Azithromycin and erythromycin ameliorate the extent of colonic damage induced by acetic acid in rats

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Abstract

Ulcerative colitis is a common inflammatory bowel disease (IBD) of unknown etiology. Recent studies have revealed the role of some microorganisms in the initiation and perpetuation of IBD. The role of antibiotics in the possible modulation of colon inflammation is still uncertain. In this study, we evaluated the effects of two macrolides, namely azithromycin and erythromycin, at different doses on the extent and severity of ulcerative colitis caused by intracolonic administration of 3\% acetic acid in rats. The lesions and the inflammatory response were assessed by histology and measurement of myeloperoxidase (MPO) activity, nitric oxide synthetase (NOS) and tumor necrosis factor alpha (TNF\textalpha) in colonic tissues. Inflammation following acetic acid instillation was characterized by oedema, diffuse inflammatory cell infiltration and necrosis. Increase in MPO, NOS and TNF\textalpha was detected in the colonic tissues. Administration of either azithromycin or erythromycin at different dosage (10, 20 and 40 mg/kg orally, daily for 5 consecutive days) significantly \((P < 0.05)\) reduced the colonic damage, MPO and NOS activities as well as TNF\textalpha level. This reduction was highly significant with azithromycin when given at a dose of 40 mg/kg. It is concluded that azithromycin and erythromycin may have a beneficial therapeutic role in ulcerative colitis.

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Introduction

An increasing number of both clinical and laboratory findings support the importance of luminal bacteria in the pathogenesis of inflammatory bowel disease (IBD) (Onderdonk, 2000). Actually, the anatomic sites of the intestine in which bacteria colonize with a high density, such as the distal ileum and colon, are sites that are frequently affected by inflammation in IBD patients. In addition, the use of antibiotics or a diversion of the fecal stream is found to reduce the activity of inflammation in patients with IBD, thus supporting the notion that intestinal bacteria play an important role in sustaining inflammation in IBD (Rutgeerts et al., 1991; Sutherland et al., 1991). Results of studies using knockout mice that had disrupted genes for cytokines or cell surface structures for immunity further support the role of luminal microorganisms in the development and perpetuation of IBD (Sadlack et al., 1993; Panwala et al., 1998). Substantial data from clinical observations and animal models incriminate normal luminal bacteria or bacterial products in the initiation and perpetuation of IBD (Farrell and LaMont, 2002; Linskens et al., 2001).

In several experimental models of IBD, intestinal inflammation either does not develop or is much less severe in animals raised in germ-free conditions (Dianda et al., 1997; Rath et al., 1996; Sartor, 1997; Sellon et al., 1998). More direct evidence to support the importance of microbacteria is provided by experimental studies where bacterial products, such as PG–PS polymers, can induce and aggravate colitis (Sartor, 1997; Yamada et al., 1993).
Based on serological responses or on the presence of organisms or specific antigens in affected tissues, a variety of specific pathogens have been proposed as etiologic agents for IBD, such as Mycobacterium paratuberculosis, Listeria monocytogenes, Chlamydia trachomatis and Escherichia coli. Despite the studies that have been done, the etiopathology is still poorly understood (Linskens et al., 2001). Other studies using in situ hybridization and polymerase chain reaction techniques have demonstrated that both Crohn’s disease and ulcerative colitis are associated with high numbers of mucosal bacteria (Schultz et al., 1999; Swidsinski et al., 2002). The concentration of the bacteria increases progressively with the severity of the disease (Swidsinski et al., 2002).

Antimicrobial agents have proved much more effective in preventive protocols than in treatment protocols in experimental colitis models. Metronidazole, an antimicrobial agent against anaerobes and parasites, had proved effective in the prevention of colitis in various models (Fukuda et al., 2002; Onderdonk et al., 1978; Rath et al., 2001). Combining metronidazole with ciprofloxacin, which eliminates aerobic gram-negative bacteria, or with neomycin, which is effective against anaerobes, had also demonstrated beneficial effects in the prevention of experimental colitis (Hans et al., 2000; Madsen et al., 2000). Other antimicrobial agents with preventive effects on experimental colitis included ciprofloxacin (Madsen et al., 2000; Rath et al., 2001) and vancomycin alone (Fukuda et al., 2002), and the broad-spectrum antibiotic combination of vancomycin and imipenem (Rath et al., 2001; Videla et al., 1994). However, various other antimicrobial agents have failed to show any preventive effects in experimental colitis models (Onderdonk et al., 1978; Videla et al., 1994).

Treatment of established experimental colitis with different antimicrobial agents has only rarely proved beneficial. Although effective in the prevention of colitis of different models, Metronidazole is not sufficient as a treatment on its own in established experimental colitis (Fukuda et al., 2002; Onderdonk, 2000). The same applies to the combination of metronidazole and ciprofloxacin (Hans et al., 2000). However, when combined with neomycin, metronidazole can effectively treat experimental colitis (Madsen et al., 2000). Ciprofloxacin alone has not been shown to treat colitis effectively in any model investigated (Madsen et al., 2000; Rath et al., 2001). A combination of vancomycin and imipenem has shown favourable effects in several models (Rath et al., 2001; Videla et al., 1994). Numerous other antimicrobial agents have failed to treat experimental colitis (Gardiner et al., 1994; Videla et al., 1994).

The aim of the present study was to assess and compare the effects of erythromycin and azithromycin on the extent and severity of ulcerative colitis induced by acetic acid in rats.

**Materials and methods**

**Materials**

Erythromycin was obtained from Abbott Laboratories while azithromycin was from Pfizer Labs, USA. Reagents for myeloperoxidase (MPO) assay were purchased from Sigma (St. Louis, MO, USA). The kits for the determination of NOS and TNF-α were purchased from Amersham (Bucks, UK). All other chemicals used were of analytical grade.

**Animals.** Male Wistar albino rats (150–200 g) were used throughout the work. They were supplied by the Animal Care Centre at the College of Medicine, 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 care and use of laboratory animals of the College of Medicine Research Center (CMRC) at King Saud University, Riyadh. The rats were housed individually in a rack mounted with wire mesh cages to prevent coprophagia. All rats were exposed to the same environmental conditions and were maintained on a proper diet and water ad libitum. The animals were randomly divided into eight groups, each consisting of eight animals as follows: normal control group, acetic acid control group and the drug-treated groups that received different doses of erythromycin or azithromycin (10, 20 and 40 mg/kg) orally in a volume of 0.5 mL/100 g body weight. Each dose was given once daily, 24 h after induction of colitis and continued for 5 consecutive days.

**Induction of experimental colitis in rats.** The rats were fasted for 24 h with access to water ad libitum. Induction of colitis was performed using a modification of the method described by Millar et al. (1996). Each rat was sedated by the intraperitoneal injection of phenobarbitone (35 mg/kg). Two milliliters of acetic acid (3% v/v in 0.9% saline) were infused for 30 s using a polyethylene tube (2 mm in diameter), which was inserted through the rectum into the colon to a distance of 8 cm. Acetic acid was retained in the colon for 30 s after which the fluid was withdrawn. Animals were weighed daily and inspected for the presence of diarrhoea. Following completion of the experiments, rats were killed by using ether anaesthesia and colonic biopsies were taken for macroscopic scoring, histopathological and biochemical studies.

**Assessment of colonic damage**

**Macroscopic scoring.** The colon was excised and opened longitudinally, rinsed with cold saline and the colonic damage was evaluated by an independent observer accord-
ing to a scale ranging from 0 to 4 (Millar et al., 1996) as follows:

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>normal appearance</td>
</tr>
<tr>
<td>1</td>
<td>mucosal erythema only</td>
</tr>
<tr>
<td>2</td>
<td>mild oedema, slight bleeding or small erosions</td>
</tr>
<tr>
<td>3</td>
<td>moderate oedema, bleeding, ulcers or erosions</td>
</tr>
<tr>
<td>4</td>
<td>severe ulcerations, erosions, oedema and tissue necrosis</td>
</tr>
</tbody>
</table>

### Histological studies

Full thickness biopsy specimens were fixed in formol saline prior to wax embedding, sectioning and staining with haematoxylin and eosin or Alcian blue for histological evaluation of colonic damage and mucus content, respectively, according to standard protocols (Torres et al., 1999). The slides were coded to prevent observer bias during evaluation. All tissue sections were examined under an Olympus BH-2 microscope for characterization of histopathological changes. Photographs taken from colon samples were digitized using Kodak D290 zoom camera, Eastman Kodak, U.S.A. and Mohi Images 2000 release 1.1 (MicroOptic Industrial Group B3, Series System Microscopes). Analysis of the figures was carried out by using Adobe photoshop Version 5.

### Biochemical study

Samples from the colon were stored immediately at \(-20^\circ C\) till analysis. Tissue samples were homogenized in 1 mL of 10 mmol/Tris–HCl buffer of pH 7.1 and homogenate was used for the measurement of myeloperoxidase (MPO) activity, NOS enzyme activity and TNFα concentration.

### Determination of myeloperoxidase (MPO) activity

Myeloperoxidase (MPO) activity was assayed in colonic tissues using the method as described by Bradley et al. (1982). In brief, tissue samples of approximately 50 mg were taken, weighed and homogenized three times for 30 s at 4 °C in 1 mL of ice-cold 0.5% hexadecyltrimethylammonium bromide in 50 mmol/L phosphate buffer (pH 6). The homogenate was subjected to three freeze–thaw cycles and centrifuged for 15 min at 40,000 \(\times g\). Then, 0.1 mL of the supernatant was added to 2.9 mL of 50 mmol/L phosphate buffer containing 0.167 mg/mL O-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. The change in absorbance was measured at 460 nm over a 5-min period at 25 °C using a Perkin-Elmer Lambda 5 UV–Vis Spectrophotometer. The data are expressed as the change in absorbance/min/g colonic tissue.

### Total NOS enzyme activity

Colonic NOS activity was determined by monitoring the conversion of L-[\(^3\)H]arginine to L-[\(^3\)H]citrulline (Chiesi and Schwaller, 1995). For this purpose, the intestinal samples were homogenized (1:5 wt/v) for 60 s in 10 mmol/L N-2-ethanesulphonic acid (pH 7.4) containing sucrose (0.32 mol/L), EDTA (100 μmol/L), phenylmethylsulfonyl fluoride (1 g/L) and leupeptin (10 mg/L); the resulting homogenate was centrifuged at 10,000 \(\times g\) for 10 min at 4 °C, and the supernatants were assayed for protein content according to the method proposed by Bradford (1976). Samples (40 μg of protein) were incubated at room temperature for 30 min in the presence of nicotinamide adenine dinucleotide phosphate (1 mmol/L), calmodulin (0.3 μmol/L), tetracydrobiopterin (10 μmol/L), CaCl\(_2\) (2 mmol/L), L-valine (10 mmol/L); to inhibit nonspecific arginase activity and L-[\(^3\)H]arginine (100 μmol/L, 10 mCi/L). Incubations were terminated by the addition of 1 mL N-[2-hydroxyethyl] piperazine-N-[2ethanesulphonic acid] (20 mmol/L, pH 5.5) containing 1 mmol/L EGTA and 1 mmol/L EDTA. L-[\(^3\)H]citrulline was separated from arginine by adding 1.5 mL of a 1:1 suspension of Dowex (50W) in water. Formation of citrulline in the presence of excess of L-NAME (10 mmol/L) was also determined to be able to subtract background citrulline formation. Radioactivity was measured in supernatants by liquid scintillation counting and the results were expressed as pmol L-citrulline/(mg protein × 30 min).

### Measurement of TNFα

TNFα was determined according to the method of Reinecker et al. (1993). Colonic samples were immediately weighed, minced on an ice-cold plate suspended in a tube with 10 mmol/L sodium phosphate buffer (pH 7.4) (1: 5 wt/v). The tubes were placed in a shaking water bath (37 °C) for 20 min and centrifuged at 9000 \(\times g\) for 30 s at 4 °C; the supernatant was frozen at \(-80^\circ C\) until assay. TNFα was quantified by enzyme-linked immunoabsorbent assay (Amersham Pharmacia Biotech, Little Chalfont, UK) and the results were expressed as picograms per gram of wet tissue.

### Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM) for eight rats per experimental group. Statistical group analysis was performed with SPSS 10.0 statistical software. One-way analysis of variance (ANOVA) was used to compare the mean values of quantitative variable among the groups. As the outcome variable for gross lesion score is a qualitative variable, a nonparametric statistical test Kruskal–Wallis test was used to compare the mean ranks of scores among the different groups. Statistically significant differences were accepted at \(P < 0.05\).

### Results

#### Histological results

### Macroscopic

The acetic acid treatment induced severe macroscopic inflammation in the colon 24 h after rectal administration, as assessed from the colonic damage score. Treatment with azithromycin significantly reduced the severity of gross lesion score in a dose dependent manner as...
shown in Table 1. On the other hand, erythromycin in the small dose used had no significant effect while the higher doses used had a significant effect on the intensity of inflammatory response. The effect of azithromycin is nearly doubled when compared with the corresponding doses of erythromycin used in the study (Table 1). Also, treated rats showed a faster weight recovery as compared to acetic acid control group ($P < 0.05$) (Table 2).

**Microscopic results**

The histopathological features of untreated rats included transmural necrosis, oedema and diffuse inflammatory cell infiltration in the mucosa. There was focal ulceration of the colonic mucosa extending through the muscularis mucosae, desquamated areas and loss of the epithelium with mucin depletion. The architecture of the crypts was distorted and the lamina propria was thickened in peripheral areas of distorted crypts, especially in basal areas. An infiltrate consisting of mixed inflammatory cells was observed (Fig. 1B). Some areas showed accumulation of mucus and cell remnants; however, Alcian-blue-positive cells were less numerous. In addition, the mucin layer of the epithelium was missing (Fig. 2B). Treatment of rats with erythromycin or azithromycin significantly attenuated the extent and severity of the histological signs of cell damage, which was more obvious with azithromycin (Figs. 1C,D). There were no inflammatory cells in the lamina propria, the epithelium remained intact and the mucin layer was clearly visible. Alcian-blue-positive cells were less numerous (Figs. 2C,D). Histological studies confirmed the intestinal anti-inflammatory effect exerted by the antibiotics used.

**Biochemical results**

**Myeloperoxidase (MPO) enzyme activity**

Tissue MPO activity showed a statistically significant difference among the groups tested ($P < 0.05$). By performing pair-wise comparisons among the groups, we can infer that, the mean value of acetic acid control is significantly increased ($P < 0.05$) as compared to the normal control group. Treatment with azithromycin (10, 20 and 40 mg/kg) significantly reduced colonic MPO activity. The reduction was highest with the 40 mg/kg dose (Fig. 3). On the other hand, after treatment with erythromycin, a significant reduction in MPO activity was observed with the three doses used (10, 20 and 40 mg/kg).

**Total NOS enzyme activity**

NOS activity was significantly increased in the inflamed colon at 24 h after acetic acid administration as compared to normal saline control $P < 0.05$. Administration of azithromycin and erythromycin resulted in a significant reduction in NOS activity in comparison with the acetic acid control group $P < 0.05$. Using pair-wise comparisons among the groups, azithromycin in a dose of 40 mg/kg was the most potent at reducing NOS activity (Fig. 4).

**TNFα activity**

The colonic inflammation induced by acetic acid was characterized by a significant increased in TNFα levels (Fig. 5) in comparison with rats that did not receive acetic acid treatment ($P < 0.05$). Administration of either azithromycin or erythromycin to colitic rats resulted in a significant reduction in colonic TNFα levels. Maximum reduction was observed with azithromycin at a dose of 40 mg/kg (mean value of 89.07 ± 1.41 pg/mL) compared to...
Fig. 1. Acute colitis model induced by acetic acid: effect of erythromycin and azithromycin on colon injury. Histological appearance of normal control rats (A), and treated with acetic acid (B), and erythromycin, azithromycin (C,D). (B) Mucosal injury was produced after acetic acid administration, characterized by necrosis of epithelium, focal ulceration of the mucosa and diffuse infiltration of inflammatory cells in the mucosa and submucosa. (C,D) Treatment with erythromycin and azithromycin reduced the morphological alterations associated with acetic acid administration, protecting the mucosal architecture. Hematoxylin and eosin stain. ×100.

Fig. 2. Acute colitis induced by acetic acid; effect of erythromycin and azithromycin on colon injury. Histological appearance of rat colonic mucosa after Alcian blue stain. (A) Normal control and (B) acetic acid control animal. (C,D) Treated with erythromycin and azithromycin. (B) Some areas showed accumulation of mucus and cell remnants; however, Alcian-blue-positive cells were less numerous, and the mucin layer of the epithelium was missing. ×100.
acetic acid control group (mean value of 89.67 ± 1.41 pg/mL).

Discussion

The results of the present study confirmed the efficacy of the macrolide antibiotics, azithromycin and erythromycin against intestinal inflammation induced by the intracolonic administration of acetic acid. This was evidenced both histologically by a significant reduction in the extent and severity of lesions in the involved tissue and biochemically by a decrease in colonic MPO, NOS and TNFα activities.

There is ample experimental and clinical evidence that suggests that the inflamed colon undergoes substantial oxidative stress by neutrophil-derived oxidants and MPO activity, both of which contribute markedly to tissue damage during chronic intestinal inflammation (Harris et al., 1992; Yamada and Grisham, 1994). The present study showed that oxidative damage resulted in an increase in MPO activity, which had also been reported to occur both in human IBD and in experimental models of colitis (Galvez et al., 2000; Loguercio et al., 1996). As a consequence, a reduction in MPO can be interpreted as a manifestation of the intestinal anti-inflammatory effect exerted by the antibiotics erythromycin and azithromycin in this model of experimental colitis. This was confirmed microscopically by amelioration of the histological lesions that characterize this experimental model of colitis. Thus, treated rats showed partial to near restoration (depending on dose given) of the intestinal epithelium. This was evidenced by the presence of mature goblet cells with their mucin content replenished and less oedema and granulocyte infiltration in comparison with non-treated rats.

Various inflammatory mediators released by activated mucosal immune cells and other cells contribute to intestinal injury either directly or through the recruitment of granulocytes or immune cells to the site of injury (Wallace, 1991). The list of inflammatory mediators suspected to participate in IBD currently includes
Eicosanoids (Wallace, 1991), numerous cytokines, such as TNFα (Stevens et al., 1992), and platelet activating factor (PAF) (Sun et al., 1995).

During the last decade, it was suggested that nitric oxide may also be included in the list of inflammatory mediators (Boughton-Smith et al., 1993). The results obtained in the present study reveal that colonic inflammation is associated with a higher iNOS activity in comparison with sham-treated rats. This result is in agreement with previous observations that had been reported for other models of experimental colitis (Hoga-boam et al., 1995; Rachmilewitz et al., 1993) and for human IBD (Kimura et al., 1997; Rachmilewitz et al., 1995), which describe the enhanced NO production in the inflamed mucosa by colonic iNOS. Daily administration of azithromycin or erythromycin for a period of 5 days after induction of colitis resulted in a significant reduction in iNOS activity. Boughton-Smith et al. (1992) demonstrated marked elevations in colonic iNOS activity at 24 h, which remained even at day 7 post-trinitrobenzenesulfonic acid (TNB) instillation into the colon. Also, using a model of TNB-induced ileal inflammation, Millar et al. (1993) addressed the beneficial effects of NOS blockade by demonstrating that the addition of L-NAME to drinking water significantly attenuated epithelial permeability, granulocyte infiltration and tissue thickness.

The effect of excessive production of nitric oxide during gastrointestinal inflammation is largely uncharacterized. Inflammatory cells such as phagocytic leukocytes express inducible nitric oxide synthase (iNOS) when appropriately stimulated by cytokines (e.g. IL-1 and TNFα) or bacterial products such as lipopolysaccharide (Moncada et al., 1991). The expression of iNOS results in the synthesis of micromolar quantities of nitric oxide, which can be deleterious to cells through the formation of nitric oxide-reactive products (Beckman et al., 1990). Considering that active ulcerative colitis is associated with augmented mucosal iNOS activity (Boughton-Smith et al., 1993), it is conceivable that nitric oxide inhibition may reverse the mucosal hyperaemia, tissue injury, reduced colonic tone and other clinical symptoms associated with colonic inflammation.

TNFα is a cytokine released by activated mononuclear cells and T cells. It seems to have a clinically important role in septic shock (Tracey et al., 1986) and in rheumatoid arthritis (Maini et al., 1993). In addition, TNFα has been implicated in the pathogenesis of IBD. An overproduction of TNFα in the affected intestinal mucosa has been well documented in different animal models of colitis (Kojouharoff et al., 1997; McDonald et al., 1997; Simpson et al., 1997) and in human patients with active Crohn’s disease (Derkz et al., 1993; MacDonald et al., 1990; Reinecker et al., 1993). In addition, administration of neutralizing anti-TNFα and lymphotoxin (LT)α antibodies to patients with active Crohn’s disease lead to a transient improvement of the disease (Derkz et al., 1993; Van Dullemen et al., 1995) and attenuated the development of colitis in some experimental mouse models (Kojouharoff et al., 1997; Powrie et al., 1994). The markedly increased production of TNFα by macrophages isolated from lamina propria of affected colons has been reported in patients with active IBDS (Reimund et al., 1996; Reinecker et al., 1993) and also in several animal models of colitis. Furthermore, infection of monolayers of colonic epithelial cell lines with invasive bacteria lead to the up-regulation of proinflammatory cytokines, including TNFα (Jung et al., 1995). These results are in agreement with our present findings, where the administration of azithromycin or erythromycin to rats with experimental colitis leads to a significant improvement in the severity of the disease, which was accompanied by a decrease in TNFα activity.

There is considerable evidence from both animal models and clinical investigations supporting a pivotal role for bacteria in the initiation or exacerbation of the intestinal inflammation. For instance, bacteria, or their products, have been detected in inflamed mucosa of patients with Crohn’s disease (Klasen et al., 1994), antibiotic treatment (Sutherland et al., 1991) or diversion...
of the fecal stream (Rutgeerts et al., 1991) can reduce disease severity in some patients with Crohn’s disease, and in the majority of the spontaneous models of murine colitis the inflammation does not occur when the animals are housed under germ-free conditions (Contractor et al., 1998; Dianda et al., 1997; Rath et al., 1996; Sellon et al., 1998). The luminal microflora in UC has been examined in many studies (Burke and Axon, 1988; Van der Wiel-Korstanje and Winkler, 1975; Von-Wulffen et al., 1989). Patients with UC have abnormally large numbers of facultative anaerobic bacteria (Van der Wiel-Korstanje and Winkler, 1975). One of these, adhesive *E. coli*, has been implicated in the pathogenesis of UC (Burke and Axon, 1988).

Recently, Swidsinski et al. (2002), reported high concentrations of mucosal bacteria in patients with UC but not in healthy controls. Therefore, it appears from our study that the beneficial effects of azithromycin and erythromycin may be due to, at least in part, to their antibacterial action.

The attenuation in the severity and extent of colonic damage observed in rats treated with macrolide antibiotics lend further support to the previous work, which elaborates the role of intestinal bacteria in the initiation and perpetuation of IBD. Furthermore, the therapeutic efficacy observed with azithromycin was significantly higher than that of erythromycin. This could be explained by its unique pharmacokinetic profile, which enables it to provide effective treatment when given once daily, for 3 days, in contrast to the multiple doses over a longer period required with erythromycin. The key pharmacokinetic properties that contribute to this are good bioavailability, rapid absorption, elevated concentrations of the drug in tissues following delivery by phagocytic cells and the long tissue half-life.

The precise mechanism leading to the beneficial effects of oral azithromycin or erythromycin therapy during experimental colitis could be explained by their activity to inhibit luminal agents that contribute in maintaining the exacerbated immune response. This is in agreement with the previous work where they proved a direct interaction between bacteria and bacterial products and the gut immune system (Duchmann et al., 1995; Elson et al., 2001; Sartor, 1997). In fact, the intestinal epithelium acts as a defense against invasion by luminal toxins and bacteria but, as a consequence of the inflammation, the barrier function of the epithelium is impaired and the subsequent translocation of endotoxins and antigens up-regulates the immune response (MacDermott, 1996). This leads to extensive production of proinflammatory cytokines such as TNFα, IFNγ and chemokines.

In conclusion, the results of present work demonstrate that azithromycin and erythromycin significantly decreased the extent and severity of colonic damage induced by acetic acid and azithromycin was more effective. Our present findings have important therapeutic implications and may lay the foundation for further clinical trials for the use of macrolides in human IBD.

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