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European Journal of Pharmacology 460 (2003) 209–218
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Effects of dosmalfate, a new cytoprotective agent, on acute and chronic trinitrobenzene sulphonic acid-induced colitis in rats

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Received 11 July 2002; received in revised form 13 December 2002; accepted 19 December 2002

Abstract

Activated neutrophils and proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) are clearly involved in the pathogenesis of bowel disease. Increased expression of epidermal growth factor-receptor (EGF receptor) has been reported for the colon mucosa surrounding areas of ulceration, suggesting a pivotal role in mucosal defence and repair. In this study, we examined the effects of dosmalfate, a new flavonoid derivative compound (diosmin heptakis) with antioxidant and cytoprotective properties, on acute and chronic experimental trinitrobenzene sulphonic acid (TNBS)-induced colitis in rats. The inflammation response was assessed by neutrophil infiltration as evaluated by histology and myeloperoxidase activity. Mucosal TNF-α production and histological analysis of the lesions was also carried out. In addition, we studied the expression of the EGF receptor inmunohistochemically during the healing of TNBS-induced chronic colitis. A 2-day treatment with 400 or 800 mg/kg of dosmalfate ameliorated the colon damage score and the incidence of adhesions. It also significantly ($P<0.05$) decreased myeloperoxidase activity and colonic mucosal production of TNF-α. Chronic treatment (14 days) with 800 mg/kg/day of dosmalfate also had significant protective effects on TNBS-induced colitis which were reflected by significant attenuation ($P<0.05$) of the damage score while the inflammatory indicators were not improved. The chronic beneficial effect of dosmalfate was apparently related to the enhancement of EGF receptor expression. These findings confirm the protective effects of dosmalfate in acute and chronic experimental colitis.

Keywords: Colitis; TNBS (trinitrobenzene sulphonic acid); Neutrophil; TNF-α (tumor necrosis factor-α); Flavonoid; Dosmalfate

1. Introduction

Intestinal bowel disease is a chronic recurrent inflammatory disorder characterised by the development of intestinal inflammation resulting from the transmural infiltration of neutrophils, macrophages, lymphocytes and mast cells, ultimately giving rise to mucosal disruption and ulceration (Fiocchi, 1998). The infiltrated and activated neutrophils are an important source of reactive oxygen and nitrogen species. These species are cytotoxic agents, inducing cellular oxidative stress by cross-linking proteins, lipids and nucleic acids, which cause cellular dysfunction and damage. In addition to free radicals, neutrophils can also release proteases, lactoferrin and lipid mediators that can contribute to intestinal injury (Grisham, 1994).

It has been suggested that the main chemoattractants for neutrophils are proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), which regulates molecule expression on endothelial cells and promotes neutrophil adherence. TNF-α has been thought to be involved in intestinal bowel disease (Guimbaud et al., 1998; Schreiber et al., 1999) also in experimental models such as trinitrobenzene sulphonic acid/ethanol-induced (TNBS) colitis (Bobin-Dubigeon et al., 2001), which is a useful animal inflammation model in the rat and of particular interest in relation to Crohn's disease and ulcerative colitis (Elson et al., 1995).

Flavonoids are polyphenolic compounds that have long been recognized as anti-inflammatory, neuroprotective, chemopreventive, immunomodulatory and gastroprotective agents. Their therapeutic effects are probably exerted through a complex mechanism involving inhibition of eicosanoid synthesis and/or antioxidant-free radical scavenger activity. Indeed, they have been shown to down-

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doi:10.1016/S0014-2999(02)02949-7

regulate lymphocyte and natural killer cell cytotoxicity, neutrophil function and adhesion molecule expression (Alarcón de la Lastra et al., 1995; Gee and Johnson, 2001; Manthey, 2000; Youdim et al., 2002). Based on these observations, studies have investigated the protective effects of certain flavonoids on the inflammatory response in various models of acute and chronic colonic injury. Several mechanisms could be involved in this activity which is possibly related with the protection against oxidative insult and/or amelioration of neutrophils toxicity (Sánchez de Medina et al., 1996; Cruz et al., 1998; Ocete et al., 1998; Kim et al., 1999; Hong et al., 2002).

Dosmalfate, a flavonoid derivative compound (diosmin heptakis (hydrogen sulfate) aluminium complex), is a new and non-systemic cytoprotective drug that recently has been developed and marketed in some countries for therapeutic use in the healing of gastric and duodenal ulcers. Previous reports have shown its efficacy and tolerability in the healing and symptomatic relief of peptic ulcers (Ucelay et al., 2000). Increased endogenous prostaglandin release, increased bicarbonate production and synthesis of endogenous antioxidant sulfhydryl groups in the mucus among others have been demonstrated for the protective effect of dosmalfate (Corcóstegui et al., 2000).

As mentioned above, oxygen free radicals, neutrophils and proinflammatory cytokines are clearly involved in the pathogenesis of intestinal bowel disease. Accordingly, in this study we examined the effects of dosmalfate on the acute and chronic experimental TNBS-induced colitis in rat. The inflammation response was assessed on the basis of histology and myeloperoxidase activity, as an index of quantitative inflammation and neutrophil infiltration in the mucosa. Mucosal TNF-α production, histological and histochemical analysis of the lesions was also carried out.

Healing of the gastrointestinal mucosa damage after exposure to damage is a complex process involving different mechanisms, the most important of which appear to be the growth factors, specially epidermal growth factor (EGF) (Beck and Podolsky, 1999). EGF is a 3-amino acid peptide that binds to one common receptor (EGF receptor). Exogenously applied EGF protects colonic mucosa against injury: increased expression of EGF and EGF receptors has been reported in colonic mucosa surrounding areas of acute and chronic ulcerations, suggesting a pivotal role in mucosal defence and repair (Hoffmann et al., 2000; Dvorak et al., 2002). These data prompted us to study inmunohistochemically the expression of the EGF receptor in rats during healing of TNBS-induced chronic colitis.

2. Materials and methods

2.1. Experimental animals

Male Wistar rats supplied by Animal Services, Faculty of Medicine, University of Seville, Spain, and weighing 180–220 g, were placed singly in cages with wire-mesh floors in a room with temperature, 24–25 °C, humidity, 70–75%, and lighting, 12-h light/12-h dark, and were fed a normal laboratory diet (Panlab, Barcelona, Spain). The rats were deprived of food for 24 h prior to the induction of colitis, but were allowed free access to tap water throughout. They were randomly assigned to groups of 8–14 animals. The experiments followed a protocol approved by the local animal Ethics Committee and the local Government. All experiments were in accordance with the recommendations of the European Union regarding animal experimentation (Directive of the European Council 86/609/EC).

2.2. Induction of colitis

Colitis was induced according to the procedure described by Morris et al. (1989). Briefly, rats were lightly anesthetized with ether following a 24-h fast, and then a medical-grade polyurethane cannula for enteral feeding (external diameter 2 mm) was inserted into the anus and the tip was advanced to 8 cm proximal to the anus verge. TNBS (Sigma, Spain) dissolved in 50% ethanol was instilled into the colon through the cannula (10 or 30 mg in a volume of 0.25 ml to induce acute or chronic colitis, respectively). Following the instillation of the hapten, the animals were maintained in a head-down position for a few minutes to prevent leakage of the intracolonic instillate. Control groups were created for comparison with TNBS/ethanol instillation: rats in the normal group received physiological saline instead of the TNBS solution, and the ethanol control group received 0.25 ml 50% ethanol.

In the acute model of colitis, dosmalfate (FAES Laboratories, Leioa, Spain) was suspended in 0.9% saline solution and administered (p.o.) at different doses, 100, 200, 400 and 800 mg/kg, 48, 24 and 1 h prior to the induction of colitis and 24 h later. Control groups received vehicle in a comparable volume (10 ml/kg body weight). The animals were killed with an overdose of anesthetic 48 h after induction of colitis. In the chronic model, the drug was only administered at two levels of dosage (400 and 800 mg/kg) 24 h after TNBS instillation and daily during the 2 weeks before killing.

In both models, the rats were checked daily for behaviour, body weight, and stool consistency.

2.3. Assessment of colitis

Severity of the colitis was evaluated by an independent observer who was blind to the treatment. For each animal, the distal 10-cm portion of the colon was removed and cut longitudinally, slightly cleaned in physiological saline to remove fecal residues and weighed. Macroscopic inflammation scores were assigned, based on clinical features of the colon (score 0–10), the presence of adhesions (score 0–2), and/or stool consistency (score 0–1) according to the

criteria of Bobin-Dubigeon et al. (2001). Pieces of inflamed colon were collected and frozen in liquid nitrogen to quantify myeloperoxidase activity and TNF-α production.

2.4. Histological studies

For light microscopy we used tissue samples from the distal colon of each animal fixed in 4% buffered paraformaldehyde, dehydrated in increasing concentrations of ethanol, and embedded in paraffin. Thereafter, sections of tissue were cut at 7 μm on a rotary microtome (Leica Ultracut), mounted on clean glass slides and dried overnight at 37 °C. The sections were cleared, hydrated, and stained with hematoxylin and eosin or with Alcian blue for histological evaluation of colon damage and mucus content, respectively, according to standard protocols, and the slides were coded to prevent observer bias during evaluation. All tissue sections were examined with an Olympus BH-2 microscope for characterization of histopathological changes.

Photographs of colon samples were digitized using a Kodak D290 Zoom camera Eastman Kodak, USA and Motic®Images 2000 release 1.1 (MicroOptic Industrial Group; B1 Series System Microscopes). Analysis of the figures was carried out with the Adobe®Photoshop® Version 5.0 (Adobe Systems) image analysis program.

2.5. Immunohistochemical study

Colon tissues were fixed in 4% buffered paraformaldehyde, dehydrated with increasing concentrations of ethanol, embedded in paraffin, and sectioned. Sections (7 μm thick) were mounted on slides, cleared, and hydrated. All sections were treated with a buffered blocking solution (3% bovine serum albumin in phosphate-buffered saline, PBS) for 15 min. The sections were then co-incubated with primary anti-EGF receptor antibody (1:400 in PBS, v/v), at room temperature for 2 h, followed by washing with PBS and co-incubation with secondary antibody (anti-sheep IgG, peroxidasic conjugated, Sigma) (1:400 in PBS, v/v), at room temperature for 1 h. Thereafter, the sections were washed as before and with Tris–HCl 0.05 M, pH 7.66, then co-incubated with a 3,3′-diaminobenzidine solution in the dark, at room temperature for 30 min. The sections were washed with Tris–HCl, mounted with glycerine and observed with an Olympus BH-2 microscope.

2.6. Assessment of leukocyte involvement

Myeloperoxidase activity was assessed as a marker of neutrophil infiltration according to the methods of Grisham et al. (1990). One sample from the distal colon was taken from all animals. Samples were excised from each animal and rapidly rinsed with ice-cold saline, blotted dry, and frozen at −70 °C. The tissue was thawed, weighed and homogenized in 10 volumes 50 mM PBS, pH = 7.4. The homogenate was centrifuged at 20,000 × *g*, 20 min, 4 °C. The pellet was again homogenized in 10 volumes 50 mM PBS, pH = 6.0, containing 0.5% hexadecyl-trimethylammonium bromide (HETAB) and 10 mM EDTA. This homogenate was subjected to one cycle of freezing/thawing and brief sonication. A sample of homogenate (0.5 μl) was added to a 0.5-ml reaction volume containing 80 mM PBS, pH 5.4, 0.5% HETAB and 1.6 mM 3,3′,5,5′-tetramethylbenzidine (TMB). The mixture was incubated at 37 °C for 5 min and the reaction was started by the addition of 0.3 mM H_2O_2.

Each tube containing the complete reaction mixture was incubated for exactly 3 min at 37 °C. The reaction was terminated by the sequential addition of catalase (20 μg/ml) and 2 ml 0.2 M sodium acetate, pH = 3.0. The changes in absorbance at 655 nm were measured with a spectrophotometer. One unit of myeloperoxidase activity was defined as the amount of enzyme present that produced a change in absorbance of 1.0 U/min at 37 °C in the final reaction volume containing the acetate. The results were quantified as U/mg protein.

Table 1
Parameters quantified after administration of dosmalfate (100–1600 mg/kg) in rats with acute colitis induced by TNBS intracolonic instillation (10 mg/kg)

Group	*n*	Body weight changes (g)	Food consumption (g/rat·day)	Adhesions (score 0–2)	Diarrhoea (score 0–1)	Colon weight/colon length (mg/cm)
Sham	8	10.6 ± 4.3	24.0	0	0	230.3 ± 4.0
EtOH	14	1.4 ± 2.9	25.7	0.6 ± 0.2[a]	0.1 ± 0.1	281.5 ± 30.1
TNBS	12	−10.6 ± 4.1[a]	13.8	1.1 ± 0.1[c,d]	0.3 ± 0.1	334.0 ± 28.3[b]
D 100	6	−16.9 ± 4.4[b]	12.5	0.8 ± 0.2[b]	0.8 ± 0.2[b,e,f]	358.3 ± 30.0[b]
D 200	12	−15.0 ± 4.8[a]	14.1	0.2 ± 0.1[g]	0.7 ± 0.1[b,d,f]	329.2 ± 22.6[b]
D 400	14	3.2 ± 4.7	17.7	0[a,g]	0.3 ± 0.1[a]	293.9 ± 33.8
D 800	14	−5.4 ± 3.6	18.3	0.2 ± 0.1[g]	0.3 ± 0.1	297.8 ± 41.6

Colon parameters were quantified in the sham group ($n = 8$), which received saline instillation. TNBS group ($n = 12$) received trinitrobenzene sulphonic acid intracolonically in a vehicle of 50% ethanol; ethanol group ($n = 14$) received 50% ethanol intracolonic injection. Data are expressed as means ± S.E.M.
[a]$P < 0.05$, [b]$P < 0.01$ and [c]$P < 0.001$ significantly different from sham, [d]$P < 0.05$ and [e]$P < 0.01$ significantly different from EtOH, [f]$P < 0.05$ and [g]$P < 0.001$ significantly different from TNBS.

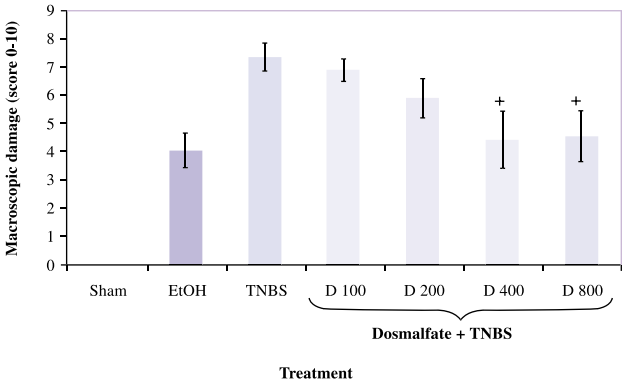

Fig. 1. Effects of acute administration of dosmalfate on the colon damage score. Colon macroscopic damage resulting from trinitrobenzene sulphonic acid (10 mg/kg) instilled into rat colon was scored as indicated in Materials and methods. Scores were quantified in the absence of treatment, but with daily administration of the vehicle saline solution (sham group, trinitobenzene sulphonic acid group and ethanol group), or in the presence of dosmalfate (100–800 mg/kg/day). The data are expressed as the means ± S.E.M. (+$P<0.05$ vs. TNBS group).

2.7. TNF-α levels

Distal colon samples were weighed (100 mg) and homogenized, after thawing, in 0.3 ml phosphate buffer saline solution (PBS pH 7.2) at 4 °C. They were centrifuged at 12 000 rpm for 10 min. Mucosal TNF-α level was assayed with a quantitative TNF-α enzyme immunoassay (ELISA) kit (Quantikine®M, R&D Systems). The TNF-α values were expressed as pg/mg protein.

2.8. Statistical analysis

All values in the figures and text are expressed as arithmetic means ± standard error of the mean (S.E.M.). The data were evaluated with Graph Pad Prism® Version 2.01 software. The statistical significance of differences for each parameter among the groups was evaluated by one-way analysis of variance (ANOVA) followed by Fisher's test. P values of <0.05 were considered statisti-

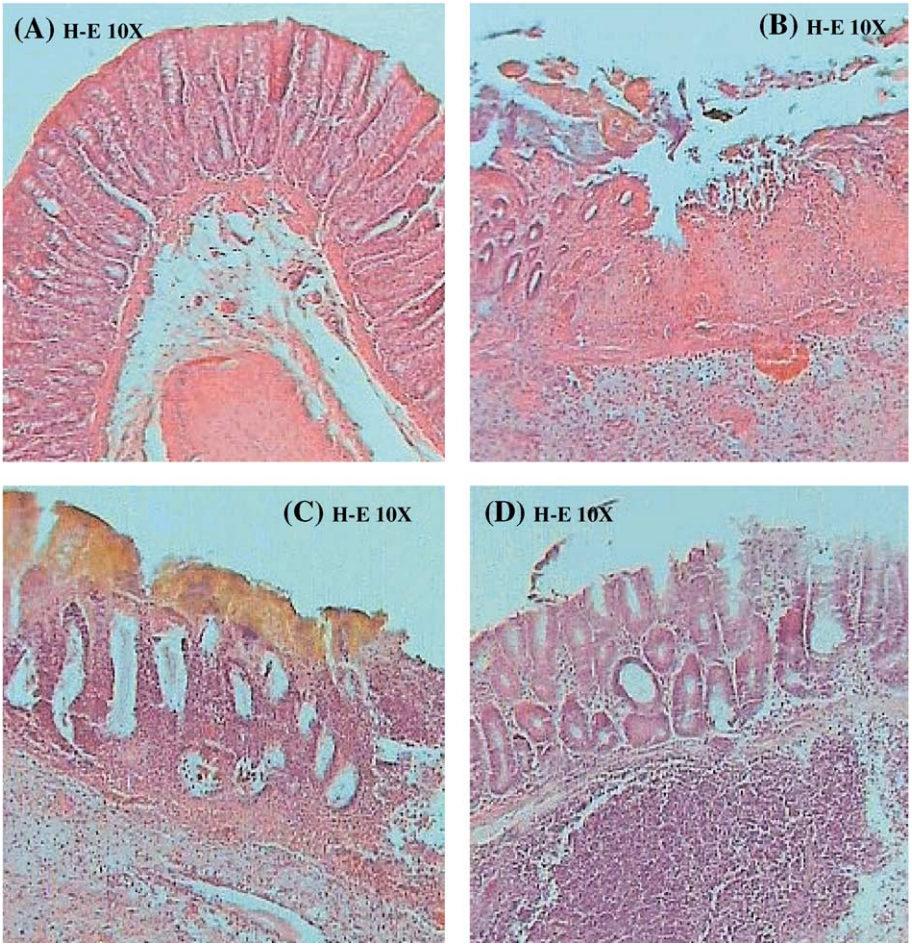

Fig. 2. Acute colitis model induced by TNBS: effect of dosmalfate on colon injury. Histological appearance of rat colon mucosa: sham (A), and treated with TNBS 10 mg/kg (B), and dosmalfate 400 or 800 mg/kg (C and D, respectively). Histopathological features of the colon in association with colitis. (A) No histological modification was present in the sham-treated animals. (B) Mucosal injury was produced after TNBS administration, characterized by necrosis of epithelium, focal ulceration of the mucosa and diffuse infiltration of inflammatory cells in the mucosa and submucosa. (C and D) Treatment with dosmalfate (400 and 800 mg/kg) reduced the morphological alteration associated with TNBS administration protecting the mucosal architecture. H-E: Hematoxylin and eosin stain. Original magnification 10 ×.

cally significant. In the experiment involving histology or immunohistochemistry, the figures shown are representative of at least six experiments performed on different days.

3. Results

3.1. Colonic inflammation induced by trinitrobenzene sulphonic acid instillation. Acute phase

Rats treated with TNBS and ethanol showed prostration, piloerection and hypomotility. Severe diarrhoea in conjunction with rectal bleeding was maximal after 48 h of the enema. In this experimental group, body weight loss was 10.6 ± 4.1 g compared with the sham-treated animals. The animals were severely anorexic, with a marked decrease in average food intake compared to that of the vehicle-treated group (Table 1). The cecum, colon and rectum showed evidence of damage with mucosal haemorrhage, oedema and deep ulceration. Some of these animals had local peritonitis compatible with transmural necrosis and inflammatory masses in the region of the descending colon. Lesions in the distal colon were quantified according to a macroscopic damage score (mean: 7.3 ± 0.5) (Fig. 1). A significant increase in the weight/length of the rat colon, an indicator of inflammation, was also observed in TNBS- and ethanol-treated rats (334.0 ± 28.3 mg/cm) in comparison with vehicle-treated rats (Table 1).

The histopathological features included 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 or loss of epithelium and mucin depletion. The architecture of the crypts was distorted and the lamina propria was thickened in peripheral areas of distorted crypts, specially in basal areas. An infiltrate consisting of polymorphonuclear leukocytes, lymphocytes, and eosinophils was observed. There was also granulation tissue in the submucosa (Fig. 2B). Some areas showed accumulation of mucus and cell remnants, however,

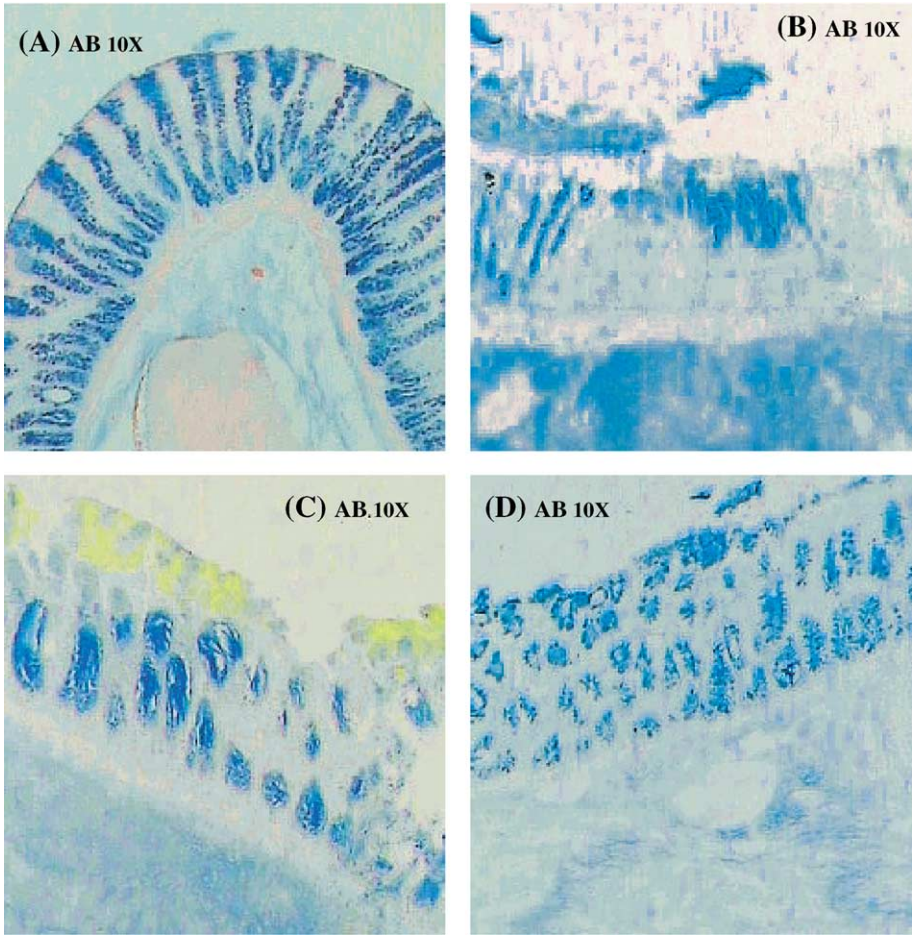

Fig. 3. Acute colitis model induced by TNBS: effect of dosmalfate on colon injury. Histological appearance of rat colon mucosa after Alcian blue stain (AB): sham (A), and treated with TNBS 10 mg/kg (B), and dosmalfate 400 or 800 mg/kg (C and D, respectively). Some areas showed accumulation of mucus and cell remnants however, Alcian blue-positive cells were less numerous, and the mucin layer of the epithelium was missing (B). Original magnification 10 ×.

Table 2
Myeloperoxidase activity (MPO, U/mg protein) and tumor necrosis factor alpha levels (TNF-α, pg/mg protein) after dosmalfate administration (100–1600 mg/kg) in rats with acute colitis produced by TNBS intracolonic instillation (10 mg/kg)

Group	*n*	MPO (U/mg protein)	*n*	TNF-α (pg/mg protein)
Sham	8	5.75 ± 0.79	6	0.07 ± 0.02
EtOH	11	6.93 ± 0.80	6	0.26 ± 0.04[b]
TNBS	10	8.01 ± 0.86[a]	6	0.71 ± 0.19[c,d]
D 100	6	8.58 ± 0.70[b]	6	0.68 ± 0.09[c,d]
D 200	8	8.36 ± 0.80[a]	6	0.53 ± 0.08[c,d]
D 400	9	5.59 ± 0.33[e]	7	0.28 ± 0.08[a,e]
D 800	11	5.72 ± 0.76[e]	9	0.34 ± 0.10[c,e]

Colon mucosal myeloperoxidase activity and tumor necrosis factor alpha levels were quantified in the absence of treatment, but with daily administration of the vehicle saline solution (sham group, trinitobenzene sulphonic acid group and ethanol group), or in the presence of dosmalfate (100–1600 mg/kg/day). Data are expressed as the means ± S.E.M. The myeloperoxidase activity and tumor necrosis factor alpha levels of colonic mucosa were quantified as described in Materials and methods and are expressed as U/mg protein and pg/mg protein, respectively.
[a]$P<0.05$, [b]$P<0.01$ and [c]$P<0.001$ significantly different from sham, [d]$P<0.05$ significantly different from EtOH, [e]$P<0.05$ significantly different from TNBS.

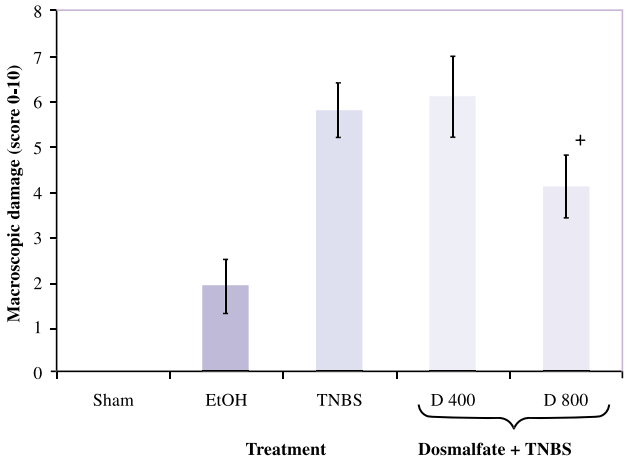

Fig. 4. Effects of chronic administration of dosmalfate on the colon damage score. Colon macroscopic damage resulting from trinitrobenzene sulphonic acid (30 mg/kg) instilled into rat colon was scored as indicated in Materials and methods. Scores were quantified in the absence of treatment, but with daily administration of the vehicle saline solution (sham group, trinitobenzene sulphonic acid group and ethanol group), or in the presence of dosmalfate (400–800 mg/kg/day). The data are expressed as the means ± S.E.M. (+$P<0.05$ vs. TNBS group).

Alcian blue positive–positive cells were less numerous. In addition, the mucin layer of the epithelium was missing (Fig. 3B).

When animals with colitis were treated with dosmalfate at doses ranging from 400 to 800 there was a significant reduction in macroscopic damage to the colon (Fig. 1). Dosmalfate was able to reduce the macroscopic damage score down to 4.3 ± 0.9 with the highest dose. In addition, no significant increases in the weight/length of the colon were produced (Table 1). Beneficial effects on body weight and the presence of adhesions were also observed; nevertheless, dosmalfate-treated rats did not have a lower incidence of diarrhoea compared to the TNBS control group. Histologically, there was attenuation of the extent and severity of the histological signs of cell damage and we saw no inflammatory cells in the lamina propria. In some areas, the epithelium remained intact and the mucin layer was clearly visible. However, in ulcerative areas, exfoliation of epithelial cells, dilated crypts and congestion vascular were observed (Figs. 2C,D and 3C,D).

Myeloperoxidase is an enzyme found in neutrophils and its activity in the colon is linearly related to neutrophil infiltration. The colitis caused by TNBS and ethanol was characterised by an increase in myeloperoxidase activity (8.01 ± 0.86 U/mg protein) (Table 2). This was consistent with the histological findings. Treatment of TNBS-treated rats with dosmalfate significantly reduced the degree of polymorphonuclear neutrophil infiltration. The levels of TNF-α were significantly elevated in the colon at 48 h after TNBS instillation. In contrast, the levels of this cytokine were significantly lower in rats treated with dosmalfate.

3.2. Colonic inflammation induced by trinitrobenzene sulphonic acid instillation. Chronic phase

The animals of the TNBS/ethanol group were severely anorexic with a 64.2% decrease in body weight gain.

Table 3
Parameters quantified after administration of dosmalfate (400–800 mg/kg) in rats with chronic colitis induced by TNBS intracolonic instillation (30 mg/kg)

Group	*n*	Body weight changes (g)	Food consumption (g/rat·day)	Adhesions (score 0–2)	Diarrhoea (score 0–1)	Colon weight/colon length (mg/cm)
Sham	10	87.5 ± 6.7	25.6	0	0	128.3 ± 4.4
EtOH	10	122.5 ± 6.0[c]	28.0	0	0	150.3 ± 9.5[a]
TNBS	16	56.3 ± 6.9[d]	14.5	1.8 ± 0.2[c,d]	0.3 ± 0.1	378.2 ± 50.6[c,d]
D 400	10	55.0 ± 12.8[a,d]	12.8	1.0 ± 0.0[c,d,e]	0	456.2 ± 95.4[c,d]
D 800	15	59.4 ± 14.3[d]	13.1	1.5 ± 0.2[c,d]	0.1 ± 0.0	405.7 ± 106.7[c,d]

Colon parameters were quantified in the sham group ($n=10$), which received saline instillation. TNBS group ($n=16$) received trinitrobenzene sulphonic acid intracolonically in a vehicle of 50% ethanol; ethanol group ($n=10$) received 50% ethanol intracolonic injection. Data are expressed as means ± S.E.M.
[a]$P<0.05$, [b]$P<0.01$ and [c]$P<0.001$ significantly different from sham, [d]$P<0.001$ significantly different from EtOH, [e]$P<0.05$ significantly different from TNBS.

Table 4
Myeloperoxidase activity (MPO, U/mg protein) and tumor necrosis factor alpha levels (TNF-α, pg/mg protein) after dosmalfate administration (400–800 mg/kg) in rats with chronic colitis produced by TNBS intracolonic instillation (30 mg/kg)

Group	*n*	MPO (U/mg protein)	*n*	TNF-α (pg/mg protein)
Sham	10	4.01 ± 0.50	6	0.11 ± 0.03
EtOH	8	5.31 ± 0.55	6	0.08 ± 0.01
TNBS	10	5.44 ± 0.82	6	0.22 ± 0.04[a,d]
D 400	7	4.66 ± 0.57	6	0.20 ± 0.11[c]
D 800	8	4.61 ± 0.44	7	0.18 ± 0.12[b]

Colonic mucosal myeloperoxidase activity and tumor necrosis factor alpha levels were quantified in the absence of treatment, but with daily administration of the vehicle saline solution (sham group, trinitobenzene sulphonic acid group and ethanol group), or in the presence of dosmalfate (400–800 mg/kg/day). Data are expressed as the means ± S.E.M. The myeloperoxidase activity and tumor necrosis factor alpha levels of colon mucosa were quantified as described in Materials and methods and are expressed as U/mg protein and pg/mg protein, respectively.
[a]$P<0.05$ significantly different from sham, [b]$P<0.05$, [c]$P<0.01$ and [d]$P<0.001$ significantly different from EtOH.

Colitis gave rise to diarrhoea in most animals. A significant increase in the weight/length of the colon was also observed (378.2 ± 50.6 mg/cm) in comparison with that of vehicle-treated rats (Table 3). Incomplete bowel obstruction and adhesions to adjacent organs were frequently observed. Damage score (6.1 ± 0.9) (Fig. 4) and the relation between weight and length of the rat colon remained significantly ($P<0.001$) above the control levels (Fig. 4). Neither myeloperoxidase activity nor tissue levels of TNF-α were significantly modified (Table 4). The histological appearance of the colon mucosa showed ulceration and inflammation extending through the mucosa and submucosa. Extensive presence of fibroblasts and lymphocytes was apparent. In some sections of ulcerated tissue thickness of the bowel wall was increased in comparison with the thickness in sections of normal tissue (Fig. 5B).

Chronic treatment with 800 mg/kg of dosmalfate also had a significant protective effect on TNBS-induced colitis which was reflected by significant attenuation ($P<0.05$) of the damage score (4.1 ± 07). No significant effects on the incidence of diarrhoea, adhesions or on colon weight/length were observed (Table 3). Histologically, the colon mucosa showed ulcers in the process of healing, evolution to a more chronic inflammatory infiltrate and initiation of a repair process (Fig. 5C and D).

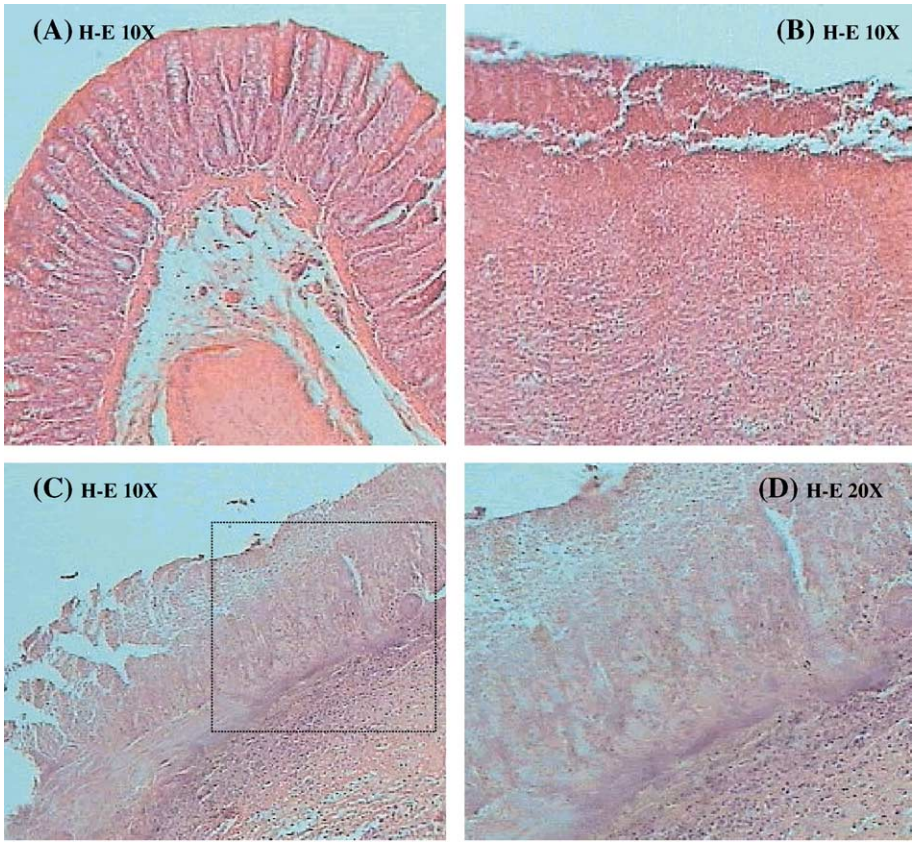

Fig. 5. Chronic colitis model induced by TNBS: effect of dosmalfate on colon injury. Histological appearance of rat colonic mucosa: sham (A), and treated with TNBS 10 mg/kg (B), and dosmalfate (C and D). Histopathological features of the colon in association with colitis. (A) No histological modification was present in the sham-treated animals. (B) After TNBS administration, histopathological features included necrosis, edema and higher fibrosis. (C and D) Crypt abscesses and regenerating epithelium were observed in the colon mucosa after treatment with dosmalfate 800 mg/kg. H-E: Hematoxylin and eosin stain. Original magnification 10× and 20×.

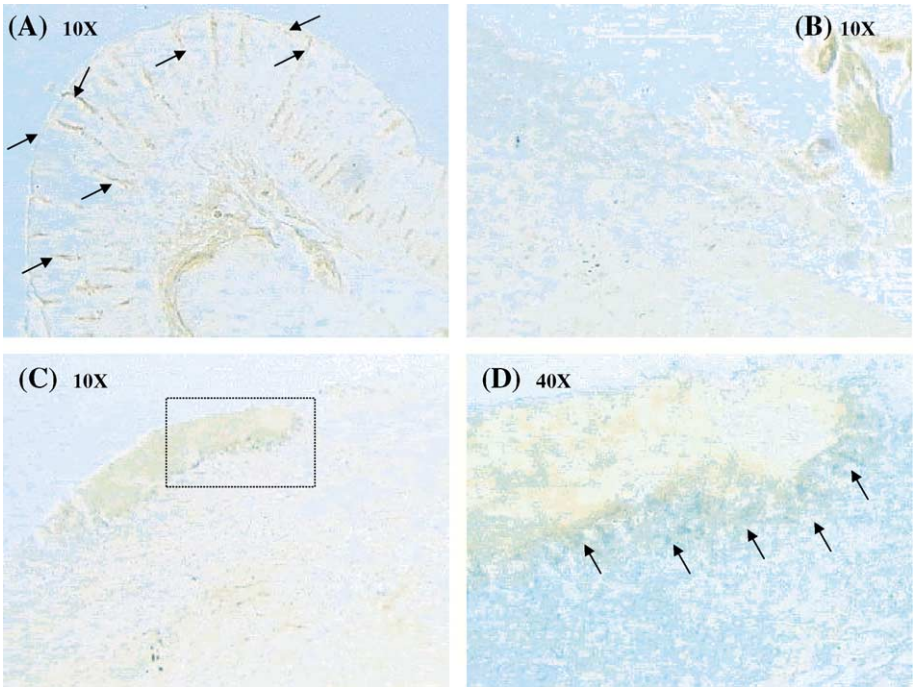

Fig. 6. Immunohistochemical localisation for epidermal growth factor receptor (EGF receptor). In normal colon mucosa, EGF-like immunoreactivity was localized in epithelial cells along the upper half of the crypts as well as the luminal surface (A). No EGF receptor expression was detected in the colon of TNBS-control rats (B). Enhanced EGF receptor expression was observed in dosmalfate-treated group. EGF receptor was mainly detected in epithelial cells of injured colon (C–D). Original magnification 10× and 40×.

In normal colon mucosa, EGF-like immunoreactivity was localized in epithelial cells along the upper half of the crypts as well as the luminal surface (Fig. 6A). No EGF receptor expression was detected in the colon of TNBS-control rats (Fig. 6B). However, in the dosmalfate-treated group, enhanced EGF receptor expression was observed. Staining for the EGF-receptor was mainly detected in epithelial cells of the injured colon (Fig. 6C and D).

4. Discussion

The present study confirmed that treatment with 400 or 800 mg/kg of dosmalfate was able to reduce the severity and extent of the acute colonic damage induced by TNBS and ethanol. The decrease in the extent of colitis induced by the flavonoid derivative was accompanied by a lower incidence of diarrhoea and of loss of weight of the animals and a decrease in the incidence of adhesions. The presence of adhesions between the colon and adjacent organs, which results from transmural inflammation, is a common feature of TNBS colitis (Morris et al., 1989; Bell et al., 1995). The reduction in the incidence of adhesions suggests a beneficial effect of dosmalfate on the extent of the inflammatory process in this experimental model.

As shown in Table 1, dosmalfate did not induce any significant effects in the colon weight/length ratio. The lack of an effect on this ratio is in agreement with findings of other authors (Ocete et al., 1998), and could be explained by the severe and over-extensive colon damage induced by TNBS and ethanol, which is difficult to overcome by pharmacological treatment as was previously suggested (Veljaca et al., 1995).

It has been proved that the gastroprotective effect of dosmalfate is related to the enhancement of several protective mucosal mechanisms such as increased endogenous prostaglandin release, increased bicarbonate production and synthesis of endogenous sulfhydryl groups in the mucus, among others (Corcóstegui et al., 2000). The protective effect of mucus as an active barrier may be attributed largely to its viscous and gel-forming properties which are derived from mucin glycoprotein constituents. Our results revealed that dosmalfate increased the amount of mucus stained by Alcian blue (acid glucoproteins such as sialomucins) in colon mucosa. Alcian blue-positive cells seem to be associated with regenerative processes of the mucosa (Alarcón de la Lastra et al., 1994), while reduction in the amount stained has been related to decreased resistance of the mucosa and paralleled by alterations in the normal pattern of maturation of the mucin in globet cells (Torres et al., 1999).

Growth factors regulate cell proliferation and also mediate processes such as extracellular matrix formation, cell migration and differentiation, immune regulation, and tissue remodelling (Beck and Podolsky, 1999). The importance of EGF in the process of epithelial repair has been reviewed recently (Howarth and Shoubridge, 2001) and is supported by results of recent studies in mice with a defective

epidermal growth factor, where increased susceptibility to experimentally induced colitis was observed, demonstrating the pivotal role of EGF receptor ligands such as EGF in protecting the integrity of the colon mucosa against mucosal injury (Egger et al., 2000). In another study, neonatal mice lacking EGF receptor ligands displayed more severe lesions in response to cysteamine treatment compared with their wild-type counterparts (Troyer et al., 2001).

EGF regulates epithelium integrity by binding to the EGF receptor, a protein tyrosine kinase that mediates signal transduction in a variety of cells. In this experimental setting, expression of the EGF receptor in normal mucosa was shown in epithelial cells along the upper half of the crypts as well as the luminal surface. These data concerning the location of this receptor are in agreement with the observations of Montaner et al. (1999) and Habel et al. (2002) who used immunohistochemistry techniques. After 14 days of induction of colitis, EGF receptor was not detected in the colon mucosa in TNBS-induced injured regions. Hoffmann et al. (2000) reported that inflammation leads to a significant increase in the EGF receptor in the early phases of colitis, supporting the hypothesis that EGF and related proteins and their common receptor play a pivotal role in mucosal defence and repair. In chronic colitis, the decreased EGF receptor immunoreactivity seems to be related, not only to the widespread epithelial necrosis and epithelial cell loss, but also to the absence of re-epithelialization. With restoration of mucosal integrity after treatment with dosmalfate, EGF receptor expression was again predominantly found in cells at the surface epithelium. One possible mechanism of dosmalfate-mediated improvement in colitis could involve EGF receptor ligands; nevertheless, this is a matter for further studies.

Infiltration of leukocytes into the mucosa has been suggested to contribute significantly to the tissue necrosis and mucosal dysfunction associated with colitis (Guo et al., 1999) as they represent a major source of reactive oxygen radicals in the inflamed colon mucosa (Grisham, 1994; Cuzzocrea et al., 2001). Quantitatively, the main free radical in tissues is superoxide anion (O_2^-), which is converted to the secondary oxidant H_2O_2 by SOD. The O_2^- can be produced by both endothelial cells, through XO and activated neutrophils through NADPH oxidase, which reduces molecular oxygen to the O_2^- radical, and through the enzyme myeloperoxidase. This enzyme catalyzes the formation of such potent cytotoxic oxidants as hypochlorous acid (HOCl) from H_2O_2 and chloride ions and *N*-chloramines. In addition, neutrophils can also release proteases, lactoferrin and lipid mediators that can contribute to colon injury. In our experiments, the level of myeloperoxidase activity was significantly increased only in TNBS control animals killed 48 h after TNBS enema, indicating that neutrophil-derived free radicals were involved in the pathogenesis of TNBS-induced colon injury. However, colons from animals treated with dosmalfate and killed 48 h after TNBS-enema had myeloperoxidase values significantly lower than those from animals who received TNBS alone. These data were also consistent with the histological findings that colon histology revealed resolution of the acute inflammatory response, suggesting an anti-inflammatory effect of this drug. In this context, previous reports have shown the anti-inflammatory action of certain flavonoids (Manthey, 2000; Bito et al., 2002), diosmin in particular, has been shown to inhibit leukocyte adhesion to the endothelial wall and subsequent migration of these cells into the interstitium (Friesenecker et al.,1994).

Of interest was our observation that the severity of the acute damage induced by TNBS was also associated with increased TNF-α levels. Our results are in line with previous reports indicating that up-regulation of TNF-α production was correlated with the development of colitis (Cuzzocrea et al., 2001; Ribbons et al., 1997). TNF-α is a cytokine with marked chemotactic activity and, in association with interleukin-1β, induces enzyme activities (nitric oxide synthase, phospholipase A_2, cyclo-oxygenases, proteases) that stimulate adhesion molecules and the production of other inflammatory cytokines contributing to the pathogenesis of colitis (Schreiber et al., 1999). In the present study, the increase in TNF-α levels in the colon was reduced by dosmalfate. A novel therapeutic approach is to down-regulate the expression or action of these cytokines, using antibodies or agents which suppress their pro-inflammatory action. In this context, promising results have been obtained with anti-TNF-α antibodies (Murthy et al., 1999) or TNF-α synthesis inhibitors in acute and chronic lesions in animal models (Videla et al., 1998; Bobin-Dubigeon et al., 2001).

In conclusion, our results demonstrate that dosmalfate is protective in acute and chronic experimental colitis. The acute anti-inflammatory effects seem to be related to impairment of neutrophil function and absence of up-regulation of TNF-α production in intestinal mucosa. The chronic beneficial effect of the flavonoid derivative, however, could not be related with a reduction of the inflammatory parameters but could apparently be associated with an increase in EGF receptor expression, improving epithelial re-growth and accelerating the healing, reparative process of colon injury. Our findings suggest that dosmalfate shows an excellent potential for therapy in the gastrointestinal area.

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