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Evaluating the prophylactic potential of zafirlukast against the toxic effects of acetic acid on the rat colon

Afaf A. Mahgoub [a,*], Azza A. El-Medany [a], Hanan H. Hager [a], Ali A. Mustafa [a], Doryia M. El-Sabah [b]

[a] *Department of Pharmacology, College of Medicine, King Saud University, P.O. Box 22452, Riyadh 11461, Saudi Arabia*
[b] *Department of Pathology, College of Medicine, King Saud University, P.O. Box 22452, Riyadh 11461, Saudi Arabia*

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Abstract

The present work was conducted to assess the possible protective effects of zafirlukast against the toxic damage induced by acetic acid in rat colon. Zafirlukast is a potent and selective cysteinyl leukotriene receptor antagonist which is used mainly in the prophylaxis of bronchial asthma. Two doses of zafirlukast were used (40 and 80 mg/kg) dissolved in gum acacia and given either orally or rectally (0.5 ml/kg). Several parameters including, macroscopic score, histopathological and biochemical such as malondialdehyde (MDA), myeloperoxidase (MPO), catalase and reduced glutathione (GSH) levels were measured using standard assay procedures. The study showed that pretreatment with zafirlukast in a dose of 80 mg/kg orally produced a significant decrease in tissue malondialdehyde, myeloperoxidase, and an increase in both reduced glutathione and catalase levels, while there was no significant changes with the rectal route. The 40 mg/kg dose had no significant protective effects when given either orally or rectally.

The available data indicate that the inhibition of leukotriene synthesis or action may have a role in inflammatory bowel disease (IBD) as they are considered as important mediators in this condition.

Keywords: Zafirlukast; Acetic acid colitis; Malondialdehyde; Myeloperoxidase; Catalase; Reduced glutathione

1. Introduction

Inflammatory bowel disease (IBD) such as ulcerative colitis (UC) and Crohn's disease (CD) are largely of unknown etiology and are among the most challenging human illness (Kirsner, 1995). There is evidence for an intense local immune response associated with recruitment of lymphocytes and macrophage followed by release of soluble cytokines.

The cysteinyl leukotrienes (LTC_4, LTD_4, and LTE_4) are lipid mediators derived from arachidonic acid via the 5-lipoxygenase pathway whereas LB_4 is identified as a potent chemotactics for neutrophils (Lewis et al., 1981). It enhances neutrophil–endothelial interactions and activates neutrophils leading to degranulation and release of mediators, enzymes and superoxides (Hoover et al., 1984).

The pathogenesis of IBD involves an interaction between genetic and environmental factors. Whatever the precise mechanism responsible for initiating and perpetuating intestinal inflammation, there is an ample evidence for an intense local immune response,

* Corresponding author. Tel.: +966-1-478-6768; fax: +966-1-478-6768.
E-mail address: afaf_mahgoub@yahoo.com (A.A. Mahgoub).

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associated with recruitment and activation of lymphocytes and macrophages. The subsequent release of soluble cytokines and other inflammatory mediators causes tissue damage and contributes to many of the clinical features of these diseases and to the amplification and perpetuation of the local immune response (Weldon and Maxwell, 1994).

Some important inflammatory mediators that have a great role in IBD include cytokines, eicosanoids and kinins (Mac-Dermatt, 1999). The identification of such mediators gives rise to new therapeutic strategies directed at the control and treatment of the disease (William, 1998). The cysteinyl leukotrienes (Cys LT_5, LTC_4, LTD_4, and LTE_4) account for the bioactivity originally termed slow-reacting substance of anaphylaxis (SRS-A), whereas LTB_4 was identified by its potent chemotactic activity for neutrophils (Lewis et al., 1981).

Zafirlukast is a potent and selective cysteinyl leukotriene antagonist that has been approved for the prophylaxis of bronchial asthma. It is the first member of a new drug family which is available for the management of asthma in more than 20 years (Smith et al., 1998). Zafirlukast blocks the action of cysteinyl leukotriene which is potent inflammatory mediators. It prevents leukotrienes from binding to their receptors (Wenzel, 1998).

It would therefore be useful to assess the possible protective effects of zafirlukast in an animal model of colitis induced by 3% acetic acid in rats.

2. Materials and methods

2.1. Materials

Zafirlukast (Zeneca Limited, Macclesfield Cheshire, UK) 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB), reduced glutathione (GSH) and 2-thiobarbituric acid were purchased from Sigma, St. Louis, MO, USA. All other chemicals used were of analytical grade.

2.2. Animals

Forty-two male Wistar albino rats (150–200 g) were used throughout this work (supplied from the Animal House, King Saud University). The animals were maintained in a room under standard conditions of light, feeding and temperature. The study was conducted in accordance with the standards established by the guide for the care and use of laboratory animals of the College of Medicine Research Council (CMRC). The rats were housed individually in a rack mounted wire mesh cages to prevent coprophagia. All rats were exposed to the same environmental conditions and were maintained on their proper diet and water ad libitum. The animals were randomly divided into seven groups. Groups from 3 to 7 received zafirlukast (40 or 80 mg/kg) either orally or rectally. The drug was suspended in 2% gum acacia, and given twice daily (0.5 mg/kg) for a period of three consecutive days before induction of colitis. Colitis was induced by intracolonic injection of 3% acetic acid (2 ml) to groups from 2 to 7. The groups were as follows:

(1) Normal control (saline p.o., 0.5 ml/kg)
(2) Gum acacia control (gum acacia p.o., 0.5 ml/kg)
(3) Acetic acid colitis control (saline p.o., 0.5 ml/kg)
(4) Zafirlukast (40 mg/kg p.o., 0.5 ml/kg)
(5) Zafirlukast (40 mg/kg rectal, 0.5 ml/kg)
(6) Zafirlukast (80 mg/kg p.o., 0.5 ml/kg)
(7) Zafirlukast (80 mg/kg rectal, 0.5 ml/kg)

2.2.1. Induction of experimental colitis in rats

Acetic acid-induced colitis was performed using a modification of the method as described by Millar et al. (1996). The animals were starved for 24 h with access to water ad libitum. Induction was performed on day three 2 h after giving saline, gum acacia or zafirlukast. Each rat was anaesthetized by brief inhalation of 20–25% CO_2 followed by intraperitoneal injection of phenobarbitone (35 mg/kg). An infant feeding tube (Pennine Health Care FT-1608/40, outside diameter 2 mm) was inserted into the colon to 8 cm and 2 ml of acetic acid (3% (v/v) in 0.9% saline) were infused into the colon. The acetic acid was retained in the colon for 30 s, after which fluid was withdrawn. The rats were killed at 24 h by CO_2 asphyxiation and colonic biopsies were taken for macroscopic scoring, histopathological and biochemical studies.

2.2.2. Assessment of colitis

2.2.2.1. Macroscopic scoring. At post-mortem laparotomy, the colon was removed by cutting at the pubic symphysis and at the caecum, and immediately transferred into Krebs buffer (pH 7.5), which was gently bubbled with 95% O_2 and 5% CO_2, and

the descending colon was cut 7 cm proximally. The colon was incised along its mesenteric border, gently washed, placed flat (mucosal surface upwards) on a glass dish containing the buffer for macroscopic evaluation (colonic damage score). The severity of inflammation was evaluated by three experienced observers unaware of the treatments, using a visual analogue scale ranging from 0 to 4, as follows:

(0) = No macroscopic change.
(1) = Mucosal erythema alone.
(2) = Mild mucosal oedema, slight bleeding or small erosion.
(3) = Moderate oedema, bleeding ulcers or erosion.
(4) = Severe ulceration, erosions, oedema and tissue necrosis.

2.3. Histopathological study

Full thickness biopsy specimens were fixed in 10% formal saline prior to wax embedding, sectioning, and staining with haematoxylin and eosin. Histological assessment by light microscopy was carried by two observers unaware of the treatments. The degree of inflammation was graded using the criteria described by Gonzalez et al. (1999), and the score represented the sum of six individual variables graded 0–3 depending upon the severity of the changes (0 = no change; 1 = mild; 2 = moderate; 3 = severe). The variables evaluated were: erosion, ulceration, necrosis, haemorrhage, oedema and inflammatory cell infiltration.

2.4. Biochemical study

Colonic samples were stored immediately at −20 °C till analysis. Tissue samples were homogenized in 1 ml of 10 mmol/l Tris–HCl buffer pH 7.1 and homogenate was used for the measurement of malondialdehyde (MDA), myeloperoxidase (MPO), catalase and glutathione activities. In colonic tissue homogenate, MDA, MPO, GSH and catalase contents were expressed per milligram tissue weight.

2.5. Determination of malondialdehyde (MDA) activity

Lipid peroxidation was measured as MDA determined by thiobarbituric acid (TBA) assay according to Buege and Aust (1978). 2.5% (w/v) homogenates of colonic tissues were prepared in phosphate buffer (pH 7.2) reacted with thiobarbituric acid reagent containing 0.37% TBA, 15% trichloroacetic acid and 0.25N HCl. Samples were boiled for 15 min, cooled and centrifuged. Absorbance of the supernatant was measured spectrophotometrically at 532 nm. MDA concentration was calculated by the use of 1,1,3,3-tetraethoxypropane as a standard, and expressed as nmol/mg tissue.

2.6. Determination of myeloperoxidase (MPO) activity

MPO activity had been used as an index of leukocyte adhesion and accumulation in several tissues including the intestine. The principle of the method depends on release of MPO enzyme in the homogenate of colonic tissue used. Its level is detected using 0.3 mmol of H_2O_2 as a substrate for MPO. A unit of MPO activity is defined as that converting U mol of H_2O_2 to water in 1 min at 25 °C (Bradley et al., 1982). In brief segments of the distal colon (0.5 g) were homogenized in 10 volumes of 50 mM sodium phosphate buffer pH 7.4 in an ice bath using polytron homogenizer (50 mg tissue/ml). The pellet (contained 95% of the total tissue MPO activity) was resuspended in an equal volume of potassium phosphate buffer pH 6. Another centrifugation step for a period of 20 min at 16,000 × g was done, the resultant supernatant was used for MPO assay using tetramethylbenzidine (TMB), and the activity of MPO was measured using Jenway 6505 UV-Vis spectrophotometer at 655 nm.

2.7. Determination of reduced glutathione (GSH)

Reduced glutathione was determined as previously described by Owens and Belcher (1965) based on the reaction of 5,5-dithiobis-(2-nitrobenzoic acid) with GSH present and the absorbance was measured at 412 nm in a Schimadzu double beam spectrophotometer UV 200 S. The amount of glutathione present in the sample was calculated using a standard solution of GSH containing 1 mg of GSH/ml 3% metaphosphoric acid. The increase in the extinction at 412 nm is proportional to the amount of GSH present.

2.8. Determination of catalase activity

Catalase activity was determined according to the method of Higgins et al. (1978). The kinetics of decrease in absorbance of hydrogen peroxide (H_2O_2) at 240 nm was followed for 1 min and the activity is expressed as nmol/(min mg) protein.

2.9. Statistical analysis

Results are expressed as mean ± S.E.M. One way analysis of variance (ANOVA) was used for multiple comparisons. For macroscopic data a non-parametric test (Kurskal–Wallis test) was used. Student's *t*-test was used to make comparison between two groups. *P*-values less than 0.05 were considered significant.

3. Results

3.1. Macroscopic features

Twenty-four hours after a single intracolonic administration of 2 ml of 3% acetic acid, there was macroscopic evidence of extensive colonic mucosal injury along the 1–3 cm segment at the site of instillation. The mucosa appeared ulcerated, oedematous and haemorrhagic compared to normal control group (Table 1).

In the group treated with zafirlukast (80 mg/kg orally) a moderate protection was noticed. Also, a mild protection was found in the group which had received zafirlukast 80 mg/kg rectally. On the other hand, no significant protection was observed in the groups which had received 40 mg/kg zafirlukast either orally or rectally (Table 1).

Table 1
Effects of zafirlukast (40 and 80 mg/kg) pretreatment given orally or rectally for three consecutive days before induction of colitis on macroscopic scoring of acetic acid-induced colitis in rats

No.	Group	Gross lesion score	Protection (%)
1	Normal control	0.0 ± 0.0	–
2	Gum acacia control	3.33 ± 0.21*	–
3	Acetic acid control	3.33 ± 0.21*	–
4	Zafirlukast 40 mg/kg oral	2.50 ± 0.22	25
5	Zafirlukast 40 mg/kg rectal	2.83 ± 0.17	15
6	Zafirlukast 80 mg/kg oral	1.60 ± 0.22**,#	52
7	Zafirlukast 80 mg/kg rectal	2.40 ± 0.22	28

Values are expressed as mean ± S.E.M.; $n = 6$.
* $P < 0.05$ in comparison to group 1.
** $P < 0.05$ in comparison to group 3.
# $P < 0.05$ in comparison to group 2.

3.2. Histopathological study

Colonic mucosa of healthy control animals showed normal appearance with intact epithelium, grade 0 (Fig. 1). In acetic acid control group the colonic mucosa showed multifocal areas of coagulative necrosis that varied in severity between rats. Also, submucosal oedema and fibrinohaemorrhagic exudate within the submucosa were found, in addition to accumulation of inflammatory cells within the mucosa, grade 3 (Fig. 2).

In zafirlukast pretreated rats, at a dose of 40 mg/kg orally or rectally, multifocal areas of coagulative necrosis of the colonic mucosa, submucosal oedema, haemorrhage and inflammatory cell infiltration were still present but not to the extent of severity as that observed with the acetic acid control group (grade 2). Administration of zafirlukast at a dose of 80 mg/kg orally revealed moderate protection of colonic mucosa. Slight submucosal oedema, mild cellular infiltration and congestion were still present, grade 1 (Fig. 3).

3.3. Biochemical study

3.3.1. Effects of zafirlukast on MDA activity

Table 2 demonstrates the mucosal MDA concentrations in colonic mucosal biopsies of rats. MDA was increased in acetic acid control (0.645 ± 0.044 nmol/mg tissue) as compared to normal controls (0.240 ± 0.019 nmol/mg tissue). Pretreatment with zafirlukast with 80 mg/kg orally for a period of 3 days resulted in a significant decrease in MDA levels ($P < 0.05$) as compared to acetic acid control, while the decrease in MDA after rectal administration was not significant.

3.3.2. Effects of zafirlukast on MPO activity

Table 3 demonstrates the mucosal MPO concentrations in colonic mucosa of rats. Tissue MPO levels significantly increased ($P < 0.05$) following intrarectal administration of acetic acid (group 3). Pretreatment of rats (group 6) with zafirlukast (80 mg/kg) orally caused

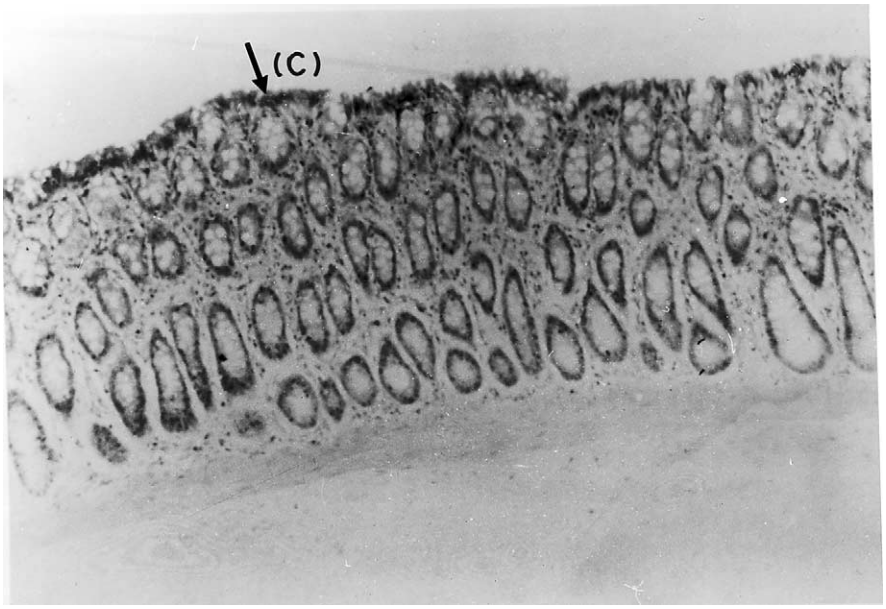

Fig. 1. Normal control. Photomicrograph of haematoxylin and eosin stained section of distal rat colon showed intact epithelial surface. No oedema, no haemorrhage or cellular infiltration (grade 0).

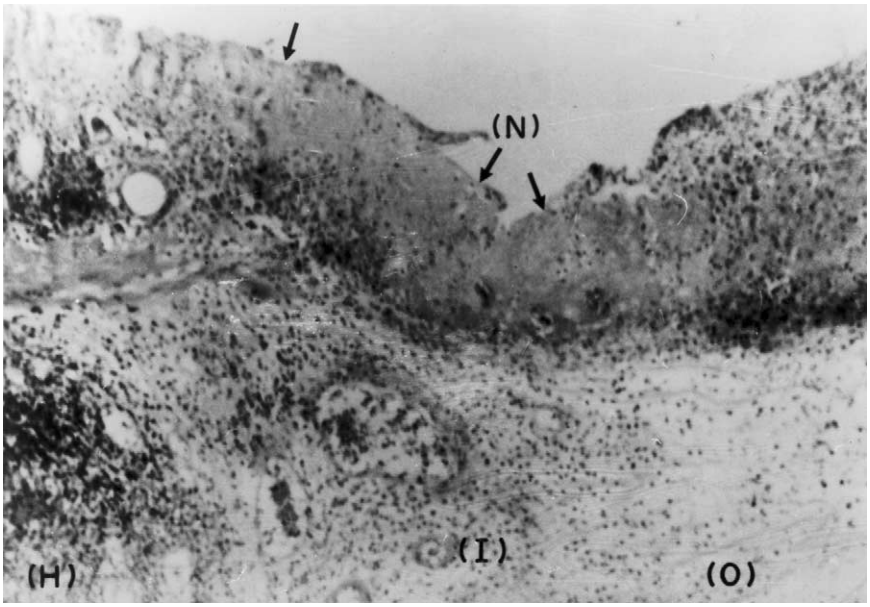

Fig. 2. Acetic acid control group. Photomicrograph of haematoxylin and eosin stained section of distal rat colon showed areas of massive necrotic destruction of epithelial surface, ulcer (N), submucosal oedema (O), scattered areas of haemorrhages (H) (grade 3).

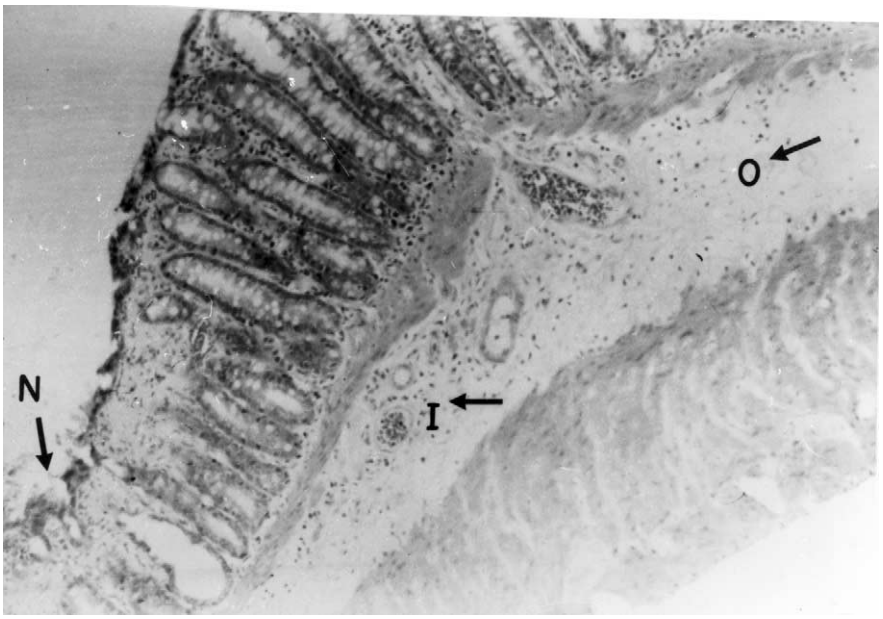

Fig. 3. Photomicrograph of haematoxylin and eosin stained section of distal rat colon pretreated with 80 mg/kg of zafirlukast orally showed areas of slight submucosal oedema (O), areas of mild cellular infiltration and congestion (I). The epithelial surface showed localized areas of necrosis (N) (grade 2).

a significant reduction ($P < 0.05$) in mean MPO activity as compared to acetic acid control (group 3). On the other hand, neither the animals (group 7) that received the same dose of zafirlukast rectally nor the animals (groups 4 and 5) pretreated with zafirlukast 40 mg/kg (both oral and rectal) showed no significant effects on MPO activities.

3.3.3. *Effects of zafirlukast on GSH level*

Reduced glutathione content was significantly decreased in acetic acid control group (690 ± 49 nmol/g tissue). Pretreatment with zafirlukast in a dose of 80 mg/kg orally resulted in a significant increase in GSH content while rectal administration failed to produce any significant effect (Table 4).

Table 2
Effects of zafirlukast (40 and 80 mg/kg) administered either orally or rectally, for three consecutive days before induction of colitis, on malondialdehyde concentrations in rat colonic mucosa

No.	Group	Malondialdehyde (MDA) (nmol/mg tissue)
1	Normal control	0.240 ± 0.019
2	Gum acacia control	0.640 ± 0.40*
3	Acetic acid control	0.645 ± 0.44*
4	Zafirlukast 40 mg/kg oral	0.519 ± 0.043
5	Zafirlukast 40 mg/kg rectal	0.569 ± 0.047
6	Zafirlukast 80 mg/kg oral	0.411 ± 0.032**,#
7	Zafirlukast 80 mg/kg rectal	0.499 ± 0.058

Values are expressed as mean ± S.E.M.; $n = 6$.
* $P < 0.05$ as compared to group 1 normal control.
** $P < 0.05$ as compared to group 3 acetic acid.
# $P < 0.05$ as compared to group 2 gum acacia.

Table 3
Effects of zafirlukast (40 and 80 mg/kg) administered orally or rectally, for three consecutive days before induction of colitis on myeloperoxidase content in colonic mucosa of rats

No.	Group	Myeloperoxidase (MPO) (U/mg colonic tissue)
1	Normal control saline	0.45 ± 0.03
2	Gum acacia control and acetic acid	0.93 ± 0.02*
3	Acetic acid control	0.97 ± 0.02*
4	Zafirlukast 40 mg/kg oral	0.92 ± 0.02
5	Zafirlukast 40 mg/kg rectal	0.91 ± 0.03
6	Zafirlukast 80 mg/kg oral	0.54 ± 0.03**,#
7	Zafirlukast 80 mg/kg rectal	0.90 ± 0.03

Values are expressed as mean ± S.E.M.; $n = 6$.
* $P < 0.05$ in comparison to group 1 normal control saline.
** $P < 0.05$ in comparison to group 3 acetic acid control group.
# $P < 0.05$ in comparison to group 2 gum acacia control.

Table 4
Effects of zafirlukast (40 and 80 mg/kg) administered, either orally or rectally, for three consecutive days before induction of colitis on glutathione concentrations in rat colonic mucosa

No.	Group	Reduced glutathione (GSH) (nmol/mg tissue)
1	Normal control	1440 ± 76
2	Gum acacia control	680 ± 52*
3	Acetic acid control	690 ± 49*
4	Zafirlukast 40 mg/kg oral	723 ± 61
5	Zafirlukast 40 mg/kg rectal	699 ± 57
6	Zafirlukast 80 mg/kg oral	989 ± 33**,#
7	Zafirlukast 80 mg/kg rectal	771 ± 48

Values are expressed as mean ± S.E.M.; $n = 6$.
* $P < 0.05$ as compared to group 1 normal control.
** $P < 0.05$ as compared to group 3 acetic acid control.
# $P < 0.05$ as compared to group 2 gum acacia control.

3.3.4. *Effects of zafirlukast on catalase activity*

The activity of catalase was significantly decreased in acetic acid control group (99 ± 9 nmol/min/mg tissue) compared to normal control (200 ± 12 nmol/min/mg tissue). Pretreatment with zafirlukast 80 mg/kg given orally resulted in a significant increase in catalase activity while the rectal dose showed no significant effect as compared to acetic acid control group (Table 5). The 40 mg/kg dose of zafirlukast, whether given orally or rectally, produced no significant effect on the levels of MDA, MPO, GSH or catalase in rat colonic tissues (Tables 2–5).

Table 5
Effects of zafirlukast (40 and 80 mg/kg) administered orally or rectally, for three consecutive days before induction of colitis, on catalase content in colonic mucosa of rats

No.	Group	Catalase (μmol/mg tissue)
1	Normal control	200 ± 12
2	Gum acacia control	90 ± 8*
3	Acetic acid control	99 ± 9*
4	Zafirlukast 40 mg/kg oral	108 ± 11
5	Zafirlukast 40 mg/kg rectal	100 ± 8
6	Zafirlukast 80 mg/kg oral	146 ± 9**,#
7	Zafirlukast 80 mg/kg rectal	116 ± 8

Values are expressed as mean ± S.E.M.; $n = 6$.
* $P < 0.05$ in comparison to group 1 normal control.
** $P < 0.05$ in comparison to group 3 acetic acid control.
# $P < 0.05$ in comparison to group 2 gum acacia control.

4. Discussion

Inflammatory bowel diseases which are chronic recurrent inflammatory disorders of the gastrointestinal tract (GIT), are considered as the most challenging of GIT diseases. The exact aetiologies of IBD, ulcerative colitis and Crohn's disease are obscure. However, recently available genetic, epidemiological data and the elevated levels of certain inflammatory mediators strongly support the idea that an interaction between the immunologic mechanisms, neuropeptides and vascular factors occurs in these conditions resulting in failure to down-regulate the usual self-limiting gut inflammatory responses (Kirsner, 1995; Kirsner and Shorter, 1982).

The cysteinyl leukotrienes are currently one target of interest for new therapeutic interventions in IBD. Available data indicate that the inhibition of leukotriene synthesis or action may play an important role in the treatment of IBD (Mac-Dermatt, 1999). Ulcerative colitis is associated with enhanced mucosal formation of cysteinyl leukotrienes (Peskar et al., 1987). Enhanced generation of leukotriene C_4 has been demonstrated in experimental models of colitis (Zipser et al., 1987), where it correlates well with the severity of inflammatory cell infiltration. However, controversial results have also been reported (Zipser et al., 1987).

Oxygen-derived species are readily available in the gastrointestinal tract. They are generated from several sources including stimulated polymorphonuclear cells, eosinophils, xanthine oxidase, colonic bacteria and epithelial lipoxygenase. All of these agents are present in the inflammed bowel of patients suffering from IBD. Xanthine oxidase, which catalyses the reduction of oxygen yielding superoxide radical (O_2^-) and hydrogen peroxide is activated by proteases that are released from either inflammatory cells or from dying epithelial cells. The antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase, further increase the vulnerability of the colon towards the deleterious actions of the oxygen-derived species (Higgins et al., 1978).

In the present study, a significant decrease in the reduced glutathione level and catalase were observed. Since GSH is an antioxidant tripeptide, its reduction signifies excessive production of reactive oxygen species and indicates its consumption in the oxidative

reaction (Frei, 1994). The reduced GSH level can be explained by the finding of Blau et al. (2000), who reported that reactive oxygen metabolites generated by oxidative metabolism of the acetic acid act as a hapten and is partly responsible for the inflammation that was produced.

Different leukotriene D_4 receptor antagonists have been shown to prevent colonic disturbances associated with ricin or trinitrobenzene sulphonic acid-induced enteritis and colitis (Pons et al., 1992). This was seen without changes in long-term motor alterations (Morteau et al., 1993), tissue morphology or the synthesis of eicosanoids (Sjogren et al., 1994). Nishikawa et al. (1995) showed a reduction in chronic trinitrobenzene sulphonic acid-induced colitis by a leukotriene C_4/D_4 receptor antagonist, praulukast (ONO-1078). In another study, amelioration of ethanol-induced gastric mucosal damage by a leukotriene C_4/D_4 receptor antagonist (L649, 923) had been demonstrated (Wallace et al., 1988). There was also a case report that the selective reversible Cys-leukotriene-1 receptor antagonist, montelukast, induced a complete resolution of the symptoms of chronic eosinophilic gastroenteritis which was resistant to other modes of treatments (Neustrom, 1999).

Jain et al. (2001) found that zafirlukast (5 and 10 mg/kg) had a significant effect on exudate formation and migration of polymorphonuclear leukocytes in the carrageenan-induced pleurisy model. Also, zafirlukast reduced myeloperoxidase activity in carrageenan-treated paw oedema model. These authors suggested that cysteinyl leukotrienes may be involved in nociceptive/inflammatory conditions. The protective effects of zafirlukast observed in the present study can be explained by the fact that it is a leukotriene antagonist especially against LTB_4. It acts as a chemoattractant and stimulant to the secretion of superoxide anion and the release of different granule constituents from leucocytes.

Kupczyk and Kuna (1999) suggested that blocking the formation or action of cysteinyl leukotrienes may be beneficial in the treatment of chronic inflammatory diseases. Additionally, Calhoun et al. (1998) reported that zafirlukast therapy alters cellular infiltration and activation associated with antigen challenge. These findings lend further support to our present data.

In a study by Zingarelli et al. (1993) the efficacy of Zileuton, a 5-lipoxygenase inhibitor, was investigated in comparison to sulphasalazine in an experimental model of rat colitis. Treatment of rats with zileuton resulted in significant reductions of colonic leukotriene B_4, 6-keto-PG F_1 alpha synthesis and myeloperoxidase. In contrast, sulphasalazine had a lesser inhibitory effect than zileuton on LTB_4 and myeloperoxidase levels. Therefore, this study showed that zileuton was effective in attenuating the lesions in an experimental model of colitis. Furthermore, the results are consistent with the hypothesis that leukotrienes play an important role in the pathogenesis of intestinal bowel diseases. These results are in concordance with our present findings on zafirlukast.

In conclusion, as cysteinyl leukotrienes play a part in inflammatory reactions such as inflammatory bowel diseases, the results of the present study indicate that zafirlukast had a protective effect against acetic acid-induced colitis in rats. Further work is needed to evaluate the role of zafirlukast in the treatment of inflammatory bowel disease and the possible mechanisms of its action.

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