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Curcumin regulates inflammation and apoptosis through PARP-1 and NF-κB in ethanol-induced gastric ulcer model

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Curcumin is a natural polyphenol with antioxidant, antiapoptotic and anti-inflammatory properties. In the present study, we explored the roles of PARP-1 and NF-κB molecules in the inflammation and apoptosis modulation caused by curcumin in the ethanol-induced gastric ulcer model. Male *wistar albino* rats (n=32) were divided into four groups. A single dose of 1 mL corn oil was given orally to the normal control and ethanol groups. After 30 minutes of after corn oil application, 1 mL of absolute alcohol was administered orally to the ethanol group. The curcumin group was given 100 mg/kg curcumin orally. In the ethanol+curcumin group, 1 mL of absolute alcohol was given orally 30 min after 100 mg/kg oral curcumin administration. The study evaluated the macroscopic and microscopic ulcer scores, as well as mucosal barrier integrity, using hematoxylin-eosin and PAS staining. Apoptotic and inflammatory pathways were examined by immunohistochemical staining, and malodialdehyde (MDA), superoxide dismutase (SOD) and myeloperoxidase (MPO) levels were examined in tissue. Compared with the ethanol group, macroscopic and microscopic mucosal lesions, MPO and MDA levels, and IL-1, IL-6, TNF-α, PARP-1, NFκB and Caspase-3, 8, 9 expressions were seen to decrease in the ethanol+curcumin group, however SOD level was observed to increase. The study demonstrated that curcumin protects the mucosal integrity by exhibiting anti-inflammatory andantiapoptotic effects through the modulation of PARP-1 and NF-κB key molecules in the ethanol-induced gastric ulcer model.

Keywords: Antiapoptotic, Anti-inflammatory, *Curcuma longa*, Caspases, Inflammatory cytokines, Turmeric

Gastric ulcer is a common health problem with a prevalence of 5-10% in the entire population and can cause mortality due to its complications. The battle between factors that form the mucosal barrier, such as regular microvascular circulation, prostaglandins, bicarbonate and nitric oxide, and factors that threaten the mucosal barrier, such as *Helicobacter pylori*, ethanol, and nonsteroidal anti-inflammatory drugs, may cause GU[1].

Ethanol-induced GU models are widely used in experimental studies, as there is now an exact relationship between alcohol consumption and the increased incidence of GU[2,3]. The reduction of mucosal blood supply by ethanol through microcirculation increases free oxygen radicals in the tissue, impairs the antioxidant mechanism, and also leads to an increase in proinflammatory cytokines such as interleukin-1-beta (IL-1β), IL-6 and tumor necrosis factor-alpha (TNF-α)[4]. The interaction of free radicals, which are formed during physiological or pathological processes in the organism, with nucleic acids, proteins and membrane lipids can trigger inflammation by activating a transcription factor, Nuclear factor kappa B (NF-κB)[5,6]. Recently, systemic inflammation has been shown to contribute to inflammatory processes by increasing the expression of transcription factors such as NF-κB, cytokines, and other inflammatory mediators through Poly [ADP-ribose] polymerase 1 (PARP-1) expression[7]. In addition, it has been shown that cytokines, such as IL-1β, TNF-α and IL-6, which increase as a result of a severe inflammatory response, can trigger apoptosis, a process that has a critical role for tissue homeostasis, especially through the caspase cascade involving caspases 3 and 9[8,9].

Curcumin, a yellowish active ingredient obtained from the rhizomes of the *Curcuma longa* L. plant, known for its anti-inflammatory, antioxidant, antiapoptotic and anticarcinogenic effects, has been used in traditional Asian medicine for centuries[10]. Curcumin, a powerful natural antioxidant, suppresses the formation of reactive oxygen species (ROS), decreases the expression of proinflammatory cytokines by inhibiting the transcription factor NF-κB, and prevents apoptosis secondary to DNA damage by inhibiting PARP-1[11,12].

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Although the protective effect of curcumin has been demonstrated in the ethanol-induced gastric ulcer model[3,13], the roles of PARP-1 and Nf-κB on inflammation and apoptosis in this protective mechanism have not been demonstrated. Here, we have studied the effect of curcumin on PARP-1, NF-κB, inflammation and intrinsic and extrinsic apoptotic pathways in this ethanol induced gastric ulcer rat model.

Materials and Methods

Animals and ethics

In our study, male wistar albino rats (n=32, weighing 300-350 g each) produced by Bülent Ecevit University Animal Care and Research Unit (Zonguldak, Turkey) were used. During the experiment, suitable environmental conditions for animal care (20 ± 1 C room temperature, 60 ± 10% humidity, and 12/12 hour light/dark cycle) were provided and subjects were allowed free access to food and water. Throughout the experiment, the Guide for the Care and Use of Laboratory Animals published by the US Public Health Service was followed and approval was obtained from the Institutional Animal Ethics Committee of Bülent Ecevit University before the study (Zonguldak, Turkey; 2020-03).

Experimental design

A total of 4 groups were formed with 8 rats in each group as follows: Gr. I, Normal control group (NC): A single dose of 10 mL/kg corn oil (cucumin's solvent) was given by gavage; Gr. II, curcumin group (CUR): A single dose of 100 mg/kg curcumin (Sigma-Aldrich, St. Louis, Missouri, USA) dissolved in 10 mL/kg corn oil was given by gavage[14]; Gr. III, ethanol group (ETH): A single dose of 10 mL/kg corn oil was given by gavage, and 30 min later, a single dose of 1 mL of absolute alcohol (Merck Millipore, Burlington, Massachusetts, USA) was given by the same route[3]; Gr. IV, ethanol + curcumin group (ETH+CUR): A single dose of 100 mg/kg curcumin dissolved in 10 mL/kg corn oil was given by gavage, 30 min later, a single dose of 1 mL of absolute alcohol was given by the same route.

Macroscopic ulcer score

One hour after absolute alcohol administration, the subjects were sacrificed and their stomach tissues were respected. Gastric tissues were opened through an incision made along the greater curvature and washed with isotonic NaCl solution, and then stretched on paraffin plates to flatten the mucosal folds. The lesion area and the total stomach surface area were determined macroscopically by fixing transparent papers in millimeter scale on the stretched stomach surface. The macroscopic ulcer score was calculated with the formula lesion area/total stomach surface area × 100[15].

Histopathological evaluations

For light microscopic examinations, stomach tissues were fixed in formalin fixator and blocked after paraffin inclusion. Hematoxylin-eosin (H-E) and periodic acid schiff (PAS) dyes were applied to 5 micron thick sections taken from these blocks in order to determine the histological features of the stomach. The stained tissues were photographed with the Axio Lab A1 microscope (Zeiss, Germany).

Each 1 cm long section was divided into 3 areas and the microscopic ulcer score for each area was calculated according to the following criteria: 0, normal mucosa; 1, epithelial cell damage; 2, glandular disruption and vasocongestion or edema of the upper parts of the mucosa; 3, vasocongestion or edema extending to the middle parts of the mucosa; and 4, mucosal damage involving the entire mucosa[16]. The average of the scores calculated for each area was accepted as the microscopic ulcer score for that section.

Evaluation of SOD, MPO and MDA levels

Stomach tissue homogenates were prepared, malon-dialdehyde (MDA) level was determined using the methods of Casini *et al.*[17], myeloperoxidase (MPO) level was determined using the methods of Bradley *et al.*[18], and superoxide dismutase (SOD) level was determined using the methods of Kakkar *et al.*[19].

Imunohistochemical evaluations

PARP-1, caspses-3, -8, -9, IL-1β, IL-6, TNF-α and NF-κB (RelA/NF-κB p65) expressions were demonstrated by immunohistochemical method to evaluate the relationship between apoptosis, inflammation and transcription factors in ethanol-induced gastric ulcer model.

Paraffin-embedded stomach sections with a thickness of 5 microns were taken. Sections were first incubated with rabbit anti-PARP-1 (1:100 dilution, Abcam, UK), anti-NF-κB (1:200 dilution, Novus biologicals, USA), anticaspase-3 (1:200 dilution, Sigma-Aldrich, Germany), anticaspase-8 (1:200 dilution, Abcam, UK), anticaspase-9 (1:100 dilution, Thermo Fisher Scientific, USA), anti-TNF-α (1:200 dilution, Sigma-Aldrich, Germany), anti-IL-6 (1:100

dilution, Novus biologicals, USA) and anti-IL-1β antibodies (1:100 dilution, Novus biologicals, USA) at +4ºC for 24 h. Subsequently, immune complexes formed on tissues incubated with a biotinylated goat anti-rabbit secondary antibody (Thermo Fisher Scientific, USA) were visualized by incubation with 3,3'-diaminobenzidine tetrachloride (Vector laboratories, DAB Substrate Kit, Peroxidase (HRP), with Nickel, USA). Finally, counterstaining was performed with hematoxylin. H-SCORE was performed on the sections using the Axio Lab A1 (Zeiss, Germany) microscope according to the following criteria: 0; no staining, 1+; weak but detectable staining, 2+; medium or pronounced staining, 3+; intense staining. The H-SCORE value for each section was obtained by multiplying the percentage of stained cells for each density category by its density. Scoring was done under the light microscope at x40 objective magnification on 20 randomly selected fields on each section and mean scores were used for statistical analysis. H-SCORE = ∑iixPi, i; density score, Pi; cell percentage[20].

Statistical analysis

SPSS 24.2 package program was used for data analysis. Immunohistochemical H-SCORE index was calculated as median (min-max). First of all, the Shapiro-Wilk test was used to examine whether the data of the subjects were suitable for normal distribution, and one-way analysis of variance was used to compare the normally distributed quantitative values. The Kruskal Wallis test was used to compare the normally distributed qualitative values. Mann Whitney U test with Bonferroni correction was used for pairwise comparison. P <0.05 was considered significant.

Results

Effect of curcumin on ethanol-induced gastric mucosal injury

The macroscopy of the stomach tissues obtained from the subjects in the NC and CUR groups were evaluated as normal. There was no statistically significant difference between the macroscopic ulcer scores of the NC and CUR groups (P >0.05). On the other hand, large bleeding areas were noted in the stomach tissues of rats given ethanol by gavage 1 hour before sacrifice. The extent of erosion areas in the stomach tissues and macroscopic ulcer scores of rats given curcumin 30 min our before ethanol administration were significantly lower compared to rats given ethanol alone (P <0.01) (Figs 1 and 2A).

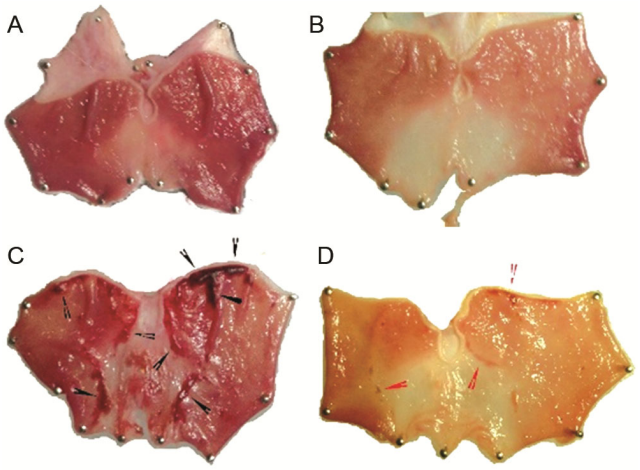


Fig. 1 — Macroscopic view of gastric mucosal lesions of rats in all groups. The gastric mucosa of the rats in the (A) normal control; and (B) curcumin groups did not show any lesions. Rats in the (C) ethanol group had extensive and severe hemorrhagic lesions on the gastric mucosa (black arrowheads) while the (D) ethanol + curcumin group had very limited and very mild hemorrhagic lesions in the gastric mucosa (red arrowheads).

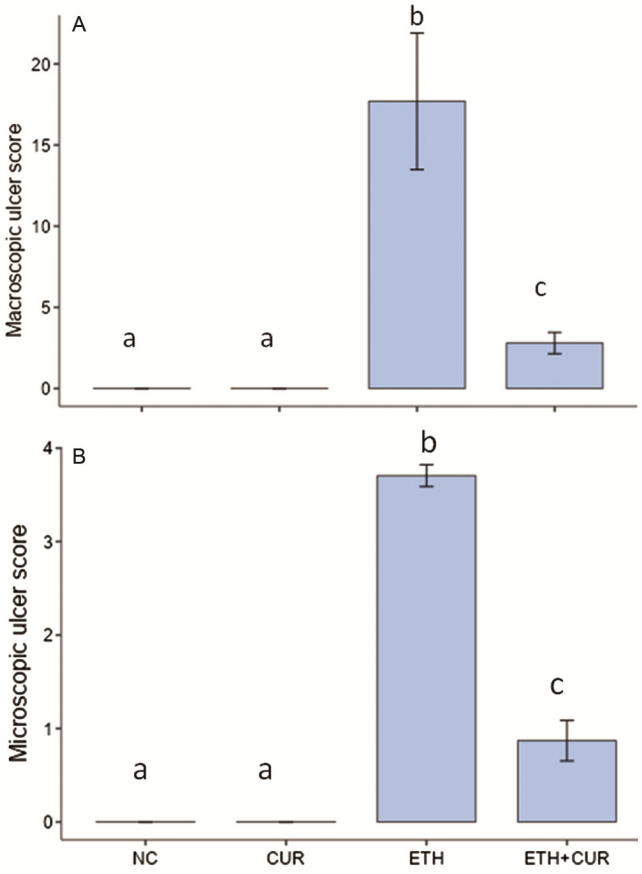


Fig. 2 — Effect of oral curcumin administration on (A) macroscopic; and (B) microscopic ulcer score. [Values are presented as means±SEM (n=8). The same letters indicate similar groups. P <0.01 for all comparisons]

There was no statistically significant difference between the microscopic ulcer scores of the NC and CUR groups (P >0.05) and both appeared normal. The microscopic ulcer score of the Gr. I (NC) and Gr. II (CUR) showed a statistically significant difference compared to both the Gr. III (ETH) (P <0.01 both) and Gr. IV (ETH+CUR) groups (P <0.01 both). In the microscopic evaluation of H-E stained sections, surface and glandular epithelial damage, vaso-congestion, edema, erythrocyte extravasation and neutrophilic infiltration were seen clearly in the entire thickness of the mucosa and submucosa in the ETH group. In contrast, both lesion width and lesion depth were significantly reduced in the ETH+CUR group. Likewise, submucosal thickness increased in the ETH group, indicating edema, however, it had a normal appearance in ETH+CUR group (P <0.01) (Figs 2B and 3).

PAS staining method was used for the evaluation of mucosal glycoproteins synthesized by surface mucus cells. The intense mauve staining observed on the mucosal surface in the ETH+CUR group compared to the ETH group indicated that the epithelium containing the surface mucus cells was intact (Fig. 4).

Effect of curcumin on SOD, MPO and MDA levels

Oxidative stress levels in gastric tissues were evaluated with SOD, MDA and MPO markers. SOD level in ETH group was significantly lower than in

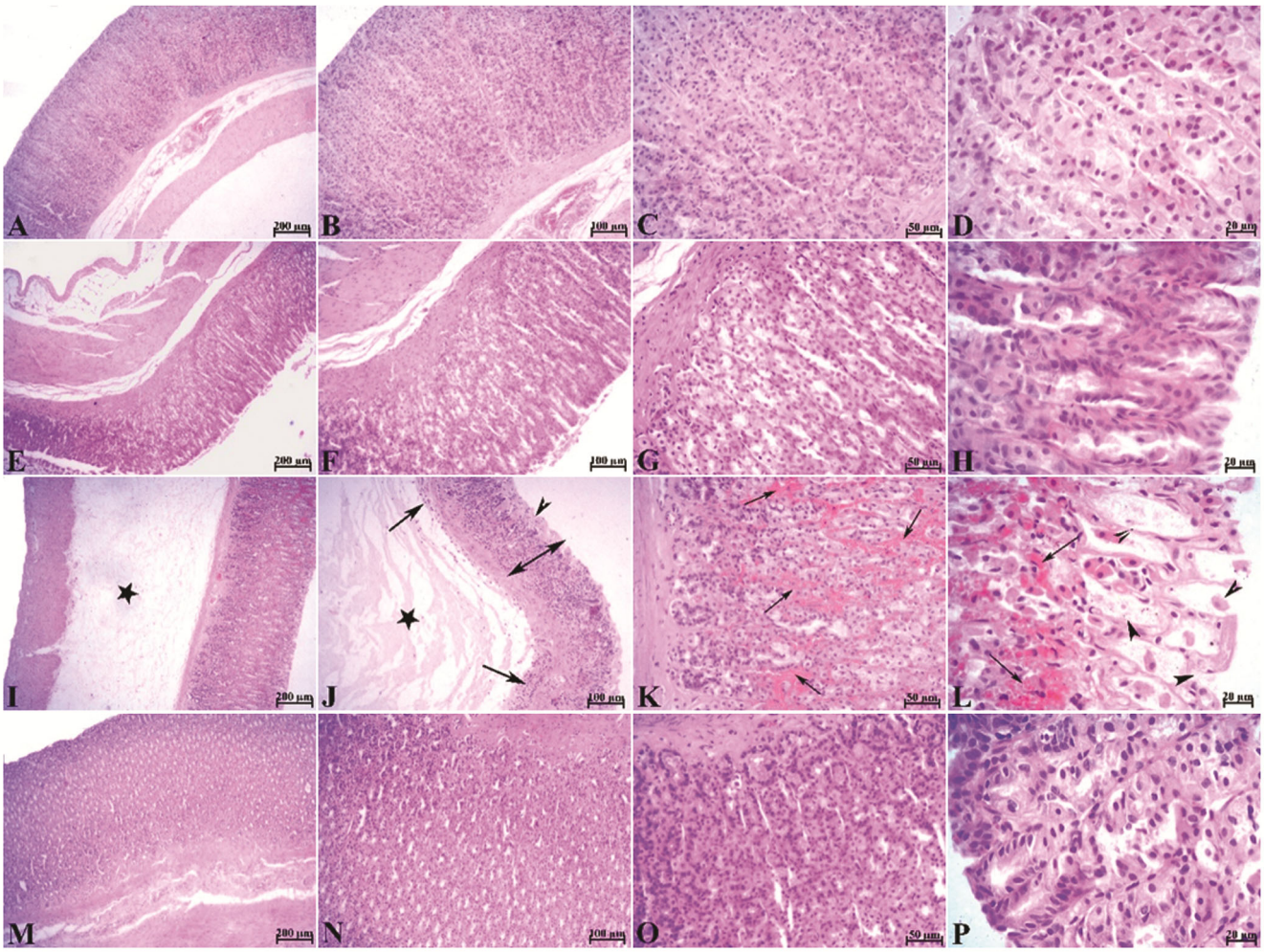


Fig. 3 — Histological evaluation of gastric mucosa of rats in all groups. In the (A-D) normal control group; and (E-H) curcumin group, the mucosa appears normal. In the (I-L) ethanol group, epithelial desquamation (arrowhead), mucosal hemorrhage and vascular congestion (arrow in K and L), inflammatory cell infiltration extending into the submucosa (arrow in J), reduction in mucosal thickness (double-headed arrow), and edema in the submucosa (star) is present. The mucosa in the ethanol+curcumin group has a normal appearance similar to the control and curcumin groups. [H+E staining, A, E, I, M scale bar; 200 µm, B, F, J, N scale bar; 100 µm, C, G, K, O scale bar; 50 µm, D, H, L, P scale bar; 20 µm]

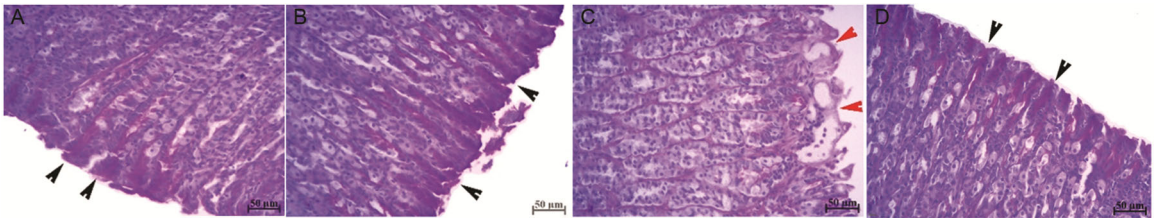


Fig. 4 — Histological evaluation of mucosal barrier integrity in gastric mucosa of rats in all groups. (A) Normal control group; (B) curcumin group; (C) Ethanol group; and (D) ethanol+curcumin group. Magenta staining on the mucosal surface in the normal control, curcumin, and ethanol+curcumin groups indicates preservation of the mucosal barrier (black arrowhead). In contrast, the mucosal barrier integrity appears to be impaired in the ulcer group (red arrowhead). [PAS staining, scale bar; 50 µm]

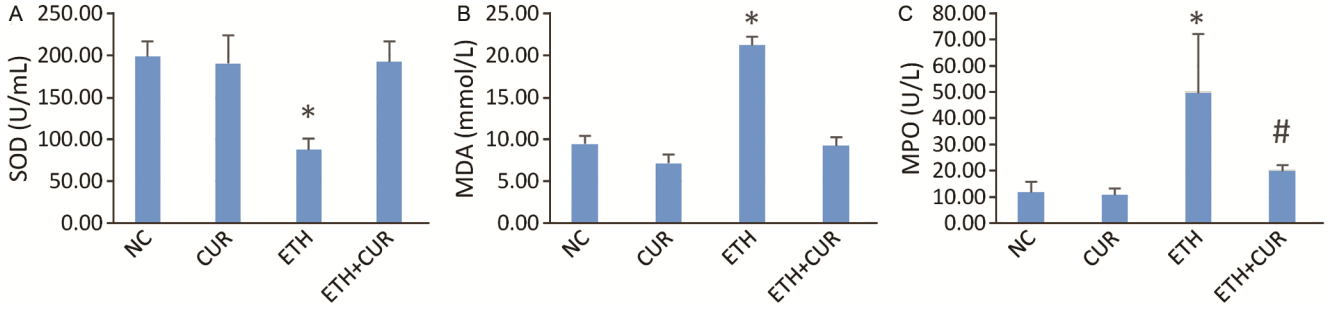


Fig. 5 — Effect of curcumin on oxidant and antioxidant parameters. (A) Superoxide dismutase (SOD); (B) Malondialdehyde (MDA); and (C) Myeloperoxidase (MPO). [All values are expressed as mean±SEM (n=8). **P* <0,01 Different from other groups. #*P*<0,01 Different from other groups]

NC, CUR and ETH+CUR groups (*P* <0.01). A significant increase was observed in the ETH+CUR group compared to the ETH group (*P* <0.01). Similarly, MDA and MPO levels in the ETH group were considerably higher than in the NC and CUR groups (*P* <0.01). On the other hand, the ETH+CUR group showed a significant decrease compared to the ETH group (*P* <0.01) (Fig. 5).

Effect of curcumin on immunohistochemical markers

Mucosal IL-1-β, IL-6, TNF-α, caspases-3, -8, -9, PARP-1 and NF-κB expressions were evaluated by immunohistochemical staining of gastric sections. Immunoreactivity for all markers in Gr. I & II was either absent or very weak, and no statistically significant difference was found between the h-score values of these two groups (*P* >0.05). A significant increase in the expression of all markers was observed in the GR. III (*P* <0.01). In contrast, the immunohistochemical staining of the Gr. IV (ETH+CUR) showed a remarkable decrease in all markers compared to the GR. III (ETH) group (*P* <0.01) (Figs 6 and 7).

Discussion

In our study, we demonstrated the protective effect of curcumin on ethanol-induced ulcer model in rats. In present experiment, it was observed that pathological changes, such as epithelial cell damage, glandular damage, vasocongestion, edema and hemorrhage, occurred in the gastric mucosa of the rats in the ETH group, Whereas the aforementioned mucosal lesions were largely eliminated in the ETH+CUR group. Furthermore, when compared with the ETH group, the increase in SOD level and the decrease in MPO and MDA levels in the gastric tissue of the rats in the ETH+CUR group were significant. Finally, we performed immunohistochemistry for proinflammatory cytokines (IL-1-β, IL-6 and TNF-α), apoptotic markers (caspases-3, -8 and -9), NF-κB, a transcription factor, and PARP-1, a DNA repair enzyme. Our immunohistochemistry study revealed that the expressions in the ETH+CUR group showed a significant decrease compared to the ETH group for all markers mentioned above.

The mucus content of the gastric mucosal surface is an important defense mechanism against the development of ulcerative lesions[21]. Previous studies have shown that curcumin can prevent the development of gastric ulcer with its antioxidant and anti-inflammatory effect, as well as its increase in gastric mucus secretion and its mucoadhesive effect. Orona-Ortiz *et al*.[22] showed that the mucoadhesive effect of curcumin plays an important role in preventing the formation of lesions in ethanol-induced

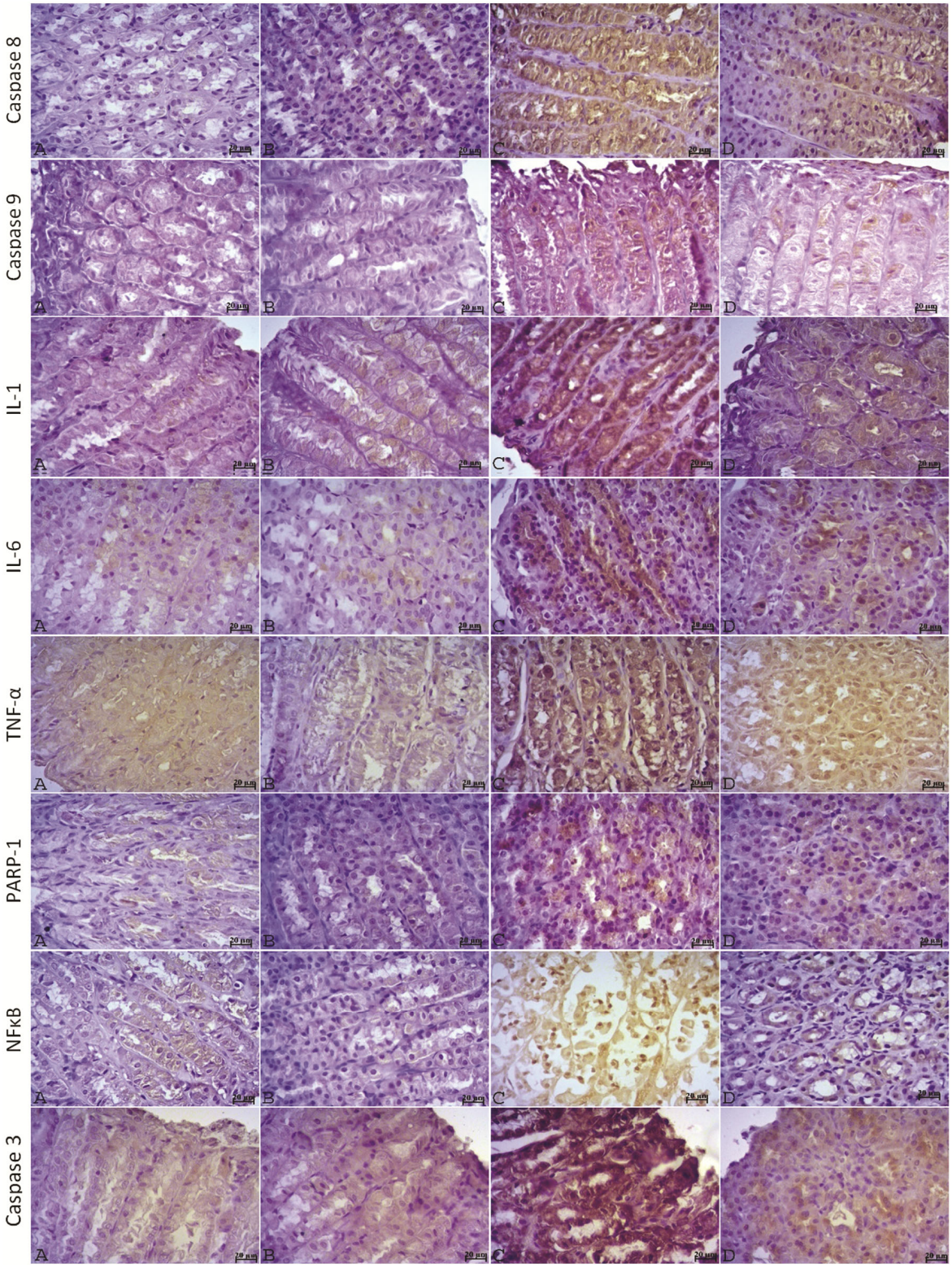


Fig. 6 — Immunohistochemical expressions of caspase 3, 8, 9, IL-1 β, IL-6, TNF-α, PARP-1 and NF-κB proteins in gastric mucosa. Expressions in the ETH group for all proteins appear to be higher compared to the other groups. However, for all proteins, it is noteworthy that the expressions in the ETH+CUR group showed a significant decrease compared to the ETH group. (A) NC group; (B) CUR group; (C) ETH group; and (D) ETH+CUR group. [Scale bar; 20 µm]

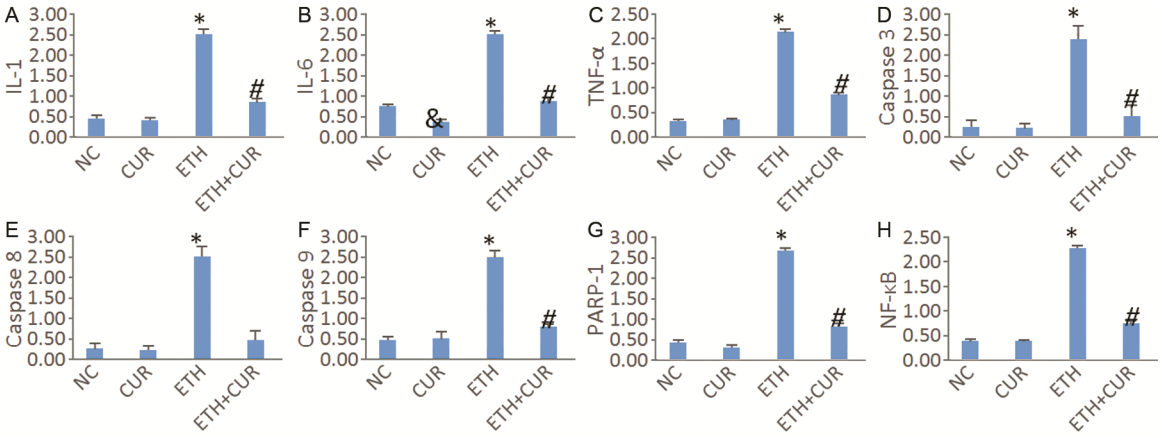


Fig. 7 — Effect of curcumin on gastric mucosal expression of (A) IL-1β; (B) IL-6; (C) TNF-α; (D-F) Caspase 3, 8 and 9; (G) PARP-1; and (H) NF-κB. [All values are expressed as mean±SEM (n=8). **P* <0,01 Different from other groups. #*P* <0,01 Different from other groups. & *P* <0,01 Different from other groups]

ulcers in rats. It has also been shown that curcumin increases the mucus level in gastric juice and suppresses ulcerative changes in gastric ulcer secondary to indomethacin administration in rats[23]. In our study, we used the PAS reaction which is widely used to display carbohydrate-containing cellular components to evaluate mucus secretion, one of the important barrier mechanisms of the gastric mucosa. Our findings revealed that the decrease in gastric mucus secretion accompanying the loss of surface and neck mucus cells in the ETH group was significantly improved in the ETH+CUR group.

The sequestration of neutrophils in the gastric mucosa plays an important role in the pathogenesis of gastric ulcer and the accumulation of neutrophils in the lesion area can be demonstrated by the MPO level. Based on this fact, it can be said that the decrease in MPO activity can be accepted as an indicator of anti-inflammatory activity[24]. A number of chemicals, such as proinflammatory cytokines and free oxygen radicals released by activated neutrophils, play a key role in tissue damage, and gastric mucosa is not exempt from this damage mechanism[25]. Infiltration of neutrophils into the gastric mucosa plays a decisive role in the development of gastric ulcer through the production of proinflammatory cytokines and ROS[26]. Cells have developed a series of antioxidant enzyme systems such as SOD, catalase (CAT), and glutathione peroxidase (GPx) against oxidative damage. In the first step of fighting free radicals, the SOD enzyme converts the superoxide radical (O_2 •-) to hydrogen peroxide(H_2O_2). Then H_2O_2 is reduced to water by the enzyme CAT. Exceeding the capacities of these antioxidant enzymes due to excessive ROS production leads to oxidative stress[27].

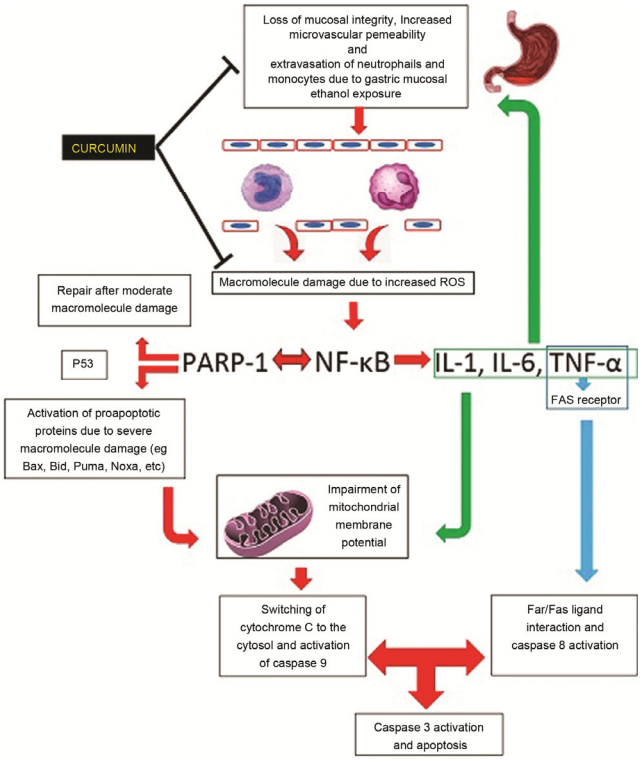
One of the most important targets of free radicals is lipids in cell membranes. MDA, the end product of ROS-membrane lipid interaction, can therefore be used as a marker of oxidative damage. The two major free radicals, the hydroxyl radical (HO·) and the hydroperoxyl (HO_2·) radical, which are the products of the Fenton reaction and Haber-Weiss reactions, specifically target lipids in the tissue[28]. Disruption of the oxidant-anti-oxidant balance in favor of oxidant agents as a result of ethanol exposure triggers the innate immune response and leads to an increase in proinflammatory cytokine expressions. It is thought that proinflammatory cytokines such as IL-1β and TNF-α initiate changes in the tissue level in gastric ulcer and also determine the course and severity of inflammation[29,30]. Although TNF-α and IL-1β are molecules that directly cause damage and determine the fate of the lesion, they also contribute to cytotoxicity and tissue damage by increasing the formation of ROS in free radical sources such as mitochondria[31]. Another factor suggesting that TNF-α plays a key role in the development and progression of gastric mucosal lesions is the presence of evidence showing that high plasma TNF-α levels facilitate leukocyte adhesion. These data suggest that TNF-α may be the conductor of gastric mucosal inflammation secondary to ethanol exposure and subsequent tissue damage[32]. Guo *et al*.[33] revealed that curcumin has a neuroprotective effect in acrylamide-induced neurotoxicity in rats by decreasing IL-1-β, TNF-α and MDA levels and increasing SOD and GPx activities in brain tissue. Celani and colleagues showed that curcumin significantly abolished the pathological changes in the colonic mucosa by increasing SOD, GPx and CAT levels while decreasing

TNF-α, IL-1-β and IL-6 levels in acetic acid-induced ulcerative colitis model in rats[34]. The findings of our study are similar to previous studies in this respect. Morover, our above study showed that IL-1β also plays a role in the pathogenesis of ethanol-induced gastric ulcer in rats and that curcumin can contribute to ulcer healing by reducing IL-1β expression.

Increased levels of reactive oxygen species (ROS) is one of the mechanisms that activate NFκB, which is the key molecule in proinflammatory cytokine expression. NFκB activation due to increased free radicals in the inflammatory process causes an increase in major proinflammatory cytokines such as IL-1β, IL-6 and TNF-α[35]. In the current study, curcumin (100 mg/kg) treatment applied to the ETH+CUR group significantly decreased TNF-α, IL-1-β, IL-6 and NFκB expressions compared to the ETH group.

Oxidative damage has devastating effects on cellular proteins and lipids and can cause single or double strand breaks in DNA, which can lead to cell apoptosis, necrosis or inflammation. Once oxidative stress begins at the cellular level, the balance between the damage caused by ROS on cellular macromolecules such as lipid, protein, DNA and the repair of this damage determines the fate of the cell. PARP-1 is a nuclear enzyme belonging to the PARP family that can repair these DNA breaks through either nucleotide excision repair or base excision repair[36-38]. If DNA damage caused by environmental factors including ROS is mild, PARP provides cell viability by repairing DNA. On the other hand, if DNA damage is severe, PARP-1 leads the cell to death by necrosis or apoptosis[39]. Mitochondria plays a key role in PARP-mediated cell death, as PARP-1 activation disrupts the mitochondrial membrane potential and initiates the migration of proapoptotic proteins such as cytochrome C(CytC) and apoptosis inducing factor (AIF) from the intermembrane space to the cytosol. As the extent of mitochondrial damage increases, more and more CytC will escape into the cytosol, resulting in impaired oxidative phosphorylation, further increasing ROS production and triggering a vicious cycle. As a result, apoptosis is triggered by a caspase-dependent or caspase-independent pathway via mechanisms triggered by Cytc and AIF, respectively[40,41]. The apoptosome complex formed as a result of the association of CytC with apaf-1 and procaspase-9 in the cytosol leads to caspase-9 activation, which in turn activates executioner caspases such as caspases-3, -6 and -7[42]. PARP, known to be a coactivator of NFκB, is involved in the inflammatory process by regulating the expression of NFκB and many other transcription factors[43]. Although there are many studies in the literature mentioning the contribution of NFκB to the antiapoptotic mechanism, the number of studies showing that NFκB can also mediate apoptotic cell death is increasing. Ryan *et al.* [44] using a human osteosarcoma cell line, showed that P53 facilitates the binding of NFκB, especially the P65/RELA subunit, to DNA, leading cells to apoptosis. Similarly, NFκB has been shown to induce death receptor-mediated apoptosis via caspase-8 activation in T-cell hybridomas by increasing the expression of Fas ligand (FasL) as a result of ionomycin treatment[40,45].

From this perspective, in our study, caspase-8 expression increased in the ETH group, while a significant decrease was observed in the ETH+CUR group. These findings are consistent with NFκB expressions in these groups. This result can be interpreted as NFκB contributes to the pathogenesis by increasing proapoptotic gene expressions in the pathogenesis of gastric ulcer induced by ethanol. On the other hand, curcumin suppressed NFκB expression and caspase-8 and caspase-9 activation with its anti-inflammatory and anti-oxidant effects in the ETH+CUR group and prevented the development of gastric mucosal lesions (Scheme 1).



Scheme 1 — Inflammation and apoptosis modulation caused by curcumin in the ethanol-induced gastric ulcer model: roles of PARP-1 and NF-κB molecules

Since this study was carried out with a limited budget, analyses such as Western blot/PCR, which we planned to do to confirm our results, could not be performed. However, the current study is the first to show that increased oxidative stress in the gastric mucosa due to ethanol exposure can trigger both intrinsic and extrinsic apoptotic pathways through inflammatory cytokines and DNA damage, and that the mucosal barrier can be protected by modulation of PARP-1 and NF-κB molecules.

Conclusion

In the view of the histological, biochemical and immunohistochemical findings of our study, it can be said that curcumin has a protective effect in ethanol-induced gastric ulcer. Our findings show that curcumin exerts anti-inflammatory and anti-apoptotic effects mainly by modulating PARP-1 and NF-κB expressions with its anti-oxidant activity, and thus has a protective effect on the gastric mucosa.

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Conflict of interest

Authors declare no competing interests.

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