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ORIGINAL ARTICLE

Bupropion Ameliorates Acetic Acid–Induced Colitis in Rat: the Involvement of the TLR4/NF-kB Signaling Pathway

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Abstract— Inflammatory bowel disease composed of ulcerative colitis and Crohn's disease is a disorder that may involve entire gastrointestinal tract. Its pathogenesis is mainly an immune-mediated inflammation. Recently, it has been indicated that bupropion possesses anti-inflammatory properties; hence, the objective of this experiment is the investigation of the anti-inflammatory influence of bupropion on colonic lesions that emerged following the intrarectal administration of acetic acid. Thirty-six male Wistar rats were allocated randomly into six groups, including control, acetic acid, dexamethasone (2 mg/kg), and bupropion (40, 80, and 160 mg/kg). Colitis was induced by intrarectal administration of acetic acid in all study groups except control group, and animals were treated by oral administration of dexamethasone and bupropion. While macroscopic and microscopic lesions were observed after colitis induction, administration of dexamethasone and bupropion 160 mg/kg led to the remarkable improvement in lesions. In addition, the expression of TLR4 and NF-κB was decreased after colitis induction; however, treatment with dexamethasone (2 mg/kg) and bupropion (160 mg/kg) resulted in a significant decrease in their expression. Regarding biochemical factors, following colitis induction, TNF-α level and MPO activity were increased; nevertheless, dexamethasone (2 mg/kg) and bupropion (160 mg/kg) decreased the TNF-α and MPO activity. In conclusion, bupropion exerts anti-inflammatory influence through suppressing the TLR4 and NF-κB expression in the rat model of acute colitis.

KEY WORDS: inflammatory bowel disease; acetic acid; bupropion; TLR4/NF-kB signaling pathway.

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INTRODUCTION

Inflammatory bowel disease (IBD) is defined as an immune-mediated inflammatory state, which consists of ulcerative colitis (UC) and Crohn's disease (CD) [1, 2]. Notwithstanding the fact that the definite pathogenesis of IBD has remained obscure, it has been indicated that the immunological response of intestinal mucosa was triggered by the interaction between genetic and environment results in immense inflammation and damages in the gastrointestinal tract [3]. One of the constituents of immune system is TLRs, which are involved in innate immunity and play a part in intestinal development and protection through the recognition of commensal bacteria [4]. TLR4 is one of the important members of TLRs and plays a key role in the pathophysiology of IBD. It has been implicated that the expression of TLR4 is in low levels in normal intestinal epithelial cells; however, its expression is increased during inflammation and colitis [5]. Indeed, TLR4 is provoked by pathogen-associated molecular patterns (PAMPs); consequently, other downstream molecules are accumulated, resulting in the expression of the NF-κB, leading to the generation of various pro-inflammatory cytokines like tumor necrosis factor-alpha (TNF-α) [6–8]. Thus, The TLR4/NF-κB signaling pathway play an essential role in the pathogenesis of IBD [9]. Therefore, it seems that the inhibition of TLR4/NF-kB signaling pathway may be one of the main targets in the treatment of IBD [10]. Current therapies such as aminosalicylates and glucocorticoids have incomplete efficiency and some adverse effects [11].

Bupropion, otherwise known as amfebutamone, is an atypical anti-depressant drug and a smoking cessation aid as well. The primary mechanism by which bupropion acts is neuronal reuptake inhibition of norepinephrine (NE) and dopamine (DA) [12]. Bupropion is also a noncompetitive nicotinic acetylcholine receptor antagonist [13]. Studies have reported that bupropion lowers the level of inflammatory cytokines such as TNF-α and interferon-gamma (INF-γ) [14–16]. In addition, one study revealed that bupropion mitigates inflammation in MDD (major depressive disorder) patients through reducing the expression of TLRs such as TLR3 and TLR4 [17]. Therefore, bupropion may be helpful in attenuating inflammation in some diseases, which arise following the activation of TLR4/NF-kB signaling pathway like IBD.

In the present experiment, we aimed to investigate the presumable anti-inflammatory influence of bupropion *via* the inhibition of the TLR4/NF-kB signaling pathway in acetic acid–induced colitis in rat.

MATERIAL AND METHODS

Animals

This experiment was acknowledged by the Ethical Committee of Tehran University of Medical Sciences (No.: 1398/006) and was performed in accordance with "Principles of Laboratory Animal Care" (NIH publication 82–23, revised in 1985). Thirty-six male Wistar rats weighing between 200 and 250 g were purchased from Department of Pharmacology, Tehran University of Medical Sciences. They were retained in plastic cages under-controlled condition of 12 h light/dark cycles, ambient temperature of 20–23 °C, and relative humidity of 50–60% with free access to water and standard rodent food.

Chemicals

Bupropion and dexamethasone were provided from Sigma (St. Louis, MO, USA) and dissolved in 0.9% saline solution. Xylazine and ketamine were bought from Alfasan Company (Woerden Holland), and formalin solution 35% w/w and acetic acid were bought from Merck Company.

Induction of Colitis and Surgical Procedure

In order to induce colitis, at first, animals were inhibited from eating rodent food overnight; meanwhile, they had free access to tap water. Thereafter, they were lightly anesthetized by intraperitoneal administration of ketamine (50 mg/kg) and xylazine (10 mg/kg). Two milliliters of 4% acetic acid was administered by an 8-cm-long and flexible catheter through the rectum. To prevent leaking acetic acid solution, rats were positioned upside down for 1 min. Two hours following colitis induction (first day), oral administration of dexamethasone (2 mg/kg) and bupropion (40, 80, and 160 mg/kg) were conducted and continued for another five consecutive days [18, 19]. In the end, animals were sacrificed by cervical dislocation on the sixth day. For extracting the samples, in the first instance, the skin of abdomen was shaved and opened by the surgical incision. Then, colon was exposed and the last 8 cm of which was excised, opened longitudinally, and washed in ice-cold normal saline. Finally, after macroscopic assessments, specimens were cut into two pieces: one of which was frozen in − 80 °C for biochemical measurements, and the other one was fixed in formalin 10% solution for histological and immunohistochemical evaluations.

Experimental Design

Animals were allocated into 6 study groups, by 6 rats in each group with the following treatment:

Control group: without colitis induction + 0.9% normal saline solution/day

Acetic acid group: 2 ml acetic acid 4% + 0.9% normal saline solution/day

Dexamethasone group: 2 ml acetic acid 4% + dexamethasone 2 mg/kg/day

Bupropion 40 group: 2 ml acetic acid 4% + bupropion 40 mg/kg/day

Bupropion 80 group: 2 ml acetic acid 4% + bupropion 80 mg/kg/day

Bupropion 160 group: 2 ml acetic acid 4% + bupropion 160 mg/kg/day

Assessment of Macroscopic Appearance

The evaluation of the macroscopic appearance of samples in each study group was performed by an observer blinded to the study in compliance with the following score: no macroscopic changes = 0; only mucosal erythema = 1; mild mucosal edema, slight bleeding, or slight erosion = 2; moderate edema, bleeding ulcers, or erosions = 3; and severe ulceration, erosion, edema, and tissue necrosis = 4 [20]. Ulcer area was estimated by 3M® surgical transparent tape consisted of 1-mm^2 cells. The number of cells, which covered the ulcerated area of each colonic sample, was counted. Ulcer index was calculated by adding the ulcer score to the ulcer area of each sample. Ulcer index was obtained in accord with the following formula [21]: Ulcer index = Ulcer area (cm^2) + macroscopic damage score.

Assessment of Histological Features

To perform histologic studies, colon tissues, which had been fixed in neutral 10% formalin solution, were embedded in paraffin wax, cut into sections of 4 μm, and ultimately stained by hematoxylin and eosin (HE). Histopathologic evaluations were conducted by a pathologist unaware to the study based on histopathologic grading (Table 1).

Measurement of TLR4 and NF-κB

In order to determine the expression of TLR4 and NF-κB by immunohistochemical (IHC) method, 4-μm colonic slices, embedded by paraffin, were firstly deparaffinized in xylene; then, they were washed with alcohol, and finally, rehydration was occurred by using phosphate buffer saline (PBS). Antigen retrieval was conducted in citrate buffer (pH = 6.0) for 15 min at 90 °C, and the slide incubation was done with blocking solution (3% BSA) at 37 °C for 1 h. Primary antibodies including mouse monoclonal IgG1 (kappa light chain) p-NFκB p65 (27. Ser 536) (SC136548) (1:100 dilution) and mouse monoclonal IgG1 (kappa light chain) TLR4 (sc-293072) (1:100) (Santa Cruz Biotechnology, USA) were incubated at 4 °C overnight. Using PBS, the slides were washed four times and incubated with 3% H_2O_2 for 12 min at room temperature. Thereafter, mouse IgG kappa-binding protein (m-IgGκ BP) conjugated to horseradish peroxidase (HRP) (1:50 dilution) antibody (Santa Cruz Biotechnology, USA) was added and the slides were incubated at 37 °C for 1 h. Afterwards, the slides were washed with PBS, and then diaminobenzidine solution (DAB; Boster Biological Technology, USA) was added for 10 min at room temperature. By using distilled water, staining was stopped; then, counterstaining was performed by means of hematoxylin. The evaluations were done by a histologist unaware to the study in accord with the previous scoring system [22]. Immunohistochemical scores were computed based on the sum of a proportion score (percentage of positive stained cells as none = 0, below 1% = 1, 1–10% = 2, 11–33% = 3, 34–66% = 4, 67–100% = 5) and an intensity score (none = 0, weak = 1, intermediate = 2, and strong = 3). The numerical score rating was as follows: negative = 0–1, weak positive = 2–3, positive = 4–6, and strong positive = 7–8.

Assessment of TNF-α Level

The level of TNF-α was assessed by means of rat TNF-α enzyme-linked immunosorbent assay (ELISA) kit, based on the manufacturer's instructions (CUSABIO Technology LLC., USA). At first, colonic tissue specimens

Table 1. Criteria of Inflammation Grading

Grades	Histological feature
Grade 0	Normal mucosa, submucosal, muscularis propria, and serosa without inflammatory cells
Grade 1	Inflammatory cells in mucosa and submucosal
Grade 2	Transmural acute inflammation
Grade 3	Small ulcers (up to 3) with acute inflammation in the wall
Grade 4	Multiple large ulcers with transmural inflammation
Grade 5	Extensive ulceration with necrosis of the entire wall or part of it, transmural inflammation, and irregular villous mucosal surface

were homogenized in 50 mmol/L ice-cold potassium phosphate buffer (pH = 6.0); then, homogenate was centrifuged at 4 °C for 20 min; finally, supernatant was elicited and stored at − 80 °C by the time of ELISA analysis. The TNF-α level were presented as picograms per milligram.

Assessment of MPO Activity

The evaluation of myeloperoxidase (MPO) activity in colonic tissues was performed by the method formerly described with some alterations [23]. A 0.1-g section from colonic tissue was homogenized in 1 ml of potassium phosphate (pH = 6), which was containing 0.5% HTAB in polytron homogenizer ice bath. Thereafter, the buffer was added to the final volume of 5 ml, and the homogenate was sonicated for 10 s in an ice bath as well as frozen and thawed three times sequentially. Afterward, the homogenate was centrifuged at 15,000 rpm, at 4 °C for 15 min. Using phosphate buffer (50 mM, pH = 6), which was containing 0.0005% hydrogen peroxide and 0.167 mg/ml o-dianisidine dihydrochloride, 0.1 ml of the supernatant was diluted to the final volume of 3 ml. Finally, the absorbance variation by the suspension at 460 nm was measured by the UV/VIS spectrophotometer (LSI Model Alfa-1502). Myeloperoxidase activity was presented as units per gram of wet colonic tissue.

Statistical Analysis

The data analysis was performed by GraphPad Prism (Ver.5.04). The differences between groups were determined by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test, and $p < 0.05$ was considered significant. All data were represented as mean ± SEM.

RESULTS

Effects of Bupropion Administration on Ulcer Index

As depicted in Fig. 1, the induction of colitis resulted in a significant increase in ulcer index compared to the control group ($p < 0.001$). Nevertheless, oral administration of dexamethasone (2 mg/kg) and bupropion (160 mg/kg) led to a notable decrease in ulcer index in comparison with the control group ($p < 0.001$). No remarkable changes were observed in ulcer index following the administration of bupropion (40 mg/kg and 80 mg/kg).

Effects of Bupropion Administration on Histologic Appearance

Figure 2 illustrates the histopathologic feature of all study groups. In the control group, mucosa, submucosal, muscularis propria, and serosa were intact and there was no evidence of inflammation; however, after the induction of colitis by intrarectal administration of acetic acid, samples showed severe damages, necrosis, and infiltration of inflammatory cells. Following the treatment of dexamethasone (2 mg/kg) and bupropion (160 mg/kg), a significant improvement in histologic necrosis and ulceration was observed and inflammatory cells remarkably were reduced. By contrast, treatment with bupropion (40 mg/kg and 80 mg/kg) did not ameliorate the necrosis and damages that arose after colitis induction.

Effects of Bupropion Administration on Protein Expression of TLR-4 and NF-κB

Evaluations delineated that the expression of TLR-4 and NF-κB was at a low level in the colonic tissue in the control group. However, following the induction of colitis by means of intrarectal administration of acetic acid, the expression of TLR4 and NF-KB markedly rose ($p < 0.01$). Treatment with dexamethasone (2 mg/kg) and bupropion (160 mg/kg) caused significant decrease in the expression

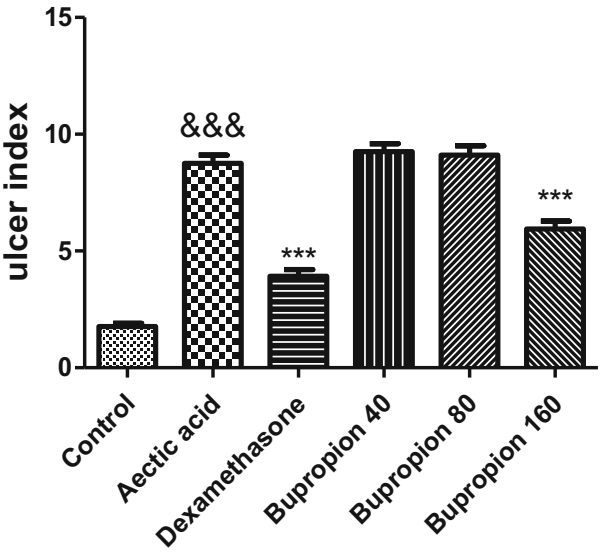

Fig. 1. Effect of acetic acid, dexamethasone (2 mg/kg), and bupropion (-40, 80, 160 mg/kg) on ulcer index. Data are expressed as mean ± SEM ($n = 6$). $^{\&\&\&}p < 0.001$ compared to control group; $^{***}p < 0.001$ compared to acetic acid group. The statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test.

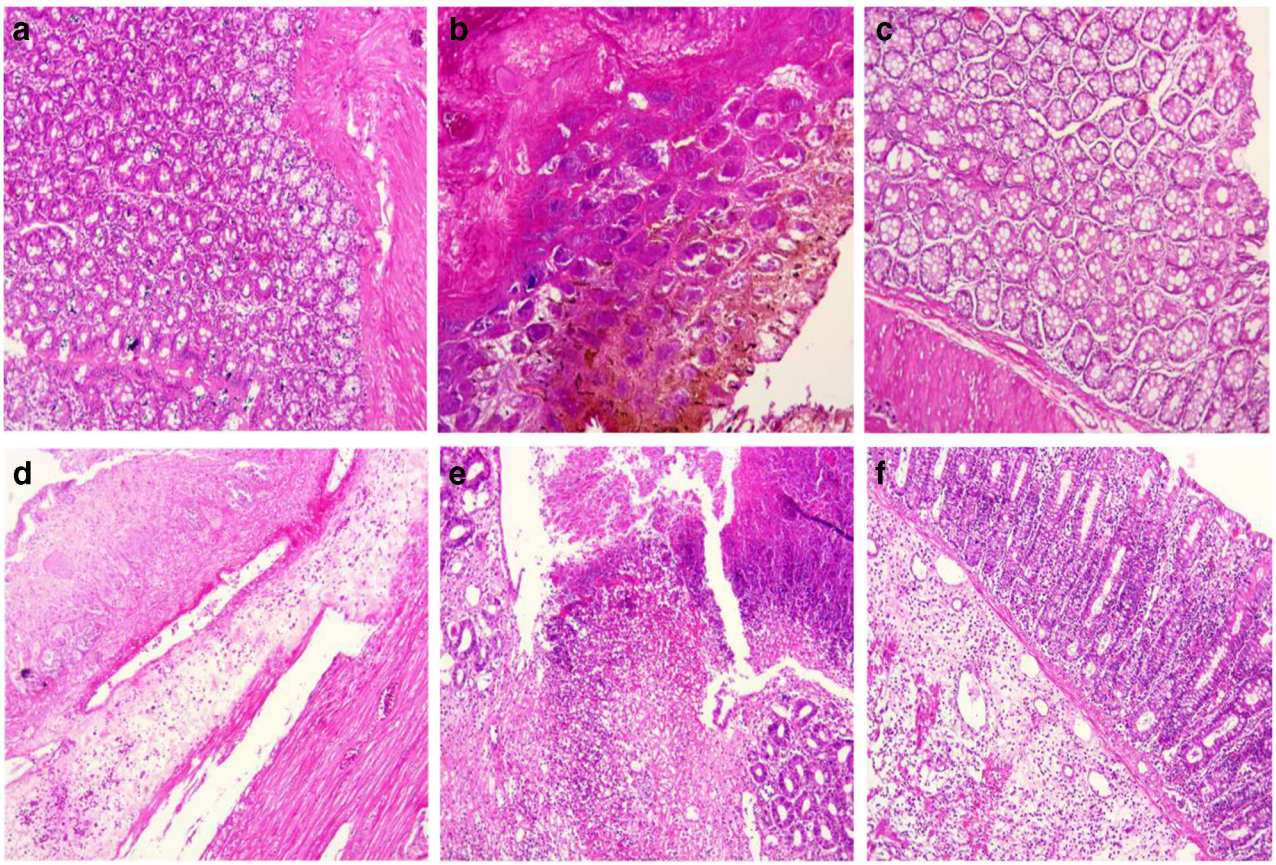

Fig. 2. Histopathological appearance of colonic tissue in all study groups. Representative hematoxylin and eosin stained of colon sections. Magnifications: × 10. **a** Control. **b** Acetic acid. **c** Dexamethasone (2 mg/kg). **d** Bupropion (40 mg/kg). **e** Bupropion (80 mg/kg). **f** Bupropion (160 mg/kg).

of TLR4 ($p < 0.05$) and NF-KB ($p < 0.01$) compared to the acetic acid group. By contrast, pretreatment with bupropion at the doses of 40 mg/kg and 80 mg/kg did not exert any attenuating effect on the expression of TLR4 and NF-KB (Figs. 3 and 4).

Effects of Bupropion Administration on the Level of TNF-α

Figure 5 demonstrates the alterations of TNF-α level after the intrarectal administration of acetic acid as well as following treatment with dexamethasone and bupropion 40, 80, and 160 mg/kg. The level of TNF-α significantly rose after colitis induction ($p < 0.001$). Conversely, after treating animals with dexamethasone (2 mg/kg) and bupropion (160 mg/kg), TNF-α level was notably decreased ($p < 0.001$ and $p < 0.01$, respectively); however, administration of bupropion 40 and 80 mg/kg did not exert any influence on the level of TNF-α in comparison with the acetic acid group.

Effects of Bupropion Administration on the Level of MPO

Measurements indicated that MPO activity significantly was enhanced after colitis induction in the acetic acid group compared with the control group ($p < 0.001$). On the contrary, following treatment with dexamethasone (2 mg/kg) and bupropion (160 mg/kg), MPO activity markedly was reduced ($p < 0.001$ and $p < 0.01$, respectively). Treatment with bupropion at the doses of 40 and 80 mg/kg did not lead to significant change in MPO activity compared to the acetic acid group (Fig. 6).

DISCUSSION

The present experiment indicated that the oral administration of bupropion significantly improved macroscopic and microscopic lesions that emerged after acetic acid–induced

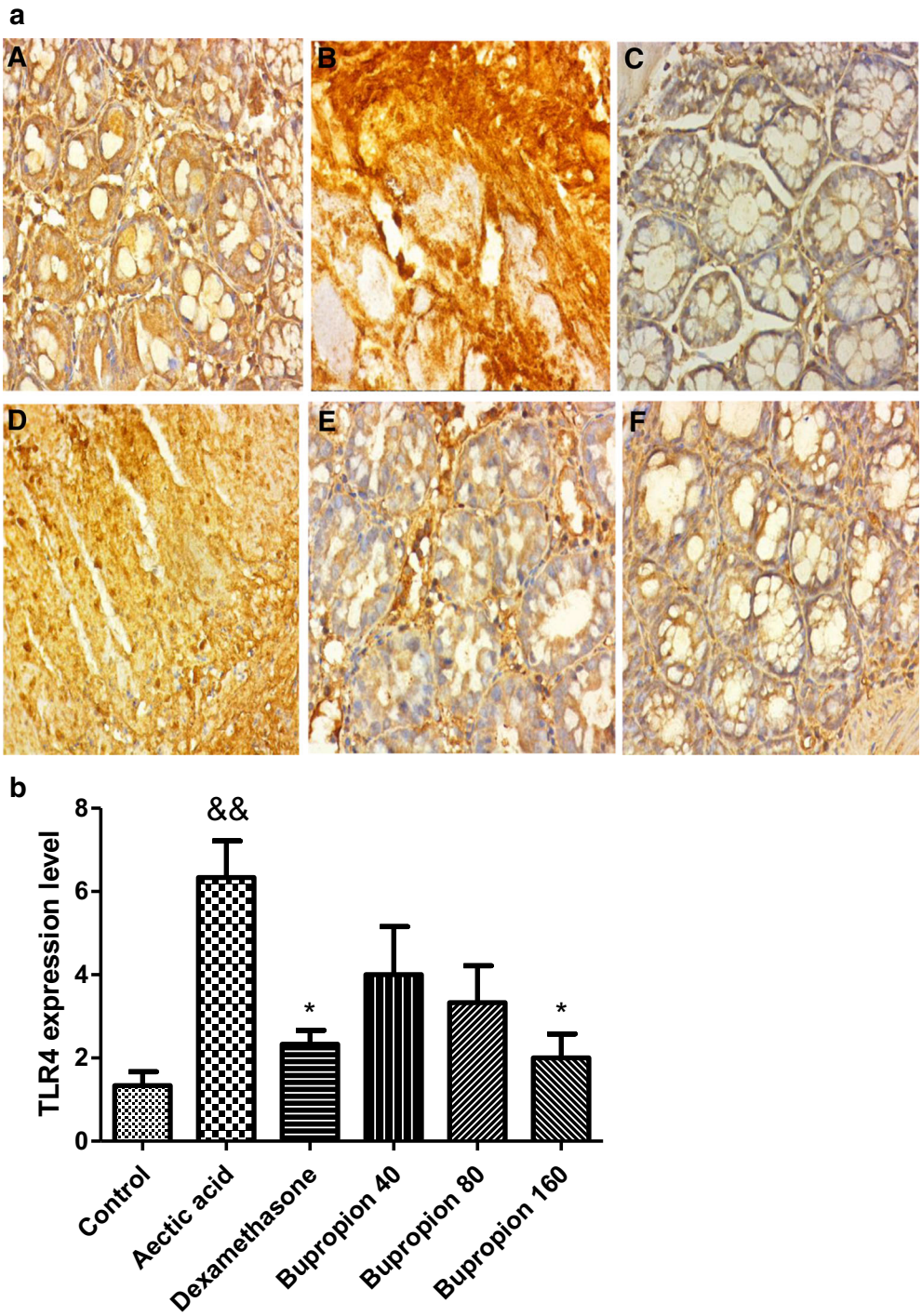

Fig. 3. **a** Immunohistochemical analysis of TLR4 expression in the colonic tissue of animals in all study groups. Immunohistochemical staining of the colon tissue sections for TLR4 (× 40). A: control; B: acid acetic; C: dexamethasone (2 mg/kg); D: bupropion (40 mg/kg); E: bupropion (80 mg/kg); F: bupropion (160 mg/kg). **b** Effect of dexamethasone (2 mg/kg) and bupropion (40, 80, and 160 mg/kg) on the level of TLR4 expression in colonic tissue. Data are expressed as mean ± SEM ($n = 6$). $^{\&\&}p < 0.001$ compared to control group and $^{*}p < 0.001$ compared to acid acetic group. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test.

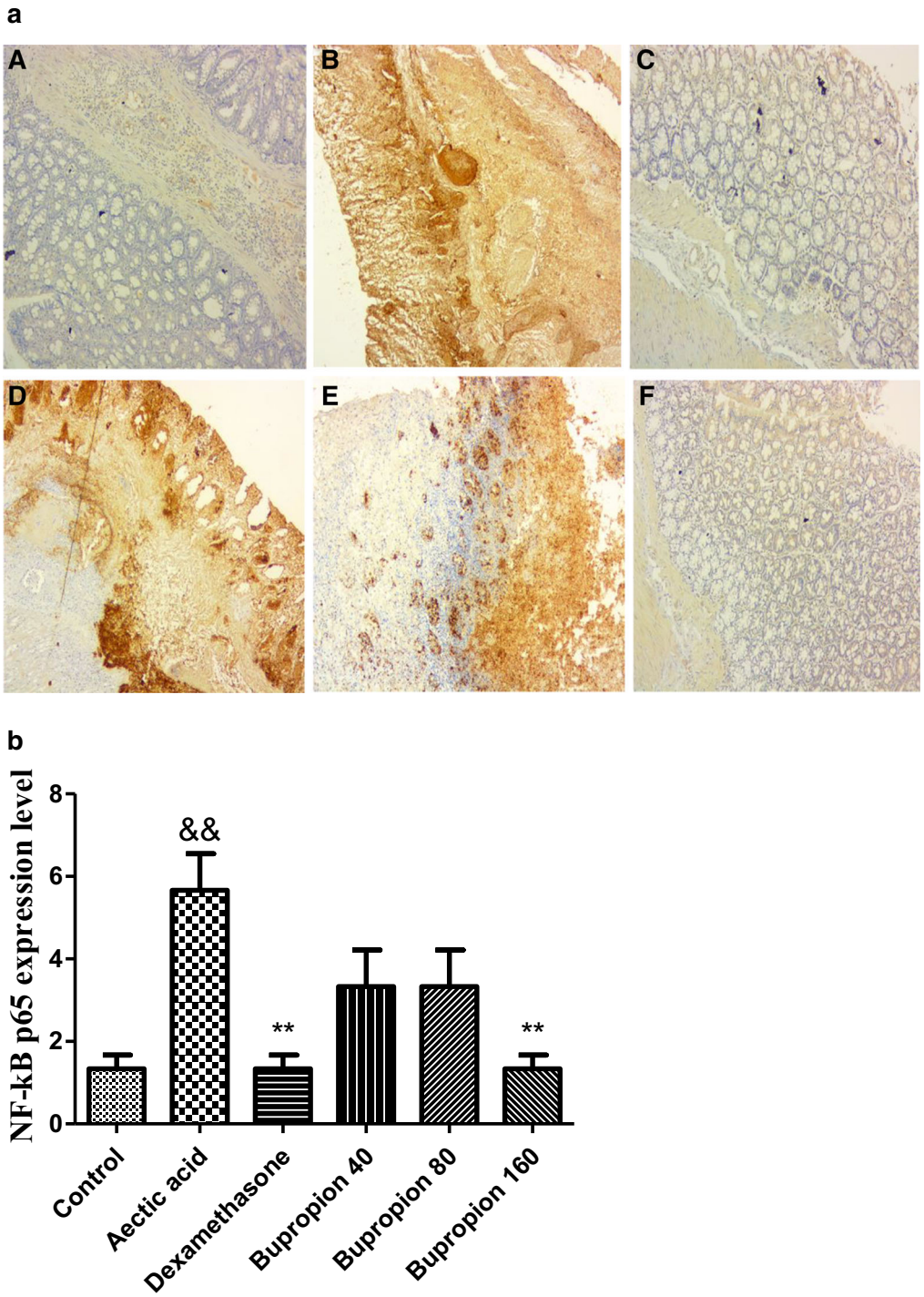

Fig. 4. **a** Immunohistochemical analysis of NF-κB expression in colonic tissue of animals in all study groups. Immunohistochemical staining of the colon tissue sections for NF-κB (× 10). A: control; B: acid acetic; C: dexamethasone (2 mg/kg); D: bupropion (40 mg/kg); E: bupropion (80 mg/kg); F: bupropion (160 mg/kg). **b** Effect of dexamethasone (2 mg/kg) and bupropion (40, 80, and 160 mg/kg) on the level of NF-κB expression in colonic tissue. Data are expressed as mean ± SEM ($n = 6$). $^{\&\&}p < 0.001$ compared to control group and $^{**}p < 0.001$ compared to acid acetic group. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test.

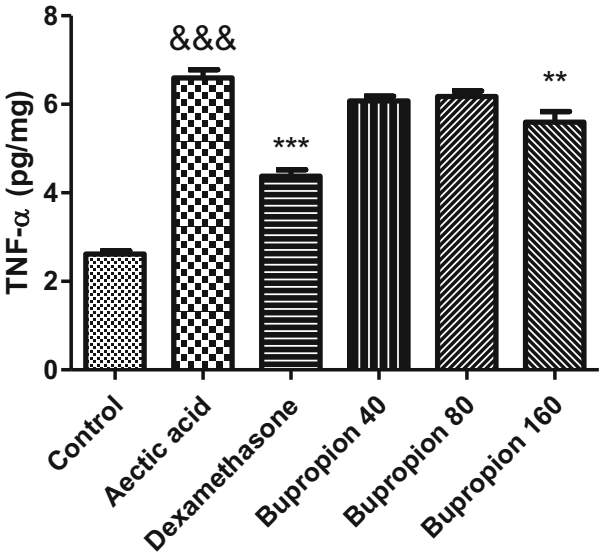

Fig. 5. Colonic tumor necrosis factor-alpha (TNF-α) levels in rats with acetic acid–induced colitis and respective treatments. Data are expressed as mean ± SEM ($n = 6$). $^{\&\&\&}p < 0.001$ compared to the control group. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to acetic acid group. The statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test.

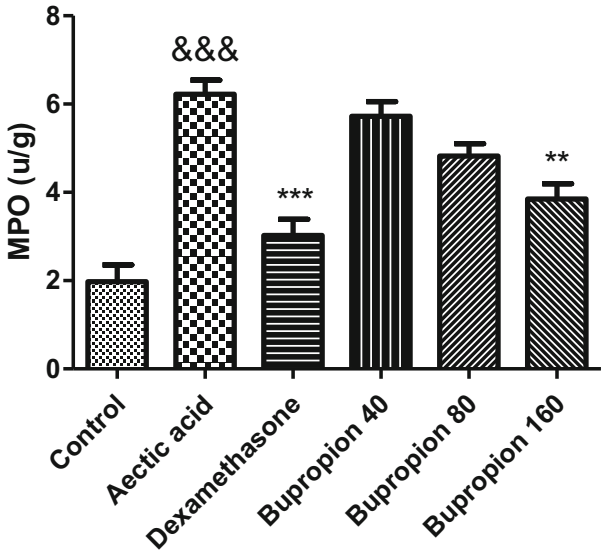

Fig. 6. Colonic activity of MPO enzyme in rats with acetic acid–induced colitis and respective treatments. Data are expressed as mean ± SEM ($n = 6$). $^{\&\&\&}p < 0.001$ compared to control group. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to acetic acid group. The statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test.

colitis through lowering the expression of TLR4 and NF-kB and decreasing in the level of TNF-α and MPO activity.

Inflammatory bowel disease is composed of Crohn's disease and ulcerative colitis, which are recurring and chronic inflammatory diseases [24]. Induction of colitis by acetic acid is a putative model of acute colitis and is compatible with those reported in human IBD [25]. Various studies reported some macroscopic lesions like severe ulcer and tissue necrosis in the intestinal tissue after induction of colitis by acetic acid [26–28]. Our macroscopic assessments also were in harmony with previous studies and showed similar damages. The injury mechanism in this model is epithelial necrosis and edema. In fact, acetic acid releases proton within the intracellular space, which results in massive intracellular acidification followed by massive epithelial damages [29]. Mucosa and submucosal injury is followed by inflammation, which is associated with the activation of NF-kB and other inflammatory mediators [30]. The other possible mechanism is that acetic acid increases the level of FFA (free fatty acid) that leads to the interaction of FFA with TLR4 followed by the activation of NF-kB [31].

Irregular innate and adaptive immune responses in the intestinal mucosa have been considered as the pathophysiology of IBD [32]. With this in mind, TLRs are one of the main mediators of innate defense in the intestine, which play a part in mucosal integrity and intestinal homeostasis. The TLR4 gene is located on chromosome 9 (q32-33) [33], a genomic region in which the CD susceptibility gene also has been localized [34]. TLR4 recognizes bacterial lipopolysaccharide (LPS) and is expressed throughout the gastrointestinal tract in an inducible or constitutive manner. Additionally, they exert cytoprotective influence on the intestinal epithelium. In fact, TLR has a dual regulatory effect on the healthy and inflamed intestinal mucosa. With this in mind, in the healthy host, basal TLR has a protective role in mucosal integrity, homeostasis, and tissue repair. On the contrary, in the susceptible host to IBD, aberrant TLR may breed mucosal destruction, chronic inflammation, disturb homeostasis, and activate pro-apoptotic-signaling pathways [35]. In addition, under healthy conditions, TLR4 expression is low in the intestinal mucosa; however, these receptors significantly are up-regulated in either nonactive and/or active human IBD, resulting in stimulation of immune response and inflammation [36]. Following the recognition of LPS, TLR4 is activated, which triggers a signaling cascade through which transcription factor NF-κB is activated. This

process also called TLR4/NF-κB pathway. It has been reported that the TLR4 and NF-κB expressions are significantly increased within the induction of colitis in rat, and this pathway plays an essential role in the pathogenesis of IBD [32]. Previous studies have indicated that anti-TLR4 therapy can ameliorate intestinal inflammation. In addition, the suppression of NF-κB activation results in the inhibition of the inflammatory signaling cascade [37]. Therefore, the TLR4/NF-κB pathway would be a therapeutic objective for the management of IBD [9, 38]. In consistency with the aforementioned evidence, we found that after induction of colitis by acetic acid, TLR4/NF-κB signaling cascade is activated and the expression of both TLR4 and NF-κB is remarkably increased. Moreover, after the activation of this pathway, the level of inflammatory cytokines such as IL-1, IL-6, IL-8, IL-27, and TNF-α is elevated [32, 39]. TNF-α plays an essential part in the pathogenesis of IBD and leads to the development of ulcerations and tissue damages in Crohn's disease [40]. The increased expression of TNF-α has been also detected in mucosal biopsies of patients with IBD [41] and in the rat model of colitis, induced by acetic acid [42]. In addition, anti-TNF-α antibody had been effective for the prevention of inflammation in patients with Crohn's disease [43]. In our experiment also after colitis induction, the level of TNF-α was significantly increased. Following the increase in the level of TNF-α, inflammatory cells such as neutrophils are provoked towards the tissue. One of the indicators for the activation of neutrophils in tissue is myeloperoxidase (MPO), which is a 140-kDa proteolytic enzyme and released from neutrophil granulocytes. Indeed, in consequence of the increase in the amount of tissue neutrophil, the activity of MPO is enhanced [44]. In our experiment, after the induction of colitis, the MPO activity also was markedly elevated, indicating an increase in the number of inflammatory cells and neutrophils in the colonic tissue.

Bupropion is an anti-depressant drug that belongs to monocyclic phenylbutylamine of the aminoketone group, in which its mechanism is the inhibition of norepinephrine (NE) and dopamine (DA) reuptake [45]. Additionally, bupropion plays a role as a noncompetitive nicotinic acetylcholine receptor antagonist [13]. This drug is metabolized into hydroxyl-bupropion (active metabolite) by CYP2B6, which is excreted by the kidney [46]. Some studies have been performed regarding the anti-inflammatory effect of bupropion. In 2005, Brustolim et al. conducted an experiment regarding the impacts of bupropion on inflammation induced by lipopolysaccharide (LPS) in mice. They concluded that bupropion by mediating signals at D1 receptors and beta-adrenoreceptors increases cAMP, resulting in suppression of TNF-α generation [14]. Another animal study revealed that bupropion *via* inhibition of prostaglandin synthesis as well as central inhibitory mechanisms exerts anti-inflammatory and analgesic properties [47].

It also has been indicated that bupropion decreased inflammation in some diseases, in which their pathogenesis is attributed to the inflammation and increase in TNF-α level, for instance, psoriasis and atopic dermatitis [48], hepatitis B [40], Crohn's disease [16, 49], and some malignancies like chronic lymphocytic leukemia [50] and multiple myeloma [51]. In accordance with the aforementioned findings, we also observed that after treatment with bupropion, the expression of TLR4 and NF-κB was decreased. In addition, the level of TNF-α and MPO activity also was lowered.

CONCLUSION

In conclusion, bupropion ameliorates colonic lesions, which were emerged following the induction of colitis by intrarectal administration of acetic acid. Moreover, this property is presumably *via* the inhibition of the TLR4/NF-KB signaling pathway and suppression of inflammation.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflict of interest.

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