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Effects of the non-peptidyl low molecular weight radical scavenger IAC in DNBS-induced colitis in rats

Valentina Vasina [a], Massimiliano Broccoli [b], Maria Grazia Ursino [a], Simona Fiò Bellot [c], Antonio Soleti [c], Moreno Paolini [a], Fabrizio De Ponti [a,*]

[a] Department of Pharmacology, Alma Mater Studiorum, University of Bologna, Bologna, Italy
[b] Faculty of Pharmacy, Alma Mater Studiorum, University of Bologna, Bologna, Italy
[c] Medestea Research, Torino, Italy

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ABSTRACT

Intestinal inflammation is accompanied by excessive production of reactive oxygen and nitrogen radical species because of the massive infiltration of polymorphonuclear and mononuclear leukocytes. Antioxidant compounds seem to protect against experimental colitis. Here we investigated the effects of the innovative non-peptidyl, low molecular weight radical scavenger bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl) decandioate (IAC), which is highly reactive with most oxygen, nitrogen and carbon centred radicals and is easily distributed in cell membranes and intra-extra cellular compartments, in the DNBS model of colitis. Colitis was induced in male SD rats by intrarectal administration of DNBS (15 mg/rat). IAC (30 mg/kg b.w., hydrophilic or lipophilic form) was administered daily (orally or i.p.) starting from the day before the induction of colitis for 7 days ($n=6$–8 per group). Colonic damage was assessed by means of macroscopic and histological scores, myeloperoxidase activity (MPO) and TNF-α tissue levels. Colitis impaired body weight gain and markedly increased all inflammatory parameters. IAC significantly counteracted the reduction in body weight gain, decreased colonic damage and inflammation and TNF-α levels in DNBS-colitis. The antioxidant IAC significantly ameliorates experimental colitis in rats. This strengthens the notion that antioxidant compounds may have therapeutic potential in inflammatory bowel disease.

1. Introduction

Inflammatory bowel disease, including Crohn's disease and ulcerative colitis, are chronic inflammatory disorders of unknown origin: multiple factors, including genetic and environmental factors, probably contribute to inflammatory bowel disease. Intestinal inflammation is accompanied by excessive production of reactive oxygen and nitrogen radical species, such as superoxide ($O\cdot_2^-$), nitric oxide ($NO\cdot$), peroxynitrite ($ONOO\cdot^-$) and hydroxyl radicals ($\cdot OH$) (Grisham, 1994; Grisham and Granger, 1988; Middleton et al., 1993; Rachmilewitz et al., 1993) because of the massive infiltration of polymorphonuclear and mononuclear leukocytes which may produce large amounts of free radicals (Kitahora et al., 1988; Kruidenier et al., 2003a; Mahida et al., 1989; Verspaget and Beeken, 1985). Large numbers of peripheral neutrophils (Crama-Bohbouth et al., 1988) producing oxygen-derived free radicals (Verspaget et al., 1988), migrate into the intestinal wall of inflammatory bowel disease patients. In addition, nitric oxide ($NO\cdot$) overproduction, due to the expression of the inducible isoform of $NO\cdot$ synthase (iNOS), plays an important role in several animal models of inflammation (Cuzzocrea et al., 1998). Inflammatory bowel disease patients show high levels of nitrite (metabolite of $NO\cdot$ in water) and increased iNOS activity(Ikeda et al., 1997; Middleton et al., 1993): inhibition of iNOS activity exerts beneficial effects in animal models of experimental colitis (Aiko and Grisham, 1995; Mourelle et al., 1996). NO-induced damage is believed to be mediated, at least in part, by peroxynitrite ($ONOO\cdot^-$), a highly reactive oxidant produced by the combination of $O\cdot_2^-$ and $NO\cdot$ at rates approaching the diffusion limit (Aiko and Grisham, 1995; Ischiropoulos et al., 1992). $ONOO\cdot^-$ can induce cytotoxicity by initiating lipid peroxidation, inactivating various enzymes (mitochondrial respiratory enzymes and membrane pumps) (Beckman, J.S., 1996) and by depleting glutathione (Phelps et al., 1995). Peroxynitrite can also cause DNA damage (Inoue and Kawanishi, 1995) resulting in the activation of the nuclear enzyme poly (ADP-ribose) synthetase and poly (ADP-ribose) synthase-driven cell death (Szabo and Dawson, 1998). Furthermore, $ONOO\cdot^-$ inhibits the activity of the endogenous superoxide dismutase enzymes, contributing to an increased $O\cdot_2^-$ production (Yamakura et al., 1998).

Recent studies indicate that antioxidant compounds, which can reduce the production or the effects of reactive oxygen species, such as melatonin (Cuzzocrea et al., 2001b), natural antioxidants (Korkina

* Corresponding author. Department of Pharmacology, Via Irnerio, 48, I-40126 Bologna BO, Italy. Tel.: +39 051 2091805; fax: +39 051 248862.
E-mail address: fabrizio.deponti@unibo.it (F. De Ponti).

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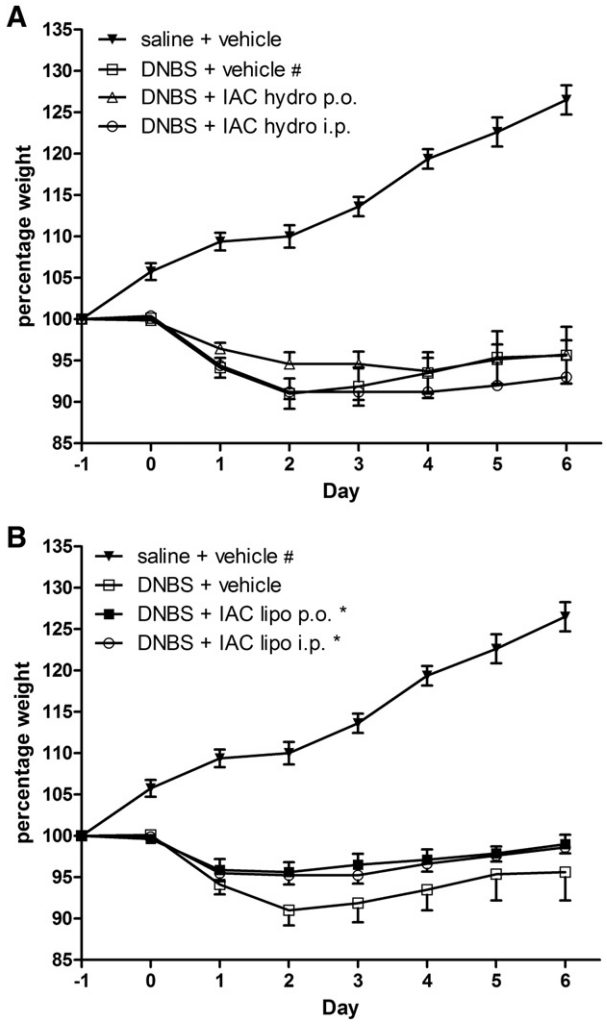

Fig. 1. Panel A: Effect of IAC 30 mg/kg b.w. (hydrophilic form) on body weight gain (%) in the different experimental groups; Panel B: Effect of IAC 30 mg/kg b.w. (lipophilic form) on body weight gain (%) in the different experimental groups. Data are expressed as means ± S.E.M.; $n = 5–8$ rats per group. # $P<0.01$ vs. saline; * $P<0.05$ vs. DNBS; IAC hydro = hydrophilic form of IAC; IAC lipo = lipophilic form of IAC. Group saline (+vehicle) and DNBS (+vehicle) in panels A and B represent the same experiments. p.o. = *per os*; i.p. = intraperitoneal administration.

et al., 2003; Oz et al., 2005) glutathione (GSH) precursors or compounds involved in GSH synthesis (Oz et al., 2005) and peroxynitrite decomposition catalysts (Salvemini et al., 1999), protect against experimental intestinal inflammation, including DNBS-induced colitis. Tempol, a well-known low molecular weight radical scavenger, exerted beneficial effects in DNBS-induced colitis (Cuzzocrea et al., 2000) as well as in gastric mucosal damage induced by ischemia/reperfusion (Abdallah et al., 2009). However, cyclic nitroxides such as tempol are very persistent in water or organic solution, but when used *in vivo* or in a biological sample they are reduced to the parent hydroxylamine by several enzymatic processes mainly involving ascorbate or glutathione (Miura et al., 1992).

In the present work we investigated the effects of the non-peptidyl, low molecular weight radical scavenger bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)decandioate (IAC) in the DNBS model of colitis, relying on its high reactivity with most oxygen, nitrogen and carbon centred radicals. Due to its peculiar physico-chemical properties, IAC is more stable in physiological solutions, its antioxidant capability is stronger than those of aforementioned cyclic nitroxides (Valgimigli et al., 2000) and, unlike peptidic antioxidants, is slightly affected by the gastric environment (low pH, peptidases) (Paolini et al., 1996). Moreover, due to its log *P* (Valgimigli et al., 2001), it is easily distributed through cell membranes and intra-extra cellular compartments, thus it can directly react with oxidant molecules within the cell, where free radicals are produced.

In the present work, we tested two different forms of IAC, the hydrophilic (protonated, as hydrochloride) and the lipophilic form (for more details on chemical structures see (Valgimigli et al., 2000)). The IAC protonated (hydrophilic) form is completely water-soluble and distributes into the extracellular compartments, but it is also in equilibrium with the free form, which is highly lipophilic (the calculated logP is 4.01) (Crippen, 1987) and readily crosses the cellular membrane, distributing into any compartment. The IAC unprotonated (lipophilic) form permeates the cellular membrane even more easily and the equilibrium between protonated and unprotonated forms is affected by the pH of the compartment, without any loss of activity.

Usually, the hydrophilicity/lipophilicity balance of a radical trap physically confines it to just one compartment and enables it to specifically react only with those radicals produced therein. On the contrary, due to its peculiar physico-chemical properties, IAC readily diffuses through the cellular membrane and can reach virtually any compartment where the production of free radicals occurs. This represents an advantage, because it can directly react with oxidant molecules within the cell in many different compartments.

2. Materials and methods

2.1. Animals

Male Sprague Dawley rats (180–200 g body weight; Harlan Italy, S. Pietro al Natisone, Udine, Italy) were used in this study. Animals were housed in a controlled environment and had free access to food and water throughout the study. Before starting any experimental procedure, in order to minimize the effects of stress *per se* on the parameters to be measured, animals were weighed and gently manipulated in the laboratory environment for 30 min everyday for at least 1 week. All experiments were carried out according to the guidelines set forth by EEC Directive 86/609 on the care and use of experimental animals. The protocol for induction of colitis was reviewed by the Institutional Committee on the care and use of experimental animals of the University of Bologna and was authorized by the Italian Ministry of Health. A persistently hunched posture and laboured respiration, a markedly erected coat and a weight loss of more than 20% were considered as humane end-points to euthanize the animals.

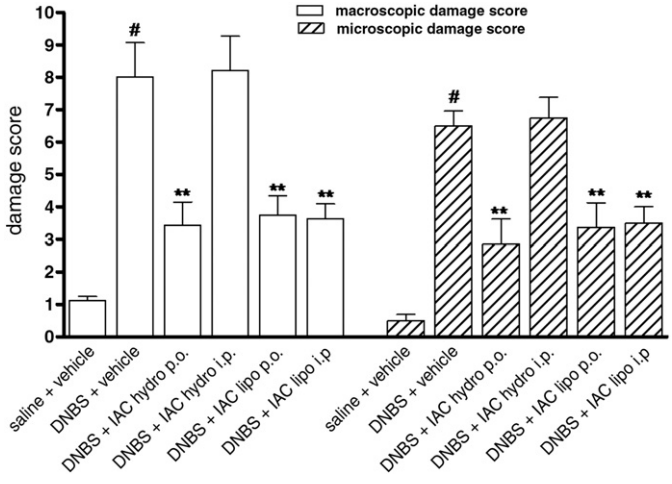

Fig. 2. Effect of IAC 30 mg/kg b.w. (hydrophilic or lipophilic form) on macroscopic and microscopic damage score. Treatment with hydrophilic IAC p.o. significantly decreased colonic damage and inflammation, while i.p. administration failed to protect the colon from DNBS-induced damage. Seven-day treatment with lipophilic IAC p.o. and i.p. significantly reduced intestinal damage induced by inflammation. Data are expressed as means ± S.E.M.; $n = 5–8$ rats per group # $P<0.01$ vs. saline; ** $P<0.01$ vs. DNBS.

2.2. Induction of colitis

Colitis was induced using a previously described method (Elson et al., 1995) with slight modifications (Vasina et al., 2008). Briefly, rats were lightly anaesthetised by inhalation of chloroform (Sigma-Aldrich, Italy); 2,4-dinitrobenzene sulfonic acid (DNBS, ICN Biomedicals, Aurora, OH, USA, 15 mg/rat) dissolved in 0.25 ml of 50% ethanol were instilled into the distal colon of each animal using a rubber catheter, so that the tip was about 8 cm proximal to the anus. Ethanol was used as an enhancer of DNBS-induced damage but, *per se*, had no effect on the parameters to be measured (Blandizzi et al., 2003). Control rats received 0.25 ml 0.9% NaCl alone intrarectally. DNBS and control rats were kept in separated cages during the study.

2.3. Experimental design

On the basis of previous experiments, in which we studied the dose-response of DNBS in inducing colitis and the time-course of inflammation, the dose of 15 mg of DNBS/rat was used because it evoked adequate inflammation without causing unnecessary distress and suffering to the animals, and day 6, when all parameters of inflammation peaked, was selected to sacrifice the animals in all remaining experiments (Vasina et al., 2008).

We studied groups of rats with and without colitis ($n = 6$–8 per group), which were treated with the radical scavenger bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)decandioate (IAC) synthesised in our laboratories (Valgimigli et al., 2001), starting the day before the induction of colitis for 7 days. IAC (30 mg/kg b.w.), both the hydrophilic (M.W. = 548) and lipophilic (M.W. = 512) form, was administered once daily at the same time (orally or i.p.) as water solution or suspension. Animals were sacrificed at the end of the treatment.

The dose of 30 mg/kg b.w. was selected on the basis of previous studies to achieve maximum antioxidant activity (Novelli et al., 2007; Zavatti et al., 2009).

2.4. Tissue collection

Rats were sacrificed on day 6 after the induction of colitis, which corresponds to the time of maximal inflammatory injury (Vasina et al., 2008; Wallace et al., 1995). The distal colon was removed, opened longitudinally and washed with phosphate buffered saline (PBS) to remove luminal contents. Whole-wall samples from distal-colon, taken from a region immediately adjacent to the gross macroscopic

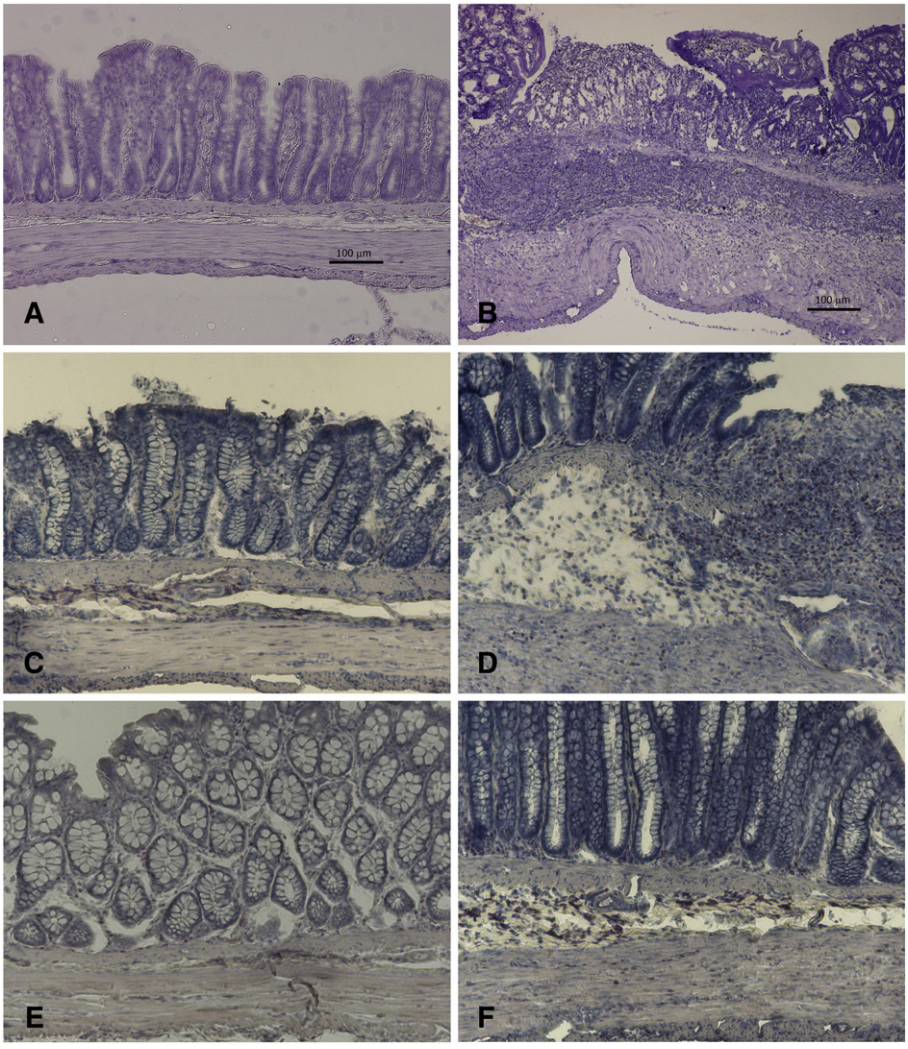

Fig. 3. Representative example of cross sections of distal colon from a non-inflamed rat (A) (intrarectal saline + IAC vehicle orally) and (B) from an inflamed rat (intrarectal DNBS 15 mg + IAC vehicle orally; H&E, scale bar: 100 µm). Note the dramatic loss of mucosal architecture with goblet cell depletion and the massive granulocyte infiltrate extending throughout the mucosa and submucosa, also involving the *muscolaris propria* which appears thickened. In C–F are represented cross sections of distal colon from an inflamed rat treated with hydrophilic (C–D) and lipophilic (E–F) IAC 30 mg/kg orally (C–E) and intraperitoneally (D–F). Lipophilic (*p.o.* and i.p.) and hydrophilic (*p.o.*) IAC 30 mg/kg decreased the microscopic damage produced by DNBS, reducing mucosal architecture loss, ulceration and muscle thickening (panels C, E and F). Hydrophilic IAC i.p. failed to protect the colon from the damage induced by DNBS (panel D).

damage, were pinned flat on wax, fixed in cold neutral 4% formalin and then placed in 25% sucrose in PBS at 4 °C for cryoprotection and embedded in Optimal Cutting Temperature tissue freezing medium. Seven-micron-thick sections of colon were cut, serially mounted on glasses and processed for routine haematoxylin-eosin (H&E) staining, naphthol as-d chloroacetate esterase assay or immunohistochemistry. Specimens of colonic tissue were also removed from the area of gross injury, snap frozen in liquid nitrogen and stored at −80 °C until subsequent assays.

2.5. Assessment of colitis

Colonic damage was assessed macroscopically and histologically using a method previously described (Sturiale et al., 1999). Briefly, the macroscopic criteria were based on the following: presence of adhesions between the colon and other intra-abdominal organs, consistency of colonic faecal material (as an indirect marker of diarrhea), thickening of the colonic wall, presence and extension of hyperaemia and macroscopic mucosal ulceration (expressed in mm^2). Microscopic criteria for damage and inflammation were assessed by light microscopy on H&E stained histological sections obtained from whole gut specimens taken from a region of the colon immediately adjacent to the gross macroscopic damage, fixed in 4% paraformaldehyde for 2 h, maintained in 25% sucrose for two days and finally processed in O.C.T. (Optimal Cutting Temperature) tissue freezing medium. Histological criteria included: degree of mucosal architecture changes, cellular infiltration, external muscle thickening, presence of crypt abscess and goblet cell depletion.

2.6. Myeloperoxidase (MPO) assay

A tissue sample (50 mg) was removed from the area of the gross injury, snap frozen in liquid nitrogen and assayed within seven days using a previously described method (Boughton-Smith et al., 1988). MPO is a granule-associated enzyme present in neutrophils and other cells of myeloid origin, and widely used as a marker of intestinal inflammation. Colonic tissue was weighed and placed in a plastic tube with hexadecyl-trimethylammonium bromide buffer (1 ml per 50 mg of tissue), homogenized and centrifuged for 10 min at 6000 g at 4 °C. Seven µl of the supernatant were then collected and assayed to assess MPO activity. MPO was expressed in units per milligram of tissue, where 1 unit corresponds to the activity required to degrade 1 µmol of hydrogen peroxide in 1 min at room temperature.

2.7. TNF-α tissue levels

Tumor necrosis factor-α (TNF-α) concentration was assessed in colonic tissues removed from the area immediately adjacent to the gross injury, snap frozen and stored at −80 °C. On thawing, samples were weighed, homogenized in a solution of protease inhibitors (pepstatin, aprotinin, leupeptin, 1 µg/ml, Sigma-Aldrich, St. Louis, MO, USA) and centrifuged at 6000 g at 4 °C. A 100 µl aliquot of supernatant was then added to the enzyme immunoassay 96-wells plate in duplicate and assayed according to the manufacturer's protocols (Rat TNF-alfa ELISA, Bender MedSystems). Absorbance was read using a spectrophotometer at 450 nm. Assessment of protein content in colonic samples was performed by using the Quick Start™ Bradford Protein Assay (BIO-RAD, Hercules, CA, USA); the protein-dye complex absorbance was read using a spectrophotometer at 595 nm. TNF-α was expressed as pg/mg of protein.

2.8. TBARS assay

Malondialdehyde (MDA) is a naturally occurring product of lipid peroxidation. Lipid peroxidation is a well-established mechanism of cell injury and is widely used as an indicator of oxidative stress in cells

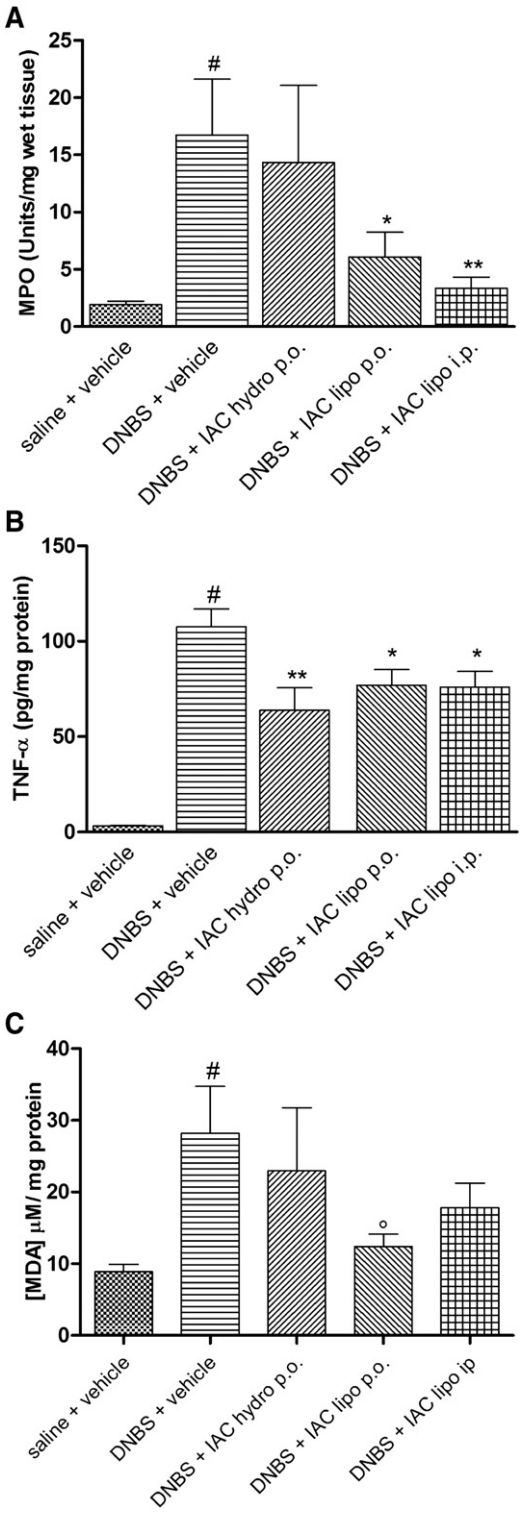

Fig. 4. Effect of IAC 30 mg/kg b.w. (hydrophilic or lipophilic form) on MPO activity (panel A), TNF-α tissue levels (panel B) and MDA activity (panel C). Colitis was characterized by a 9-fold increase in MPO activity (panel A), a more than 32-fold increase in TNF-α tissue levels (panel B) and a more than 3-fold increase in lipid peroxidation, as measured by the formation of MDA (panel C), compared with non-inflamed rats. Seven-day treatment with lipophilic IAC p.o. and i.p. induced a down-regulation in myeloperoxidase activity, TNF-a tissue levels and lipid peroxidation observed in experimental colitis. Treatment with hydrophilic IAC p.o. significantly reduced TNF-α tissue levels and with a trend to a decreased MDA activity, although statistical significance was not achieved. Data are expressed as means ± S.E.M.; $n = 5–8$ rats per group. # $P<0.01$ vs. saline; * $P<0.05$ vs. DNBS; ** $P<0.01$ vs. DNBS;° $P<0.05$ vs. DNBS.

and tissues (Armstrong and Browne, 1994). Colonic samples (25 mg) removed from the area of the gross injury, snap frozen in liquid nitrogen, were homogenized on thawing in 250 µl of RIPA buffer containing protease inhibitors (pepstatin, aprotinin, leupeptin, 1 µg/ml, Sigma-Aldrich, St. Louis, MO, USA) and centrifuged at 1600 g at 4 °C for 10 min. Tissue homogenates were then assayed according to the manufacturer's protocols (TBARS Assay Kit, Cayman Chemical, Ann Arbor, MI, USA). Absorbance was read using a spectrophotometer at 540 nm.

Assessment of protein content in colonic samples was performed by using the Quick Start™ Bradford Protein Assay (BIO-RAD, Hercules, CA, USA); the protein-dye complex absorbance was read using a spectrophotometer at 595 nm. MDA concentration was expressed as µM/mg of protein.

2.9. Naphthol as-d chloroacetate esterase assay

In order to identify neutrophil infiltration within the tissue, we used a commercially available kit (naphthol as-d chloroacetate esterase; 91C, Sigma-Aldrich). This enzyme is considered specific for cells of granulocytic lineage and its sites of activity show bright red granulation. Briefly, tissue sections were fixed in citrate-acetone-formaldehyde solution and assayed according to the manufacturer's protocols. Specimens were mounted with mounting media (glycerol-PBS, 9:1), examined by light microscope (ECLIPSE 90i, Nikon Instruments, Calenzano, Italy) and representative photomicrographs were taken by DS-5M digital camera (Nikon Instruments, Calenzano, Italy).

2.10. Immunofluorescence

Anti-intercellular adhesion molecule 1 (ICAM-1) mouse monoclonal antibody (MAB 1391Z, 1:100; Chemicon International, Temecula, CA, USA) was employed to detect intercellular adhesion molecule. Briefly, cryostat sections were washed three times with PBS and then incubated with 5% normal donkey serum in PBS containing 0.1% Triton® X-100 to block non-specific binding. Sections were incubated with anti-ICAM-1 primary antibody in a humid chamber at 4 °C overnight, rinsed with PBS, and incubated at room temperature with Alexa Fluor® 488 donkey anti-mouse IgG (1:600, Molecular Probes, Eugene, OR, USA) for 2 h. Specimens were mounted with Mowiol® 4-88 reagent (Calbiochem, San Diego, CA, USA) and examined by fluorescence microscope (ECLIPSE 90i, Nikon Instruments, Calenzano, Italy) and representative photomicrographs were taken by DS-5M digital camera (Nikon Instruments, Calenzano, Italy). To verify the specificity of immunohistochemical detections, omission of the primary antibody was performed.

2.11. Statistical analysis

Results are expressed as means ± standard error mean (S.E.M.). Statistical analysis was performed using analysis of variance (one-way or two-way, as appropriate, with the Bonferroni's correction for multiple comparisons). A P value <0.05 was considered significant. N refers to the number of animals used for each experiment ($n = 8–16$). Calculations were performed using GraphPad Prism™ (version 4.0, GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Assessment of colitis

DNBS significantly reduced body weight gain (Fig. 1) and increased inflammatory damage, as indicated by macroscopic and microscopic damage score (Figs. 2 and 3B) and MPO activity (Fig. 4A) with respect to non-inflamed controls.

At day 6 after DNBS administration, the distal colon was thickened and ulcerated with evident areas of transmural inflammation. Adhesions were often present and the bowel was occasionally dilated

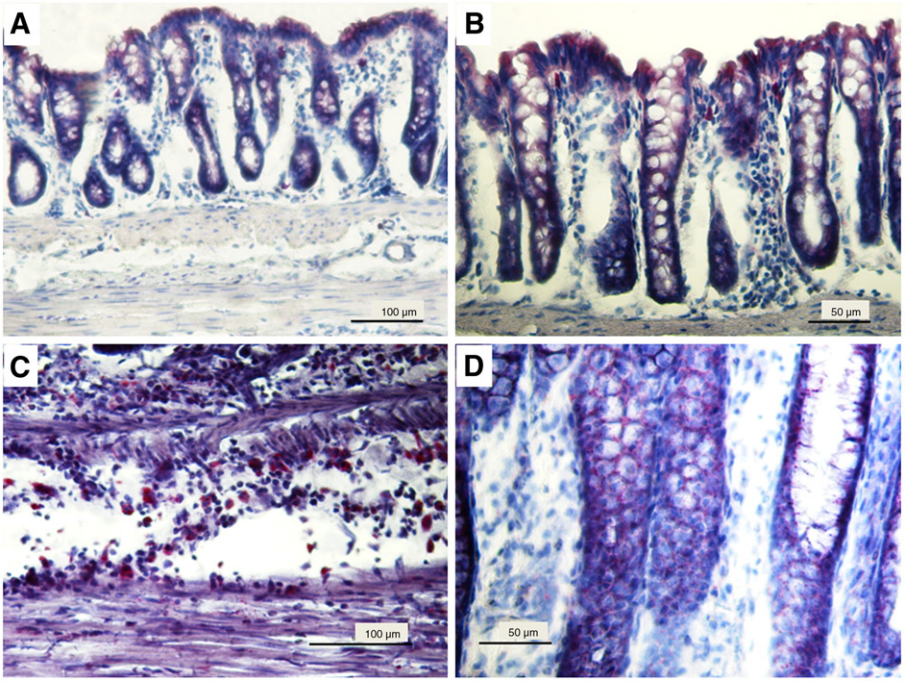

Fig. 5. Naphthol as-d chloroacetate esterase positivity (red) in cross section of distal colon from a non-inflamed rat (panels A–B; scale bar: 100 and 50 µm respectively) and from an inflamed rat (C–D); scale bar: 100 and 50 µm respectively). (A–B) Tissue sections obtained from non-inflamed rats showed occasional red staining, indicating a low presence of neutrophils within the bowel wall in physiological conditions. (C–D) Compared to non-inflamed rats, tissues from rats with colitis showed a massive neutrophil infiltration (panel C) extending throughout the mucosa (in panel D, note the scattered degranulation within the crypts D) and submucosa, also involving the *muscularis propria*.

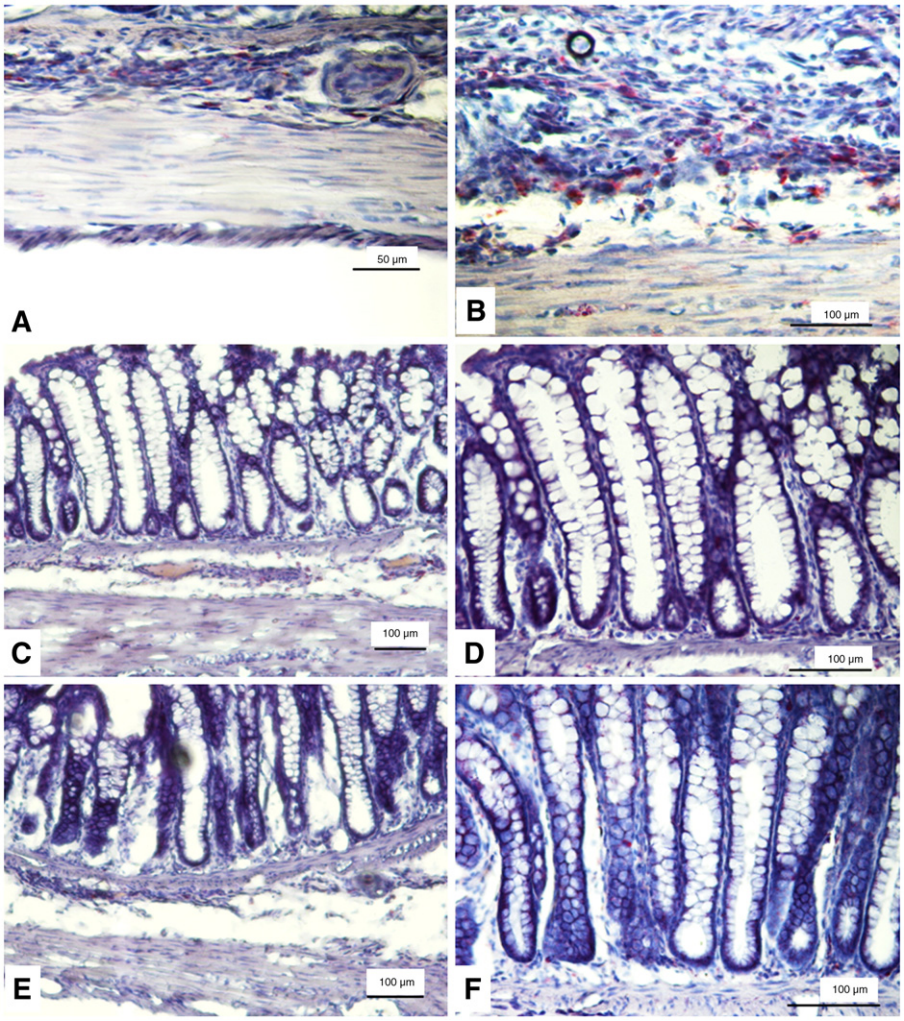

Fig. 6. Naphthol as-d chloroacetate esterase positivity (red) in cross sections of distal colon from a rat with colitis treated with IAC 30 mg/kg b.w. (hydrophilic form p.o., panels A–B; lipophilic form, p.o. or i.p., panels C–F). (A–B) Treatment with IAC 30 mg/kg hydrophilic form p.o. significantly improved architecture structure loss and reduced smooth muscle thickening, as already shown in Fig. 3; however, it was unable to completely suppress neutrophil infiltration, especially in the mucosa and submucosa (scale bar: 50–100 µm). (C–F) Treatment with lipophilic IAC both p.o. (panels C–D) and i.p. (panels E–F) almost completely suppressed neutrophil infiltration within the colonic wall (scale bar = 100 µm).

and there was a greater than 7-fold increase in macroscopic damage score (Fig. 2). Colitis was characterized by a massive granulocyte infiltrate extending throughout the mucosa and submucosa, often involving the muscularis propria, which invariably appeared thickened (Fig. 3B) and by a 13-fold increase in microscopic damage score over the non-inflamed control animals (Fig. 2). DNBS treatment induced a 9-fold increase in MPO activity (Fig. 4A), a more than 32-fold increase in TNF-α tissue levels (Fig. 4B) and a more than 3-fold increase in lipid peroxidation, as measured by the formation of MDA (Fig. 4C), compared with non-inflamed rats. In inflamed tissues (DNBS-treated rats), we observed a massive neutrophil infiltration (red cells, Fig. 5, panel C) with respect to non-inflamed controls (Fig. 5, panels A-B), extending throughout the mucosa (note the scattered degranulation within the crypts in Fig. 5, panel D) and submucosa, also involving the muscularis propria (Fig. 5, panels C-D).

3.2. Effect of IAC on DNBS-induced colitis

Treatment with hydrophilic IAC 30 mg/kg b.w. orally significantly decreased colonic damage and inflammation (Fig. 2), partially reduced the inflammatory cell infiltrate (Fig. 3C) and decreased TNF-a tissue levels in DNBS-colitis (Fig. 4B), but was unable to counteract the increased MPO and MDA levels induced by DNBS (Fig. 4A and C) and failed to completely suppress neutrophil infiltration, especially in the mucosa and submucosa (Fig. 6, panels A–B); i.p. administration failed to protect the colon from DNBS-induced damage (Figs. 2, 3D and 4).

Seven-day treatment with lipophilic IAC (30 mg/kg b.w.) orally and i.p. significantly counteracted the reduction in body weight gain (Fig. 1), reduced intestinal damage induced by inflammation (Figs. 2 and 3E-F), induced a down-regulation in myeloperoxidase activity, TNF-α tissue levels and lipid peroxidation (Fig. 4, panels A-C) and also minimized DNBS-induced neutrophil infiltration within the colonic wall (Fig. 6C–F).

3.3. Immunofluorescence

In normal animals, tissue sections treated with anti-ICAM-1 antibody showed specific staining within the mucosa and along blood vessels, indicating that ICAM-1 is constitutively expressed in endothelial cells (Fig. 7A). DNBS-induced colitis was associated with increased ICAM-1 expression in blood vessels of the lamina propria and the submucosa; ICAM-1 immunoreactivity was also observed in colonic epithelial cells and infiltrated inflammatory cells (Fig. 7B).

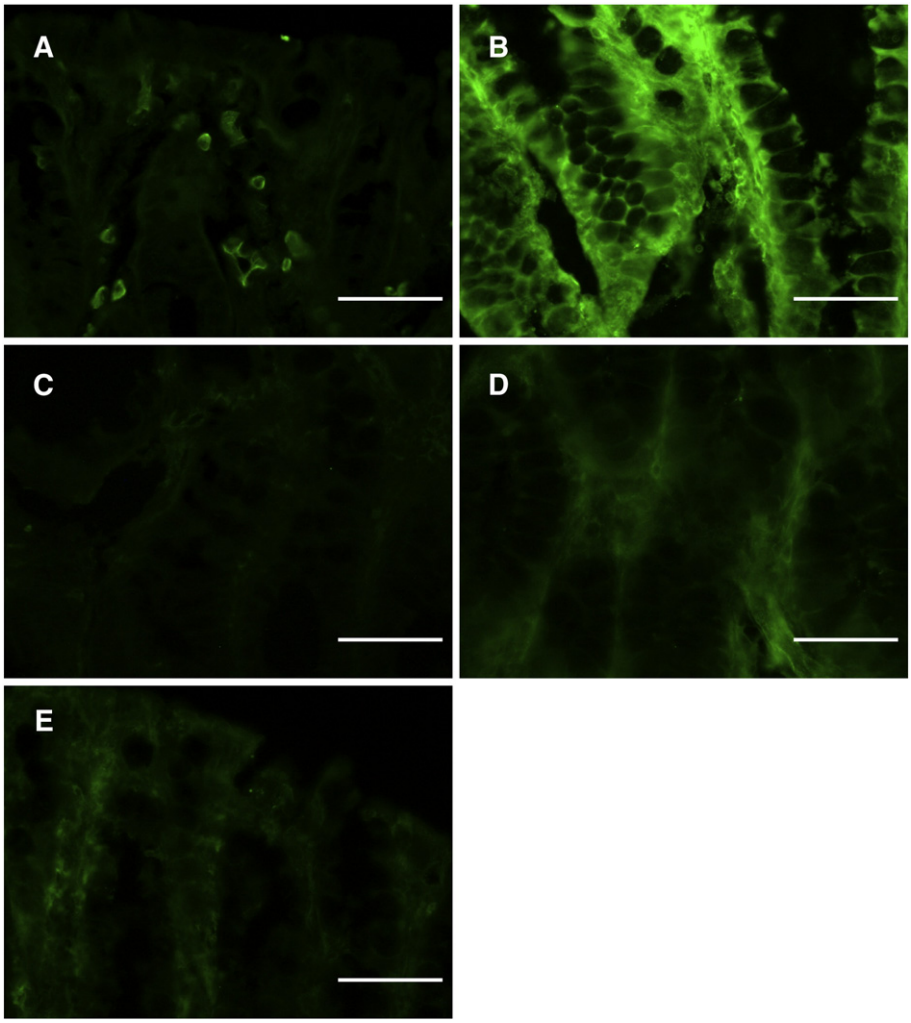

Fig. 7. ICAM-1 immunoreactivity in cross sections of distal colon from a normal rat (A), a rat with colitis (B) and a rat with colitis treated with IAC 30 mg/kg b.w. (hydrophilic form, orally or lipophilic form, orally or i.p., C–E). (A) Tissue sections obtained from normal rats showed a specific immunoreactivity, indicating that ICAM-1 is constitutively expressed in this tissue. (B) Compared to non-inflamed rats, tissues from rats with colitis showed an increased ICAM-1 immunoreactivity on blood vessels as well as in inflammatory cells infiltrating the mucosa, the submucosa and throughout the muscular layer. (C–E) Treatment with IAC 30 mg/kg hydrophilic form orally (C) as well as lipophilic form (D and E, orally or i.p. respectively) markedly reduced ICAM-1 positive staining. Scale bar = 50 μm.

Tissue sections from rats treated with hydrophilic (30 mg/kg b.w., orally) and lipophilic (30 mg/kg b.w., orally and i.p.) IAC did not reveal any upregulation of the constitutive ICAM-1 (Fig. 7C–D).

4. Discussion

Oxidative damage may represent a pathogenic factor in inflammatory bowel disease because intestinal inflammation is accompanied by increased production of reactive oxygen and nitrogen species and an imbalanced antioxidant response (Grisham, 1994; Kruidenier et al., 2003b; Kruidenier et al., 2003a). Indeed, free radical production is a key mechanism for the appearance of colonic inflammation in experimental models of colitis (Cuzzocrea et al., 2000; Keshavarzian et al., 1990; Segui et al., 2005).

This study shows that DNBS caused a substantial degree of inflammation and tissue injury in the rat colon, which was associated with polymorphonuclear colonic infiltrate (histology and myeloperoxidase activity) and an increase in TNF-α tissue levels as well as lipid peroxidation. Modulation of oxidative stress by the radical scavenger IAC ameliorated DNBS-induced colitis in rats. Indeed, treatment with hydrophilic (30 mg/kg b.w., orally) and lipophilic IAC (30 mg/kg b.w., orally and i.p.) significantly decreased colonic damage and inflammation and TNF-α tissue levels. Treatment with lipophilic IAC (30 mg/kg b.w., orally and i.p.) was also able to decrease neutrophil infiltration, MPO activity and lipid peroxidation.

IAC is a low molecular weight radical scavenger which reacts rapidly with most carbon-, nitrogen- and oxygen-centered radicals of biological interest — including peroxyl, superoxide, and peroxynitrite radicals (Valgimigli et al., 2001). The physico-chemical properties of IAC affecting its partition properties in cell membranes and intra-extra-cellular compartments deserve some comments: the protonated form administered to a biological system is completely water-soluble and distributes in the extra-cellular compartments, but it is also in equilibrium with the free form, which is highly lipophilic (the calculated logP is 4.01) and readily crosses the cell membrane to allow distribution to any compartment where the production of free radicals occurs (Valgimigli et al., 2001). Recently, IAC was used in a nonobese mouse diabetes model (Novelli et al., 2007) and in a rat model of transient middle cerebral artery occlusion (tMCAO), with positive results in both models. Notably, in our hands, hydrophilic IAC failed to protect the rat colon from DNBS-induced colitis when administered intraperitoneally: this can tentatively be ascribed to the limited local availability of the hydrophilic form at the site of inflammation. Likewise, treatment with hydrophilic IAC p.o. significantly reduced morphological damage and smooth muscle thickening, but was unable to counteract lipid peroxidation and neutrophil infiltration,

as assessed by MDA, MPO activity and naphthol as-d chloroacetate esterase. We ascribe this different activity on inflammatory cell infiltration to the different ability of the two different forms of IAC (hydrophilic vs lipophilic) to distribute in cell membranes and intra-extra cellular compartments. Hydrophilic IAC, when administered p.o., may have a particular profile of absorption and distribution, leading to a lower concentration within areas of major damage.

There is substantial evidence that proinflammatory cytokines and adhesion molecules, including TNF-α and ICAM-1, play a key role in the inflammatory process (Podolsky, 2002); moreover, increased TNF-α tissue levels and ICAM-1 expression have been observed in inflammatory bowel disease patients as well as in experimental models of colitis (Inoue et al., 1999; Li et al., 2005). Functional and in vitro studies have shown that stimulation with cytokines, such as TNF-α, induced ICAM-1 and VCAM-1 up-regulation (D'Alessio et al., 1998) and that this phenomenon was oxidant dependent, since treatment with antioxidants inhibits expression of adhesion molecules (Sasaki et al., 2003; Sasaki et al., 2002).

In our study, DNBS treatment induced a marked increase in TNF-α colonic levels, which was counteracted by IAC, in both hydrophilic and lipophilic forms. The decreased cytokine production observed in our study could be ascribed to scavenging of reactive oxygen species derived from the xantine-oxydase system, vascular endothelial cells or neutrophils, as hypothesised by Isosaki et al. (Isozaki et al., 2006).

The vascular endothelium is a major target of oxidant stress, which increases vascular endothelial permeability and promotes leukocyte adhesion and neutrophil recruitment (Crama-Bohbouth et al., 1988; Lum and Roebuck, 2001). Previous *in vitro* studies suggested that during inflammation endothelial cell adhesion molecules are modulated by reactive oxygen species (Lum et al., 2001) and that treatment with antioxidants is able to significantly reduce adhesion molecules expression and leukocyte recruitment into inflamed tissues (Segui et al., 2004; Segui et al., 2005).

Recently, Cuzzocrea et al. demonstrated that treatment with antioxidants such as tempol (Cuzzocrea et al., 2000) and M40403, a superoxide dismutase mimetic (Cuzzocrea et al., 2001a), significantly reduced expression of ICAM-1 in experimental colitis, probably by limiting leukocyte recruitment. Indeed, several studies suggested that immuno-neutralization of adhesions molecules such as ICAM-1 and VCAM-1 (Sans et al., 1999) reduces leukocyte adhesion in colonic venules and that regulating the expression of endothelial adhesion molecules is important in vascular inflammatory processes during experimental colitis (Segui et al., 2004; Segui et al., 2005).

In conclusion, our data show that treatment with IAC reduced colonic polymorphonuclear infiltrate (histology and myeloperoxidase activity) in colonic tissue and was associated with a decrease in ICAM-1 up-regulation induced by inflammation. This strengthens the notion that antioxidant agents such as IAC may have a therapeutic potential in inflammatory bowel disease.

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