International Immunopharmacology 76 (2019) 105884

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Febuxostat attenuates ulcerative colitis by the inhibition of NF-κB, proinflammatory cytokines, and oxidative stress in mice

Keyvan Amirshahrokhi

Department of Pharmacology, School of Pharmacy, Ardabil University of Medical Sciences, P. O. Box 5618953141, Ardabil, Iran

ARTICLE INFO

Keywords:
Colitis
Febuxostat
Inflammation
NF-κB
Oxidative stress

ABSTRACT

Ulcerative colitis is a chronic inflammatory disorder characterized by oxidative stress and upregulation of proinflammatory mediators in colonic tissue. Febuxostat, a xanthine oxidase inhibitor has been shown to exert anti-inflammatory and antioxidant effects. The aim of this study was to investigate the protective effect of febuxostat against ulcerative colitis, and to elucidate the potential mechanisms involved. Experimental colitis was induced in mice by intrarectal administration of 5% acetic acid. Mice were treated with febuxostat (10 and 20 mg/kg/day, orally) for three days. Results showed that body weight loss, colon shortening, macroscopic damage and histopathological changes of colonic mucosa were reduced in mice treated with febuxostat. Treatment of mice with febuxostat significantly increased the levels of glutathione (GSH) and superoxide dismutase (SOD), and decreased the levels of malondialdehyde (MDA), carbonyl protein, xanthine oxidase, nitric oxide (NO) and myeloperoxidase (MPO) activity of colon tissue compared with those in the acetic acid-induced colitis group. The expression of nuclear factor kappa B (NF-κB) as a key regulator of inflammation in the colonic tissue was decreased by febuxostat. Furthermore treatment with febuxostat significantly reduced the levels of proinflammatory cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and interferon (IFN)-γ, while increased the levels of IL-10 compared with the colitis group. These results suggest that febuxostat is able to decrease the severity of acetic acid-induced colitis by inhibition of oxidative stress and inflammatory responses through NF-κB pathway.

1. Introduction

Ulcerative colitis is a chronic inflammatory gastrointestinal disorder characterized by intestinal inflammation and damage of the intestinal mucosa. The main clinical symptoms are diarrhea, abdominal pain, rectal bleeding, and weight loss. Colorectal cancer is also a serious complication of chronic ulcerative colitis. Although the pathophysiology of ulcerative colitis is not completely understood, it is believed that several factors are involved in the pathogenesis of ulcerative colitis, such as environmental factors, genetic, and intestinal microbiota [1]. One of the important pathogenic factors associated with ulcerative colitis is oxidative stress. Excessive production of reactive oxygen species (ROS) can produce direct oxidative damage to cellular proteins, membrane lipids and DNA, leading to cell death. Sustained production of ROS including the superoxide, hydrogen peroxide and peroxynitrite may diminish endogenous antioxidants that remove these toxic species [2–4].

Chronic inflammation of the intestinal mucosa is the typical feature of ulcerative colitis and results from activation of the immune system. Dysregulation of the immune system results in the production of proinflammatory mediators, such as TNF-α, IL-1, IL-6, cyclooxygenase (COX)-2, prostaglandins and leukotrienes [2,5]. Production of the inflammatory mediators is controlled by NF-κB, which is an important transcription factor that regulates inflammation process in ulcerative colitis [6].

Febuxostat is a non-purine xanthine oxidase inhibitor that was clinically approved for the treatment of gout through reducing uric acid in the body. Febuxostat has been found to exert potent antioxidant and anti-inflammatory effects in several experimental models. It has been suggested that the antioxidant and anti-inflammatory properties of febuxostat may result from its ability to inhibit xanthine oxidase-induced oxidative stress and inflammation. It has been reported that febuxostat reduces doxorubicin-induced cardiotoxicity, cisplatin-induced renal injury, and hepatic and pulmonary inflammation [7–9]. Xanthine oxidase plays an important role in the inflammatory presses and then xanthine oxidase inhibitors can modulate the inflammatory responses. Febuxostat is able to inhibit the production of proinflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-8, TGF-β1, chemokine MCP-1 and COX-2 [10,11].

The most common drugs for the treatment of ulcerative colitis

E-mail address: k.amirshahrokhi@pharmacy.arums.ac.ir.

https://doi.org/10.1016/j.intimp.2019.105884
Received 12 May 2019; Received in revised form 3 September 2019; Accepted 4 September 2019
Available online 06 September 2019

include aminosalicylates, glucocorticoids, immunosuppressive drugs and TNF-α inhibitors; however, these agents have many adverse effects and drug interactions. It has been proposed that drugs that modulate both inflammatory and oxidative mediators may be a good choice for the treatment of ulcerative colitis. Because of the antioxidant and anti-inflammatory effects of febuxostat, we hypothesized that febuxostat may attenuate intestinal mucosal injury in ulcerative colitis. Therefore the present study was conducted to investigate the possible effectiveness and the mechanisms of action of febuxostat on acetic acid-induced colitis in mice.

2. Materials and methods

2.1. Animals

Male BALB/c mice (25–30 g) were used for experiments. Animals were allowed free access to tap water and standard mouse food. Mice were fasted 24 h with free access to water before the induction of colitis. All animal procedures were performed in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals and with the approval of our institutional Ethics Committee.

2.2. Induction of colitis

Acetic acid-induced colitis has long been used as an experimental mouse model of ulcerative colitis. This model exhibits many clinical and pathophysiological features of the human ulcerative colitis [1]. Mice were slightly anesthetized with ether and a flexible catheter was inserted into the rectum and the tip was approximately 4 cm proximal to the anus of mice. Colitis was induced by single intra-colonic instillation of 5% acetic acid (0.1 ml) in 0.9% saline. After administration of acetic acid, animals were suspended by the tail for 60 s to prevent leakage of the solution. In our preliminary experiment, macroscopic and microscopic evaluations of the colonic tissues shows that this model of colitis was completely induced 24 h after administration of acetic acid in mice.

2.3. Experimental design

Mice were randomly divided into four groups with 7 mice in each group; (1) Normal control group, mice received saline solution. (2) Colitis group, mice received acetic acid (0.1 ml, intrarectally). (3) Feb 10 group, mice received acetic acid (0.1 ml, intrarectally) and febuxostat (10 mg/kg/day, orally). (4) Feb 20 group, mice received acetic acid (0.1 ml, intrarectally) and febuxostat (20 mg/kg/day, orally). Febuxostat was suspended in 0.9% saline and administered by gavage once daily for 3 consecutive days. The doses of febuxostat were selected based on previous studies [7,8] and our preliminary experiments. Body weight was measured daily throughout the experiment. On the fourth day after the induction of colitis, mice from each group were anesthetized with ketamine and xylazine and the colon specimens were collected for the following experiments. Colonic tissue samples were frozen in liquid nitrogen and kept at −70 °C until analysis.

2.4. Macroscopic examination

After measurement of colon length, specimens were opened by a longitudinal incision and washed with saline solution. The severity of the colonic damage was evaluated macroscopically using the following scores: 0 = no macroscopic changes; 1 = mucosal erythema only; 2 = mild edema, slight bleeding or small erosions; 3 = moderate edema, bleeding ulcers or erosions; 4 = severe edema, ulceration and tissue necrosis [12].

2.5. Histological examination

The colon specimens were fixed in 10% formalin, embedded in paraffin and sections were stained with hematoxylin and eosin (H&E). The severity of colonic inflammation was examined by an experienced pathologist according to the method previously reported [4]: 0 = no signs of inflammation; 1 = low leukocyte infiltration; 2 = moderate leukocyte infiltration; 3 = high leukocyte infiltration, high vascular density, colonic wall thickening; 4 = transmural infiltration, loss of goblet cells, high vascular density, colonic wall thickening.

2.6. Preparation of colon tissue homogenate

Frozen tissue samples were cut into small pieces and homogenized with a homogenizer (Heidolph, Germany) in ice-cold Tris–HCl buffer (50 mM, pH 7.4, containing protease inhibitor cocktail). The ratio of tissue to buffer was 100 mg/mL. Tissue homogenates were centrifuged at 20,000 ×*g* for 20 min at 4 °C and the supernatants were collected and stored at −70 °C until analysis.

2.7. Assessment of colonic malondialdehyde

The level of MDA in the colonic tissue was determined by the thiobarbituric acid (TBA) method [13]. Briefly, the supernatant was added to a reaction mixture which containing trichloroacetic acid (20% *w/v*) and thiobarbituric acid (0.1 M). The mixture was vortexed and heated at 90 °C for 1 h. After cooling, mixture was centrifuged at 10,000 *g* for 5 min and the supernatant was collected. The absorbance of the supernatant was measured at 532 nm using a microplate reader (BioTek, USA). A calibration curve using tetramethoxypropane as MDA standard was made and the results were expressed as nmol per mg of tissue.

2.8. Assessment of colonic carbonyl proteins

The content of protein carbonyls as a marker for protein oxidation was measured by a colorimetric method [14]. An aliquot of the sample was added to a solution of 2,4-dinitrophenylhydrazine (10 mM) in 2 M HCl and incubated for 1 h at room temperature. After adding trichloroacetic acid (20% w/v), the mixture was centrifuged at 15,000 *g* for 5 min. The supernatant was discarded, and the pellet was washed with 1 ml ethanol-ethyl acetate (1:1 *v/v*). The final pellet was resuspended in guanidine hydrochloride (3 M) in potassium phosphate buffer (10 mM, pH 2.3). The absorbance was measured at 370 nm and carbonyl content was calculated using a molar absorption coefficient of 22 mM^{-1} cm^{-1}. The result was expressed as nmol carbonyl per mg tissue.

2.9. Assessment of colonic glutathione

Colonic GSH level was determined using a modified Ellman's method. An aliquot of the sample was precipitated with trichloroacetic acid (10% w/v), centrifuged (10,000*g*, 4 °C) for 5 min and the supernatant was separated. The resulting supernatant was added to a solution of 5,5′-dithiobis-2-nitrobenzoic acid (1 mM) in phosphate buffer (0.2 M, pH 7.8, 1 mM EDTA). The absorbance was measured at 412 nm using a microplate reader and the GSH content was calculated using a glutathione standard curve. The result was expressed as μmol GSH per mg tissue.

2.10. Assessment of colonic superoxide dismutase and xanthine oxidase

The activity of SOD and xanthine oxidase enzymes in the colonic tissue was measured using a SOD assay kit (Cayman Chemical) and xanthine oxidase assay Kit (Cayman Chemical) according to the manufacturer's protocol. The values were expressed as U/mg tissue.

2.11. Assessment of colonic nitric oxide

Colonic tissue supernatants were used for the measurement of nitrite and nitrate concentrations as an index of NO production. The level of NO was measured using a Nitric Oxide Assay Kit (Cayman Chemical) and the result was expressed as nmol/mg tissue.

2.12. Assessment of colonic myeloperoxidase activity

In order to assess granulocyte infiltration into the colon tissue, the activity of MPO was determined by measuring the hydrogen peroxide–dependent oxidation of tetramethylbenzidine (TMB). In a 96-well microplate, 50 μL of the supernatant was pipetted and mixed with 50 μL of TMB solution (15 mM). Reaction was initiated by addition of H_2O_2 (25 mM) diluted in phosphate buffer (50 mM, pH 5.4). The rate of change in absorbance was measured at 370 nm using a microplate reader. One unit of MPO activity was defined as the quantity of enzyme able to convert 1 μmol of H_2O_2 to water in one minute at room temperature. The result was expressed as mU MPO/mg tissue.

2.13. Assessment of colonic inflammatory cytokines

The levels of inflammatory cytokines TNF-α, IL-1β, IL-6, IL-10 and IFN-γ in the colonic tissue were measured by using mouse ELISA kits from eBioscience according to the manufacturer's instructions. The results were expressed as pg/mg tissue.

2.14. Quantitative real-time PCR analysis of NF-κB

Total RNA was extracted from colonic tissue samples using TRIzol Reagent (Roche). The RNA samples were treated with DNase I to remove DNA contamination. The first-strand cDNA was synthesized using a First-Strand cDNA Synthesis Kit (Thermo Scientific, USA) according to the manufacturer's instructions. The cDNA was used as the template for SYBR Green-based quantitative real-time PCR using the LightCycler (Roche). The thermocycling conditions were as follows: an initial denaturation at 94 °C for 5 min, followed by 40 cycles of denaturing at 94 °C for 10 s, annealing at 59 °C for 15 s, and a final extension at 72 °C for 15 s. Target gene expression was normalized to GAPDH. The primer sequences were as follows: GAPDH forward, TGAAGCAGGCATCTGA GGG and reverse, CGAAGGTGGAAGAGTGGGAG; NF-κB forward, ACC TTTGCTGGAAACACACC and reverse, ATGGCCTCGGAAGTTTCTTT.

2.15. Statistical analysis

Data were presented as means ± standard error (SEM). Statistical significance between groups was evaluated by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Macroscopic and histopathological scores were analyzed by non-parametric Kruskal–Wallis test followed by Dunn's post hoc test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of febuxostat on acute acetic acid-induced colitis in mice

Administration of acetic acid caused a significant body weight loss (Fig. 1A), colon shortening (Fig. 1B) and reduced survival rate (Fig. 1C). These symptoms indicate sever intestinal inflammatory damage in mice of the colitis group. Treatment of mice with febuxostat at the dose of 20 mg/kg/day significantly improved body weight loss ($P < 0.01$) and colon shortening ($P < 0.01$) and increased survival rate (30%) compared to the colitis group.

3.2. Effects of febuxostat against acetic acid-induced macroscopic changes in the colon of mice

The colonic tissue evaluation of mice treated with saline in the control group showed a normal appearance of the colonic mucosa without any damage (Fig. 2A). Macroscopic examination of the colonic mucosa in the colitis group showed that intrarectal administration of acetic acid produced marked edema, hyperemia, hemorrhage and extensive mucosal ulcerations. In addition, mice in the colitis group showed a significant increase in the macroscopic score as compared to the other groups (Fig. 2B). Treatment of mice with febuxostat (20 mg/kg/day) significantly reduced colonic mucosal damage and the macroscopic score as compared to the colitis group. Statistically, no significant change was observed in the macroscopic appearance in mice treated with low dose febuxostat (10 mg/kg/day) compared with colitis group.

3.3. Effects of febuxostat against acetic acid-induced pathological changes in the colon of mice

Histopathological changes of the colonic tissues were evaluated by H&E staining to estimate the score and level of inflammatory damage in the colon (Fig. 3A). Mice in control group showed normal colonic histological architecture with no inflammation or leukocyte infiltration. In contrast, in colitis group administration of acetic acid induced a significant inflammatory response characterized by severe leukocyte infiltration, submucosal edema, high vascular density and loss of goblet cells. Treatment of mice with febuxostat (20 mg/kg/day) significantly reduced these histopathological changes and preserved intestinal mucosal epithelial integrity. As shown in Fig. 3B the total histopathological score was significantly elevated in the colitis group as compared with the control group. However, treatment with febuxostat (20 mg/kg/day) showed remarkably ameliorated colonic inflammatory damage and significantly decreased histological scores.

3.4. Effects of febuxostat on the levels of MDA and protein carbonyl

Oxidative stress was evaluated by measuring the levels of MDA as a marker of lipid peroxidation and protein carbonyl content as an indicator of protein oxidation in the colonic tissue (Table 1). Acetic acid administration remarkably increased the content of MDA and protein carbonyl in colon tissue. Treatment with febuxostat (10 and 20 mg/kg) significantly reduced the levels of MDA ($P < 0.001$ for 20 mg/kg and $P = 0.018$ for 10 mg/kg) and protein carbonyl ($P < 0.01$for 20 mg/kg and $P = 0.045$ for 10 mg/kg) compared to those in the colitis group.

3.5. Effects of febuxostat on the levels of GSH and SOD in the colon tissue

Oxidative stress induced by acetic acid leads to the depletion of GSH and SOD as endogenous antioxidants in the colon tissue when compared with those in the control group (Fig. 4A and B). Treatment with febuxostat (20 mg/kg/day), significantly restored the levels of GSH ($P = 0.001$) and SOD ($P < 0.001$) in colon tissue as compared with those in the colitis group. Consequently, febuxostat at dose of 20 mg/kg/day improved colon antioxidant status in the acetic acid-induced colitis model.

3.6. Effects of febuxostat on the levels of xanthine oxidase and NO in the colon tissue

Intrarectal administration of acetic acid resulted in significant increase in xanthine oxidase concentration ($P < 0.01$) and NO production ($P < 0.05$) in the colon tissue of mice as compared to the control group (Fig. 5A and B). Treatment with febuxostat (10 and 20 mg/kg) produced a significant and dose dependent reduction ($P < 0.01$ for 20 mg/kg and $P = 0.04$ for 10 mg/kg) in xanthine oxidase activity as

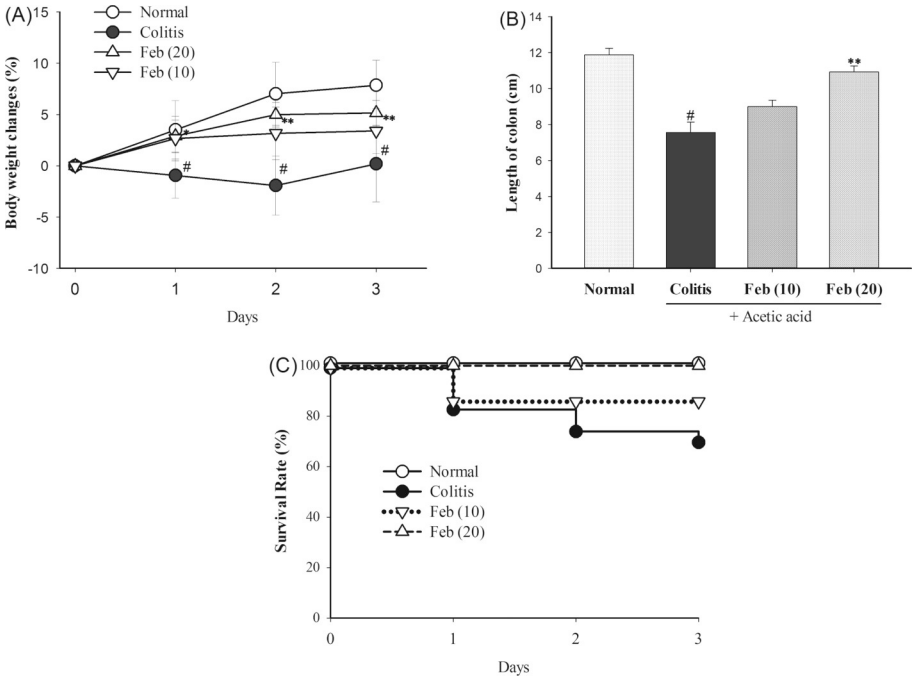

Fig. 1. Effects of febuxostat treatment (10 and 20 mg/kg/day) on body weight changes (A), colon length (B) and survival rate (C) in mice with acetic acid-induced colitis. Febuxostat at a dose of 20 mg/kg/day significantly attenuated acetic acid-induced weight loss and colon shortening and increased survival rate in mice. The survival rate in normal and Feb (20) groups was 100%. Data are means ± SEM. # $P < 0.001$ compared with normal group; * $P < 0.05$, ** $P < 0.01$ compared with colitis group.

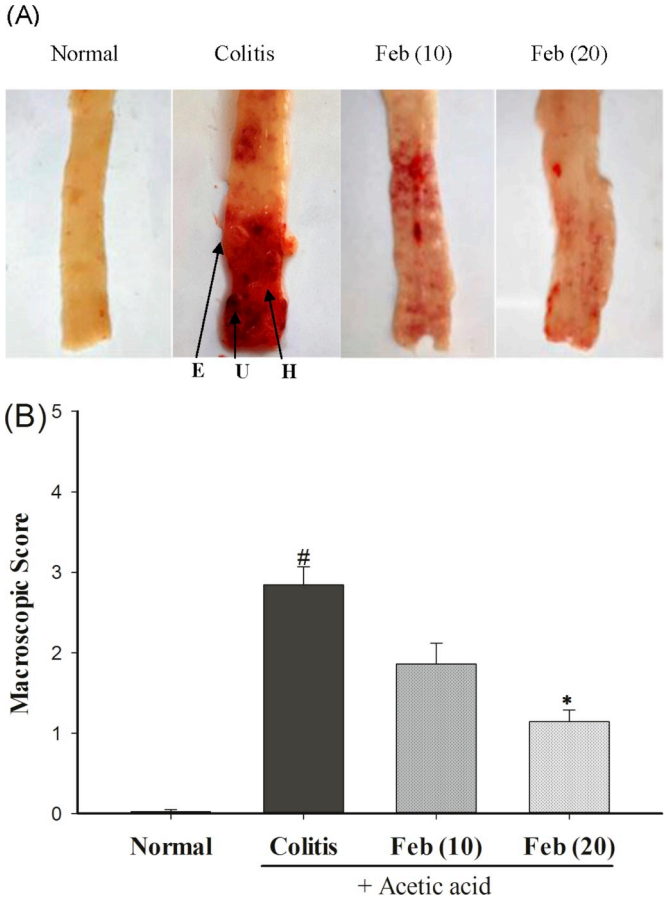

Fig. 2. (A) Representative macroscopic appearance of the colonic mucosa in the experimental groups; (a) normal, (b) colitis, (c) febuxostat (10 mg/kg/day) and (d) febuxostat (20 mg/kg/day). E: edema; H: hemorrhage; U: ulcerations. (B) Effects of febuxostat treatment (10 and 20 mg/kg/day) on the macroscopic score of colonic mucosal damage induced by acetic acid. Data were analyzed by non-parametric Kruskal–Wallis test. Data are means ± SEM. # $P < 0.05$ compared with normal group; * $P < 0.05$ compared with colitis group.

compared to the colitis group. Febuxostat only at dose of 20 mg/kg/day significantly reduced the formation of NO ($P < 0.05$) in the colon tissue of mice as compared to the colitis group.

3.7. Effect of febuxostat on the MPO activity in the colon tissue

MPO activity was measured to evaluate the degree of neutrophil infiltration in the colon tissue as a marker of colonic inflammation (Fig. 6). Administration of acetic acid significantly increased the MPO activity as compared with the control group ($P < 0.001$). Whereas, treatment with febuxostat (20 mg/kg/day), significantly reduced the activity of MPO in colon tissue as compared to the colitis group ($P < 0.001$). This result was consistent with the finding of histological changes.

3.8. Effects of febuxostat on the colonic inflammatory cytokines

The production of inflammatory cytokines during the development of colitis was evaluated by measuring the levels of TNF-α, IL-1β, IL-6, IL-10 and IFN-γ in the colonic tissues (Fig. 7). Administration of acetic acid caused significant increase in the colonic levels of proinflammatory cytokines TNF-α, IL-1β, IL-6, and IFN-γ, while decreased the level of anti-inflammatory cytokine IL-10 as compared with those in the normal group. Treatment of mice with febuxostat (20 mg/kg/day) significantly decreased the production of TNF-α, IL-1β, IL-6, and IFN-γ, while increased the production of IL-10 as compared with those in colitis group. As shown in Fig. 7, the effect of low dose febuxostat (10 mg/kg/day) on the colonic inflammatory cytokines was not statistically significant when compared with the colitis group. These findings suggest that febuxostat (20 mg/kg/day) may reduce colonic inflammation by influencing the balance between proinflammatory and anti-inflammatory cytokines.

3.9. Effect of febuxostat on the expression of NF-κB in the colon tissue

Analysis the gene expression of NF-κB mRNA by real-time PCR showed that the level of NF-κB in the colitis group was significantly increased when compared with normal control group ($P = 0.001$) (Fig. 8). Treatment with febuxostat (20 mg/kg/day) caused significant reduction in NF-κB mRNA expression ($P < 0.01$) as compared to the

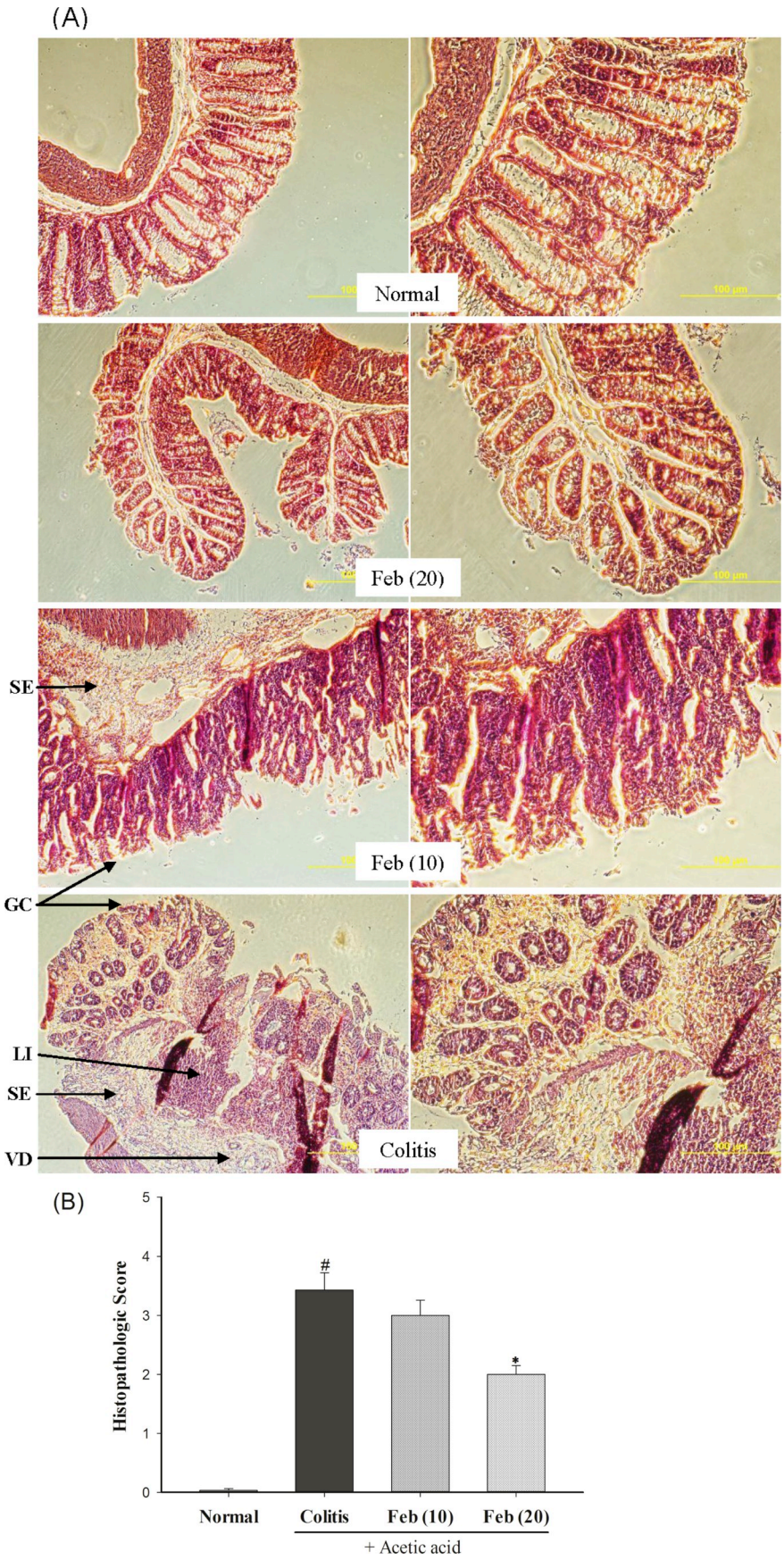

Fig. 3. (A) Histological images of the colonic tissue in the experimental groups; (a) normal, (b) colitis, (c) febuxostat (10 mg/kg/day) and (d) febuxostat (20 mg/kg/day). LI: leukocyte infiltration; SE: submucosal edema; VD: vascular density; GC: goblet cells loss. (B) Effects of febuxostat treatment on the histopathological score of colon tissue in mice with acetic acid-induced colitis. Treatment with febuxostat (20 mg/kg/day) significantly reduced the histopathological alterations induced by acetic acid. Data were analyzed by non-parametric Kruskal–Wallis test. Data are means ± SEM. # $P < 0.05$ compared with normal group; * $P < 0.05$ compared with colitis group.

Table 1
Effects of febuxostat treatment on the levels of MDA and protein carbonyl in the colonic tissue of mice.

Groups	MDA (nM/mg tissue)	Protein carbonyl (nM/mg tissue)
Normal	12.7 ± 1.3	0.26 ± 0.04
Colitis	41.9 ± 3.8$^{\#\#}$	0.58 ± 0.05$^{\#}$
Feb (10 mg/kg)	27.3 ± 4.3*	0.34 ± 0.07*
Feb (20 mg/kg)	17.9 ± 1.8***	0.26 ± 0.04**

Data are means ± SEM.
$^{\#}$ $P < 0.01$.
$^{\#\#}$ $P < 0.001$ compared with normal group.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$ compared with colitis group.

colitis group. However the effect of low dose febuxostat (10 mg/kg/day) on the level of NF-κB was not statistically significant ($P = 0.08$).

4. Discussion

Ulcerative colitis is a type of inflammatory bowel disease (IBD) characterized by ulcers and severe inflammation in the inner lining of the colon. It is demonstrated that oxidative and inflammatory mediators play a vital role in the pathogenesis of ulcerative colitis. Acetic acid-induced colitis is used as an experimental model of ulcerative colitis in mice and shows many clinical symptoms similar to human ulcerative colitis.

The present study revealed that, treatment with febuxostat (20 mg/kg/day, orally) significantly ameliorated colonic mucosal damage induced by acetic acid. Histopathological examination showed that administration of acetic acid induced a significant inflammatory response characterized by severe leukocyte infiltration, submucosal edema, high vascular density and loss of goblet cells. Treatment of mice with febuxostat (20 mg/kg/day) significantly reduced these histological alterations and pathologic score in the colonic tissue.

It has been well known that reactive oxygen species are involved in the initiation and progression of ulcerative colitis [15]. Oxygen derived reactive molecules such as superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl free radical ($HO^{\bullet}$) and peroxinitrite ($ONOO^-$) attack to cellular membrane lipids, proteins and nucleic acids and eventually leading to cell death. Free radicals induce lipid peroxidation by abstracting a hydrogen atom from polyunsaturated fatty acids in cell membranes. Lipid peroxidation is considered as an important step in the mechanism of acetic acid-induced colonic mucosal injury. MDA is the end-product of lipid peroxidation and commonly used as a biomarker of lipid peroxidation process. The lipid peroxidation products such as unsaturated aldehydes and MDA are able to react with multiple biomolecules such as proteins and nucleic acids [2,13]. In the development of colitis, accumulated ROS can oxidize proteins and lead to the generation of protein carbonyl derivatives [16]. In the present study it has been shown that acetic acid administration significantly increased the levels of MDA and carbonyl proteins as biomarkers of oxidative damage in the colon tissue. However, treatment with febuxostat considerably decreased MDA and carbonyl proteins concentrations. Inhibition of lipid peroxidation and protein carbonylation by febuxostat could prevent oxidative induced damage to the colonic tissue.

The intestinal cells possess more efficient antioxidant defense mechanisms to detoxify the harmful effects of oxidants. Reduced glutathione (GSH) is considered as an important and essential endogenous antioxidant in the cells. It occurs abundantly in reduced form in tissues and plays an important role in several physiological processes, including conjugation of metabolites, detoxification of xenobiotics, regulation of cell signaling pathways and DNA repair mechanisms [17–19]. It has been shown that glutathione supplementation and increase the level of GSH improve oxidative colonic damage in an experimental colitis [20]. SOD is the main antioxidant enzyme responsible for scavenging superoxide anions. SOD exerts a crucial protective effect in the colonic tissue against oxidative induced injury and inflammation. Several studies have shown that the levels of GSH and SOD in the colonic tissue decreased in acetic acid-induced colitis [2,4,21]. The present study also showed that administration of acetic acid significantly decreased the level of GSH and SOD activity in the colonic tissue. It can be suggested that the reduction in the colonic content of GSH and SOD may result from consumption of these antioxidants during the scavenging of reactive oxygen radicals in the colon. Depletion of GSH and SOD leads to the accumulation of ROS which eventually result in cell damage and death. Treatment with febuxostat (20 mg/kg/day) significantly restored the levels of GSH and SOD in the colon tissue. These findings demonstrate the antioxidant activity of febuxostat and may explain its protective effect against acetic acid-induced colitis.

Nitric oxide is an important biological mediator and involved in many physiological functions of the gastrointestinal tract. However, excessive production of NO can be toxic to cells and has been implicated in the pathogenesis of ulcerative colitis. NO may react with superoxide anion ($O_2^{\bullet -}$) to generate peroxynitrite ($ONOO^-$) and subsequently peroxynitrous acid (ONOOH). Peroxynitrite and its conjugate acid are highly toxic oxidant that can cause cell death by lipid peroxidation, protein oxidation, enzymes inactivation, DNA damage, and disruption of cellular signaling pathways [16,22]. Excessive NO production by induce nitric oxide synthase (iNOS) leads to colonic mucosal damage through activation of NF-κB signaling pathway [23]. Xanthine

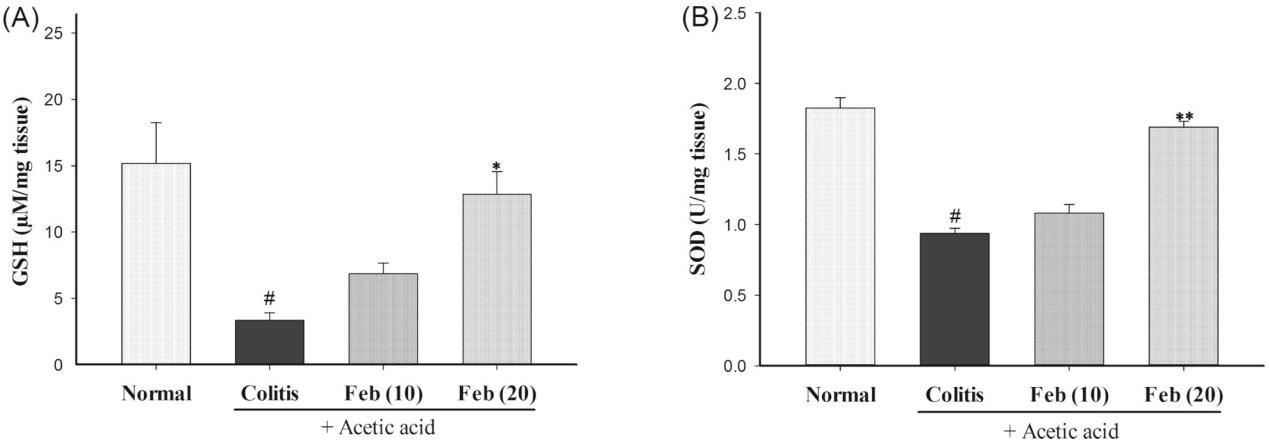

Fig. 4. Effects of febuxostat treatment (10 and 20 mg/kg/day) on the levels of GSH (A) and SOD (B) in the colonic tissue. Data are means ± SEM. # $P < 0.001$ compared with normal group; * $P < 0.01$, ** $P < 0.001$ compared with colitis group.

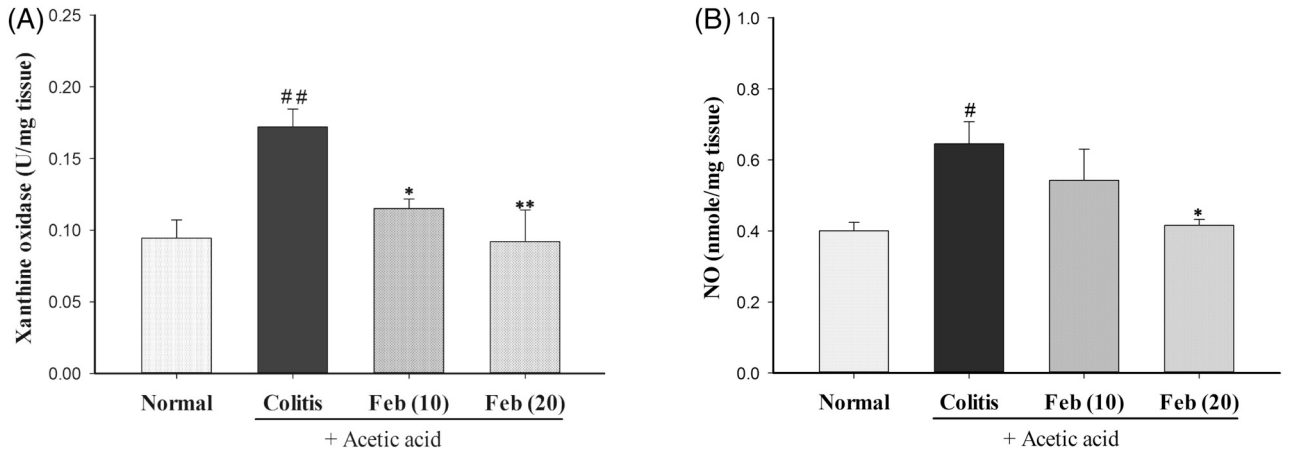

Fig. 5. Effects of febuxostat treatment (10 and 20 mg/kg/day) on the levels of xanthine oxidase (A) and NO (B) in the colonic tissue. Data are means ± SEM. # $P < 0.05$, ## $P < 0.01$ compared with normal group; * $P < 0.05$, ** $P < 0.01$ compared with colitis group.

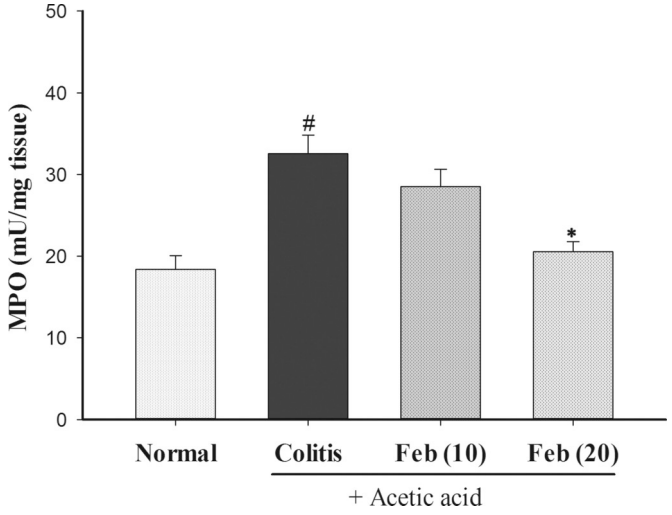

Fig. 6. Effects of febuxostat treatment (10 and 20 mg/kg/day) on the levels of MPO in the colonic tissue. Data are means ± SEM. # $P < 0.001$ compared with normal group; * $P < 0.001$ compared with colitis group.

oxidase is one of the major enzymes to generate superoxide anion when it catalyzes the oxidation of hypoxanthine to xanthine and subsequently to uric acid. This enzyme plays an important role in the progression of oxidative and inflammatory disorders such as IBD. Xanthine oxidase enzyme is found normally in the intestinal tissue, and its activity is increased in pathological conditions. Toxic oxygen radicals produced by activated xanthine oxidase can trigger intestinal tissue damage [24]. It has also been shown that the activity of xanthine oxidase can be induced by activated neutrophils and inflammatory cytokines, such as TNF-α, during ulcerative colitis. It has been demonstrated that xanthine oxidase inhibition may be a potential treatment for ROS-related diseases including IBD [24–26]. Consistent with previous findings, the results of the present study revealed that the levels of xanthine oxidase and NO in colon tissue were significantly increased during colitis. Treatment with febuxostat (20 mg/kg/day) significantly decreased xanthine oxidase activity and NO overproduction in the colon tissue. These findings suggest that febuxostat may ameliorate acetic acid-induced colitis by inhibiting xanthine oxidase and NO-mediated colonic mucosal damage.

Neutrophil infiltration into the colonic mucosa plays an important role in the progression of colonic inflammation and injury. During ulcerative colitis, activated neutrophils migrate from the circulation into the colonic tissue, where they release inflammatory mediators and ROS. MPO as a biomarker of neutrophil infiltration has been widely used to assess colonic inflammation [2,3]. MPO catalyzes the oxidation of the chloride ion with hydrogen peroxide to form hypochlorous acid (HClO), which is toxic to cells [19]. The present study showed that acetic acid administration significantly increased the MPO activity in the colonic tissues. Febuxostat (20 mg/kg/day) was able to decrease MPO activity and neutrophil infiltration into the colonic mucosa as indicated by the histological findings. The ability of febuxostat to inhibit neutrophilic inflammatory response, MPO activity and xanthine oxidoreductase-mediated NO formation has been shown in other studies [9,27].

Ulcerative colitis is associated with inflammatory responses which increase the production of pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6 and IFN-γ leading to tissue damage and progression of the disease [28,29]. TNF-α is a primary inflammatory cytokine and plays a fundamental role in the pathogenesis of IBD. Anti-TNF-α agents have been commonly used to treat patients with ulcerative colitis [30]. TNF-α is thought to be produced primarily by macrophages in response to various pathological processes and has multiple biological activities involved in colitis. TNF-α stimulates immune cells to produce and release of other cytokines, chemokines, inflammatory lipid prostaglandin E2, and reactive oxygen and nitrogen species [2,13]. IL-1β causes an increase in intestinal epithelial permeability and stimulates the recruitment of neutrophils to the inflamed colonic tissue [5]. IL-6 is a potent pleiotropic cytokine that acts as a regulator of the adaptive immune response in IBD. IL-6 induces the expression of acute phase proteins during acute inflammation. It also regulates the differentiation of the immune cells including macrophages and T cells. Moreover, IL-6 has been involved in the pathogenesis of colorectal cancer [31]. Inhibition of IL-6 signaling pathway prevents T cell-mediated colitis [32]. It has been shown that the levels of IL-1β and IL-6 in serum and colon tissue correlate with the severity of intestinal inflammation and disease activity [4,28]. Another pleiotropic proinflammatory cytokine, IFN-γ is produced by activated T lymphocytes and thought to be involved in the disruption of intestinal epithelial barrier function. It has been shown that IFN-γ is increased in human IBD and inhibition of IFN-γ significantly ameliorates chronic intestinal inflammation in murine models of colitis [33,34]. IL-10 is an important anti-inflammatory cytokine and plays essential role in controlling immune responses in the intestinal mucosa. IL-10 inhibits the synthesis of proinflammatory cytokines such as TNF-α, IL-1β and IFN-γ and blocks NF-κB activity. It has been reported that IL-10-deficient mice develop enterocolitis, and administration of IL-10-producing *Lactococcus lactis* protects mice from colitis. IL-10 also reduces colitis severity in mice through inhibition of macrophage NO and ROS production [35–37].

In the present study administration of acetic acid caused a significant increase in the colonic levels of proinflammatory cytokines

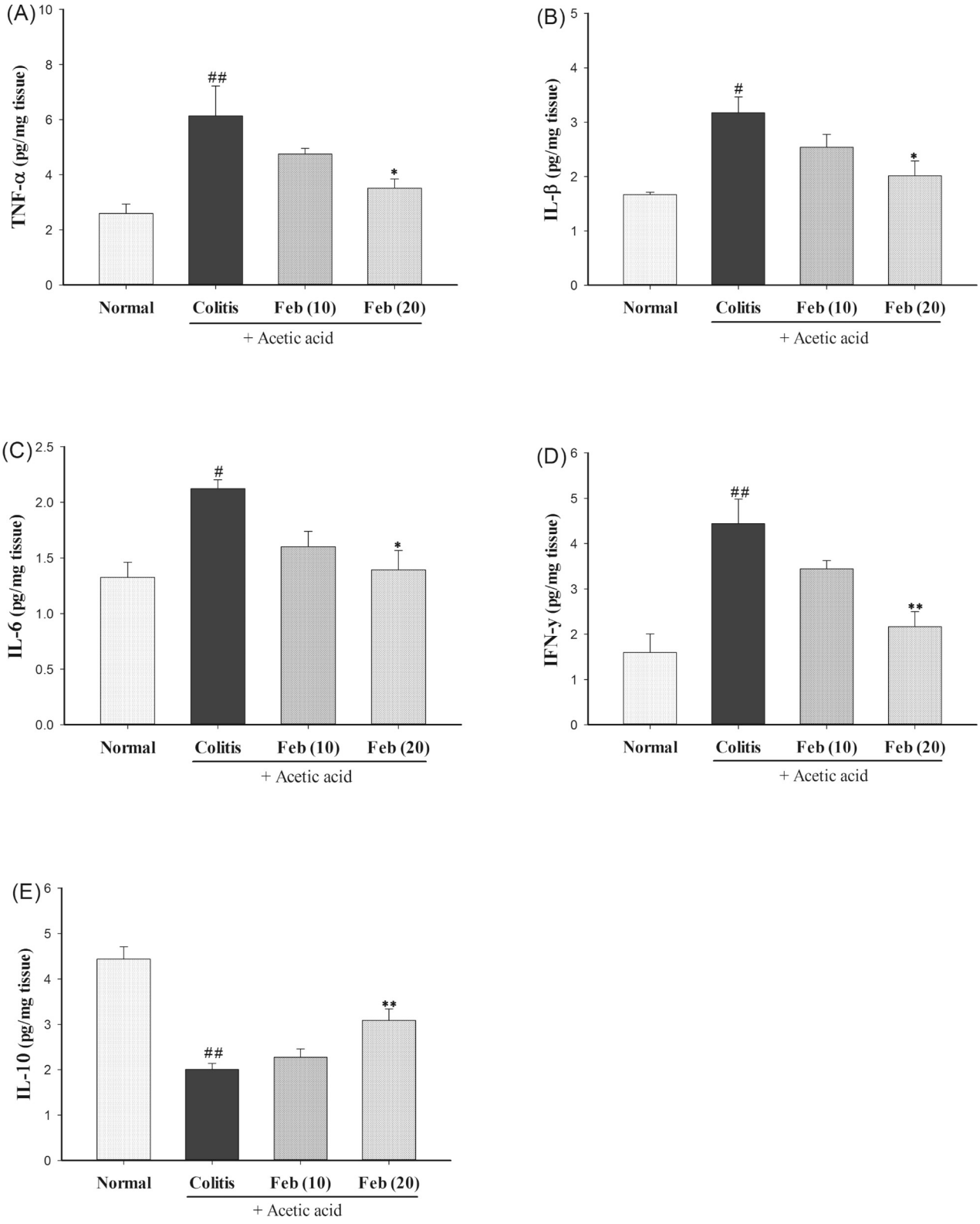

Fig. 7. Effects of febuxostat treatment (10 and 20 mg/kg/day) on the production of cytokines TNF-α (A), IL-1β (B), IL-6 (C), IFN-γ (D) and IL-10 (E) in the colonic tissue of mice. Data are means ± SEM. # $P < 0.05$, ## $P < 0.01$ compared with normal group; * $P < 0.05$, ** $P < 0.01$ compared with colitis group.

TNF-α, IL-1β, IL-6, and IFN-γ, while decreased IL-10 level. Acetic acid-induced proinflammatory cytokines were reduced by febuxostat (20 mg/kg/day) treatment. However, the level of anti-inflammatory cytokine IL-10 was elevated in response to febuxostat (20 mg/kg/day). These findings demonstrated that febuxostat exerts a remarkable anti-inflammatory property. In fact, febuxostat attenuates acetic acid-induced colitis in mice by affecting the balance between anti-inflammatory and proinflammatory cytokines.

NF-κB is a key regulator of inflammation and has an important role in the pathogenesis of ulcerative colitis. It has been well-known that the expression and activation of NF-κB is markedly induced in patients with IBD. NF-κB signaling pathway can be activated by various stimuli,

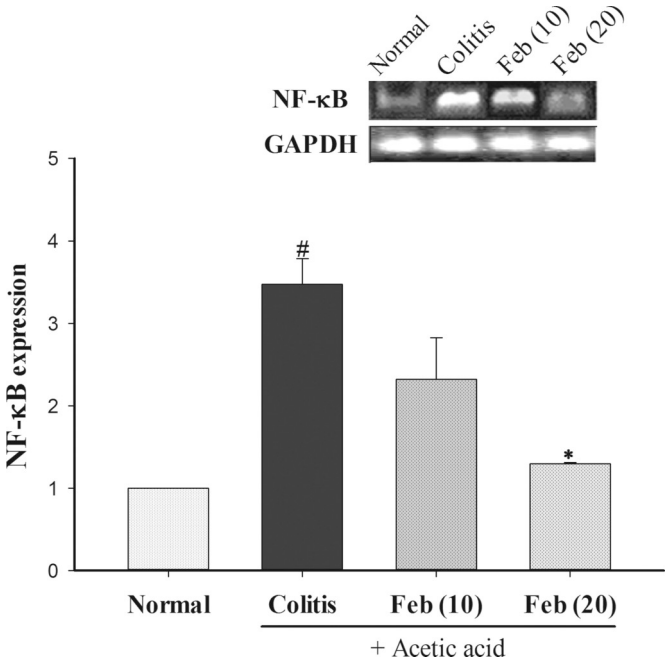

Fig. 8. Effects of febuxostat treatment (10 and 20 mg/kg/day) on the expression of NF-κB mRNA in the colonic tissue of mice with acetic acid-induced colitis. The mRNA levels were normalized by the expression of GAPDH. Data are means ± SEM of three experiments. # $P = 0.001$ compared with normal group; * $P < 0.01$ compared with colitis group.

including cytokines, lipopolysaccharide and ROS. Activation of NF-κB induces the production of inflammatory cytokines (TNF-α, IL-1β, IFN-γ and IL-6), chemokines, and enzymes (COX-2) leading to inflammation and tissue damage. Immunosuppressive and anti-inflammatory drugs that are widely used in the treatment of IBD are known to mediate their therapeutic effects at least partly by inhibition of NF-κB activity [38–40]. Indeed, NF-κB pathway inhibition is an attractive potential therapeutic strategy in IBD. The results of the present study showed that, acetic acid administration induced NF-κB expression and treatment with febuxostat (20 mg/kg/day) significantly reduced acetic acid-induced NF-κB activation in the colon tissue. It can be suggested that NF-κB inhibition is the key molecular mechanism for the anti-inflammatory effects of febuxostat in colitis.

In conclusion, this work indicates that febuxostat treatment is able to attenuate acetic acid-induced colitis in mice by inhibition of inflammatory and oxidative stress mediators.

Declaration of competing interest

There is no conflict of interest.

References

[1] D.C. Rubin, A. Shaker, M.S. Levin, Chronic intestinal inflammation: inflammatory bowel disease and colitis-associated colon cancer, Front. Immunol. 3 (2012) 107 May 8.
[2] K. Amirshahrokhi, S. Bohlooli, M.M. Chinifroush, The effect of methylsulfonylmethane on the experimental colitis in the rat, Toxicol. Appl. Pharmacol. 253 (3) (2011) 197–202 Jun 15.
[3] M. Ferrat, H. Moulahoum, B.M.A. Boumaza, S. Mouzaoui, A. Périanin, B. Djerdjouri, Gadolinium chloride attenuates acetic acid-evoked colitis in mice by reducing neutrophil infiltration and pro-oxidative enzyme activity, Naunyn Schmiedeberg's Arch. Pharmacol. 392 (3) (2019) 299–311 Mar.
[4] X. Niu, H. Zhang, W. Li, Y. Wang, Q. Mu, X. Wang, Z. He, H. Yao, Protective effect of cavidine on acetic acid-induced murine colitis via regulating antioxidant, cytokine profile and NF-κB signal transduction pathways, Chem. Biol. Interact. 239 (2015) 34–45 Sep 5.
[5] Z. Gong, S. Zhao, J. Zhou, J. Yan, L. Wang, X. Du, H. Li, Y. Chen, W. Cai, J. Wu, Curcumin alleviates DSS-induced colitis via inhibiting NLRP3 inflammsome activation and IL-1β production, Mol. Immunol. 104 (2018) 11–19, https://doi.org/10.1016/j.molimm.2018.09.004 Dec.
[6] D.K. McDaniel, K. Eden, V.M. Ringel, I.C. Allen, Emerging roles for noncanonical NF-κB signaling in the modulation of inflammatory bowel disease pathobiology, Inflamm. Bowel Dis. 22 (9) (2016) 2265–2279 Sep.
[7] B. Krishnamurthy, N. Rani, S. Bharti, M. Golechha, J. Bhatia, T.C. Nag, R. Ray, S. Arava, D.S. Arya, Febuxostat ameliorates doxorubicin-induced cardiotoxicity in rats, Chem. Biol. Interact. 237 (2015) 96–103 Jul 25.
[8] A.N. Fahmi, G.S. Shehatou, A.M. Shebl, H.A. Salem, Febuxostat exerts dose-dependent renoprotection in rats with cisplatin-induced acute renal injury, Naunyn Schmiedeberg's Arch. Pharmacol. 389 (8) (2016) 819–830. Aug.
[9] H. Kataoka, K. Yang, K.L. Rock, The xanthine oxidase inhibitor Febuxostat reduces tissue uric acid content and inhibits injury-induced inflammation in the liver and lung, Eur. J. Pharmacol. 746 (2015) 174–9.0 Jan 5.
[10] G. Hao, W. Duan, J. Sun, J. Liu, B. Peng, Effects of febuxostat on serum cytokines IL-1, IL-4, IL-6, IL-8, TNF-α and COX-2, Exp Ther Med. 17 (1) (2019 Jan) 812–816.
[11] J. Nomura, N. Busso, A. Ives, S. Tsujimoto, M. Tamura, A. So, Y. Yamanaka, Febuxostat, an inhibitor of xanthine oxidase, suppresses lipopolysaccharide-induced MCP-1 production via MAPK phosphatase-1-mediated inactivation of JNK, PLoS One 8 (9) (2013 Sep 25) e75527.
[12] A.D. Millar, D.S. Rampton, C.L. Chander, A.W. Claxson, S. Blades, A. Coumbe, J. Panetta, C.J. Morris, D.R. Blake, Evaluating the antioxidant potential of new treatments for inflammatory bowel disease using a rat model of colitis, Gut 39 (3) (1996) 407–415 Sep.
[13] K. Amirshahrokhi, A.R. Khalili, Methylsulfonylmethane is effective against gastric mucosal injury, Eur. J. Pharmacol. 811 (2017) 240–248 Sep 15.
[14] K. Amirshahrokhi, A.R. Khalili, Carvedilol attenuates paraquat-induced lung injury by inhibition of proinflammatory cytokines, chemokine MCP-1, NF-κB activation and oxidative stress mediators, Cytokine 88 (2016) 144–153 Dec.
[15] C.F. Babbs, Oxygen radicals in ulcerative colitis, Free Radic. Biol. Med. 13 (2) (1992) 169–181.
[16] A.C. Blackburn, W.F. Doe, G.D. Buffinton, Protein carbonyl formation on mucosal proteins in vitro and in dextran sulfate-induced colitis, Free Radic. Biol. Med. 27 (3–4) (1999) 262–270. Aug.
[17] S. Bohlooli, S. Mohammadi, K. Amirshahrokhi, H. Mirzanejad-Asl, M. Yosefi, A. Mohammadi-Nei, M.M. Chinifroush, Effect of Methylsulfonylmethane pretreatment on Aceta-minophen induced hepatotoxicity in rats, Iran J Basic Med Sci 16 (8) (2013) 896–900. Aug.
[18] H.M. Hamer, D.M. Jonkers, S.A. Vanhoutvin, F.J. Troost, G. Rijkers, A. de Bruïne, A. Bast, K. Venema, R.J. Brummer, Effect of butyrate enemas on inflammation and antioxidant status in the colonic mucosa of patients with ulcerative colitis in remission, Clin. Nutr. 29 (6) (2010) 738–744 Dec.
[19] K. Amirshahrokhi, A.R. Khalili, Gastroprotective effect of 2-mercaptoethane sulfonate against acute gastric mucosal damage induced by ethanol, Int. Immunopharmacol. 34 (2016) 183–188 May.
[20] C. Loguercio, G. D'Argenio, M. Delle Cave, V. Cosenza, N. Della Valle, G. Mazzacca, C. Del Vecchio Blanco, Glutathione supplementation improves oxidative damage in experimental colitis, Dig. Liver Dis. 35 (9) (2003) 635–641 Sep.
[21] N.A. Soliman, W.A. Keshk, F.H. Rizk, M.A. Ibrahim, The possible ameliorative effect of simvastatin versus sulfasalazine on acetic acid induced ulcerative colitis in adult rats, Chem. Biol. Interact. 298 (2019) 57–65 Jan 25.
[22] N. Kannan, C. Guruvayoorappan, Protective effect of Bauhinia tomentosa on acetic acid induced ulcerative colitis by regulating antioxidant and inflammatory mediators, Int. Immunopharmacol. 16 (1) (2013) 57–66 May.
[23] X. Tun, K. Yasukawa, K. Yamada, Involvement of nitric oxide with activation of toll-like receptor 4 signaling in mice with dextran sodium sulfate-induced colitis, Free Radic. Biol. Med. 74 (2014) 108–117 Sep.
[24] J. Fang, H. Yin, L. Liao, H. Qin, F. Ueda, K. Uemura, K. Eguchi, G.Y. Bharate, H. Maeda, Water soluble PEG-conjugate of xanthine oxidase inhibitor, PEG-AHPP micelles, as a novel therapeutic for ROS related inflammatory bowel diseases, J. Control. Release 223 (2016) 188–196 Feb 10.
[25] S. Bibi, Y. Kang, M. Du, M.J. Zhu, Dietary red raspberries attenuate dextran sulfate sodium-induced acute colitis, J. Nutr. Biochem. 51 (2018) 40–46 Jan.
[26] J.K. Ko, F.Y. Lam, A.P. Cheung, Amelioration of experimental colitis by Astragalus membranaceus through anti-oxidation and inhibition of adhesion molecule synthesis, World J. Gastroenterol. 11 (37) (2005) 5787–5794 Oct 7.
[27] Z. Zhuge, L.L. Paulo, A. Jahandideh, M.C.R. Brandão, P.F. Athayde-Filho, J.O. Lundberg, V.A. Braga, M. Carlström, M.F. Montenegro, Synthesis and characterization of a novel organic nitrate NDHP: role of xanthine oxidoreductase-mediated nitric oxide formation, Redox Biol. 13 (2017) 163–169 Oct.
[28] G. Rogler, T. Andus, Cytokines in inflammatory bowel disease, World J. Surg. 22 (4) (1998) 382–389. Apr. (Review).
[29] L. Zhu, P. Gu, H. Shen, Protective effects of berberine hydrochloride on DSS-induced ulcerative colitis in rats, Int. Immunopharmacol. 68 (2019) 242–251 Mar.
[30] M.Z. Cader, A. Kaser, Recent advances in inflammatory bowel disease: mucosal immune cells in intestinal inflammation, Gut 62 (11) (2013) 1653–1664 Nov.
[31] M.J. Waldner, M.F. Neurath, Master regulator of intestinal disease: IL-6 in chronic inflammation and cancer development, Semin. Immunol. 26 (1) (2014) 75–79 Feb.
[32] D. Noguchi, D. Wakita, M. Tajima, S. Ashino, Y. Iwakura, Y. Zhang, K. Chamoto, H. Kitamura, T. Nishimura, Blocking of IL-6 signaling pathway prevents CD4+ T cell-mediated colitis in a T(h)17-independent manner, Int. Immunol. 19 (12) (2007) 1431–1440 Dec.
[33] Min Cao, Pei Wang, Chunhong Sun, Wen He, Fengjun Wang, Amelioration of IFN-γ and TNF-α-induced intestinal epithelial barrier dysfunction by Berberine via suppression of MLCK-MLC phosphorylation signaling pathway, PLoS One 8 (5) (2013) e61944.
[34] R. Ito, M. Shin-Ya, T. Kishida, A. Urano, R. Takada, J. Sakagami, J. Imanishi,

M. Kita, Y. Ueda, Y. Iwakura, K. Kataoka, T. Okanoue, O. Mazda, Interferon-gamma is causatively involved in experimental inflammatory bowel disease in mice, Clin. Exp. Immunol. 146 (2) (2006) 330–338 Nov.

[35] K. Amirshahrokhi, M. Ghazi-khansari, A. Mohammadi-Farani, G. Karimian, Effect of captopril on TNF-α and IL-10 in the livers of bile duct ligated rats, Iran J Immunol 7 (4) (2010) 247–251 Dec.

[36] D.S. Shouval, J. Ouahed, A. Biswas, J.A. Goettel, B.H. Horwitz, C. Klein, A.M. Muise, S.B. Snapper, Interleukin 10 receptor signaling: master regulator of intestinal mucosal homeostasis in mice and humans, Adv. Immunol. 122 (2014) 177–210.

[37] B. Li, R. Alli, P. Vogel, T.L. Geiger, IL-10 modulates DSS-induced colitis through a macrophage-ROS-NO axis, Mucosal Immunol. 7 (4) (2014) 869–878 Jul.

[38] C. Luo, H. Zhang, The role of Proinflammatory pathways in the pathogenesis of colitis-associated colorectal Cancer, Mediat. Inflamm. 2017 (2017) 5126048.

[39] D.K. McDaniel, K. Eden, V.M. Ringel, I.C. Allen, Emerging roles for noncanonical NF-κB signaling in the modulation of inflammatory bowel disease pathobiology, Inflamm. Bowel Dis. 22 (9) (2016) 2265–2279 Sep.

[40] I. Atreya, R. Atreya, M.F. Neurath, NF-kappaB in inflammatory bowel disease, J. Intern. Med. 263 (6) (2008) 591–596 Jun.