



Anti-inflammatory Effects of Ivermectin in the Treatment of Acetic Acid-Induced Colitis in Rats: Involvement of GABA_B Receptors

Armin Aryannejad^{1,2} · Mohammadreza Tabary^{1,2} · Nafise Noroozi^{1,2} · Baharnaz Mashinchi^{1,2} · Setare Iranshahi³ · Seyed Mohammad Tavangar⁴ · Razieh Mohammad Jafari¹ · Amir Rashidian^{1,2} · Ahmad Reza Dehpour^{1,2} 

Received: 22 June 2021 / Accepted: 13 September 2021 / Published online: 21 October 2021
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Abstract

Background Recent investigations have proposed the potential role of gamma-aminobutyric acid (GABA) in regulating motility and immunity of the gastrointestinal system.

Aims We aimed to investigate the anti-inflammatory effects of ivermectin (IVM) through GABA_B receptors following acetic acid-induced colitis in rats.

Methods In a controlled experimental study, we enrolled 78 male Wistar rats (13 groups; 6 rats/group). After colitis induction using acetic acid (4%), IVM, baclofen (a standard GABA_B agonist) or the combination of both agents was delivered to rats orally (by gavage), with the same dosage continued for 5 days. The control group received the vehicle, and prednisolone (a standard anti-inflammatory agent) was administered in a separate group as the positive control. Colon samples were collected on the sixth day for histopathological evaluations and measurement of myeloperoxidase (MPO) activity, TNF- α levels, and p-NF- κ B p65, COX-2 and iNOS expression levels.

Results The greatest recovery was found after administering IVM 0.5, baclofen 0.5, or IVM 0.2 + baclofen 0.2 mg/kg/day (ulcer index [UI] = 1.4 ± 0.4 , 1.7 ± 0.6 , and 1.4 ± 0.3 , respectively; $p < 0.001$ vs. the control [UI = 6.5 ± 0.7]). Histopathological evaluations revealed a significant decrease in the inflammation severity in the three above-mentioned groups. P-NF- κ B p65, COX-2, and iNOS expression, MPO activity, and TNF- α levels also decreased dramatically following treatment with IVM 0.5, baclofen 0.5, or the combination therapy ($p < 0.001$ vs. the control).

Conclusions IVM exerted promising anti-inflammatory effects in treating acetic acid-induced colitis in rats. Its synergistic effect with baclofen also signified the possible involvement of GABA_B receptors in this process.

Keywords Inflammatory bowel disease · Ivermectin · Gamma-aminobutyric acid · Rats

Introduction

Inflammatory bowel disease (IBD) is a chronic bowel relapsing–remitting inflammatory condition within the gastrointestinal (GI) tract [1–3]. Although the etiology of IBD is still unclear [4], recent extensive studies have indicated that a complex interaction between the ambient environment, genetic susceptibility, microbial agents, and immunoregulatory factors might be involved in the pathogenesis of the disease [5–7]. Concerning immunoregulatory factors, some evidence has revealed that nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) is overexpressed in IBD, which in turn induces a wide variety of proinflammatory cytokines, including tumor necrosis factor-alpha (TNF- α) [8]. Myeloperoxidase (MPO) activity also increases significantly in IBD, indicative of neutrophil accumulation [8].

Armin Aryannejad and Mohammadreza Tabary have contributed equally to this work.

✉ Ahmad Reza Dehpour
dehpour@yahoo.com

¹ Experimental Medicine Research Center, Tehran University of Medical Sciences, 13145-784 Tehran, Iran

² Department of Pharmacology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

³ Shahid Beheshti University of Medical Sciences, Tehran, Iran

⁴ Department of Pathology, Shariati Hospital, Tehran University of Medical Sciences, Tehran, Iran

Recent investigations have proposed a potential role of gamma-aminobutyric acid (GABA) in the neuroimmune dialogue between the enteric nervous system and the intestinal mucosal immune system. GABA seems to have a significant role in regulating motility, secretion, and immunity of the GI system [9, 10]. Emerging evidence indicates that GABA has many other beneficial effects, especially ameliorating immune and inflammatory responses. In the colon tissue, epithelial cells are enriched with a functional GABAergic signaling system. Studies also report that many species of commensal bacteria residing in the gut can produce GABA [11]. In addition, lower concentrations of GABA have been found in the colonic mucosa of patients diagnosed with ulcerative colitis [9].

Ivermectin (IVM), a broad-spectrum anthelmintic agent, has been found to possess distinct anti-inflammatory properties, and it is known to affect cellular and humoral immune responses. Evidence suggests that IVM could act through GABA receptors and ligand-gated channels, such as glutamate-gated chloride channels [10]. In addition, IVM has been utilized in a wide variety of conditions, from regulating glucose and cholesterol levels in diabetic mice to suppressing malignant cells proliferation and inhibition of viral replication. Studies have revealed that IVM-mediated inhibition of inflammatory cytokines production may occur through the suppression of the NF- κ B pathway [12]. Concerning the potential anti-inflammatory role of IVM, the present study aimed to evaluate its beneficial effects in treating acetic acid-induced colitis in rats and to investigate the possible role of the GABAergic signaling pathway in this respect. This study will also provide a better understanding of the role of the GABA_B receptors in IBD and could pave the way for new clinical applications of IVM to modulate inflammatory conditions by interacting with the GABA signaling pathway.

Materials and Methods

Animals

Male Wistar rats weighing 200–220 g were obtained from the animal house of the Pharmacology Department, Tehran University of Medical Sciences (Tehran, Iran). Animals were housed under controlled environmental conditions; they were kept in translucent polycarbonate cages at an ambient temperature of 23 ± 2 °C in a 12-h/12-h light/dark cycle, and had free access to clean tap water and adequate food before any experiment. Rats underwent general anesthesia before any painful procedure, surgery, or sampling. All experiments were carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8523, revised in 2011) and Institutional Animal Care and Use Committee (IACUC) guidebook

[13]. In addition, the Ethics in Medical Research Committee of Tehran University of Medical Sciences approved all the study protocols (No. IR.TUMS.MEDICINE.REC.1399.135). Animals were euthanized at the sampling time by administering lethal doses of anesthetic agents, as described later in the manuscript.

Reagents

Ketamine 10% and xylazine 2% were purchased from Alfasan (Woerden, Netherlands) and were injected intraperitoneally to induce general anesthesia in rats. Acetic acid was purchased from Merck (Darmstadt, Germany), and prednisolone was obtained from Sigma-Aldrich (St. Louis, MO, USA). IVM was received as a gift from Gilaranco (Rasht, Iran), and baclofen was purchased from Shimi Darou Kowsar (Tehran, Iran). To dissolve IVM, normal saline (0.9% NaCl) and propylene glycol (total concentration of 10% [v/v]), as solvent, were utilized. Baclofen was dissolved in dimethyl sulfoxide (DMSO), purchased from Sigma-Aldrich (St. Louis, MO, USA) and subsequently diluted with normal saline (final concentration of DMSO: 0.4% v/v). Prednisolone was dissolved in normal saline. Prednisolone, IVM, and baclofen were delivered to rats orally through a flexible plastic gavage tube.

Acetic Acid-Induced Colitis Model

The model was performed as described previously by Rashidian et al. [14]. Briefly, rats were starved for 24 h before colitis induction while having free access to clean tap water. On the day of colitis induction, rats were given general anesthesia, and 2 mL of 4% acetic acid solution was delivered into the rat colons through a rectal tube in an upside-down position, using an 8-cm flexible plastic catheter with a diameter of 2 mm, inserted into the rectum. Rats were kept in this position for an extra minute to prevent any leakage. Subsequently, they were kept warm under a lamp until complete recovery from anesthesia. Then, the first dose of each reagent was administered orally (by gavage) to each rat based on the experimental groups.

Experimental Design and Sampling Procedure

Rats were randomly divided into different experimental groups (six rats each). After the induction of colitis, treatment with different doses of reagents was initiated. The first doses were administered at around the second hour post-induction, and drug administrations were then continued for 5 days (one bolus dose each day with the same dose as the day of colitis induction). On the sixth day, rats were euthanized by administering high doses of ketamine and xylazine [15], and tissue sampling was performed as follows:

the ventral surface of each rat was incised at the midline; the colon was explored, and the last 8 cm of the colon, including the rectum, was transected. The 8-cm section was incised at the midline and washed with ice-cold normal saline, and macroscopic photographs were obtained. The ulcerated area was detected by a dark-colored change and thickening of the tissue as compared to the normal colon; a full-thickness sample (1.5 cm in length) was then obtained, including the intermediate margin of the ulcerated/necrotic area and the grossly healthy tissue. This segment was then halved in width into two segments. One section was frozen immediately using a liquid nitrogen tank and transferred to a -80°C freezer to be used for further investigations, including western blot analysis, enzyme-linked immunosorbent assay (ELISA), and colorimetric assay. The other section was stored in 10% formaldehyde (for at least 48 h) to be prepared for histopathological studies. Figure 1 shows the timeline of the current study and the experimental groups.

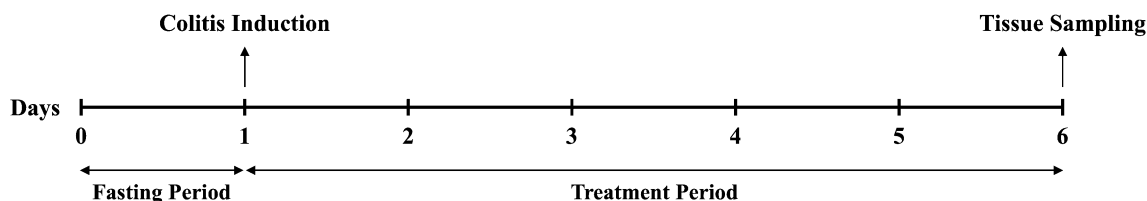
Accordingly, the final experiments consisted of 13 groups (6 rats each) considering different doses of reagents:

- Sham group: no colitis induction + vehicle/day (normal saline + propylene glycol 10% v/v + DMSO 4% v/v)
- Control group (acetic acid): colitis induced + vehicle/day
- Prednisolone group (standard positive control): colitis induced + prednisolone 1 mg/kg/day

- IVM dose–response groups: colitis induced + IVM 0.05, 0.1, 0.2, 0.5, or 1 mg/kg/day in five separate groups
- Baclofen (as a standard GABA_B agonist) dose–response groups: colitis induced + baclofen 0.2, 0.5, 1, or 2 mg/kg/day in four separate groups
- IVM + baclofen group: colitis induced + coadministration of sub-effective dose of IVM and sub-effective dose of baclofen—this group was designed to investigate any possible synergistic effect (described in the results section and Online Resource 1).

Although the main aim of the present study was investigating the anti-inflammatory role of IVM through the GABA_B signaling pathway, we examined the role of GABA_A in this treatment separately by injecting bicuculline—a standard GABA_A antagonist—intraperitoneally before administering the effective dose of IVM (0.5 mg/kg/day), presented briefly in Online Resource 1.

We chose IVM and baclofen dosage based on previous experiments in rats suggesting the most effective doses; they were then adjusted based on the experiments performed in this study. IVM was initiated with a dosage of 0.05 mg/kg and then increased to investigate the dose–response pattern; baclofen was initiated with 1 mg/kg, and the effectiveness of higher and lower doses was explored [10, 16]. Prednisolone was also administered based on previous reports [17].



Day 0: The fasting day; rats had free access to only clean tap water.

Day 1: General anesthesia with ketamine 10% and xylazine 2% and colitis induction with the insertion of 4% acetic acid (2 ml) through a rectal catheter.

Day 1 (About the 2nd-hour post-induction): The first dose of each drug was delivered orally to each rat based on the treatment groups:

- **Control:** Vehicle (normal saline + propylene glycol 10% v/v + DMSO 4% v/v)
- **Prednisolone group:** Prednisolone 1 mg/kg
- **Ivermectin groups:** Ivermectin 0.05, 0.1, 0.2, 0.5, or 1 mg/kg in 5 separate groups
- **Baclofen groups:** Baclofen 0.2, 0.5, 1, or 2 mg/kg in 4 separate groups
- **Ivermectin + Baclofen group:** Ivermectin 0.2 + baclofen 0.2 mg/kg

Days 2–5: Drug delivery was continued with the same dosage as the first day on each day in each treatment group.

Day 6: Rats were euthanized by administering high doses of ketamine and xylazine, and tissue sampling was performed.

Fig. 1 Timeline and the study design explaining the treatment period in detail

Disease Activity Index (DAI)

During the experiments and treatment period, body weight changes, diarrhea, and rectal bleeding were evaluated in all groups. We scored each parameter based on a previously described method for calculating the disease activity index (DAI) [18, 19]. Briefly, changes in body weight were scored as 0=no weight loss, 1=1–5%, 2=6–10%, 3=11–15%, and 4=>15% weight loss. Stool consistency was also scored as 0=normal, 2=loose, and 4=diarrhea, and rectal bleeding was scored as 0=normal, 2=occult bleeding, and 4=gross bleeding. These three scores were recorded for each rat in each study group. Subsequently, DAI was calculated for each rat by averaging these three scores. The mean of DAIs was then calculated and compared among the study groups.

Macroscopic Evaluation of Rats’ Colons and Ulcer Index (UI) Calculation

After colon dissection, an observer blinded to the treatments evaluated each colon and scored the severity of ulcers based on the following scale: 0=no macroscopic change observed; 1=only mucosal erythema could be seen; 2=mild mucosal edema, slight bleeding, or slight erosion; 3=moderate edema, bleeding ulcers or prominent erosions; 4=“severe” ulceration, erosions, edema, and tissue necrosis [20]. The ulcerated/necrotic area was calculated based on the obtained macroscopic photographs using ImageJ software version 1.53a (National Institutes of Health, USA), reported as cm². The ulcer index (UI) was then calculated as follows [21]:

$$\text{Ulcer index (UI)} = \text{Ulcer area (cm}^2\text{)} + \text{Ulcer severity grade.}$$

Histopathological Examination and Scoring

As described earlier, colon specimens were fixed using 10% formalin and were dehydrated, embedded in paraffin wax, processed, and cut into 4-µm-thick sections. They were then stained with hematoxylin and eosin (H&E). Colon sections were assessed by an expert pathologist, blinded to the

treatments, who scored tissues based on the inflammation severity. Inflammation severity was graded on a scale of 0–5 as described previously [22] (Table 1). Random measurements were also repeated regarding inflammation severity to assess intra-observer reliability, calculated to be 0.91 (95% CI 0.88–0.93).

Western Blot Analysis for p-NF-κB p65, COX-2, and iNOS Expression

To investigate the inflammatory response in the intestinal tissue of rats subjected to colitis induction and treated with IVM, baclofen, or a combination of these two drugs, western blot analysis for p-NF-κB p65, prostaglandin-endoperoxide synthase 2 (COX-2), and inducible nitric oxide synthase (iNOS/NOS2) was performed via the following protocol:

Frozen colon samples were defrosted and homogenized in lysis buffer (500 µL Tris-HCL, pH=8; 0.003 g EDTA; 0.08 g NaCl; 0.025 g sodium deoxycholate; 0.01 g sodium dodecyl sulfate [SDS]; protease inhibitor cocktail [one tablet]; 10 µL Triton [NP40 1%]). Tissues were then centrifuged at 4 °C and 12,000×g for 10 min in an Eppendorf 5415R centrifuge (Hamburg, Germany), and the supernatant consisting of protein was extracted and stored in a –20 °C freezer. Protein concentration was assessed by bicinchoninic acid assay (BCA; Bradford), and bovine serum albumin (BSA) was utilized as the standard protein in the procedure. Briefly, after preparing the samples using a sample buffer (0.6 mL Tris; pH=6.8; 2.5 mg glycerol; 0.5 mg β-mercaptoethanol; 0.01 g bromophenol blue; 0.2 g SDS), electrophoresis was performed on polyacrylamide gels. Following electrophoresis, protein bands were transferred to polyvinylidene fluoride (PVDF) membranes, and blocking was performed using a blocking solution consisting of 2% non-fat dried milk in TBS-T buffer for 1 h 15 min at room temperature. After blocking, incubation with primary antibodies, including β-actin (C4) mouse monoclonal antibody (1:300 dilution; sc-47778; Santa Cruz Biotechnology, Inc., TX, USA), p-NF-κB p65 (27.Ser 536) mouse monoclonal antibody (1:300 dilution; sc-136548; Santa Cruz Biotechnology), COX-2 (H-3) mouse monoclonal antibody (1:300

Table 1 Grading of inflammation (histopathological score) based on histopathological findings in rat colon sections

Grade of inflammation	Histopathological findings
Grade 0	Mucosa, submucosa, muscularis propria, and serosa could be seen with normal histological characteristics with no inflammatory cell infiltration
Grade 1	Infiltration of inflammatory cells in mucosa and submucosa could be seen
Grade 2	Transmural inflammatory cells infiltration (acute inflammation)
Grade 3	Small ulcers along with acute inflammation in the wall
Grade 4	Extensive ulceration and necrosis of the entire wall or partial transmural inflammation and irregularity of villous mucosal surface

dilution; sc-376861; Santa Cruz Biotechnology), and anti-NOS2 (C-11) mouse monoclonal antibody (1:300 dilution; sc-7271; Santa Cruz Biotechnology), was performed for 16 to 18 h. After subsequent washing with TBS-T buffer (20 mL Tris–HCL; 8 g NaCl; 1 mL Tween 20 10% v/v; 100 mL distilled water) three times for 15 min each, secondary antibodies were added, including mouse m-IgGκ BP-HRP antibody (1:1000 dilution; sc-516102; Santa Cruz Biotechnology) and mouse anti-rabbit IgG-HRP antibody (1:1000 dilution; sc-2357; Santa Cruz Biotechnology), and the mixture was shaken at room temperature, followed by washing three times with TBS-T buffer (15 min each time). The protein bands were then detected via the chemiluminescent western blotting technique using photographic films.

Myeloperoxidase (MPO) Activity Colorimetric Assay

MPO activity was assessed using the MPO Colorimetric Activity Assay Kit (Sigma-Aldrich, St. Louis, MO, USA; Catalog Number: MAK068). In this assay, MPO catalyzes the formation of hypochlorous acid, which then forms taurine chloramine by reacting with taurine. Taurine chloramine subsequently reacts with the chromophore TNB and results in the formation of DTNB. Each unit (1 U) of MPO activity is defined as the amount of enzyme that hydrolyzes the substrate and forms taurine chloramine to consume 1.0 μmol of TNB per minute at room temperature. The sample preparation and MPO activity assay were performed according to the protocol provided by the manufacturer, the final absorbance was measured at 412 nm, and the final MPO activity was adjusted for each sample's protein concentration, reported as mU/mg protein in this manuscript.

ELISA for Measurement of TNF-α Levels

Quantitative assessment of TNF-α was performed using a rat TNF-α ELISA kit (DuoSet ELISA Development Systems, R&D Systems, Minneapolis, MN, USA; Catalog Number: DY510), according to the protocol provided by the manufacturer. The optical density was then determined with a microplate reader set at 450 nm. The final concentrations were adjusted for the protein concentration of the samples, and the final results are reported as pg/mg protein.

Statistical Analysis

The final results are reported as mean ± standard deviation (SD). Group differences regarding UI, DAI, p-NF-κB p65, COX-2, and iNOS expression levels, MPO activity, and TNF-α level were analyzed using parametric one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. A *p* value < 0.05 was considered statistically significant. All the statistical analyses were performed using GraphPad

Prism version 8.2.1 software for macOS (GraphPad Software, San Diego, CA, USA).

Results

Disease Activity Index (DAI)

We found severe diarrhea and weight loss in rats who underwent acetic acid-induced colitis in the control group. Rectal bleeding was also noticeable in the control group compared to the others. The highest DAI was found in this group; however, prednisolone 1 mg/kg/day, IVM 0.1 mg/kg/day or more, baclofen 0.2 mg/kg/day or more, and the combination therapy of IVM 0.2 + baclofen 0.2 mg/kg/day significantly improved the DAI compared to the control group (*p* < 0.001) (Fig. 2).

Macroscopic Evaluation of Rats' Colons and Ulcer Index (UI)

In the control group, signs of significant mucosal injury could be seen grossly in the colon tissue after dissection, including edema, hyperemia, wall thickness, ulceration, and necrosis (UI = 6.5 ± 0.7; Fig. 3). However, colitis signs improved markedly in groups treated with effective doses of IVM and baclofen, almost the same as the prednisolone group. The dose–response pattern of IVM and baclofen are illustrated in Fig. 3. IVM 0.2 and baclofen 0.2 were considered sub-effective doses that led to a relative improvement in the colon tissue, based on macroscopic evaluations and the UIs of 3.0 ± 0.9 and 2.4 ± 0.6, respectively. However, the IVM 0.5 (UI = 1.4 ± 0.4) and baclofen 0.5 (UI = 1.7 ± 0.6) mg/kg/day were the optimum effective doses (*p* < 0.001

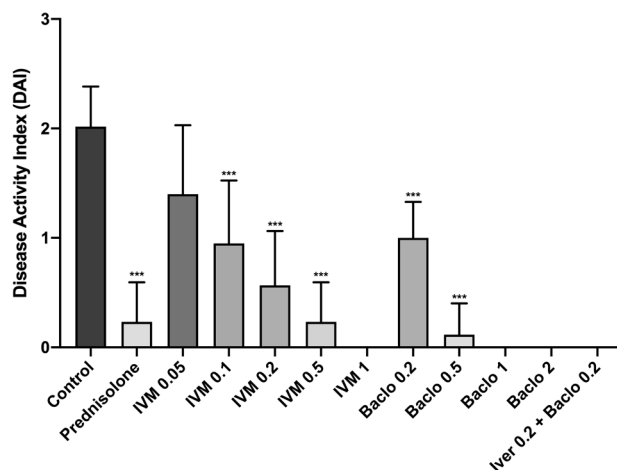


Fig. 2 Evaluation of the disease activity index (DAI) in different study groups; ****p* ≤ 0.001 compared to the control group

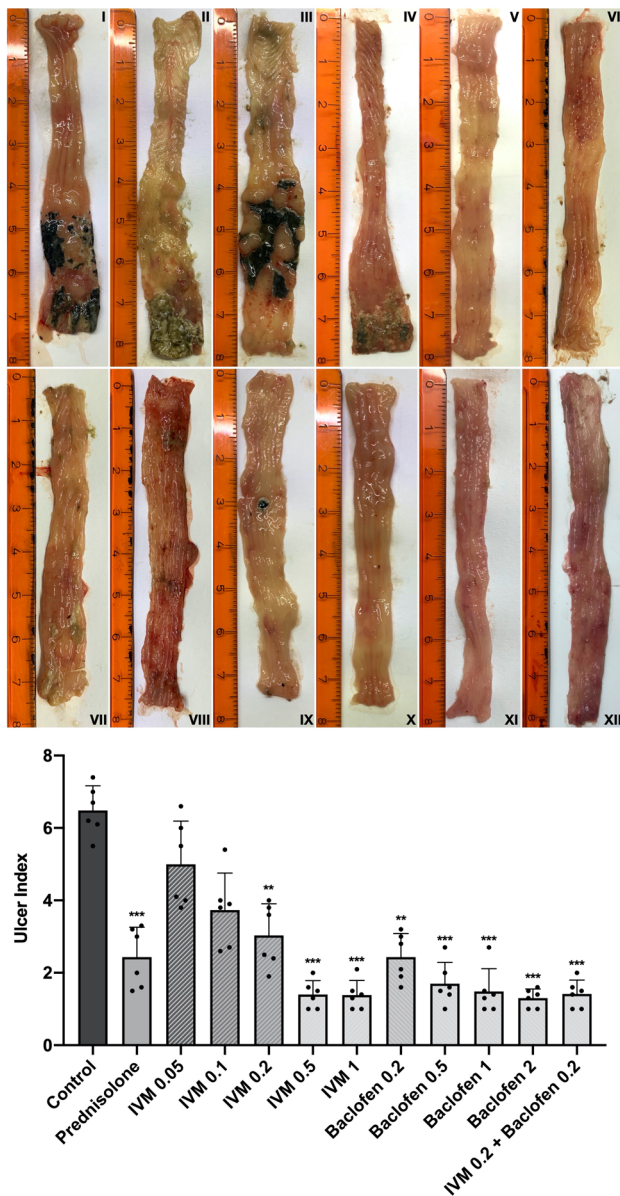


Fig. 3 Macroscopic appearance of the last 8 cm of the rat colons (including rectum) at the sixth day following acetic acid-induced colitis, treated with vehicle (I, control), ivermectin (IVM) 0.05 mg/kg/day (II), IVM 0.1 mg/kg/day (III), IVM 0.2 mg/kg/day (IV), IVM 0.5 mg/kg/day (V), IVM 1 mg/kg/day (VI), prednisolone 1 mg/kg/day (VII), baclofen 0.2 mg/kg/day (VIII), baclofen 0.5 mg/kg/day (IX), baclofen 1 mg/kg/day (X), baclofen 2 mg/kg/day (XI), and IVM 0.2 + baclofen 0.2 mg/kg/day (XII). Ulcer indices are shown in the chart; ** $P \leq 0.01$ and *** $P \leq 0.001$ compared to the control group

vs. the control group) and showed no necrosis or severe ulceration, and no edema or wall thickening could be found grossly. A further increase in the dosage of IVM or baclofen did not produce a substantial change in the final results, and no significant difference was found between higher doses and the IVM 0.5 and baclofen 0.5 ($p > 0.05$). The difference between effective doses of IVM or baclofen and the

prednisolone group was not statistically significant (prednisolone group $UI = 1.3 \pm 0.5$; $p > 0.05$). Coadministration of IVM 0.2 and baclofen 0.2 also significantly affected the colon tissue, leading to recovery from colitis ($UI = 1.4 \pm 0.3$; $p < 0.001$ compared to the control group), showing a synergistic pattern (Online Resource 1).

Histopathological Examination

Sample tissues obtained from the control group (acetic acid) indicated extensive ulceration and necrosis of either the entire colon wall or part of it, along with irregular villus mucosal surface and extensive transmural inflammatory cells infiltration (score = 4.8 ± 0.4). In addition, colon layers were almost indistinguishable from each other under a light microscope. In contrast, the IVM 0.5, baclofen 0.5, and IVM 0.2 + baclofen 0.2 groups showed the most significant effect in lowering histopathological scores and the grade of inflammation, with scores of 0.3 ± 0.5 , 0.2 ± 0.4 , and 0.3 ± 0.5 , respectively ($p < 0.001$ compared to the control group). Histopathological scores in the IVM 0.5, baclofen 0.5, and IVM 0.2 + baclofen 0.2 groups were lower than those in the prednisolone group ($p = 0.04$, 0.01 , and 0.04 , respectively), indicating better improvements. In these three groups, mucosa, submucosa, muscularis propria, and serosa were distinguishable almost as well as the normal colon tissue, with no or mild inflammatory cells infiltration. The IVM 0.2 + baclofen 0.2 group had significantly lower scores than either IVM 0.2 or baclofen 0.2 administration alone ($p < 0.001$) (Fig. 4).

Western Blot Analysis for p-NF-κB p65, COX-2, and iNOS Expression

Following the induction of colitis with acetic acid, the expression level of p-NF-κB p65 increased significantly compared to that in the sham group ($p < 0.001$). After the administration of the effective dose of IVM (0.5 mg/kg/day) or baclofen (0.5 mg/kg/day), p-NF-κB p65 decreased markedly in comparison with the control group ($p < 0.05$ and < 0.001 , respectively). The lowest expression level was recorded in the combination therapy with IVM 0.2 + baclofen 0.2 mg/kg/day ($p < 0.001$ vs. the control and $p < 0.05$ vs. the baclofen 0.5 group) (Fig. 5).

Almost the same pattern was found in the expression of COX-2 and iNOS. After colitis induction, the expression level of both factors increased significantly compared to the sham group ($p < 0.001$). However, COX-2 and iNOS expression decreased markedly in the intestinal tissue of rats receiving IVM (0.5 mg/kg/day), baclofen (0.5 mg/kg/day), or a combination of IVM 0.2 + baclofen 0.2 mg/kg/day ($p < 0.001$ vs. the control).

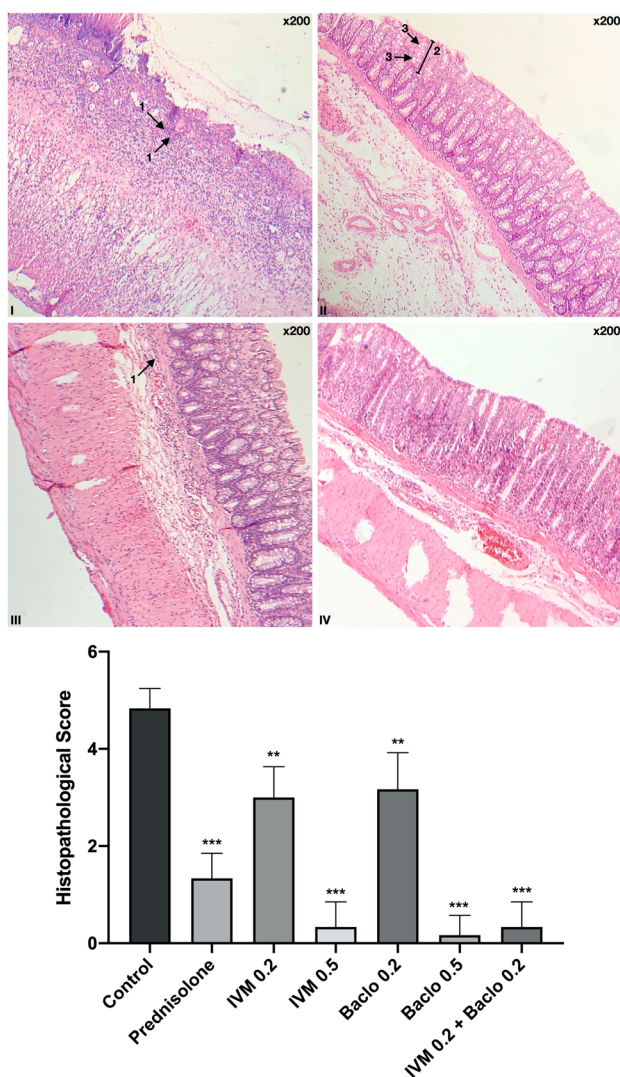


Fig. 4 Histopathological sections of rat colons following acetic acid-induced colitis treated with vehicle (I, control), prednisolone 1 mg/kg/day (II), IVM 0.5 mg/kg/day (III), and IVM 0.2 + baclofen 0.2 mg/kg/day (IV) at $\times 200$ magnification stained with hematoxylin and eosin (H&E). Acetic acid stimulation led to extensive mucosal damage (control group), such that colon layers could not be distinguished from each other. Significant transmural inflammatory cells infiltration was also observed, and the normal structure of crypts and goblet cells was disrupted. Effective doses of IVM (0.5 mg/kg/day) and prednisolone (1 mg/kg/day) attenuated histopathological damage by diminishing infiltration of inflammatory cells, and crypt and goblet cell structures were almost normal in these groups. Mucosa, submucosa, muscularis propria, and serosa could be seen with almost normal structures. The same was found with the coadministration of IVM and baclofen simultaneously (IVM 0.2 + baclofen 0.2 mg/kg/day). The chart provides histopathological scores (based on the inflammatory responses) for the study groups; lower scores indicate better recovery from colitis injury and milder inflammation. The results are illustrated based on the mean \pm SD for each group; ** $p \leq 0.01$ and *** $p \leq 0.001$ compared to the control group. Arrows: (1) inflammatory cells, (2) depth of a crypt, and (3) goblet cells

Myeloperoxidase Activity and TNF- α Levels in Colon Tissues

Colorimetric assessment of MPO activity, as an indicator of granulocytic infiltration, revealed a considerable decrease in MPO activity in groups treated with IVM 0.5 or baclofen 0.5 or the combination therapy, in comparison with the control group ($p < 0.001$). MPO activity in the sham group was calculated as 163.2 ± 8.6 mU/mg protein. It was measured as 160.3 ± 9.6 mU/mg protein in the prednisolone group, in contrast to the control group with estimated MPO activity of 317.9 ± 7.7 mU/mg protein ($p < 0.001$ vs. the control group and $p > 0.5$ vs. the sham group). Meanwhile, in the IVM 0.5, baclofen 0.5, and IVM 0.2 + baclofen 0.2 groups it was 220.7 ± 10.5 , 155.0 ± 6.5 , and 167.1 ± 8.6 mU/mg protein, respectively ($p < 0.001$). The IVM 0.2 + baclofen 0.2 group also showed markedly lower MPO activity than both the IVM 0.2 and IVM 0.5 groups ($p < 0.001$), but no difference was found in comparison with baclofen alone (0.2 or 0.5; $p > 0.05$) (Fig. 6).

TNF- α level, a pivotal proinflammatory cytokine, showed the same pattern as MPO activity among different groups, and the control group had extremely high levels of TNF- α (116.2 ± 14.0 pg/mg protein) compared to the treatment groups. Effective doses of IVM and baclofen, administered separately or in combination therapy, both significantly decreased TNF- α levels following colitis induction. Estimated TNF- α levels were 6.5 ± 3.3 pg/mg protein in the prednisolone group and 11.1 ± 4.2 , 4.6 ± 3.3 , and 9.8 ± 3.4 pg/mg protein in the IVM 0.5, baclofen 0.5, and IVM 0.2 + baclofen 0.2 groups ($p < 0.001$ vs. the control group), respectively (Fig. 6).

Discussion

Acetic acid-induced colitis can be used as a standard experimental model of ulcerative colitis [23]. Additionally, corticosteroids have been used in numerous animal studies as a standard treatment to reverse the devastating effect of acetic acid [24, 25]. In this study, IVM showed a promising effect in treating acetic acid-induced colitis in rats in terms of inflammatory biomarkers and both macroscopic and microscopic features. This study revealed that IVM 0.5 mg/kg/day could decrease DAI, UI, p-NF- κ B p65, COX-2, and iNOS expression, MPO activity, and TNF- α levels. These desired effects were also observed with baclofen (a standard GABA_B agonist) 0.5 mg/kg/day and synergistically with the combination of IVM 0.2 mg/kg/day and baclofen 0.2 mg/kg/day.

IVM, an old anthelmintic drug, was recently studied by Tabary et al. in an experimental model of skin flap, showing a robust anti-inflammatory effect and improving flap survival [10]. The authors also proposed the GABAergic

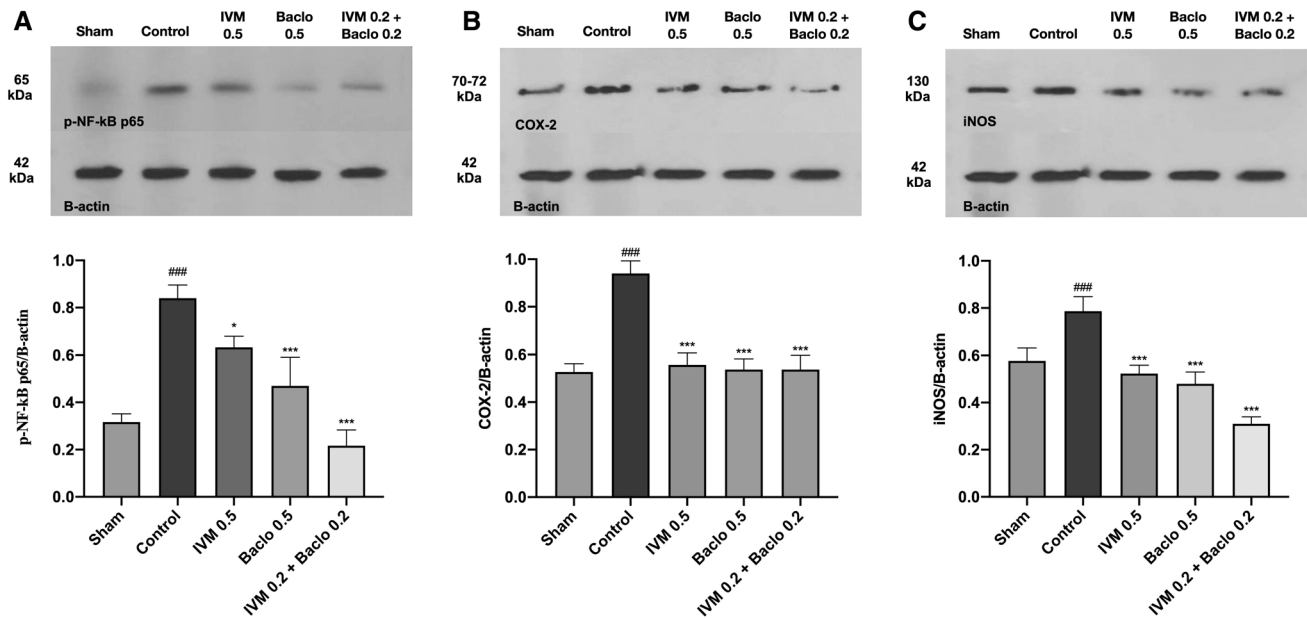


Fig. 5 Western blot analysis for **a** p-NF-κB p65, **b** COX-2, and **c** iNOS expression in the sham (no colitis induction) and control groups compared to the effective therapies ivermectin (IVM) 0.5 mg/kg/day, baclofen 0.5 mg/kg/day, and IVM 0.2 + baclofen 0.2 mg/kg/day.

Densities were normalized to B-actin band density. The results are illustrated based on the mean ± SD for each group; * $p \leq 0.05$ and *** $p \leq 0.001$ compared to the control group; ### $p \leq 0.001$ compared to the sham group

pathway as a possible mechanism for the beneficial anti-inflammatory effect of IVM on skin flap survival. GABA is the major inhibitory neurotransmitter in the mature central nervous system, acting on two distinct subtypes of receptors: GABA_A and GABA_B [26, 27]. GABA_A receptors are heteropentameric chloride ion channels, whereas the GABA_B subtype is a G protein-coupled receptor [27]. The presence of both subunits was previously shown in colon tissue [28, 29].

Recent studies have also indicated that GABA is involved in inflammatory events and immune cell activity, since its receptors have been found on macrophages, dendritic cells, and T-cells [30]. Both GABA_A and GABA_B seem to play a pivotal role in attenuating the inflammatory condition; GABA_A reduces macrophage cytokine production and inhibits T-cell proliferation, while GABA_B reduces TNF-α production and decreases interleukin (IL-6 and IL-12) release from microglial cells. Concerning IBD, the role of GABA_A is still a matter of controversy. In a study by Dudley et al., it was found that the GABA_A agonist topiramate could alleviate colitis in rats. In contrast, activation of GABA_A led to worsening colitis in mice models in a study by Ma et al. [28]. However, concerning the role of GABA_B in treating colitis, there are still too few data available. In the present study, we targeted GABA_B receptors by administering baclofen (GABA_B agonist) to investigate the possible involvement of the GABA_B pathway in the anti-inflammatory effect of IVM in colitis and the simultaneous coadministration of these two agents. Both agents showed a significant and promising

effect in the treatment of acetic acid-induced colitis in rats. Also, in a separate group investigating the probable role of GABA_A, we found no reliable or significant effect regarding the macroscopic and microscopic parameters used in the present study, and it did not significantly reverse the positive effects of IVM (see Online Resource 1).

It is worth noting that inflammation is among the most important events justifying the beneficial role of IVM in the treatment of colitis. We found lower levels of TNF-α and NF-κB, as well as reduced MPO activity, after the administration of effective doses of IVM or baclofen, all of which are major inflammatory factors known to be involved interactively in the pathogenesis and treatment of IBD [31]. The anti-inflammatory effect of IVM was described previously by Tabary et al. [10]. Briefly, they showed that IVM could diminish the inflammatory condition by decreasing the production and release of IL-1b and TNF-α following skin flap surgery in rats. They also indicated that GABAergic pathway activation could be a possible mediator for this anti-inflammatory effect. A study by Duthey et al. also demonstrated a new role for GABA_B receptor activation in alleviating inflammation [32]. They used baclofen in both in vitro and in vivo models of allergic contact dermatitis. In vitro, baclofen reduced chemotaxis of human peripheral blood mononuclear cells. In addition, baclofen significantly reduced signs of inflammation and recruitment of neutrophils, monocytes, and lymphocytes into the skin in vivo. The

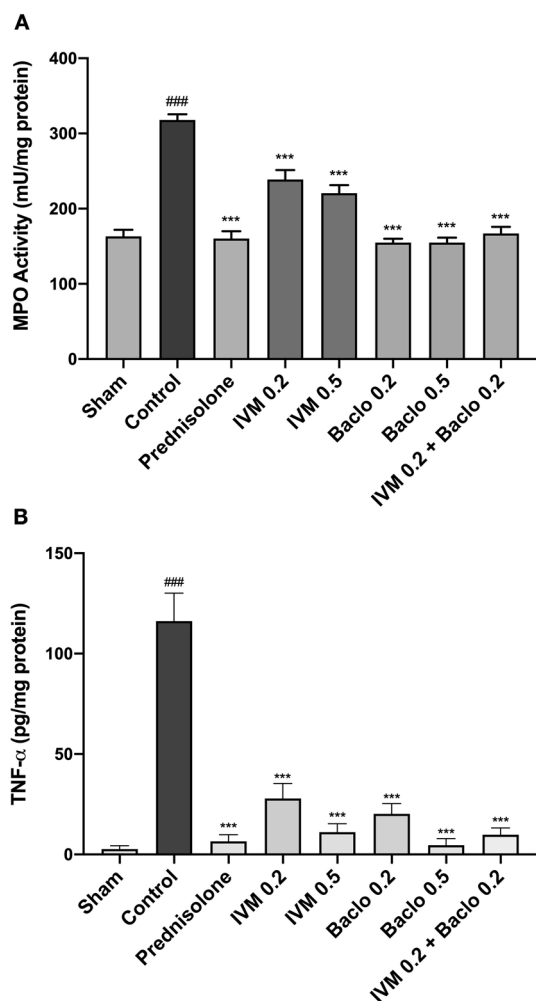


Fig. 6 Colorimetric myeloperoxidase (MPO) activity (a) and tumor necrosis factor-alpha (TNF- α) quantitative level (ELISA) (b) in each treatment group. The results are illustrated based on the mean \pm SD for each group; *** $p \leq 0.001$ compared to the control group and ### $p \leq 0.001$ compared to the sham group

current study also indicated that IVM exerts an acceptable anti-inflammatory effect after induction of colitis in rats.

TNF- α is believed to play an important role in the pathogenesis of colitis, as many drugs target TNF- α or its receptor in IBD treatment [33–35]. We found lower levels of TNF- α after the administration of IVM or baclofen, which implies that TNF- α might be a crucial target. In vitro, TNF- α secretion was the only reduced cytokine, among numerous others, after the administration of baclofen in treating allergic contact dermatitis, as described previously [32].

Transcription factor NF- κ B seems to be one of the major regulatory components of dysregulated cytokine production and signaling mechanisms in the pathogenesis of IBD. Macrophages and epithelial cells isolated from inflamed gut specimens from IBD patients indicated higher levels of NF- κ B p65 [36]. A study by Liu et al. showed that baclofen

suppressed the increased expression of p-NF- κ B p65 in satellite glial cells (SGCs) treated with lipopolysaccharide (LPS) in vitro [37]. They also concluded that baclofen could attenuate inflammatory orofacial pain by modulating IL-1 β . These findings are in line with ours, indicating that NF- κ B expression is reduced after administration of IVM or baclofen in acetic acid-induced colitis models. It is worth mentioning that although prednisolone revealed better MPO and TNF- α activity than IVM 0.2 + baclofen 0.2 mg/kg/day, this difference did not reach statistical significance. On the other hand, the prednisolone mechanism might differ from that of IVM; for example, prednisolone inhibited NF- κ B in patients with inflammatory bowel disease [38]. However, there might be considerable differences between prednisolone and IVM on their downstream action. Finally, a longer duration of treatment might be needed for the maximal effect of IVM, which needs further investigation in future studies.

COX-2, a proinflammatory enzyme, is induced in both animal models of IBD and patients with IBD [39]; the same has been observed for iNOS [40]. Experimental evidence also shows that simultaneous inhibition of COX-2 and iNOS might have a promising role in treating chemically induced colitis [41]. In line with previous studies, our findings revealed that COX-2 and iNOS expression increased in rats following colitis induction, while a significant reduction was observed after treatment with IVM, baclofen, or the combination of both drugs.

This study was designed as an experimental study, and all the limitations related to experimental and animal studies are also applicable to ours. In addition, some recent animal colitis models in the literature have utilized agents other than acetic acid, especially 2,4,6-trinitrobenzene sulfonic acid (TNBS) [42]; however, at the time it was only possible for us to use acetic acid to perform the colitis model. Studies have also reported that the acetic acid-induced colitis model is comparable to TNBS in the case of colitis induction [43]. The effect of IVM and baclofen could be investigated in TNBS-induced colitis in rats in further studies. Moreover, evaluation of the role of the GABA_A receptor in the anti-inflammatory role of IVM in colitis was not within the main scopes of our study. The role of this receptor can be investigated in further studies, and its controversial role based on previous studies necessitates the conduct of a comprehensive study at the molecular level to investigate its role in greater depth.

Conclusions

This study showed that controlling the inflammatory condition in acetic-acid colitis in rats could be achieved through activation of the GABA_B signaling pathway by administering IVM or baclofen or the combination therapy using both

agents. The combination therapy helps us administer lower doses of these agents, which can lower the risk of any possible side effects of higher doses of IVM or baclofen. This could also pave the way to utilize these agents in the clinical setting safely. Further studies still need to be conducted to investigate the possibility of utilizing these agents in the clinic. This study also signified the anti-inflammatory role of GABA, which allows researchers to investigate its role in other organs.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10620-021-07258-x>.

Acknowledgment This study was supported by a grant from the Experimental Medicine Research Center, Tehran University of Medical Sciences (Grant No. 99-1-209-48299). The authors also want to thank the Iran National Science Foundation (INSF) for their kind support.

Authors' contributions Study conception and design: AD; acquisition of data: AA, MT, NN, SMT; analysis and interpretation of data: AA, MT, AR, RM; drafting of the manuscript: AA, MT, BM, AD, SI; critical revision: AD, AR.

Funding This study was supported by a grant from the Experimental Medicine Research Center, Tehran University of Medical Sciences (Grant No. 99-1-209-48299).

Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

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

Ethics approval All experiments were carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8523, revised in 2011). All the study protocols were also approved by the Ethics in Medical Research Committee of Tehran University of Medical Sciences (No. IR.TUMS.MEDICINE.REC.1399.135) and were therefore performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

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