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ORIGINAL ARTICLE

Fisetin Attenuates Gastric Mucosal Lesions through Modulating Nuclear Factor-Kappa B and Peroxisome Proliferator-Activated Receptor-γ in Rats

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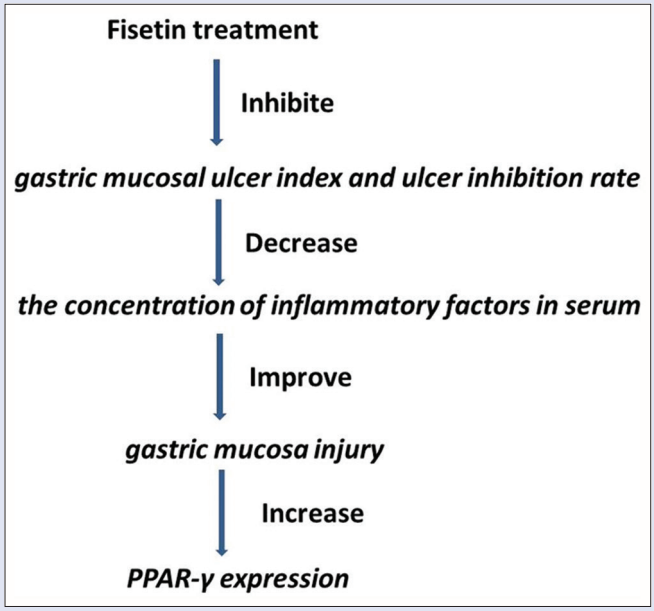
ABSTRACT

Background: Nowadays, the use of plant extracts is increasing in the world for the prevention and treatment of ulcer. **Objective:** The objective of this study was to explore the underlying mechanism of action of fisetin on ethanol-induced gastric ulcer model. **Materials and Methods:** In this study, gastric mucosal lesions were induced by ethanol in rats. Five groups of rats were formed based on the treatment administered: model group (model), omeprazole (40 mg/kg) group (omeprazole), high-dose fisetin group (100 mg/kg, H-fisetin), medium-dose fisetin group (50 mg/kg, M-fisetin), and low-dose fisetin group (25 mg/kg, L-fisetin). Interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α levels were assessed in serum. The expression of peroxisome proliferator-activated receptor (PPAR)-γ, nuclear factor-kappa B (NF-κB), and p38-mitogen-activated protein kinase (p38-MAPK) in the gastric mucosa was also measured. **Results:** In the case of the high-dose fisetin group, the level of TNF-α, IL-1β, and IL-6 decreased from 9.57 pg/mL to 5.19 pg/mL, from 0.59 pg/mL to 0.27 pg/mL, and from 37.96 pg/mL to 21.09 pg/mL, respectively. In the case of the omeprazole group, the level of TNF-α, IL-1β, and IL-6 decreased to 4.38 pg/mL, 0.27 pg/mL, and 18.58 pg/mL, respectively. The expression of PPAR-γ protein in the high-dose fisetin and omeprazole groups was about 1.5 times higher than that in the model group. Compared with the model group, the expression of NF-κB protein reduced to 0.34 level and 0.47 level in the omeprazole and high-dose fisetin groups, respectively. Compared with the model group, the expression of p38-MAPK protein reduced to 0.55 level and 0.68 level in the omeprazole and high-dose fisetin groups, respectively. **Conclusion:** Fisetin might relieve the symptoms of ethanol-induced gastric ulcer in rats through the regulation of NF-κB pathway.

Key words: Fisetin, gastric mucosal lesions, nuclear factor-kappa B, p38-mitogen-activated protein kinase, peroxisome proliferator-activated receptor-γ

SUMMARY

- Administration of fisetin or omeprazole suppressed the levels of tumor necrosis factor-α, interleukin-1β (IL-1β), and IL-6. Fisetin reduced the acidity of the stomach and significantly activated the expression of peroxisome proliferator-activated receptor-γ and inhibited the expression of nuclear factor-kappa B and p38-mitogen-activated protein kinase in the gastric mucosa in rats.



Abbreviations used: IL: Interleukin; TNF: Tumor necrosis factor; SOD: Superoxide dismutase; NO: Nitric oxide; MDA: Malondialdehyde; MPO: Myeloperoxidase; PPAR: Peroxisome proliferator-activated receptor; NF-κB: Nuclear factor-kappa B; p38-MAPK: p38-mitogen-activated protein kinase.

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INTRODUCTION

Worldwide, gastric mucosal lesions affect about 4.6 million people, which makes it the most common disorder of the gastrointestinal tract.[1] An imbalance in homeostasis and increased level of oxidative stress induces gastric mucosal lesions, which is characterized by erosion, ulceration, and hemorrhage of the gastric mucosa.[2] Many factors can increase the incidence rate of gastric mucosal lesions, for example, alcohol consumption and unhealthy eating habits. Most of the drugs are effective in treating gastric lesions, including proton-pump inhibitors, gastric mucosal protectors, and antibiotics;[2] however, the majority of

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them have side effects, such as altered heartbeat, hemopoietic changes, and systemic alkalosis.[3] Nowadays, a greater amount of research has been devoted toward the development of natural therapeutic agents that show fewer side effects.[1,4] The plant-based therapeutic agents are mostly derived from medicinal plants and their extracts, and plant-based active ingredients are being increasingly studied throughout the world for the prevention and treatment of gastric ulcer.

Fisetin (3,3',4',7-tetrahydroxyflavone) is widely present in fruits and vegetables, such as grape seed, strawberries, apple, and onion.[5] Previous studies have reported that fisetin shows antioxidant and anti-inflammatory activity and is widely used in the treatment of various diseases, such as cancer,[6,7] depression,[8] cardiac problems, and autoimmune disorders.[5,9] Fisetin has shown to protect against hepatic steatosis in high-fat diet-induced obese mice through sirtuin 1/mitogen-activated protein kinase (Sirt1/MAPK) and fatty acid β-oxidation pathways.[10] It inhibited the growth of cancer cells in gastric cancer by suppressing the extracellular-regulated protein kinase (ERK) ½ pathway.[11] In the case of human laryngeal cancer, fisetin controlled cancer by inducing tumor cell apoptosis and autophagy regulated by ERK1/2 and by nuclear factor-kappa B (NF-κB) signaling pathways.[12] Moreover, a recent study has shown that fisetin significantly decreases the levels of malondialdehyde (MDA) and myeloperoxidase (MPO) in the gastric mucosa of ethanol-induced gastric ulcer model.[13] Fisetin also improves the histopathology of gastric lesions.[13] However, the mechanism of action of fisetin in ameliorating gastric mucosal lesions in ethanol-induced rats is still unclear.

Omeprazole is now globally available for the treatment of gastrointestinal diseases. It can inhibit the secretion of gastric acid. More importantly, it regulates endogenous levels of oxidative stress and prevents the release of inflammatory cytokines.[14] In this study, we used omeprazole as a reference to assess the protective activity of fisetin in ethanol-induced gastric ulcers in rats. Furthermore, the mechanism of action of fisetin will also be studied.

MATERIALS AND METHODS

Animals

Fifty male Sprague Dawley rats (200–220 g) were used in this study (SCXK (Lu) 20140007, Jinan PengYue Experimental Animal Breeding Co., Ltd., China). The environmental temperature and humidity were in the range of 20°C–26°C and 50%–70% in the room with a 12 h light/dark cycle and free access to water. This study was approved by the Institutional Animal Care and Use Committee of the Affiliated Yantai Yuhuangding Hospital of Qingdao University.

Model and groups

Before the start of the experiments, rats were fed with free access to food and water for 1 week. Rats were randomly assigned to five groups ($n = 10$): model group, omeprazole (40 mg/kg) group, low-dose fisetin (L-fisetin, 25 mg/kg, purity ≥98%, St. Louis, MO, USA) group, medium-dose fisetin (M-fisetin, 50 mg/kg) group, and high-dose fisetin (H-fisetin, 100 mg/kg) group. All reagents were orally administered. Fisetin and omeprazole were dissolved in dimethyl sulfoxide (DMSO, 2 mL/kg). Rats in the model group were administered with DMSO (2 mL/kg) for 14 days. Rats in the omeprazole group were administered with omeprazole (40 mg/kg) for 14 days, which was equivalent to the dose effective in humans (20–40 mg). Rats in the fisetin groups were administered with different concentrations of fisetin (25, 50, and 100 mg/kg for low-, medium-, and high-dose groups, respectively) for 14 days. All rats were fasted for 24 h after the 13th day of administration of treatment.

Next, 2 h after the last administration, absolute ethanol (0.5 mL/100 g) was administered to all the rats.[14] The effects of daily administration of fisetin or omeprazole on body weight and food intake were recorded for the next 13 days.

Sample collection

Next, 1 h after ethanol administration, rats were anesthetized with 10% chloral hydrate (0.3 mL/100 g, Lan Bright Chemical Co., Ltd., Wuhan, China) by intraperitoneal injection. Then, 5 mL of blood was withdrawn from the abdominal aorta, and the serum was collected by centrifugation (3000 ×*g*, 10 min). The serum was frozen at −80°C until further use. The rats were sacrificed, and the abdominal cavity was dissected. Next, 2 mL of 1% formaldehyde solution was injected into the stomach cavity. The stomach was taken out and immersed in 1% formaldehyde solution.

To determine the effect of consecutive administration of omeprazole and fisetin on the levels of inflammatory mediators and oxidative stress in rats without the administration of ethanol, we collected the blood from the abdominal aorta on the 13th day. The drug administration in groups was consistent with the above description.

Ulcer index and inhibition rate

After 30 min, the stomach wall was cut along the big curve of the stomach and washed with frozen saline. The degree of gastric mucosal injury was observed using an operating microscope with a magnification of 10×. Some gastric sections were cut out and placed in liquid nitrogen and stored in a refrigerator at −80°C. A part of the gastric section was fixed in 4% polyformaldehyde solution.

Erosion, ulcer, and bleeding in the gastric epithelium were scored. The scoring was conducted as per the details specified in a previous study. The injury index of the animal is its total score.[14]

The ulcer inhibition rate = (ulcer index of the model group − ulcer index of the drug group)/ulcer index of the model group × 100%.

Acidity of the gastric juice

The gastric content was collected and centrifuged (800 ×*g*, 8 min) to obtain the supernatant. Then, the supernatant was dissolved in distilled water and titrated using a 0.01 M NaOH solution to the endpoint. Total gastric acidity was expressed in μEq/200 g.[15]

ELISA

The levels of TNF-α (ab100785, Abcam, Shanghai, China), IL-6 (ab100772, Abcam, Shanghai, China), MDA (ab238537, Abcam, Shanghai, China), superoxide dismutase (SOD) (24787, R&D, Shanghai, China), nitric oxide (NO) (SBJ-R0010, SenBeijia Biological Technology Co., Ltd, Nanjing, China), MPO (ab155458, Abcam, Shanghai, China), and IL-1β (ab100704, Abcam, Shanghai, China) were measured in serum by ELISA kits.

Histopathological evaluation

Tissues were immobilized in 4% neutral formaldehyde at 25°C for overnight, and the tissues were dehydrated with different concentrations of ethanol. Subsequently, the tissues (5 μm) were stained by hematoxylin and eosin staining (Thermo Fisher, Beijing, China).

Immunohistochemistry

Tissue slices (5 μm) were dewaxed and hydrated by successive grading of ethanol. After washing with phosphate-buffered saline (PBS, Thermo Fisher, Beijing, China), antigen was heated at 95°C for 60 min, then cooled for 3 min at 25°C, repeated 3 times. The primary antibodies of peroxisome proliferator-activated receptor (PPAR-γ) (1:800, Thermo Fisher, Beijing, China), NF-κB (1:800, Thermo Fisher, Beijing, China),

and p38-MAPK (1:1200, Thermo Fisher, Beijing, China) were added and incubated overnight at 4°C. The tissue slices were incubated at 20°C–25°C for 30 min and then washed with PBS-Tween. The secondary antibody labeled with horseradish peroxidase (1:600) was added and incubated at 37°C for 30 min. Next, the tissue specimens were washed with distilled water until it turns blue. Finally, the tissue specimens were dehydrated for 5 min in the ascending order of ethanol series (70%, 95%, and 100%). After washing the specimens for 5 min with PBST, the slices were incubated with DAB Horseradish Peroxidase Color Development Kit (Beyotime Biotechnology, Shanghai, China) for 3–15 min.

Western blot

The total protein was quantified using BCA Protein Quantification Kit (23225, Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific, Waltham, MA, USA). The protein was extracted according to the instructions provided in the kit. From each group, a 40-μg sample was loaded and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Mini-Protean-3, Bio-Rad, Hercules, CA, USA). The separated proteins were transferred onto a PVDF membrane (Millipore, Massachusetts, USA). Then, the samples were blocked by incubating with 5% skim milk powder for 1 h, and then, 5% bovine serum albumin was added to dilute the primary antibody of each protein. The primary antibodies were rabbit anti-human anti-PPAR-γ antibody (1:800, PP-K8713-00, R&D, Shanghai, China), anti-NF-κB antibody (1:800, AF5078, R&D, Shanghai, China), anti-p38-MAPK antibody (1:1000, AF-1519, R&D, Shanghai, China), and glyceraldehyde-3phosphate dehydrogenase (GAPDH) (1:1000, AF5718, R&D, Shanghai, China) polyclonal antibody. Then, the samples were incubated with goat anti-rabbit IgG (1:2000, ab6721, Abcam, UK) for 1 h. Then, electrogenerated chemiluminescence was detected, and the grayscale scanning and quantification were performed by ImageJ software (NIH).

Statistical analysis

All data were analyzed with SPSS 19.0 (IBM SPSS, Armonk, NY, USA) and expressed as the mean ± standard deviation. The differences among groups were analyzed by one-way analysis of variance, and the Tukey test was used for subsequent analysis. $P < 0.05$ was indicated as statistically significant.

RESULTS

General observation

We analyzed the effects of daily administration of fisetin or omeprazole on the body weight and amount of food intake for up to 14 days. During the study period, there was no significant change. There were no weight loss, low food intake, and slow movement observed in rats. These results show that fisetin or omeprazole treatment had no adverse effect during this short treatment duration.

Fisetin inhibited gastric mucosal ulcer index and ulcer inhibition rate

To reveal the effect of pretreatment of fisetin on gastric mucosal lesions, we analyzed the gastric mucosal ulcer index and ulcer inhibition rate. As shown in Figure 1a, the ulcer index was significantly decreased in different doses of the fisetin group and the omeprazole group compared with the model group ($P < 0.05$). When compared with the omeprazole group, the ulcer index was obviously increased in the low-dose fisetin group ($P < 0.05$). The gastric mucosal injury was found to be improved after the treatment of rats with fisetin and omeprazole [Figure 1b]. The results were similar between the high-dose fisetin group and the omeprazole group. The inhibition rate was 52.75% and 55.61% in the high-dose fisetin and omeprazole groups, respectively. These data revealed that fisetin pretreatment inhibited gastric mucosal injury.

Fisetin decreased the gastric acidity

Next, we explored the effect of fisetin on gastric acidity. As shown in Table 1, the gastric acidity decreased after pretreatment with fisetin when compared with the model group ($P < 0.05$). Omeprazole, as a positive group, produced obvious differences in contrast to the model group ($P < 0.05$). There was an obvious difference between the low-dose fisetin group and the omeprazole group ($P < 0.05$), but there was no significant difference between the high-dose fisetin, medium-dose fisetin, and omeprazole groups. These results show that fisetin administration decreased the acidity of the stomach in a dose-dependent manner.

Fisetin decreased the concentration of inflammatory cytokines in serum

Next, we examined the effect of administration of omeprazole and fisetin on the levels of tumor necrosis factor (TNF)-α, interleukin-6 (IL-6), and IL-1β without administration of ethanol. According to the results, there was no significant difference between groups [Figure 2a]. Furthermore, the effect of pretreatment of omeprazole and fisetin on mucosal lesions was analyzed [Figure 2b]. Compared with the model group, the levels of inflammatory cytokines were downregulated after treatment with fisetin ($P < 0.05$). With a higher dosage of fisetin, the effect was higher. There was no significant difference between the high-dose fisetin, medium-dose fisetin, and omeprazole groups. However, the levels of inflammatory cytokines were higher in the low-dose fisetin group than that of the omeprazole group ($P < 0.05$).

Fisetin suppressed oxidative stress response in serum

Next, we examined the effect of consecutive administration of omeprazole and fisetin on the levels of MDA, MPO, SOD, and NO without ethanol administration. According to the results, there was no significant difference between different groups of fisetin [Figure 3a]. We also analyzed the effect of pretreatment with omeprazole and fisetin on mucosal lesions [Figure 3b]. According to the results, the contents of MDA and MPO decreased and the activities of SOD and NO increased after the administration of fisetin or omeprazole when compared with the model group ($P < 0.05$). The effects were similar between the high-dose fisetin and omeprazole groups. The contents of MDA and MPO were notably higher in the low-dose fisetin group than that in the omeprazole group ($P < 0.05$). These results show that fisetin suppresses oxidative stress response in ethanol-induced gastric mucosal lesions.

Table 1: The effect of fisetin or omeprazole preadministration on gastric acidity (n=5)

Groups	Dosage (mg/kg)	Gastric acidity (μEq/200 g)
Model	-	3.84±0.61
Omeprazole	40	1.38±0.23**
Low-dose fisetin	25	2.56±0.46*,#
Medium-dose fisetin	50	1.85±0.38*
High-dose fisetin	100	1.45±0.19**

Versus model group, *P<0.05, **P<0.05. versus omeprazole group, #P<0.05

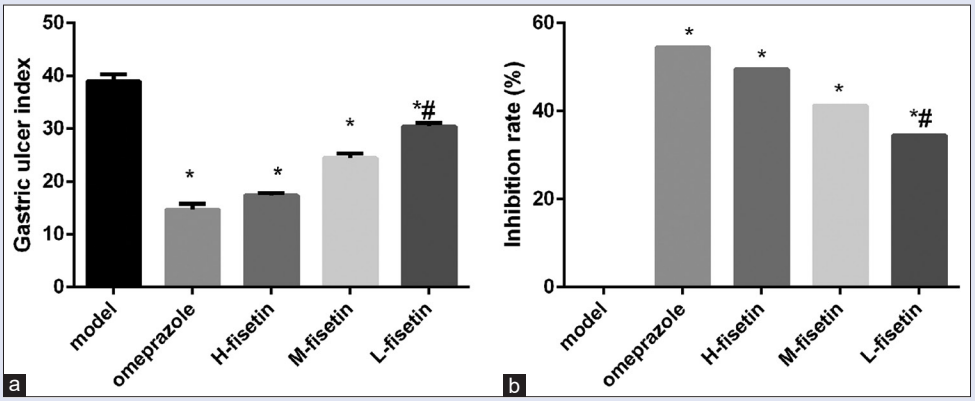

Figure 1: Effects of fisetin or omeprazole pretreatment on gastric mucosal lesion index (a) and inhibition rate (b) in rats ($n = 5$). H-fisetin, high dose of fisetin group; M-fisetin, medium dose of fisetin group; L-fisetin, low dose of fisetin group versus model group, $^{*}P < 0.05$ versus omeprazole group, $^{\#}P < 0.05$

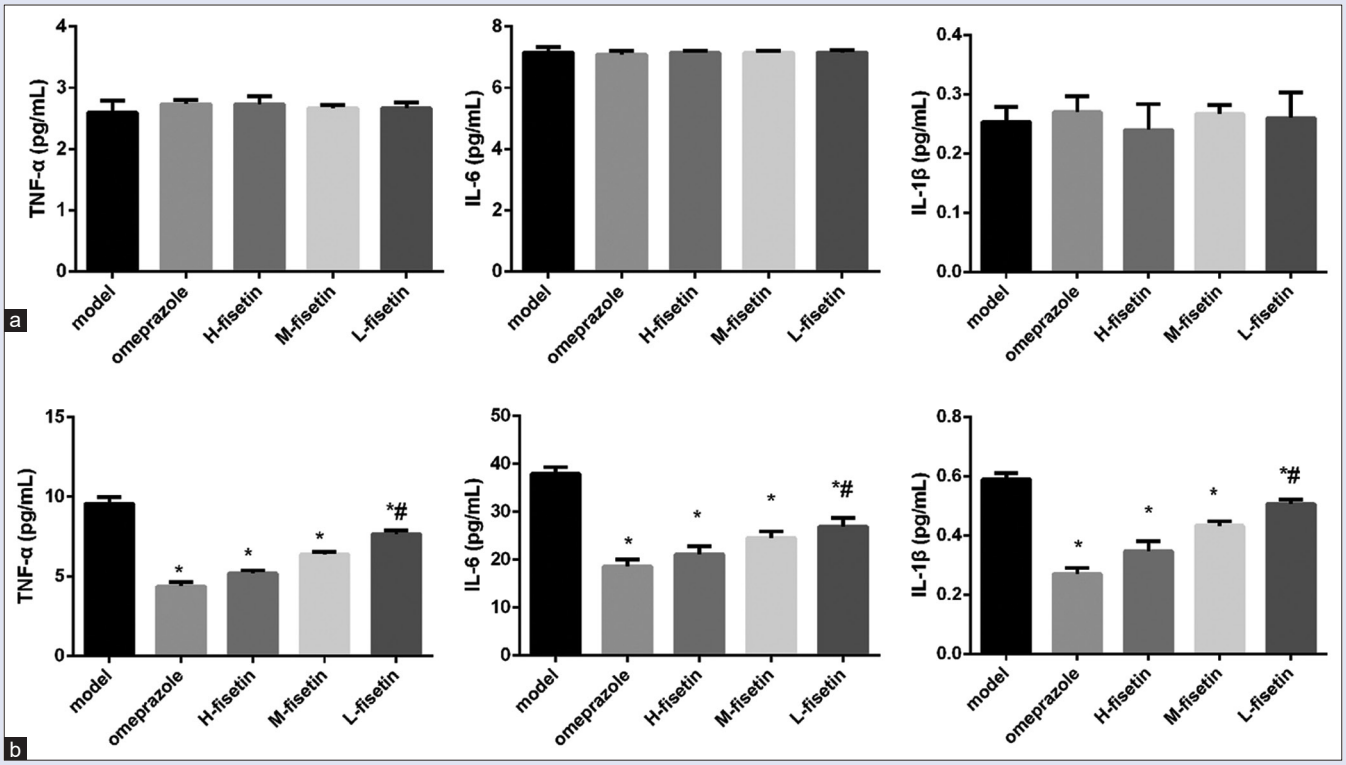

Figure 2: Effect of fisetin or omeprazole pretreatment on expression of tumor necrosis factor-α, interleukin-6, and interleukin-1β in serum. (a) The effect of consecutive administration of omeprazole or fisetin on levels of tumor necrosis factor-α, interleukin-6, and interleukin-1β without ethanol administration; (b) The effect of consecutive administration of omeprazole or fisetin on levels of tumor necrosis factor-α, interleukin-6, and interleukin-1β with ethanol administration versus model group, $^{*}P < 0.05$ versus omeprazole group, $^{\#}P < 0.05$

Fisetin improved gastric mucosal injury

Tissue specimens of the gastric mucosa were histologically analyzed in each group under a microscope [Figure 4]. The surface epithelium showed severe disruption in the gastric mucosa of the model group. In the omeprazole and high-dose fisetin groups, there was no disruption of the surface epithelium. Medium-dose fisetin showed a slight disruption of surface epithelium when compared with the high-dose fisetin group. Low-dose fisetin showed more disruption of the surface epithelium, and the healing was worse when compared with the medium-dose fisetin group.

Fisetin upregulated the expression of peroxisome proliferator-activated receptor-γ and downregulated the expression of nuclear factor-kappa B and p38-mitogen-activated protein kinase in the gastric mucosa

The expression of PPAR-γ, NF-κB, and p38-MAPK was measured in the gastric mucosa by conducting immunohistochemistry [Figure 5]. The expression of PPAR-γ was the lowest in the model group, whereas the expression of PPAR-γ increased gradually with the increase in the

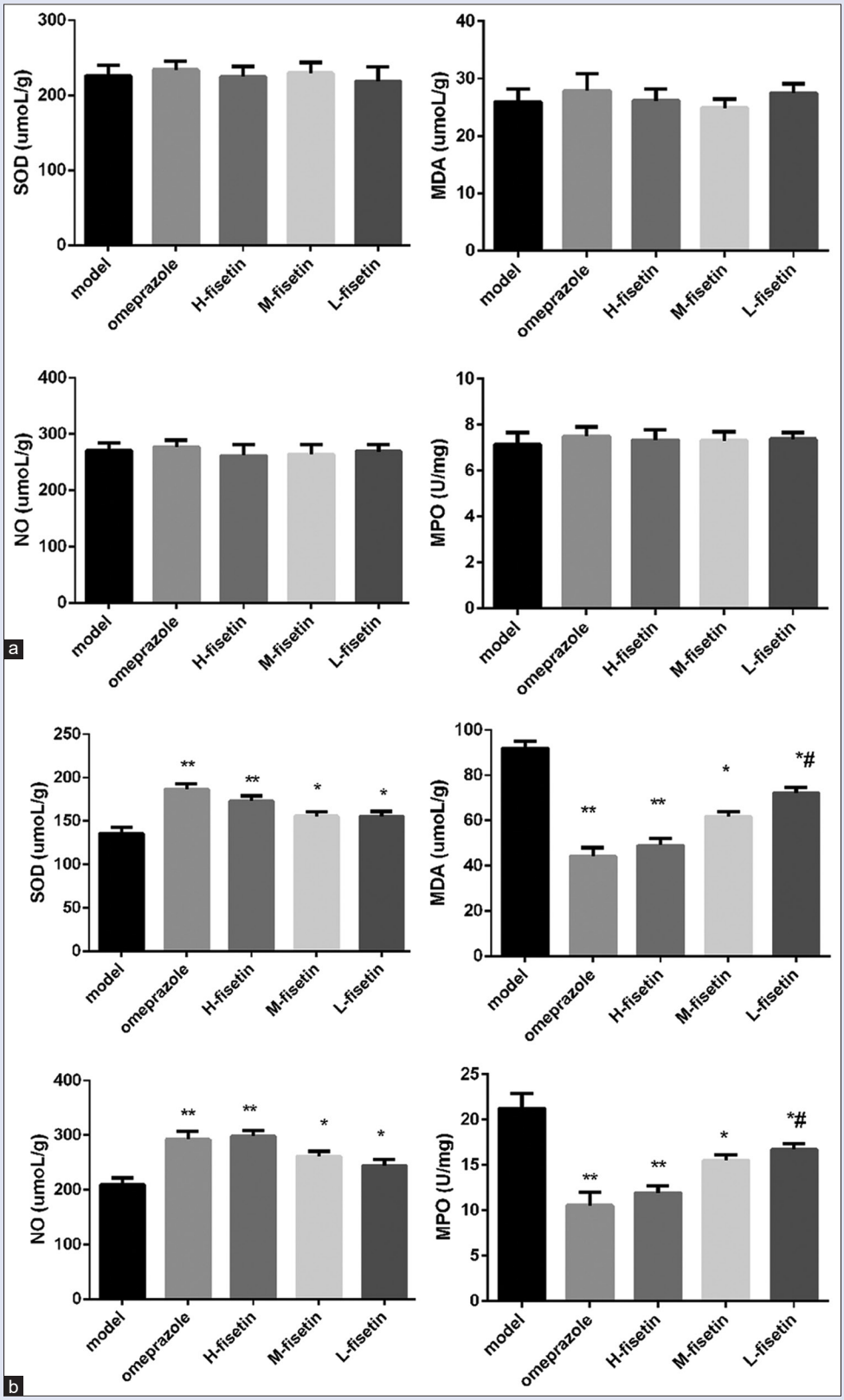

Figure 3: Effect of fisetin or omeprazole pretreatment on contents of superoxide dismutase, MDA, nitric oxide, and myeloperoxidase in serum. (a) The effect of consecutive administration of omeprazole and fisetin on superoxide dismutase, MDA, nitric oxide, and myeloperoxidase expression without ethanol administration; (b) the effect of consecutive administration of omeprazole and fisetin on superoxide dismutase, MDA, nitric oxide, and myeloperoxidase expression with ethanol administration. Data are presented as mean ± standard deviation ($n = 5$) versus model group, $^{*}P < 0.05$, $^{**}P < 0.05$ versus omeprazole group, $^{\#}P < 0.05$

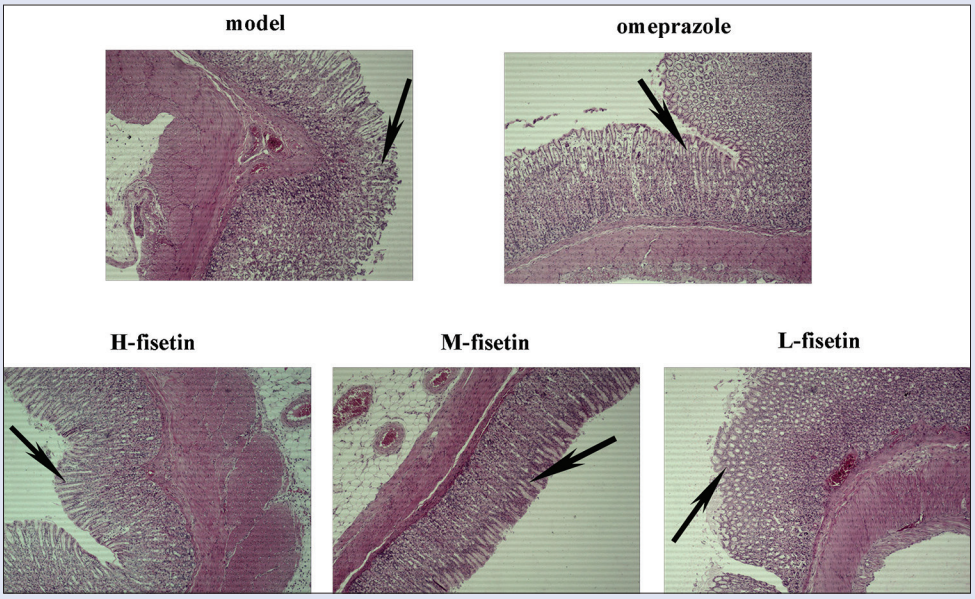

Figure 4: Effect of fisetin or omeprazole pretreatment on gastric mucosal injury (n = 5). Histological damage of the gastric mucosa in each group was observed by H and E, ×40

concentration of fisetin [Figure 5a]. The positive expression of PPAR-γ was similar between the high-dose fisetin and omeprazole groups. In the case of the fisetin and omeprazole groups, the NF-κB and p38-MAPK expression was obviously decreased when compared with the model group [Figure 5b and c]. When compared to other groups, the expression of NF-κB and p38-MAPK was the lowest in the omeprazole group. In this study, the results of Western blot analysis were consistent with the results of immunohistochemistry [Figure 6]. The effects of fisetin on the expression of PPAR-γ, NF-κB, and p38-MAPK were in a dose-dependent manner.

DISCUSSION

In this study, we demonstrated that fisetin pretreatment had a protective effect against ethanol-induced gastric mucosal lesions in rats. Furthermore, a high dose of fisetin and omeprazole had a similar effect. The safety profile of omeprazole is extremely favorable with minor side effects, such as headache and diarrhea; however, there are reports that show that omeprazole induces galactorrhea and delusional ideas.[16,17] Therefore, we consecutively preadministered fisetin in rats without ethanol administration. According to the results, fisetin pretreatment in rats before the exposure to ethanol did not cause any side effects. This suggested that fisetin played an improving role in ethanol-induced gastric mucosal lesions.

Inflammation is a complex process causing damage to the tissue by increasing the secretion of inflammatory cytokines, such as TNF-α, IL-6, and IL-1β. According to a previous study, fisetin shows significant cardioprotective effects against doxorubicin through the inhibition of expression of TNF-α and IL-1β.[5] In this study, pretreatment with fisetin also helped to decrease the levels of TNF-α and IL-1β in ethanol-induced gastric mucosal lesions in rats. TNF-α is one of the most important cytokines that not only promotes the production of IL-6 and IL-1β but also activates the NF-κB pathway.[18,19] NF-κB plays a key role in regulating inflammatory processes by activating transcriptional capacity. It is activated by p38-MAPK and is transferred to the nucleus to activate gene transcription of proteins involved in inflammatory processes.[4,20] Previous studies have shown that fisetin inhibits inflammatory response through the regulation of NF-κB and MAPK activation in rats.[8,10] In this study, consistent with the aforementioned studies, fisetin pretreatment suppressed the expression of NF-κB and p38-MAPK in damaged gastric mucosa. PPAR-γ inhibits the phosphorylation of NF-κB in cells.[21] Similarly, fisetin ameliorated the liver disease of obese mice through the regulation of NF-κB and PPAR-γ expression.[10] The results of this study are consistent with those of previous studies, who show that fisetin blocks inflammation through downregulating the expression of NF-κB pathway.

Oxidative stress is associated with the pathogenesis of inflammatory ulcerative diseases. In addition, the activation of inducible nitric oxide synthase and cyclooxygenase-2 and subsequent upregulation of end products (e.g., NO) destroy the large intestinal mucosa by inhibiting the functioning of the antioxidant system.[22] A previous study showed that the administration of fisetin suppressed the doxorubicin-induced oxido-nitrosative stress, which is reflected by SOD, glutathione, MDA, and NO.[5] To identify the effect of fisetin on antioxidative potential in gastric mucosal lesions, we studied the activity of MDA, SOD, NO, and MPO in different groups. The results showed that the levels of MDA and MPO were reduced and the levels of SOD and NO were increased after fisetin pretreatment in gastric mucosal lesions. Our results were consistent with previous reports.[5,13] Fisetin pretreatment ameliorated the alcohol-induced oxidative stress in rats.

In this study, the mechanism of action of fisetin in attenuating gastric mucosal lesions was investigated by measuring the biomarkers of oxidative stress and inflammatory response. However, it should be emphasized that the mechanism of induction of gastric mucosal lesions by alcohol is very complicated. Many other reasons might lead to the development of gastric lesions, including apoptosis, intercellular junction disorders, and alterations in epithelial transport. Future studies should aim to explore other potential targets of fisetin against gastric mucosal lesions.

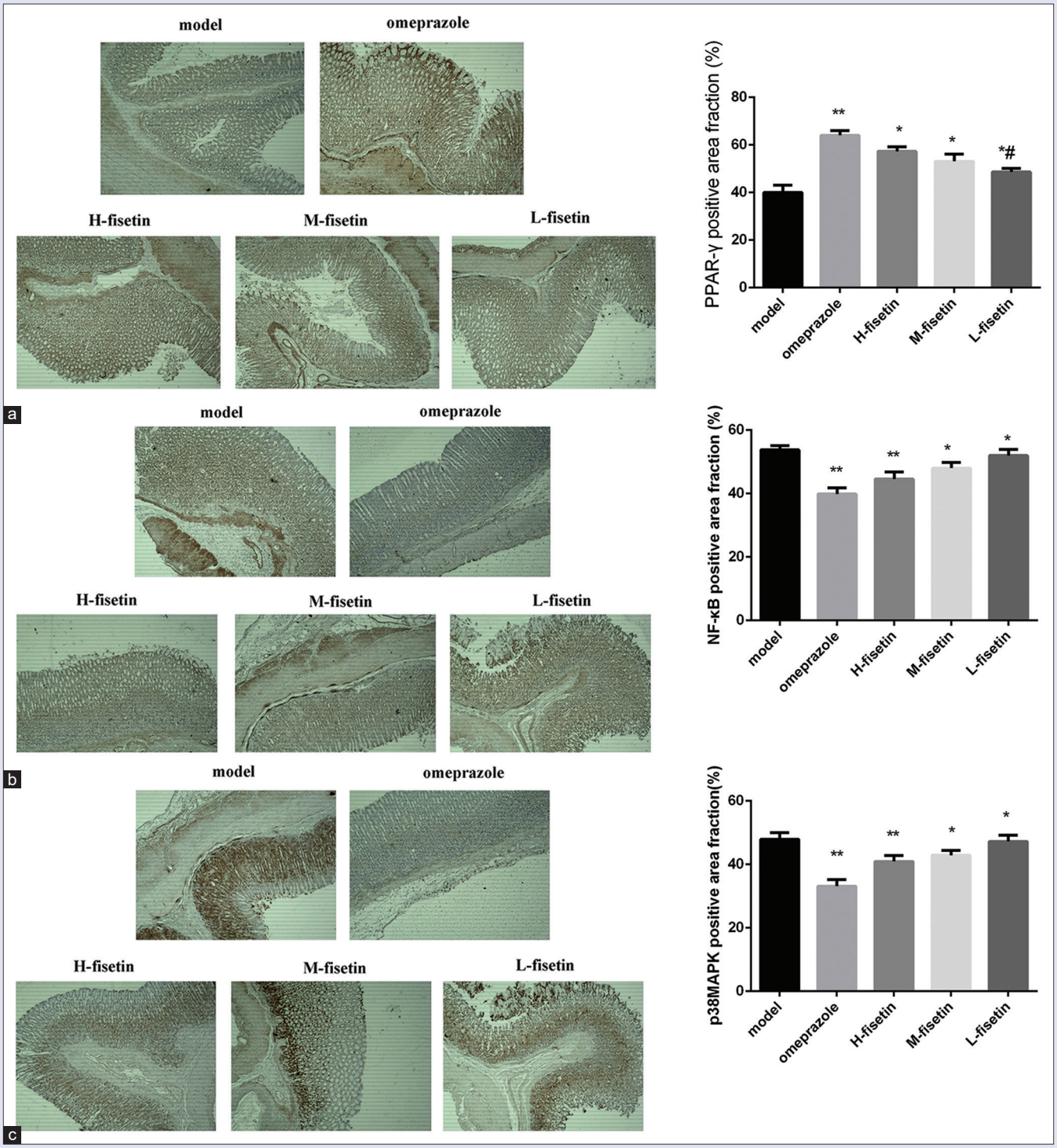

Figure 5: Effect of fisetin or omeprazole pretreatment on peroxisome proliferator-activated receptor-γ (α), nuclear factor-kappa B (b), and p38-MAPK (c) expression in the gastric mucosa ($n = 5$). The expression in the gastric mucosa was analyzed by immunohistochemistry, ×40. Brown represented positive staining cells versus model group, $^{*}P < 0.05$, $^{**}P < 0.05$ versus omeprazole group, $^{\#}P < 0.05$

CONCLUSION

Fisetin pretreatment significantly relieved ethanol-induced gastric mucosal lesions in rats. The possible mechanism of action of fisetin is inhibition of activation of NF-κB pathway and by decreasing oxidative stress in gastric tissue. Fisetin demonstrated a protective role against ethanol-induced formation of gastric mucosal lesions.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

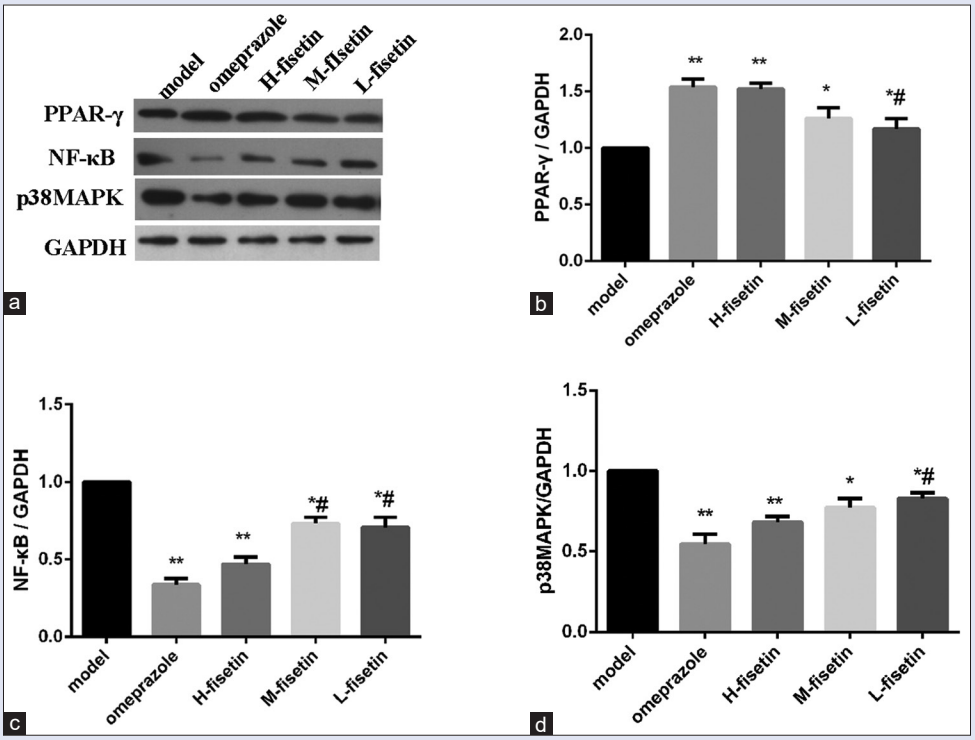

Figure 6: Effect of fisetin or omeprazole pretreatment on peroxisome proliferator-activated receptor-γ, nuclear factor-kappa B, and p38-MAPK expression in the gastric mucosa ($n = 5$). (a) The expression in the gastric mucosa was analyzed by Western blot. (b) The relative expression of PPAR-γ. (c) The relative expression of NF-κB. (d) The relative expression of p38MAPK versus model group, $^{*}P < 0.05$, $^{**}P < 0.05$ versus omeprazole group, $^{\#}P < 0.05$

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