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Research Article

Ibudilast ameliorates experimentally induced colitis in rats via down-regulation of proinflammatory cytokines and myeloperoxidase enzyme activity

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Abstract

Objectives: This study was carried out to explore the possible anti-inflammatory effect of ibudilast on acetic acid-induced colitis in rats.

Methods: Fifty adult Wistar rats were separated into 5 groups, including the control group, acetic acid group, acetic acid + vehicle, acetic acid + sulfasalazine (100 mg/kg/day)group, and acetic acid + ibudilast (30 mg/kg/day) group. Colitis was induced in rats by the inter-rectal installation of 2 ml of 4% (v/v) acetic acid. Sulfasalazine and ibudilast were administered orally for ten days after 2 hours of induction.

Results: The treatment with ibudilast significantly reduced disease activity index (DAI), macroscopic colonic scores (MAC), and histopathological changes induced by acetic acid. Also, ibudilast markedly decreased the expression of proinflammatory markers (TNF-α and IL-1β) in colonic tissue. Moreover, ibudilast inhibited myeloperoxidase (MPO) enzyme activity that was increased by acetic acid.

Conclusion: Therefore, ibudilast may have a therapeutic effect in the management of ulcerative colitis.

Keywords

Ibudilast, colitis, antinflammatory, IL-1β, and TNF-α

Introduction

Inflammatory bowel diseases (IBD) refer mainly to two idiopathic gastrointestinal diseases, Crohn's disease (CD) and ulcerative colitis (UC), characterized by diarrhea, abdominal pain, weight loss, and in ulcerative colitis, perianal bleeding (Firouzabadi et al. 2021). Furthermore, severe UC will increase the risk of many complications, such as toxic megacolon and colorectal cancer (Almuttairi et al. 2020). UC is described as a mucosal inflammation

that starts in the rectum and may spread anteriorly and continuously to affect other parts of the colon with a recurrence pattern of activity (Gajendran et al. 2019). The cause of ulcerative colitis remains unknown. However, various factors have been linked to its pathogenesis, including microbes, genetic background, environmental factors, and immune response abnormalities (Chung et al. 2014). Neutrophil infiltration, excessive secretion of inflammatory cytokines such as TNF-α and IL-1β, and excessive production of oxidative stress byproducts generally participate in colitis pathogenesis (Balmus et al. 2016). Increasing oxidative stress promotes intestinal mucosal inflammation, which in turn activates nuclear factor-kappa B (NF-kB) and the upregulation of proinflammatory cytokines and adhesion molecules in a vicious cycle (Zhu and Li 2012; Liu et al. 2017). In addition, the activation of Toll-like receptors (TLRs), mainly TLR4, stimulates the proinflammatory signaling pathway and plays an essential role in initiating the inflammatory response (Xia et al. 2012).

Therefore, controlling inflammation and oxidative stress is a critical therapeutic goal for UC (Hazel and O'Connor 2020). Although significant developments have been made in managing ulcerative colitis, the adverse effects of medications during extended treatment durations and the high relapse rate diminish their effectiveness. Therefore, it is necessary to explore new strategies to restore the altered immune response that arises in the inflamed intestine (Leppkes and Neurath 2020). Recently, phosphodiesterase enzyme 4 (PDE4) inhibitors have demonstrated remarkable therapeutic efficacy in treating UC symptoms (Spadaccini et al. 2017). PDE4 is a specific enzyme for cyclic adenosine monophosphate (cAMP) breakdown. Therefore, inhibiting PDE4 increases intracellular cAMP, reducing cell migration and releasing chemokines and cytokines from inflammatory cells (Crilly et al. 2011). UC was associated with lower colon cAMP levels and increased PDE4 expression, which led to aberrant cytokine production in the inflamed gut (Schafer et al. 2016). PDE4 has been established as a new therapeutic approach for treating inflammatory disorders such as asthma, chronic obstructive pulmonary disease, psoriatic arthritis, dermatitis, and inflammatory bowel disorders (Li et al. 2018).

Ibudilast inhibits PDE4 mainly and other PDE subtypes to various degrees (Grodin et al. 2021). It possesses anti-inflammatory and pain-modulating properties as both a TLR4 antagonist and a PDE inhibitor (Korhonen and Moilanen 2014). Ibudilast has long been used in Japan for treating asthma and post-stroke dizziness (Fox et al. 2016). Recently, due to its immunomodulatory, anti-inflammatory, and potentially neuroprotective actions, ibudilast has been evaluated in neurological disorders (Goodman et al. 2016). They show that ibudilast reduces astroglial reactivity and the generation of proinflammatory mediators in the striatum in a mouse model of Parkinson's disease (Schwenkgrub et al. 2017). In addition, it reduces the release of neuropeptides from bronchial sensory neuron endings (Ichinose et al. 1993). It has also been observed that ibudilast diminishes the acute inflammatory effects of methamphetamine therapy (Li et al. 2020). Moreover, ibudilast was reported to alleviate severe arthritis by lowering inflammatory mediator levels (Clanchy and Williams 2019). Therefore, this study aimed to investigate the potential anti-inflammatory effect of ibudilast on experimentally induced colitis.

Materials and methods

Animals

Fifty male albino Wister rats (200 ± 20gram) were obtained from the College of Science/Babylon University. The rats were housed in standard cages for seven days before starting the study for acclimation to laboratory conditions. The rats were housed at a temperature (25–27 °C) and 50% humidity, with 12 hours of light/dark cycle, had free access to a commercial diet and were allowed to drink tap water. The institutional ethics committee at Al-Nahrain university/college of medicine approved this study proposal.

Drugs and chemicals

Glacial acetic acid, dimethyl sulfoxide solvent (DMSO) and diethyl ether were obtained from BDH Chemical Ltd., England. Immunohistochemistry kits of TNF-α and IL-1B (Abcam, UK), ibudilast and sulfasalazine (Hangzhou Jinlan Pharm-Drugs Technology, China) were purchased.

Induction of colitis

The colitis was produced in rats using acetic acid following the procedure suggested by Atarbashe and Abu-Raghif (2020) with slight modifications. Rats were fasted for at least 24 hours to dispose of the colon faeces to get proper colitis induction but were permitted to tap water. Before inducing colitis, the water was interrupted for 2 hours. Briefly, under anaesthesia with light ether, rats received 2 ml of (4% v/v) acetic acid (AA) solution as a single transrectally infusion into the colon 8 cm using a flexible silicone tube with a 2 mm diameter. After acetic acid administration, rats were held in head down position for 2 min to prevent the outflow of acetic acid. The same procedure using normal saline (0.9%) instead was installed for the control group.

Experimental design

The animals were separated into five groups (10 rats in each). **Group 1** (control group) received 2 ml of 0.9% normal saline transrectally on 1st day, while other groups received 2 ml of 4% v/v acetic acid transrectally on 1st day. **Group 2** (AA group) received normal saline orally; **Group 3** received 1 ml of a vehicle 1% dimethyl sulfoxide p.o. (DMSO). **Group 4** (positive control) received

sulfasalazine 100mg/kg/day p.o. (Morsy et al. 2021); **Group 5** received ibudilast 30mg/kg/day p.o. (Inaba et al. 2015). All treatments started 2 hours after acetic acid installation and continued for 10 days (Morsy et al. 2021). All drugs used in this study were freshly prepared before administration. Sulfasalazine was suspended in normal saline, while ibudilast was suspended in 1% DMSO.

After 24 hours from the final oral dose of the treatments, rats were anaesthetized with diethyl ether to sacrifice them. The colons were collected from all animals and cleaned with chilled normal saline during the abdomen dissection. The excised colon of all rats was assessed macroscopically. Then, the samples were divided into two pieces: one for fixation in 10% formalin for histopathological and immunohistochemical study, and the second was kept at – 80 °C for tissue homogenization until myeloperoxidase activity could be measured.

Methods

Assessment of disease activity index (DAI)

The disease activity index was used to evaluate colitis severity clinically, as previously defined by Niu et al. (2015). DAI involved the following parameters: body weight loss, 0 = (weight gain or no reduction); 1 = 1–5%; 2 = 6–10%; 3 = 11–15%; 4 more than 15%); the stool consistency: 0 = normal, 2 = loose stool (don't stick to the anus), 4 = diarrhea (liquid stools that stick to the anus); and bleeding of the rectum (0 = normal; 1,2 = mild; 3,4 = severe). The DAI scoring was performed by combining the sums of the preceding parameters' scores.

Assessment of macroscopic colonic score (MAC)

According to Wallace et al. (1989), the degree of macroscopic alteration was evaluated by awarding scores. Briefly, the grading system is as follows: no macroscopic change (grade 0); no ulcer, mucosal hyperemia only (grade 1); hyperemia and mild edema with little erosion, no ulcers (grade 2); one ulcer or inflammation at one site (grade 3); ulceration or inflammation at two or more site (grade 4); severe ulceration extending > l cm along the entire colon and tissue necrosis (grade 5); and damage covered > 2 cm along the whole colon and tissue necrosis (grade 6).

Measurement of myeloperoxidase (MPO) activity

Myeloperoxidase (MPO) enzyme activity was detected in colonic samples using a modified form of a previously described method (Bradley et al. 1982). 0.1g of colon tissue was homogenized with 1 ml potassium phosphate buffer (50 mM pH=6). The homogenate was blended with 5 ml of buffer solution in an ice bath and was centrifuged at 4 °C for 15 minutes at 15,000 rpm. The final volume was created by combining 3 ml of phosphate buffer (50 mM, pH=6), 0.167 mg/mL of O-dianisidine dihydrochloride, and 0.0005% hydrogen peroxide. Finally, the absorbance at 460 nm was detected by a spectrophotometer (Shimadzu/ Japan), and MPO activity was calculated as U/g (units/gram).

Histopathological and immunohistochemical (IHC) study

For histological evaluation, colons were preserved in 10% neutral formalin and incorporated in paraffin blocks. Then, 5-µm thick sections were sliced and stained with hematoxylin and eosin (H&E). Evaluation of microscopic abnormalities of a colonic lesion according to previously reported grading criteria (Wang et al. 2019): loss of the architecture of mucosa (0–3), leukocyte infiltration (0–3), muscle thickening (0–3), generation of crypt abscess (0–1), and goblet cells loss (0–1). For a scale of (0–3) (absent, mild, moderate, and severe) and scores (0–1) = (absent or present), the maximum score was 11.

An immunohistochemical (IHC) study was conducted on tissue in a paraffin block using TNF-α polyclonal antibody(Abcam/ab220210) and IL-1β antibody (Abcam/ab200478). Immunohistochemical evaluation of TNF-α and IL-1β was determined according to semi-quantitative scores (Hernandez-Rodriguez et al. 2004) that were based on the percentage of positively stained cells as follows: 0, no staining; 1 ≤ 25%; 2 (26–50%); 3 (51–75%); and 4 (76–100%).

Statistical analysis

Data were presented as mean ± SD and analyzed using SPSS version 23. One-way ANOVA followed by LSD was used to determine the differences among groups. P value < 0.05 was deemed statistically significant throughout the study.

Results

Effect of ibudilast on DAI

Colitis severity was estimated for each animal according to DAI criteria. The three criteria that made up the DAI scores were rectal bleeding, stool consistency, and body weight reduction. The AA group and AA + vehicle group have significantly higher DAI scores than the control group ($p < 0.001$). However, the oral treatment with ibudilast and sulfasalazine significantly decreased DAI scores than the untreated AA group (Fig. 1A; $p < 0.001$).

Effect of ibudilast on the macroscopic colonic score (MAC)

According to Wallace's score, the macroscopic feature was assessed with a grading system (0–6). The control group did not have a score, while the AA group manifested significant damage, such as thickening of the colon and massive tissue necrosis and ulceration. Treatment with ibudilast and sulfasalazine significantly lowered the extent of the macroscopic damage (Fig. 1B; $p < 0.001$).

Effect of ibudilast on MPO activity

The acetic acid installation increased MPO activity in colonic tissue compared to the control group. Ibudilast and sulfasalazine significantly reduced the increase in MPO activity upon comparison with the acetic acid group. (Fig. 1C; $p < 0.001$)

Effect of ibudilast on histopathological changes in rat colonic tissues

As shown in Fig. 2, specimens from the control group exhibited a typical appearance of colonic layers, intact mucosa, submucosa, and muscularis, as well as a few inflammatory cells appearing in the submucosa. Rectal injection of acetic acid caused diffuse damage and necrosis with severe mucosal ulceration and little crypt remnants and was associated with massive inflammatory cell infiltration. Therefore, the acetic acid group showed a significantly higher histopathological score than the control group (Fig. 1D; $p < 0.001$). Treatment with ibudilast and sulfasalazine resulted in an improved histological pattern, less mucosal ulcers, preservation of crypts, and markedly decreased inflammatory cell infiltration (Fig. 2). Therefore, ibudilast and sulfasalazine treatment had lower microscopic scores than the acetic acid group (Fig. 1D; $p < 0.001$).

Effect of ibudilast on proinflammatory cytokines (TNF-α and IL-1β)

As shown in Figs 3, 4, rectally installation of acetic acid significantly increased inflammatory mediators (TNF-α and IL-1β) expression in colonic tissue than in the control group. Treatment with ibudilast and sulfasalazine significantly decreased the TNF-α and IL-1β levels increment upon comparison with the acetic acid group (see Table 1; $p < 0.001$).

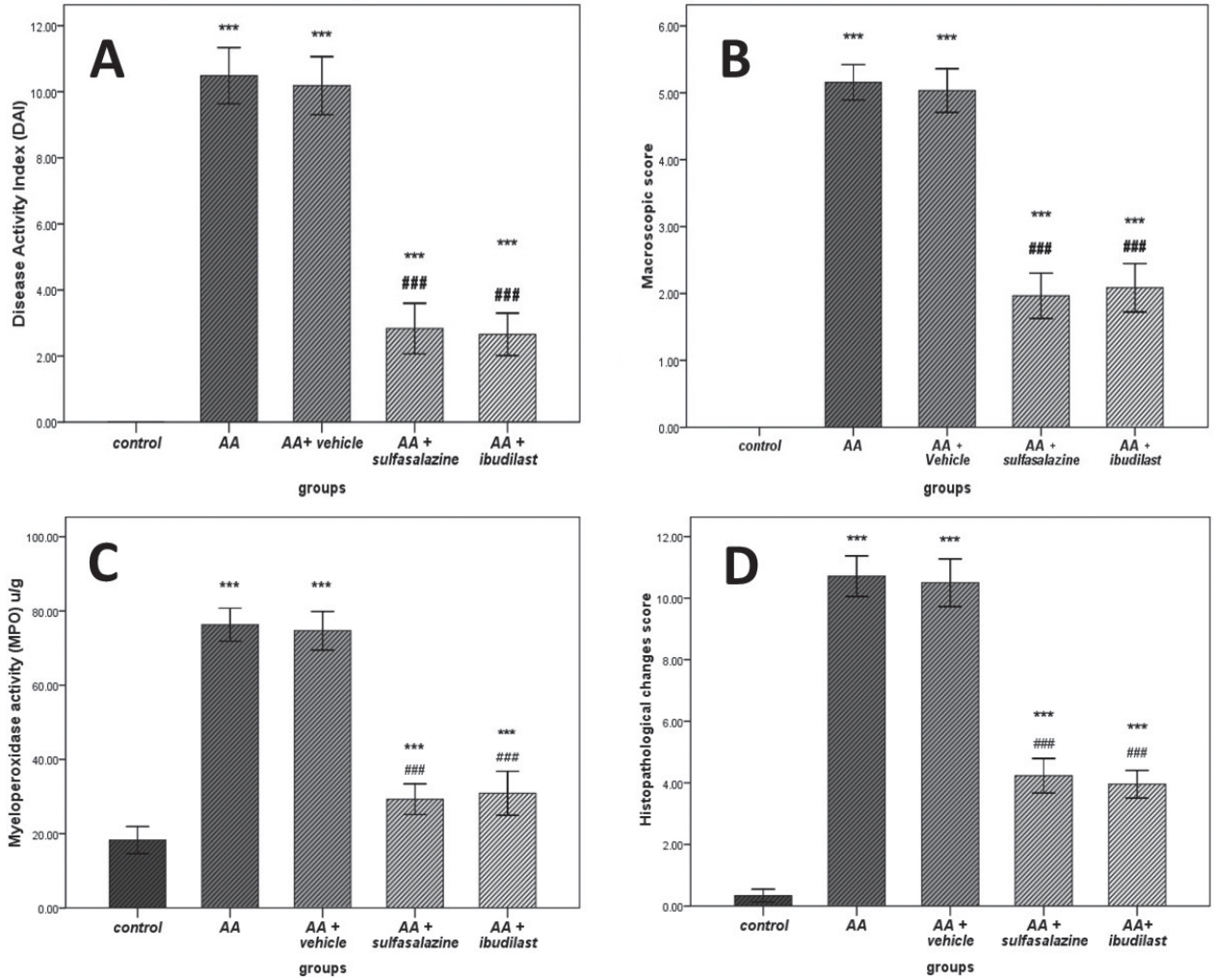

Figure 1. A, B. Effect of ibudilast and sulfasalazine on **(A)** disease activity index (DAI) and **(B)** macroscopic colonic scores (MAC). Data presented as mean ± SD for experimental groups. (***$p < 0.001$versus control group); and (###$p < 0.001$versus AA group). AA= acetic acid; **C, D.** Effect of ibudilast and sulfasalazine on **(C)** myeloperoxidase activity (MPO), and **(D)** histopathological changes score. Data presented as mean ± SD for experimental groups. (***$p < 0.001$versus control group); and (###$p < 0.001$versus AA group). AA= acetic acid.

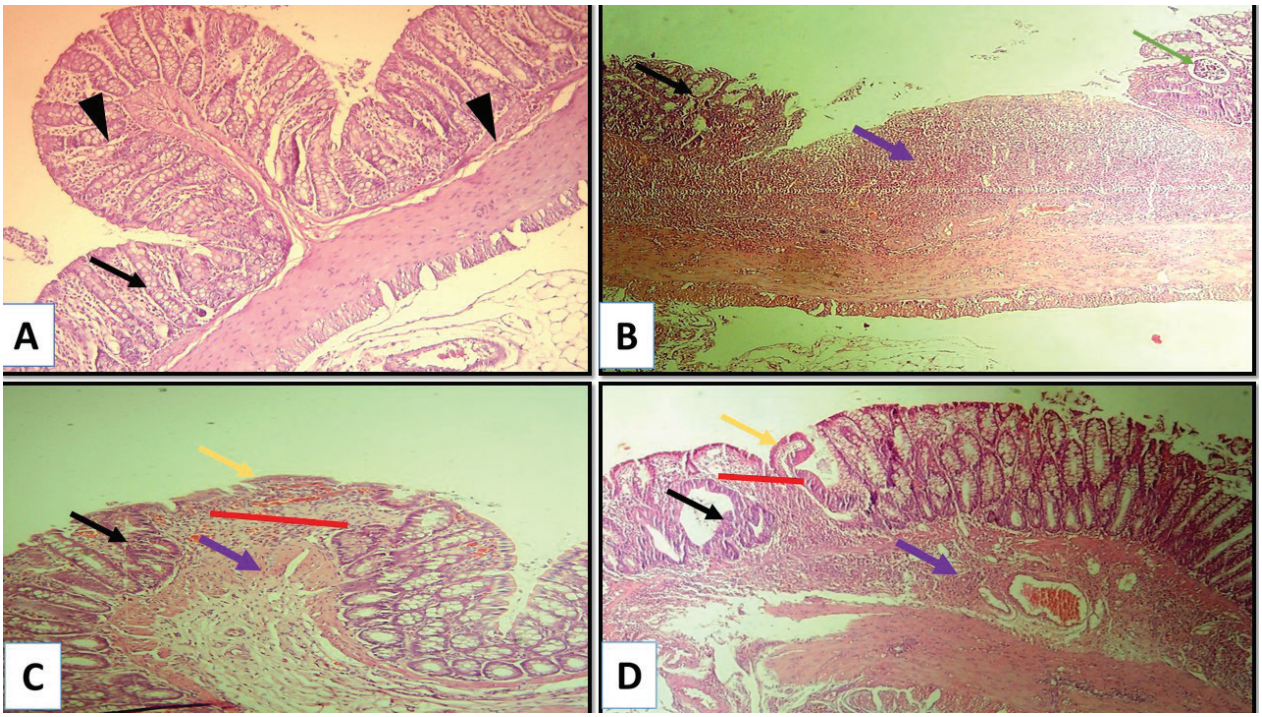

Figure 2. Photomicrographs of colon tissue sections from experimental groups. **A.** Control group shows intact colonic mucosa and submucosa (arrowhead); **B.** Untreated AA group shows features of colitis, including extended ulcerated area (black arrow), showing mixed inflammatory cells infiltration and edema (purple arrows), and crypt abscess (green arrow); **C.** AA + sulfasalazine group shows an improved histological picture with a decreased ulcerated area (red line), less inflammatory cell infiltration (purple arrow) and goblet cells regeneration (black arrow); **D.** AA + ibudilast group shows improved histologic features with a decreased ulcerated area (red line), a marked decrease in the inter-glandular inflammatory cell infiltration (purple arrow), and goblet cell regeneration (black arrow). H&E stain, X 10.

Table 1. Immunohistochemical expression of proinflammatory cytokines (TNF-α and IL-1β) in colonic tissue of experimental groups.

Parameter	Control group	AA group	AA+ vehicle	AA+ Sulfasalazine	AA+ Ibudilast
TNF-α	0.4±0.08	3.9± 0.11***	3.9±0.09***	1.56±0.08###	1.47±0.15###
IL-1β	0.9± 0.10	4.00±0.08***	3.9± 0.08***	1.90±0.15###	1.71±0.17###

† values expressed as mean ± Standard deviation (SD) (***p < 0.001versus control group); and (###p < 0.001versus AA group). AA= acetic acid.

Discussion

Ulcerative colitis has become a global healthcare problem that strikes people of all ages. In addition, current treatment for UC exhibits several adverse effects with decreasing efficacy in prolonged usage, which creates a need for alternative and more effective therapies (Li et al. 2019). In this study, we investigated the effect of ibudilast against colitis induced by acetic acid in rats. Our results found that ibudilast attenuated colonic tissue damage by controlling inflammation and inhibiting oxidative stress. To our knowledge, this is the first study investigating the role of ibudilast against acetic acid-induced colitis.

Ibudilast is a PDE4 inhibitor. PDE4 is a member of the phosphodiesterase enzyme subtypes expressed preferentially in immunocytes, including T lymphocyte cells, macrophages, monocytes, and neutrophils (El-Ashmawy et al. 2018a). The inhibition of PDE4 results in the accumulation of intracellular cAMP and subsequently regulates the synthesis of proinflammatory mediators by activating its receptor on protein kinase A (PKA) (Raker et al. 2016). PKA activation by initiating multiple downstream elements reduces the proinflammatory cytokines and increases the anti-inflammatory cytokines (Schafer 2012; Hernández-Flórez and Valor 2016). Previous studies have suggested that targeting PDE4 may be a potential therapeutic strategy for human IBD (Martinez and Gil 2014).

Several experimental models of UC match essential immunological and histological characteristics of human UC. The acetic acid-induced UC model closely resembles human UC in terms of clinical signs such as weight reduction, rectal bleeding, and macroscopic and histopathological changes (Matsuoka et al. 2018). In the present study, inter-rectal installation of acetic acid resulted in histopathological changes like mucosal ulceration, edema, lymphoid follicle hyperplasia, and inflammatory cell infiltration. These histological alterations were coupled with bloody diarrhea, weight loss, and high macroscopic scores. Our results paralleled those of earlier studies (Manna et al. 2017; Morsy et al. 2021). In this study, treatment with ibudilast mitigated the clinical feature of colitis induced by acetic acid. Furthermore, ibudilast improved the histological picture by decreasing ulcerated areas, regenerating goblet cells, and reducing

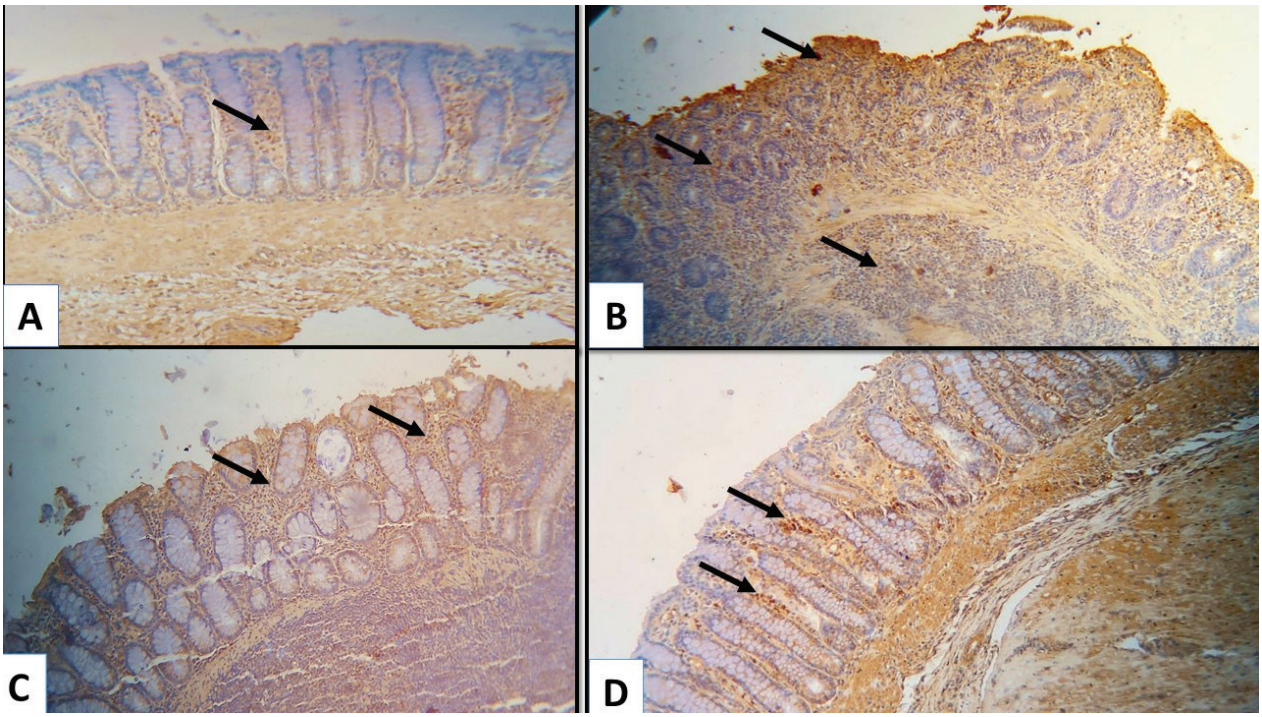

Figure 3. Photomicrographs of colon tissue sections from experimental groups presenting the immunohistochemical expression of TNF-α. **A.** The control group specimens display a little membranous expression of TNF-α in the in-between the crypts (black arrow); **B.** The untreated AA group specimens display the increased expression of TNF-α in cells infiltrating mucosa and submucosa (black arrow); **C.** AA+ sulfasalazine group displays a decreased expression of TNF-α in some cells infiltrating mucosa (black arrow); **D.** AA+ ibudilast group displays a marked decrease of TNF-α in a few cells infiltrating mucosa (black arrow).

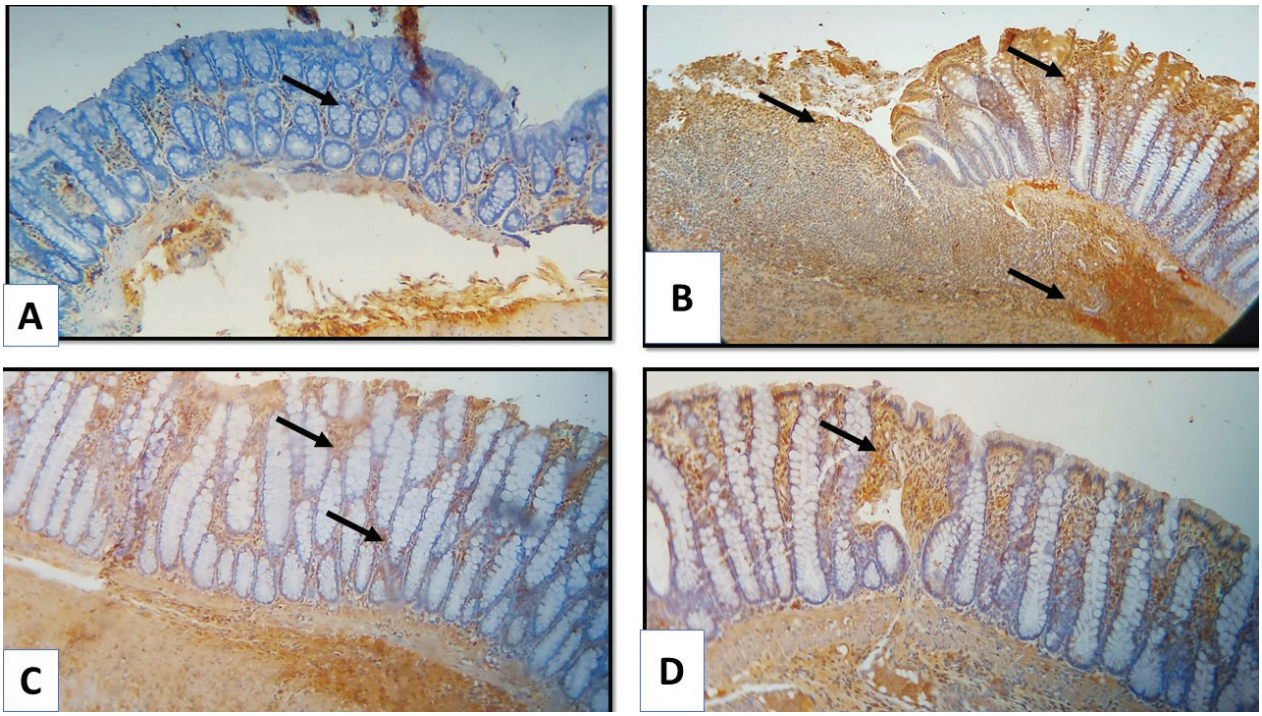

Figure 4. Photomicrographs of colon tissue sections of experimental groups presenting the immunohistochemical expression of IL-1β. **A.** control group specimen shows a positive cytoplasmic expression of IL-1β in a few cells in the mucosa layer (black arrow); **B.** untreated AA group specimen shows an increased expression of IL-1β in cells infiltrating mucosa and submucosa (black arrow); **C.** AA+ sulfasalazine group displays a decreased expression of IL-1β in some cells within the crypts in the mucosa (black arrow); **D.** AA+ ibudilast group displays a decrease of IL-1β in some cells within the crypts in the mucosa (black arrow).

inflammatory cell infiltration. Our findings were in accordance with studies previously clarifying that other PDE4 inhibitors, rolipram, tetomilast, and roflumilast, ameliorated experimental colitis (El-Ashmawy et al. 2018a; Li et al. 2018).

MPO activity is a marker of leukocyte infiltration in colon tissue (El-Ashmawy et al. 2018b). Leukocytic infiltration is an essential contributor to oxidative stress, which is the primary cause of cell damage (Aprioku et al. 2013). In this study, MPO activity was elevated in the acetic acid group and dramatically decreased with the ibudilast treatment. Jones et al. (2005) found that the PDE4 inhibitors, roflumilast, cilomilast, and rolipram, efficiently decreased myeloperoxidase activity in vitro from human neutrophils. Our results were consistent with these findings.

TNF-α is a major cytokine that has a crucial role in ulcerative colitis. Previous studies reported that TNF-α and IL-1β levels were increased in colonic tissue, serum, and faeces for UC patients, which relates to disease severity (McAlindon et al. 1998; Korolkova et al. 2015). In this study, acetic acid increased TNF-α and IL-1β expression in colonic tissue, whereas ibudilast treatment for 10 days significantly reduced tissue levels. These results are consistent with those of other studies. According to Hutchinson et al. (2009), ibudilast downregulates proinflammatory cytokines (TNF-α, IL-1β, and IL-6) in activated glial cells or brain tissue by increasing cAMP. Moreover, Yang et al. (2020) discovered that ibudilast alleviates neonatal respiratory distress syndrome by lowering proinflammatory mediators (TNF-α and IL-1β) in the alveoli.

Conclusion

The results suggest that ibudilast has a therapeutic effect on ulcerative colitis. It may, to a large extent, be due to its anti-inflammatory effects upon comparison with sulfasalazine in experimentally induced colitis.

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