EZETIMIBE AMELIORATES CLINICAL PARAMETERS, OXIDATIVE STRESS, AND ADHESION MOLECULES IN EXPERIMENTALLY INDUCED COLITIS IN MALE RAT MODELS

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Abstract. Ulcerative colitis is a persistent and recurrent medical condition for which current treatments have shown limited efficacy, necessitating the exploration of alternative drugs with minimal side effects. This study aimed to examine the anti-oxidative and antiadhesive effects of ezetimibe compared to sulfasalazine in experimentally induced colitis in male rats. A total of 40 adult male Wistar rats were divided into 4 groups: a control group (negative control), an acetic acid group (positive control), an acetic acid + sulfasalazine (100 mg/kg/day) group, and an acetic acid + ezetimibe (10 mg/kg/day) group. Colitis was induced in rats by the inter-rectal administration of 2 ml of 4% (v/v) acetic acid. Sulfasalazine and ezetimibe were administered orally for seven days 1 hour after induction. Malondialdehyde (MDA), myeloperoxidase (MPO), e-selectin, and intracellular adhesive molecule 1 (ICAM-1) were measured in tissue homogenate upon euthanizing the animals. The results showed that the treatment with ezetimibe significantly reduced disease activity index (DAI) and macroscopic colonic scores (MAC) compared to the positive control group. Moreover, ezetimibe notably decreased MDA, MPO, e-selectin, and ICAM-1 in tissue homogenates of treated animals compared to the positive control group. In most comparisons, there were no significant differences between ezetimibe and sulfasalazine effects. These findings suggest that ezetimibe may have a therapeutic effect in the management of ulcerative colitis by reducing oxidative stress and adhesive molecules.

Keywords: ezetimibe, ulcerative colitis, acetic acid, oxidative stress, adhesion molecules.

List of Abbreviations

AMPK – AMP-activated protein kinase
ARE – Antioxidant response element
DAI – Disease activity index
ELISA – Enzyme-linked immune-sorbent assay
HO – Heme-oxygenase
ICAM-1 – Intercellular adhesion molecule-1
LSD – Least significant difference
MAC – Macroscopic colonic scores
MCP-1 – Monocyte chemoattractant protein-1
MDA – Malondialdehyde
MPO – Myeloperoxidase
Nrf2 – Nuclear factor-E2-related factor 2
PBS – Phosphate-buffered saline
TLR – Toll-like receptor
TNF-α – Tumor necrosis factor-alpha
UC – Ulcerative colitis

Introduction

One of the most common inflammatory, intermittent, and chronic intestinal diseases is ulcerative colitis (UC). Although the cause of UC is uncertain, several studies have shown that genetic, environmental, and altered immune variables all contribute to the disease's pathophysiology (Chung *et al.*, 2014). A curative medical treatment for UC has not yet been found, though new therapeutic strategies addressing specific pathogenetic mechanisms of disease are emerging (Ferreti *et al.*, 2022). Increasing oxidative stress promotes intestinal mucosal inflammation, which in turn activates nuclear factor-kappa B-cell (NF-kB) and the upregulation of proinflammatory cytokines and adhesion molecules in a vicious cycle (Lingappan, 2018). In addition, the activation of Toll-like receptors (TLRs), mainly TLR4, stimulates the pro-inflammatory signaling pathway and plays an essential role in initiating the inflammatory response (Wang *et al.*, 2019). Therefore, controlling inflammation and oxidative stress is a critical therapeutic goal for UC (Ungaro *et al.*, 2017). Although significant developments have been made in managing UC, 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 (Keane *et al.*, 2017).

Ezetimibe is a medication used to control and treat hypercholesterolemia. It belongs to a brand-new group of drugs called selective cholesterol-absorption inhibitors (Ritter *et al.*, 2014). Besides these principal effects, ezetimibe was found to reduce inflammatory cytokines, such as monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor-alpha (TNF-α) and inhibit macrophage accumulation in wound lesions wounds (Zheng, 2014). Furthermore, it can affect the production of reactive oxygen species (Qin *et al.*, 2018). Nevertheless, no adequate data are accessible concerning the therapeutic outcome of ezetimibe in induced colitis.

The current study aimed to assess the antioxidative and antiadhesive effects of ezetimibe in experimentally induced colitis in male rats.

Materials and Methods

Source of experimental animals

Forty Wistar albino adult male rats (200–220 g) were used throughout this study. The rats were supplied by the animal house of the National Center for Drug Control and Research, Baghdad, Iraq. Before the experiment, the animals were placed in five cages that were supplied with a large wire–mesh floor for 7 days and were allowed water and laboratory chow pellet. The study protocol was approved by the institutional animal ethics committee from Al-Nahrain University in the College of Medicine.

Experimental design

The rats were divided into four groups (n = 10 in each group). Group I had no intervention and received no treatment and served as control while in the other groups, colitis was induced intrarectally by 4% acetic acid (v/v). Group II was administered orally normal saline, while group III was administered ezetimibe (10 mg/kg) orally and lastly, group IV was treated orally with sulfasalazine (Salazosulfapyridine; 100 mg/kg), 30 minutes after the induction of colitis for 7 days. The duration of treatment depended on previous studies of experimental colitis.

Induction of ulcerative colitis

Before the colitis induction, rats were fasted for at least 24 hours to get proper induction of colitis by evacuating the colon from feces but were permitted to drink tap water. Experimental colitis was accomplished according to the procedure reported by Mousavizadeh et al. (2009) with modifications. Briefly, rats received a single intrarectal infusion of 4% acetic acid in a dose of 5 ml/kg (8 cm into the colon) under light ether anesthesia in a flexible plastic tube (2 mm extrinsic diameter). The rats were positioned in a horizontal direction for 2 min to prevent the discharge of acetic acid. The control rats went through a similar procedure by using the same amount of normal saline as an alternative to acetic acid (Atarbashe *et al.*, 2020).

Preparation of drugs

The sulfasalazine and ezetimibe were freshly prepared before administration. Estimated drugs were prepared as suspensions in distilled water. Sulfasalazine (100 mg/kg) was served as standard therapy.

Assessment of colitis

After the experiment ended, rats inhaled an excessive dose of diethyl ether to sacrifice them. The colon was removed rapidly after dissection of the abdomen. The specimen of the colon was opened longitudinally and gently cleaned with normal saline. Then, the assessment of macroscopical features was achieved by normal observation. Finally, the samples were assessed for tissue homogenate analysis.

Macroscopic evaluation

Disease activity index: the disease activity index (DAI) was calculated by the following parameters: bodyweight reduction {0 = weight gain or no reduction, 1 = 1-5% reduction, 2 = 6-10% reduction, 3 = 11-15% reduction, 4 = more than 15% reduction}; the consistency of faeces {0 = normal, 2 = loose faeces, 4 = diarrhea}; and bleeding of rectum {0 = normal, 2 = mild bleeding, 4 = severe bleeding}. The calculation was made by a combination of the total scores of DAI (Ikeda *et al.*, 2023).

Colon edema: it was utilized as an indicator of edematous tissue and the intensity of colitis. After the incision was done along the mesenteric margin of each colonic specimen and washed gently, the colon edema was determined by measuring the colon weight by sensitive balance (Mehesen *et al.*, 2015).

Macroscopic colonic score: the colonic samples were examined visually. The macroscopic score based on the clinical features of the colon according to the scoring system ranging from 0–6 as follows: 0 = absence of inflammation; 1 = redness or swelling; 2 = swelling and redness; 3 = one or two ulcers; 4 = one large ulcer or more than two ulcers; 5 = initial necrosis; 6 = severe necrosis (Mirshahrokhi *et al.*, 2011).

Tissue samples

The colon tissues were sliced into small pieces and homogenized in a certain amount of phosphate-buffered saline (PBS; usually 1 g tissue to 9 ml of PBS) with homogenizer on ice. The resulting suspension was subjected to two freezing-thawing cycles to further break the cell membranes. After that, the homogenate was centrifuged for 15 minutes at 5,000 rpm (Herminghaus *et al.*, 2018). Ready commercial kits (ELK Biotechnology) were used to measure homogenate levels of myeloperoxidase (MPO), malondialdehyde (MDA), intercellular adhesion molecule-1 (ICAM-1), and E-selectin using enzyme-linked immunesorbent assay (ELISA). The manufacturer's instructions were followed precisely.

Statistical analysis

A statistical package for social science (SPSS; version 23) software was used to summarize, analyze, and present the data. Quantitative (numeric) variables were expressed as mean and standard deviation. One-way analysis of variance (ANOVA) was used to compare the difference in the mean of quantitative variables among groups. This was then followed by a post hoc least significant difference (LSD) test to evaluate the mean difference within groups. The significant level was set at $p \leq 0.05$.

Results

Effect of ezetimibe on macroscopic score

Rats in group II (induced colitis without treatment) showed considerable damage, including thickening of the colon with tissue necrosis and ulceration, while those in group I had normal colon appearance (Fig. 1). The proportion of macroscopic damage was dramatically reduced after administering sulfasalazine and ezetimibe (1.83 ± 0.41 and 2.17 ± 0.75, respectively) compared with induced colitis (5.67 ± ± 0.52) as shown in (Fig. 2).

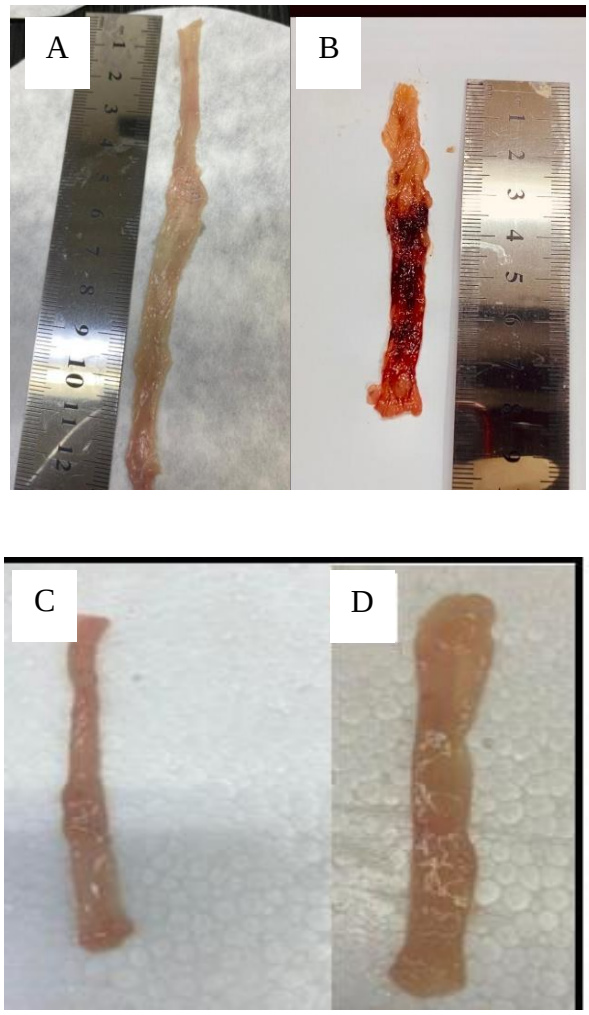

Fig. 1. Gross features of colonic wall in different study groups. A: a control group (negative control); B: an acetic acid group (positive control); C: an acetic acid + sulfasalazine (100 mg/kg/day) group; D: an acetic acid + ezetimibe (10 mg/kg/day) group

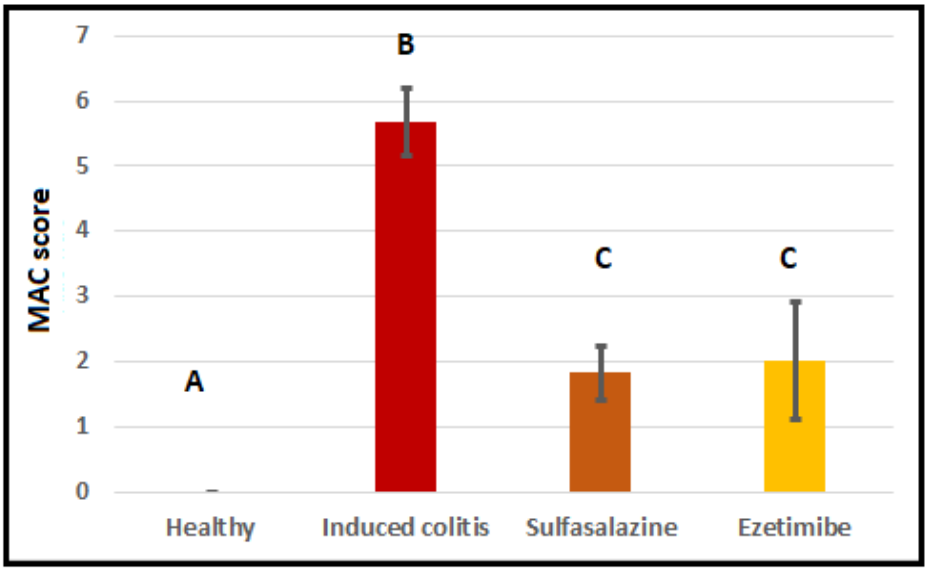

Fig. 2. Mean macroscopic scores in different experimental groups
The results are expressed as mean ± standard deviation.
Different letters indicate significant differences

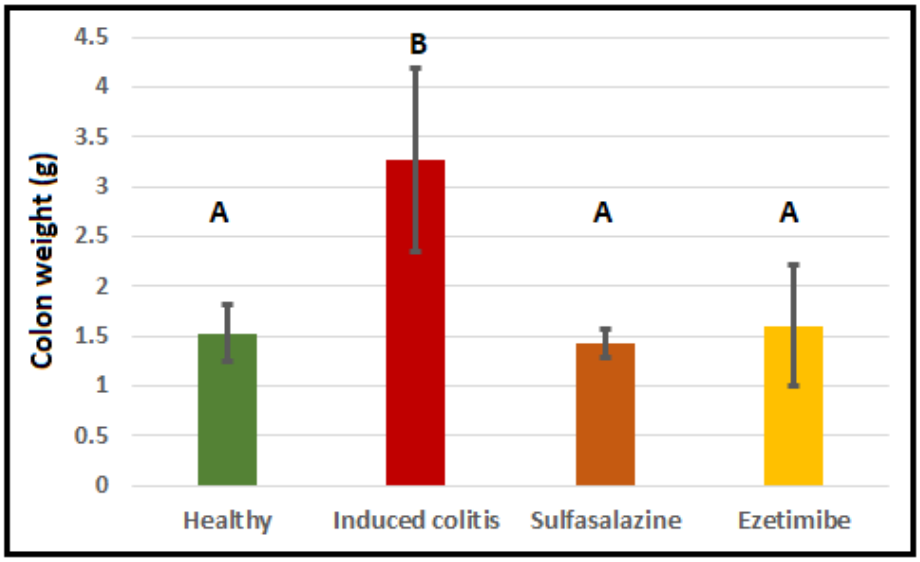

Fig. 3. Mean colon weight (g) in the experimental groups
The results were expressed as mean ± standard deviation (SD).
Different letters indicate significant differences

The influence of ezetimibe on colon weight

The colon weight (1.43 ± 0.14 and 1.59 ± 0.61) was reduced significantly in the two treated groups (sulfasalazine and ezetimibe, respectively) compared with induced colitis (3.27 ± 0.93 g), but did not differ significantly from each other (Fig. 3).

The influence of ezetimibe on the disease activity index

Rats with induced colitis have significantly higher DAI scores (11.0 ± 0.89) than those treated t with ezetimibe (2.0 ± 0.63) or sulfasalazine (1.17 ± 0.41) with highly significant differences as highlighted in Figure 4.

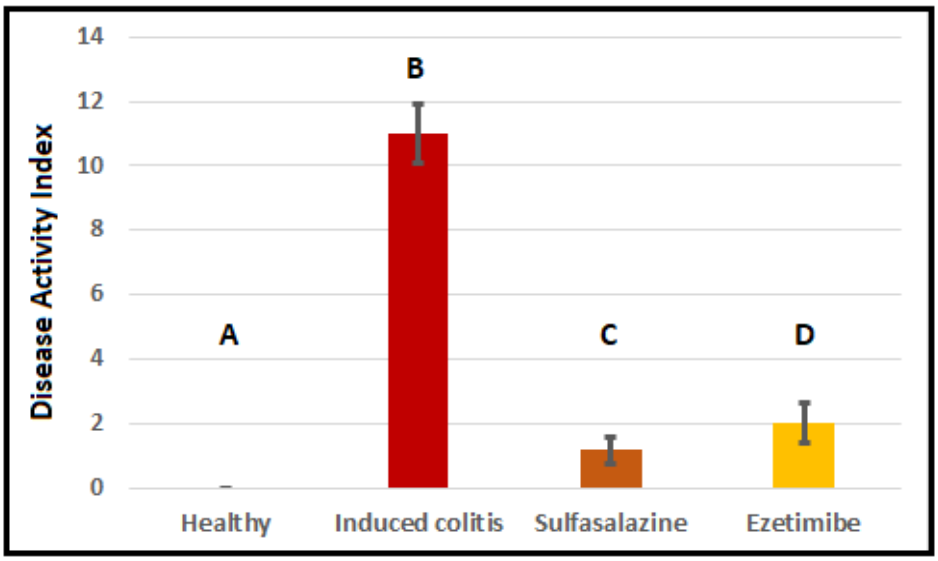

Fig. 4. Mean disease activity index (DAI) in the different study groups
The results are expressed as mean ± standard deviation (SD). Different letters indicate significant differences

Table 1

Multiple comparisons of adhesion molecules and oxidative stress between different groups

Variables	NC (n = 10)	PC (n = 10)	Sulfa (n = 10)	Ezet (n = 10)	P-value
ICAM-1	1.33 ± 0.58 A	7.43 ± 0.53 B	2.01 ± 0.67 A	2.82 ± 0.99 C	**<0.001**
E-selectin	165.70 ± 65.12 A	432.5 ± 71.3 B	194.37 ± 50.60 C	272.07 ± 81.60 D	**<0.001**
MPO	1.37 ± 0.39 A	7.47 ± 1.21 B	1.80 ± 0.62 A	2.60 ± 1.12 C	**<0.001**
MDA	105.70 ± 19.70 A	250.4 ± 30.27 B	137.56 ± 24.69 C	161.63 ± 15.91 D	**<0.001**

Note: different letters indicate significant differences; ICAM: Intercellular adhesion molecule; MPO: Myeloperoxidase; MDA: Malondialdehyde

The influence of ezetimibe on oxidative stress markers and adhesion molecules

Animals treated with sulfasalazine or ezetimibe had significantly lower levels of MPO and MDA than those in induced colitis groups with highly significant differences. In this regard, sulfasalazine treatment had a superiority over ezetimibe treatment with significant differences. Furthermore, treatment with ezetimibe or sulfasalazine reduced the homogenate concentration of ICAM-1 and E-selectin compared with induced colitis with highly significant differences. Also, sulfasalazine treatment had a superiority over ezetimibe treatment with significant differences (Table 1).

Discussion

A common healthcare problem concerning people of all ages is UC. The present UC treatment has several side effects and loses effectiveness with time, prompting the use of alternate and more potent medicines (Li *et al.*, 2014). In the present study, colon weight was inversely related to the severity of inflammation of the colon. The observed findings showed an increased DAI score and an increase in the colon weight of acetic acid-induced colitis rats. The observed increased colon was due to severe edema, necrosis, and inflammatory cell infiltration (Gopu *et al.*, 2015; Araújo *et al.*, 2016; Suluvoy *et al.*, 2017). However, treatment of colitis rats with oral administration of ezetimibe

(10 mg/kg) resulted in a significant decrease in the DAI score, macroscopic score, and colon weight compared to induced colitis. In most cases, the ameliorative effect of ezetimibe was comparable to sulfasalazine.

One of the main causative factors of inflammatory diseases is oxidative stress and suppressed antioxidant defense systems that regulate the production of reactive oxygen species (ROS) (Leong *et al.*, 2021), which initiates a reactive oxygen metabolites (ROM) cascade and causes lipid peroxidation (LPO) (Hu *et al.*, 2021). In the present study, increased oxidative stress was observed in acetic acid-induced colitis rats, which was confirmed by increased MDA levels and antioxidant (MOP) levels in colon tissues. The findings from the present study confirmed that ezetimibe was capable of protecting mucosal damage by significantly reducing the MDA levels and promoting the MOP levels in the colon tissues of colitis rats. The findings of the present study corroborate the reports of earlier studies. The complex mechanism of the anti-inflammatory effect of ezetimibe is attributed to a reduction in oxidative stress through the activation of the AMP-activated protein kinase (AMPK)/Nrf2 pathway in animal models (Lee *et al.*, 2016). Nuclear factor-E2-related factor 2 (Nrf2) is a master transcription factor that targets genes coding for antioxidant proteins and detoxification enzymes (He *et al.*, 2020). It also controls the basal and induced expression of antioxidant response element (ARE)-dependent genes, such as heme-oxygenase (HO)-1 and glutamate-cysteine ligase catalytic subunit, to regulate the physiological and pathophysiological outcomes of oxidant exposure (Taguchi *et al.*, 2011). Previous reports have shown that Nrf2 activation attenuates the injuries caused by IR, since the generation of ROS has been implicated in the cell injury, reducing oxidative stress may be a potential therapeutic approach to the prevention of such injuries (Ashrafian *et al.*, 2012).

Lipids induce inflammation by increasing proinflammatory cytokine levels. Another possible mechanism is that ezetimibe reduces the blood cholesterol level by preventing cholesterol absorption in the small intestine by blocking the intestinal cholesterol transporter Niemann-Pick C1-Like 1 (van de Peppel *et al.*, 2019; Yan *et al.*, 2019). Oxidized low-density lipoprotein influences the Th17/Treg balance. A high serum concentration of oxidized low-density lipoprotein is negatively correlated with the number of Treg cells and positively correlated with the number of Th17 cells (Li *et al.*, 2014). Therefore, it is expected that the reduction of blood lipid levels using ezetimibe would ameliorate inflammation by reducing the number of Th17 cells (Qin *et al.*, 2014).

Ezetimibe decreased the expression levels of proinflammatory cytokines in intestinal immune cells and enterocytes. Spleen tissue immunofluorescence staining and flow cytometry of mouse splenocytes showed that ezetimibe regulated intestinal inflammatory cells and inflammatory cytokines, as well as systemic immune cells. The current investigation verified an increase in E-selectin expression, which may indicate its function in tempting leukocytes in a colitis model that was induced acutely by acetic acid. Other investigations have also shown that E-selectin is expressed more frequently (Manna *et al.*, 2017). Inter-cellular adhesive molecule engages the leukocytes' association with endothelial cells and simplifies their penetration to the inflammatory site (Mulle, 2013). Selectins take part in the initial phases of rolling of leukocytes to the blood vessel epithelium, and endothelial selection plays a major role in the chemotaxis of leukocytes to the vascular wall and their adhesion to the endothelium (El-Akabawy *et al.*, 2019).

The current study demonstrated that ezetimibe elicited a significant reduction in tissue homogenate of adhesion molecules (E-selectin and ICAM-1) in rat colonic mucosa compared to the untreated colitis group. Such a finding is in agreement with the study of Barbosa *et al.* (2017), in which the treatment with ezetimibe reduced both leukocytes rolling behavior and the adhesion of leukocytes to the mucosa on day 7 of drug treatment. Some researchers have shown that ezetimibe effectively improved leukocyte/endothelium interactions by decreasing leukocyte rolling and adhesion and increasing leukocyte rolling velocity (Hernandez-Mijares *et al.*, 2016). These effects ap-

pear to be at least partially mediated by the downregulation of the expression of P-selectin in endothelial cells. This finding is supported by evidence of macroscopic and DAI improvement following administration of improve-ment and microscopic colonic injury in colitis in rats after treatment with the ezetimibe derivative.

Conclusion

The findings of the present study suggest a therapeutic impact of ezetimibe on ulcerative colitis. It could, in part, be because of its anti-oxidative and anti-adhesion molecular properties when compared to sulfasalazine in experimentally induced colitis.

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