quantities assays samples based on colorimetric methods that should be read by

ELISA reader (For MDA: 535 nm, CAT: 405 nm, GSH: 412 nm, and SOD: 420 nm). For the determination of MDA and GSH levels, standard curves were prepared and MDA and GSH levels measured according to the standard curve. For SOD measurement, enzyme activity in zero and 2 min was read and calculated.

PGE2 measurement

Stomach tissue (100 mg) were weighted, added PBS buffer (100 mM, PH 7.4, 1 mL/100 mg tissue with antiprotease cocktail), homogenized by an electrical homogenizer (IKA-3420000, Germany), and centrifuged at 4000-6000 rpm for 10 min. The supernatants were then collected and assayed for PGE2 levels using an immunometric enzyme immunoassay (EIA) kit; according to the manufacturer's instruction and read by an ELISA reader in 450 nm.

Statistical analysis

Data normality was checked by the Kolmogorov-Smirnov test and expressed as mean ± SEM. Statistical analysis was performed by one-way analysis of variances (ANOVA) and Tukey's post hoc test using SPSS software (version 25). $P < 0.05$ was statistically considered significant.

RESULTS

Rosmarinic acid increased mucosal thickness in the ethanol-induced gastritis

The mucosal layer thickness was measured from the mucosal muscle to the apex of the epithelial cells (μm) in four microscopic fields and five sections of each sample and shown in magnification of 100× (Fig. 1A).

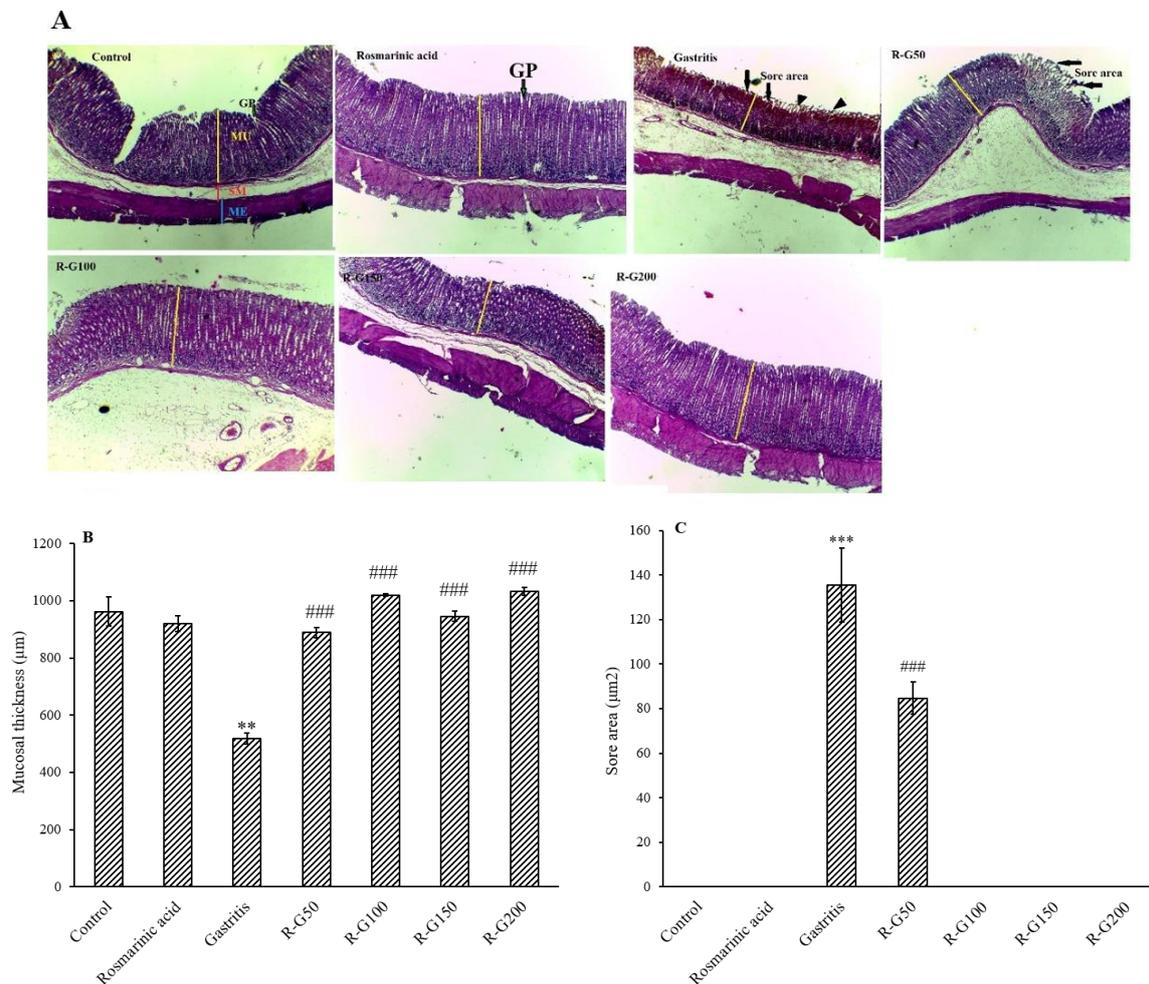**Fig. 1.** (A) Histopathological evaluation of the gastric tissue in different groups (H&E staining, 40×). The control and rosmarinic acid groups: showing a normal mucosal layer of the gastric tissue. The gastritis group: showing detachment, loss of mucosal layer, and ulcer areas. The R-G50, R-G100, R-G150, and R-G200 groups (protective groups): showing improvement of ulcers and increase of mucosal layers. GP, gastric pit; MU, mucosal layer; SM, submucosal layer; ME, muscular layer; head of arrow, necrosis; and black arrow, sore area. (B) Mucosal layer thickness and (C) ulcer areas of the gastric tissue in different groups, data represent mean ± SEM, n = 6. **$P < 0.01$ and ***$P < 0.001$ indicate significant differences compared to the control; ###$P < 0.001$ versus gastritis.

On this base, the mucosal thickness significantly decreased in the gastritis group compared to the control group (518.1 ± 18.7 *vs* 962.8 ± 50, *P* < 0.01). However, RA administration increased mucosal thickness in all treatment groups compared with the gastritis group (R-G50, R-G100, R-G150, and R-G200 groups; 889.2 ± 17.5, 1020.3 ± 4, 946.2 ± 18, and 1033.1± 12.5 *vs* 518.1 ± 18.7, *P* < 0.001, respectively; Fig. 1B).

Rosmarinic acid decreased gastric ulcers in the ethanol-induced gastritis

For the ulcer area measurement, in four microscopic fields and the five sections of each sample, the length and width of the ulcer were measured in μm². Our results showed that ulcer areas significantly increased in the gastritis group compared to the control group (135.5 ± 16.6 *vs* 0 μm², *P* < 0.001). However, RA treatment significantly decreased ulcer areas in the R-G50, R G100, R-G150, and R-G200 groups compared to the gastritis group (84.7 ± 7.32, 0 ± 0, and 0 ± 0, and 0 ± 0 *vs* 135.5 ± 16.6 μm², *P* < 0.001, respectively; Fig. 1C).

Rosmarinic acid increased mucosal, chief and parietal cells in the ethanol-induced gastritis

For counting the cells, the average values of the cells at four microscopic fields of five stomach sections in each sample were considered by the Image J software at a magnification of 400× (Fig. 2A).
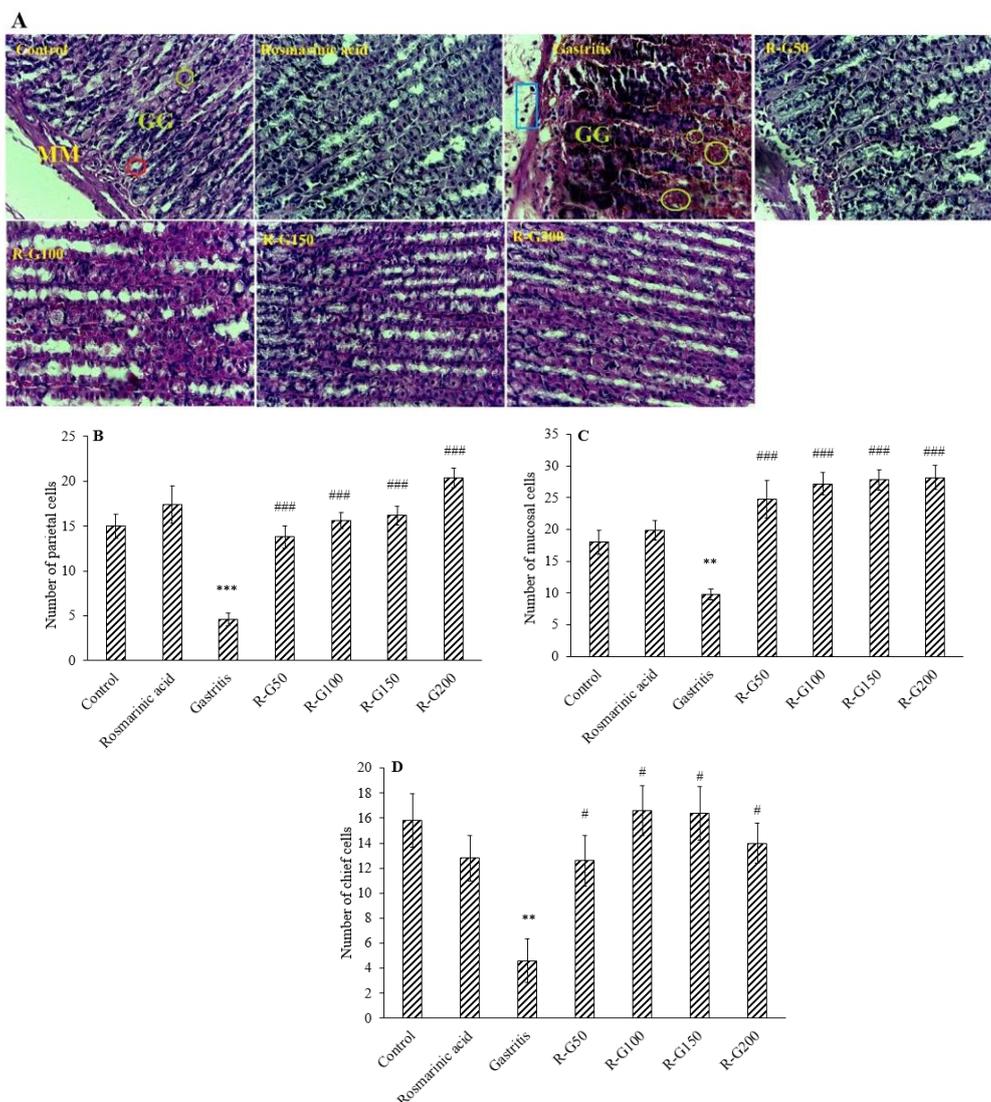

Fig. 2. (A) Histopathological evaluation of the cells in the gastric tissue of different groups (H&E staining, 400×). The control and rosmarinic acid groups: showing normal cells and glands. The gastritis group: showing cells and glands injury, hemorrhage in the mucosal layer. The R-G50, R-G100, R-G150, and R-G200 groups (protective groups): showing improvement of cells and glands injury and less hemorrhage in the mucosal layer. GG, gastric glands; MM, muscularis mucosa; red hexagonal, chief cell; green hexagonal, parietal cell; blue rectangle, leucocyte infiltration; green circle, hyperemia. (B) Number of parietal cells, (C) number of mucosal cells, and (D) number of chief cells in different groups. Data are represented as mean ± SEM, n = 6. **P < 0.01 and ***P < 0.001 indicate significant differences compared to the control; #P < 0.05 and ###P < 0.001 versus gastritis.

Histological evaluations revealed that ethanol administration significantly decreased the number of parietal, mucosal, and chief cells compared to the control group (4.6 ± 0.65 *vs* 15 ± 1.34, *P* <0.001; 9.8 ± 0.87 *vs* 18 ± 1.85, *P* < 0.01; and 4.6 ±1.74 *vs* 15.8 ± 2.15, *P* < 0.01, respectively). Meanwhile, the number of parietal cells increased in the R-G50, R-G100, R-G150, and R-G200 groups compared to the gastritis group (13.8 ± 1.21, 15.6 ± 0.9, 16.2 ±1.04, and 20.4 ± 1.12 *vs* 4.6 ± 0.65, *P* < 0.001, respectively) (Fig. 2B). Similarly, RA administration increased the mucosal cells in the R-G50, R-G100, R-G150, and R-G200 groups compared to the gastritis group (24.8 ± 2.91, 27.2 ± 1.76, 27.8 ± 1.62, and 28.2 ± 1.95 *vs* 9.8 ± 0.87, *P* < 0.001, respectively; Fig. 2C). Moreover, the number of chief cells increased after RA administration in the R-G50, R-G100, R-G150, and R-G200 groups compared to the

gastritis group (12.6 ± 2.04, 16.6 ± 1.96, 16.4 ± 2.13, and 14 ± 1.63 *vs* 4.6 ± 1.74, *P* < 0.05, respectively; Fig. 2D).

Rosmarinic acid decreased mucosal and submucosal leucocytes in the ethanol-induced gastritis

The obtained findings revealed that the leukocyte numbers in both mucosal and submucosal layers increased in the gastritis group compared to the control group (20.2 ± 2.18 *vs* 4.8 ± 1.26, *P* < 0.001 and 22.4 ± 2.62 *vs* 6.4 ± 1.52, *P* < 0.001, respectively). However, the leukocyte numbers decreased in the R-G50, R-G100, R-G150, and R-G200 groups compared to the gastritis group in both mucosal layer (7.6 ± 1.18, 7.4 ± 1.73, 7.4 ±1.69, and 5 ± 1.23 *vs* 20.2 ± 2.18, *P* < 0.001, respectively) and submucosal layer (8.6 ± 1.32, 8 ± 0.87, 7.6 ± 1.45, and 6.6 ± 0.34 *vs* 22.4 ± 2.62, *P* < 0.001, respectively) (Fig. 3A and B).
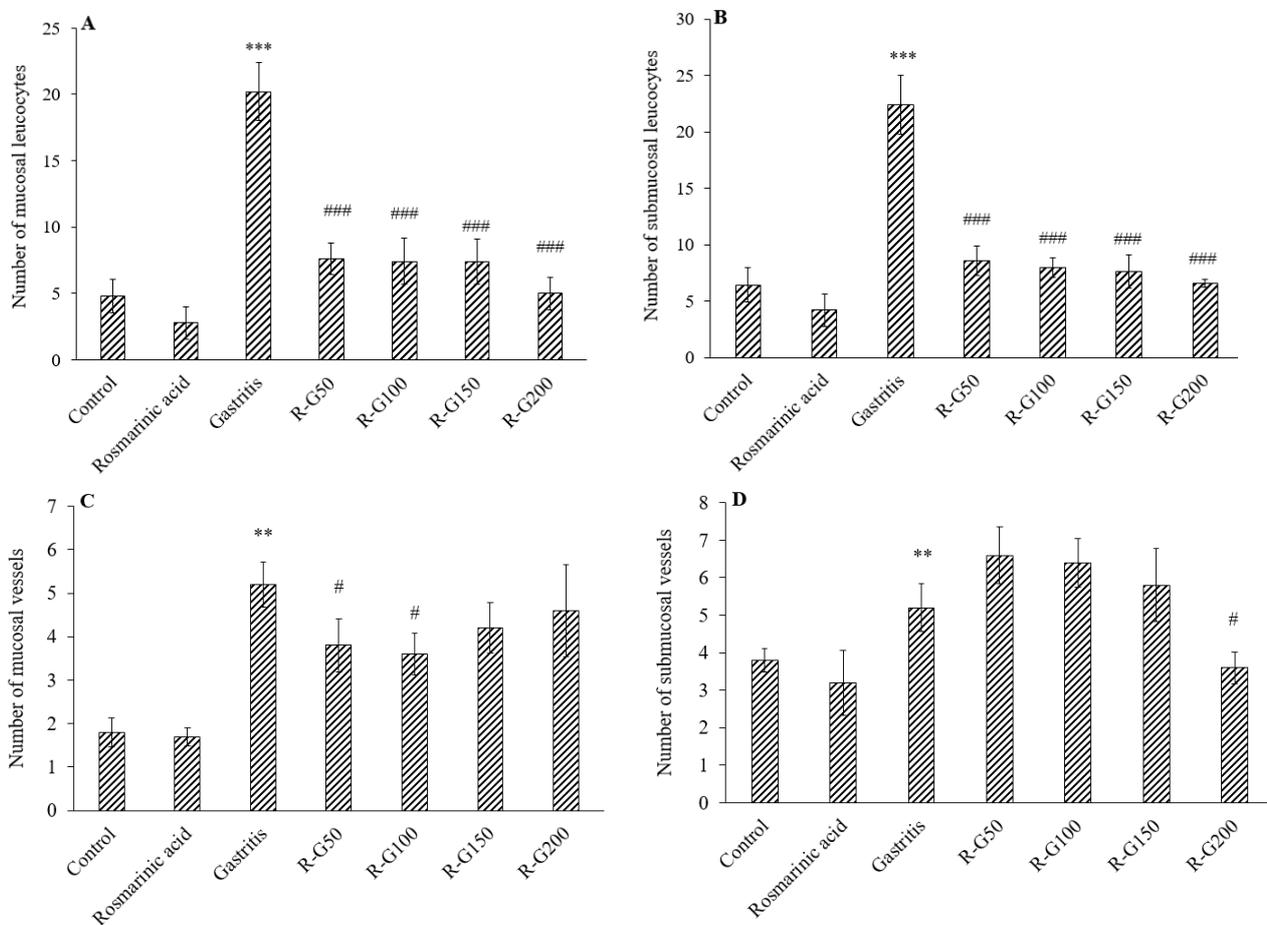

Fig. 3. (A and B) Leukocyte numbers of mucosal and submucosal layers in different groups; (C and D) number of vessels of mucosal and submucosal layers in different groups. Data are presented as mean ± SEM, n = 6. **P < 0.01 and ***P < 0.001 indicate significant differences compared to the control; #P < 0.05 and ###P < 0.001 versus gastritis.

Rosmarinic acid decreased gastric blood vessels in the mucosal and submucosal layers in the ethanol-induced gastritis

For counting lamina propria and submucosa vessels, as noted in Fig. 3C and D, gastritis markedly increased the number of vessels in the mucosal and submucosal layers compared to the control group (5.2 ± 0.52 *vs* 1.8 ± 0.33, $P < 0.01$ and 5.2 ± 0.52 *vs* 3.8 ± 0.32, $P < 0.01$, respectively). While, in the mucosal layer, the number of vessels had a significant decrease in the R-G50 and R-G100 groups compared to the gastritis group (3.8 ± 0.61 and 3.6 ± 0.47 *vs* 5.2 ± 0.52, $P < 0.05$, respectively) and in the submucosal layer, it significantly lowered in the R-G200 group only (3.6 ± 0.42 *vs* 5.2 ± 0.52, $P < 0.05$).

Rosmarinic acid decreased gastric MDA level in the ethanol-induced gastritis

The present results revealed that stomach MDA level significantly increased in the gastritis group compared with the control group (8.29 ± 0.57 *vs* 7.04 ± 0.3 μM, $P < 0.05$) meanwhile, RA dose-dependently decreased MDA levels in the R-G100, R-G150, and R-G200 groups (6.3 ± 0.35, 6.01 ± 0.45, and 4.11 ± 0.3 μM, $P < 0.05$, respectively; Fig. 4A).

Rosmarinic acid increased gastric SOD, CAT, and GSH levels in the ethanol-induced gastritis

Our findings also showed that SOD, CAT, and GSH levels in the gastric tissue markedly decreased in the gastritis group compared with the control group (SOD: 0.28 ± 0.003 *vs* 0.32 ± 0.01 IU, $P < 0.05$; CAT: 0.027 ± 0.0006 *vs* 0.031 ± 0.001 IU, $P < 0.01$; and GSH: 0.42 ± 0.004 *vs* 0.52 ± 0.01 μM respectively, $P < 0.05$). However, RA administration provides a significant increase in the antioxidant indices including SOD: 0.37 ± 0.002, 0.41 ± 0.005, 0.41 ± 0.02 and 0.45 ± 0.04 *vs* 0.28 ± 0.003 IU, respectively, $P < 0.01$; CAT: 0.033 ± 0.0002, 0.033 ± 0.0001, 0.032 ± 0.0008 and 0.031 ± 0.0006 vs 0.027 ± 0.0006 IU, respectively, $P < 0.01$; and GSH: 0.48 ± 0.005, 0.47 ± 0.01 and 0.52 ± 0.007 *vs* 0.42 ± 0.004 μM, respectively, $P < 0.05$ (Fig. 4B-D).
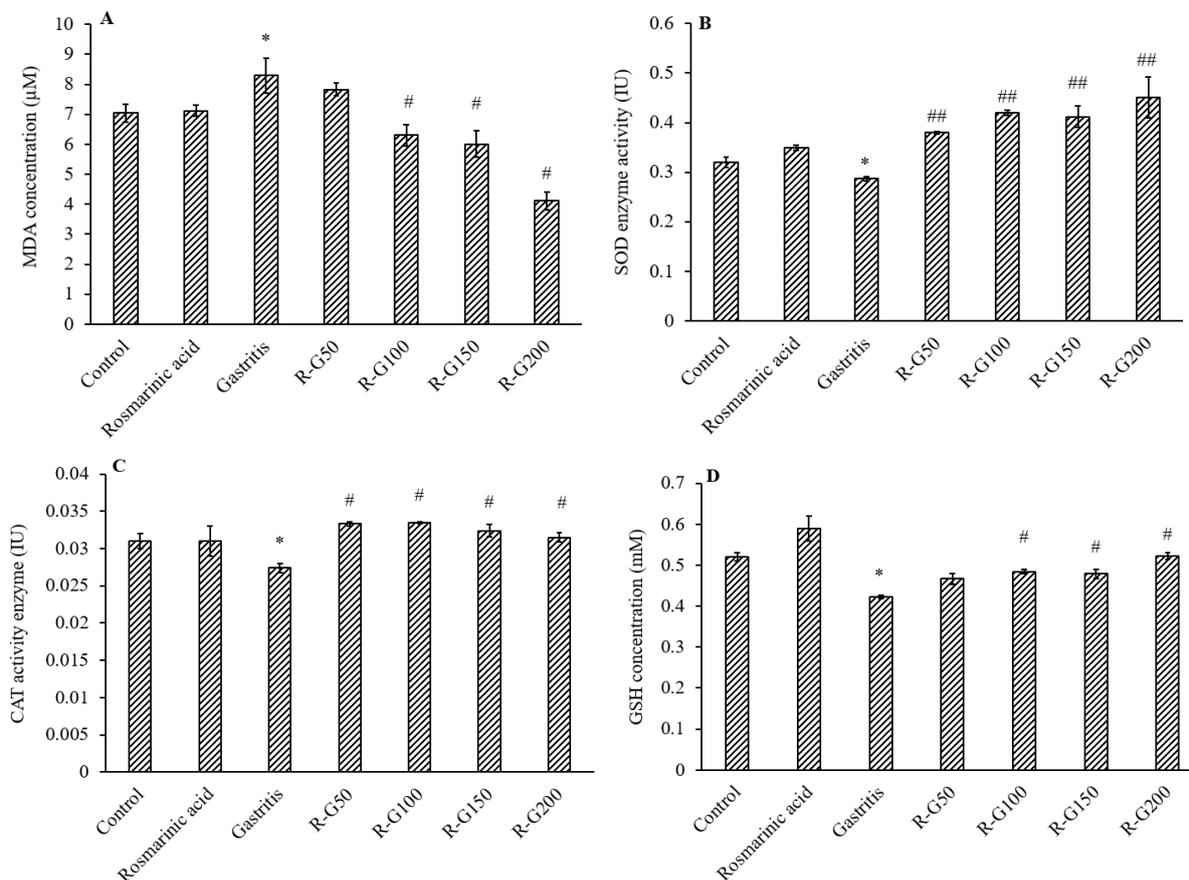

Fig. 4. (A) MDA, (B) SOD, (C) CAT, and (D) GSH levels of the gastric tissue in different groups. Data are presented as mean ± SEM, n = 6. *$P < 0.05$ indicates significant differences compared to the control; #$P < 0.05$ and ###$P < 0.001$ versus gastritis. CAT, Catalase; GSH, glutathione; MDA, malondialdehyde; SOD, superoxide dismutase.
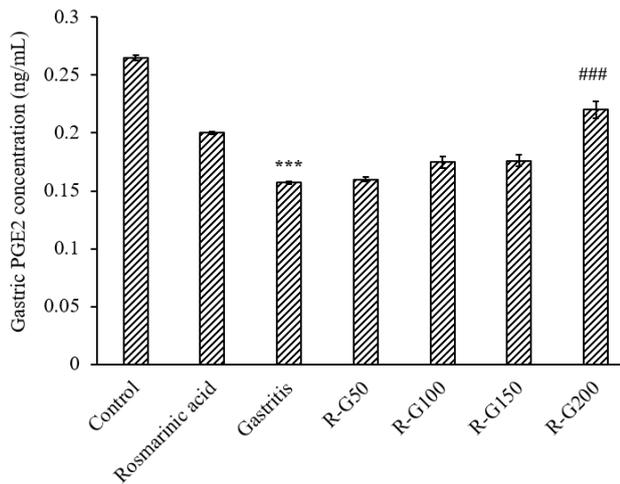
Fig. 5. PGE2 levels of the gastric tissue in different groups. Data are presented as mean ± SEM, n = 6. ***$P < 0.001$ indicates significant differences compared to the control; ###$P < 0.001$ versus gastritis. PGE2, prostaglandin E2.

Rosmarinic acid increased gastric PGE2 level in the ethanol-induced gastritis

The obtained results displayed that gastric PGE2 levels significantly decreased in the gastritis group compared with the control group (0.157 ± 0.0015 *vs* 0.265 ± 0.002 ng/mL, $P < 0.001$). However, RA administration could increase PGE2 levels in RG200 group in comparison with the gastritis group (0.22 ± 0.007 *vs* 0.157 ± 0.0015 ng/mL, $P < 0.001$; Fig. 5).

DISCUSSION

The present study showed that RA improved gastric ulcers, increased mucosal layer, preserved mucosal, parietal, and chief cells, decreased leucocytes infiltration, and blood vessel numbers in both of mucosal and submucosal layers. Furthermore, RA increased SOD, CAT, and GSH and decreased MDA levels in the gastric tissue. It also elevated the PGE2 level in the stomach.

Excessive alcohol consumption is considered one of the principal factors in gastric injuries. Indeed, alcohol causes epithelial layer destruction and mucosal edema, leukocyte infiltration, hyperemia, hemorrhage, and gastric ulceration (7). Several investigations have also demonstrated that alcohol weakens antioxidant/oxidant balance and PG synthesis in the stomach (10,23,24), therefore widely

used to study new agents and treatment procedures in animal models.

RA is a flavonoid compound, which is found in many plants. The beneficial effects of RA have been studied on inflammation, oxidative stress, and apoptosis in the brain, liver, cardiac and thyroid damages, and diabetes (14-16). To the best of our knowledge, this is the first study that evaluates the effects of RA on gastric glands, vessels, and antioxidant defense.

Histopathological evaluations revealed that RA attenuated ethanol-induced gastric ulcers and increased mucosal layer. Moreover, gastric glands were preserved after RA administration. In this regard, Govindaraj *et al.* have shown that RA improved pancreatic β-cell dysfunction induced by glucolipotoxicity (25). In another study, RA could protect the parotid gland against harmful damage from ionizing radiation (26). These results are in agreement with our findings and the possible mechanism could be as follows; since gastric mucosal barrier and bicarbonate-rich mucus is the first defense line against ulceration, its destruction permit to penetrate more acid and pepsin into the stomach tissue and ulcer development. Gastric ulcers then destroy the mucosal layer and glands, decrease mucus synthesis and provide additional factors for more ulceration. Herein, it seems that RA could protect mucosal, parietal, and chief cells from destructive effects of alcohol, keep the mucosal layer on the health status and attenuate gastric ulcers.

Our findings also showed leucocyte infiltration and blood vessel numbers ameliorated in both mucosal and submucosal layers after RA treatment. Similarly, it has been reported that some herbal and chemical agents inhibit neutrophil infiltration and migration after gastritis (27,28). Moreover, allantoin decreased gastritis-induced blood vessel numbers and leucocyte infiltration in male rats (29). Compatible with our results, many reports have demonstrated beneficial effects of RA on the inflammatory process in different disorders (14,16,30-32). Indeed, increased blood vessel numbers and their permeability lead to hyperemia, leucocyte accumulation, and infiltration, which both result from inflammation and cellular damages in gastritis. It seems that administration with RA lowered

both and attenuated inflammation and hyperemia in the gastric tissue. Of course, in our study decrease of blood vessels in the mucosal layer occurred in lower doses of RA, and in the submucosal layer occurred in higher doses of RA, which may be due to better penetration of RA into the mucosal layer than the submucosal layer.

In the following, to gain more insight into the probable mechanisms of RA effect on ethanol-induced gastritis, we evaluated oxidative stress indexes in the stomach tissue. Oxidative stress is a major factor in the pathophysiology of gastric injuries. MDA is an end product of membrane lipid peroxidation and is considered an important indicator for determining oxidative stress. In the current study, we found RA administration significantly decreased gastric MDA level. In agreement with our result, several researchers have shown that RA reduced MDA levels in the liver, heart, brain, and kidney damages (11-13). These reports reveal the strong effect of RA in the decrement of lipid peroxidation and cell injury. It seems that RA could also attenuate cell membrane peroxidation in the stomach.

It has demonstrated that in response to gastric ethanol toxicity, SOD and CAT enzymes decrease. These enzymes are the endogenous antioxidant defense in the stomach cells, which protect the cells from reactive oxygen species (ROS) (33). Sulfhydryl (SH) groups also in the GSH structure act as scavengers for ROS and inhibitors for lipid peroxidation (34). Different studies have proven the role of GSH in the regulation of cell signaling and DNA repair mechanisms (4,5,31). It has been revealed that GSH content decreases after ethanol-induced gastritis (35) which possibly results from the detoxification effect of GSH in confronting ROS. Our study showed that gastric SOD and CAT activities and GSH level increased after RA administration. In this regard, Zhang et al. have reported that RA increased SOD and CAT in the liver and kidney of aging mice (13). In another study, Khamse et al. have indicated the RA effect in the rise of SOD, CAT, and GSH in CA1 and CA3 neurons (36). There are several piece of documents in this context, which have demonstrated a powerful antioxidant effect for

RA in elevation of SOD, CAT, and GSH against ROS. Altogether, it seems that notable effects of RA in decreasing MDA level and increasing SOD, CAT, and GSH levels lead to an increased ability of gastric cells to counteract destructive effects of oxidative stress and protect stomach cells and glands against ethanol-induced gastritis, and eventually improve ulceration and inflammation in the stomach.

Prostaglandins are active molecules with a variety of effects in different organs. In the stomach, PGE2 plays a major role in maintaining mucosal barrier integrity, mucus secretion, regulation of gastric blood flow (7), reducing gastric acid secretion, suppresses inflammation and oxidative stress in the stomach (29). Alcohol administration decreases PGs synthesis and secretion and inhibits its protective effect and is identified as a principal mechanism in gastric ulcer pathogenesis (33). Many studies have indicated that some agents and herbal extracts increase the synthesis and secretion of PGE2 and improve gastric ulcers induced by ethanol (29,33). Consistent with previous studies, our findings revealed that gastric PGE2 levels increased after RA administration. In contrast with our result, Huang et al. have reported that RA decreased PGE2 levels in lipopolysaccharide-induced macrophages (37) which may be due to different PGs effects in different types of tissues. Taken together, it appears that another mechanism in the positive effects of RA on ethanol-induced gastritis is involved in increasing PGE2 secretion that raises secretion and growth of mucus glands and epithelial layer, attenuates leukocyte infiltration, regulates mucosal and submucosal blood flow, and eventually, improves gastric ulcers.

CONCLUSION

In summary, this study demonstrated notable effects of RA on the preservation of gastric mucosal layer integrity and glands and protection of gastric ulcers in ethanol-induced gastritis. These effects may be due to the high antioxidant effect of RA and its role to raise PG secretion. Future studies can help to confirm this opinion.

Conflicts of interest statement

The authors declared no conflicts of interest in this study.

Authors' contribution

F. Heidari, T. Komeili-Movahhed, and Z. Hamidi-zad performed experimental procedures. A. Moslehi did the study design and wrote the manuscript. All authors approved the manuscript.

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