



Original Article

Antioxidant effect of mesalazine in the experimental colitis model induced by acetic acid



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ABSTRACT

Introduction: Inflammatory bowel disease (IBD) is characterized by a chronic inflammation of the gastrointestinal tract, without specific cause or pathogen.

Objective: The effect of mesalazine in a colitis model induced by acetic acid (AA) was evaluated.

Methods: We used 40 Wistar rats, ± 350 g, divided into 4 groups: control (CO); control+mesalazine (CO+M); colitis (CL) and colitis+M (CL+M) at 24 and 48 h of treatment. The animals received the substances by an intracolonic enema of AA 4% and treatment with mesalazine PO 20 mg/kg after colitis induction.

Results: Mesalazine reduced tissue damage in the gut, normalized sphincter anal pressure levels and decreased lipid peroxidation, metabolites of nitric oxide and iNOS and NF- κ B expression in the treated groups in both treatment time points (24 and 48 h), as well as the activity of antioxidant enzymes.

Conclusion: Mesalazine was effective in reducing tissue damage and oxidative and inflammatory damage, restored antioxidant capacity and increased anal sphincter pressure levels, possibly due to its antioxidant effect.

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Efeito antioxidante da mesalazina no modelo de colite experimental induzida por ácido acético

R E S U M O

Palavras-chave:

Estresse oxidativo
Inflamação
Óxido nítrico

Introdução: A doença inflamatória intestinal (DII) caracteriza-se por uma inflamação crônica do trato gastrointestinal sem uma causa ou patógeno específico.

Objetivo: Foi avaliado o efeito da mesalazina no modelo de colite induzida por ácido acético (AA).

Material e métodos: Foram utilizados 40 ratos wistar, ± 350 gramas, divididos em 4 grupos: Controle (CO); Controle + Mesalazina (CO + M); Colite (CL) e Colite + M (CL + M) nos tempos de 24 e 48 horas de tratamento. Os animais foram submetidos à administração intracolônica por enema com solução de AA a 4% e tratamento com mesalazina na dose oral de 20 mg/kg após a indução da colite.

Resultados: A mesalazina reduziu as lesões teciduais no intestino, normalizou os níveis de pressão anal esfinteriana, reduziu a lipoperoxidação, metabólitos do óxido nítrico e expressão da iNOS e do NF- κ B nos grupos tratados em ambos os tempos de tratamento (24 e 48 horas), bem como a atividade das enzimas antioxidantes.

Conclusão: A mesalazina demonstrou eficácia na redução das lesões teciduais, danos oxidativos e inflamatórios, restabeleceu a capacidade antioxidante e aumentou os níveis de pressão anal esfinteriana, possivelmente pelo seu efeito antioxidante.

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Introduction

Inflammatory bowel disease (IBD) is characterized by chronic inflammation of the gastrointestinal tract without a specific cause or pathogen and affects millions of people worldwide. In recent years, there has been an increase in the number of diagnosed cases of IBD in young people; this increase has been accompanied by significant progress in the investigation of its pathophysiology.¹⁻³

Unspecific ulcerative colitis (UUC) is included in this classification and is characterized by mucosal ulceration, a submucosal inflammatory infiltrate and eventually extra-intestinal manifestations.⁴⁻⁶

An uncontrolled inflammation in UUC is probably the result of the interaction between genetic predisposition, changes in the innate immune system and an exaggerated response of adaptive immunity. The inflammatory infiltrate that occurs in UUC possibly is due to a rupture of the colonic barrier followed by bacterial invasion; thus, pro-inflammatory cytokines are released, with the generation of free radicals and increased production of nitric oxide (NO).⁷⁻¹⁰ These factors can cause injury and tissue destruction.¹⁰⁻¹²

The enzyme nitric oxide synthase (NOS) is responsible for NO synthesis, through the conversion of L-arginine and oxygen into L-citrulline and NO. The generation of nitric oxide (NO) is critical for vascular integrity, bowel motility and relaxation of the anal sphincter muscle.^{7,8,13} The enzyme inducible nitric oxide synthase (iNOS) has a cytostatic effect on inhibiting enzymes containing iron and also by causing DNA fragmentation. The synthesis of iNOS is induced by lipopolysaccharides (LPS), free radicals or any other detectable change in the intra- or extracellular environment, activating the transcription of pro-inflammatory genes.¹⁴

Activation of nuclear factor kappa B (NF- κ B) is restricted to areas with inflammatory activity of mononuclear cells and epithelial cells in the lamina propria, an effect that was demonstrated in animal models of inflammatory bowel disease. When activated, NF- κ B induces transcription of pro-inflammatory genes, including interleukins (IL-1 β , 6, 8) and iNOS.⁷

Mesalazine or 5-aminosalicylic acid (5-ASA) has chemopreventive effects against colorectal cancer by increasing p53 protein gene expression through epigenetic mechanisms. The anticancer effects of mesalazine can also be mediated by its ability to remove molecules that cause oxidative damage to mucosa.¹⁵ Studies of Ikeda et al. suggest that mesalazine can reduce tumor cells, but not the proliferation of normal epithelial cells.¹⁶

Another mechanism of action of 5-ASA is related to its ability to interfere with the production of pro-inflammatory cytokines by inhibition of NF- κ B activity, inhibiting natural killer cells of lymphocytes and monocytes in the mucosa and reducing the production of free radicals.¹⁷

The pathophysiology of UUC is not yet fully understood. To date, few studies have evaluated the antioxidant effects of 5-ASA in experimental models of colitis. In view of these considerations, this study aims to evaluate the antioxidant action of mesalazine in reducing oxidative damage in an experimental model of colitis induced by acetic acid.

Material and methods

Animals

Procedures with animals are in accordance with the ethical principles defined by the Ethics Committee on Animal Use

– ULBRA, Canoas, RS, under the approval of this Committee (project 2015-22P), and with the guidelines of the European Union of Animal Experimentation 86/609 EEC.¹⁸

Forty male Wistar rats weighing approximately 300 g were used; the animals were divided into four groups according to the time of treatment (24 and 48 h) – control (CO) (n=5), control + mesalazine (CO + M) (n=5), colitis (CL) (n=5) and colitis + mesalazine (M + CL) (n=5). The animals were kept in a vivarium of the Universidade Luterana do Brasil, in plastic boxes of 47 cm × 34 cm × 18 cm lined with wood shavings in a 12-h light/dark cycle (illumination from 7 to 19 h) and temperature between 20 and 25 °C. Water and food were given *ad libitum*. The model chosen for the induction of colitis was adapted from those described by Yamada et al. and Tannahill et al.^{19,20} All animals were previously anesthetized and CL and CL + M groups were subjected to intracolonic administration of a solution of acetic acid diluted to 4% and with a volume of 4 mL by enema; and animals of CO and CO + M groups were submitted to the intracolonic administration of saline 0.9% with a volume of 4 mL, also by enema. The administration of mesalazine in CO + M and CL + M groups was carried out PO at a dose of 20 mg/kg diluted in 1 mL of saline 0.9%, and the other groups received 1 mL of saline 0.9% (adapted from Hirovani et al.²¹). The drug administered is marketed as Mesacol® (Nycomed Pharma). Each tablet contains 1200 mg of mesalazine.

After the period of treatment of each group (24 and 48 h), the animals were anesthetized with xylazine 2% at a dose of 8 mg/kg and ketamine 98 mg/kg intraperitoneally. Following this procedure, the measurement of anal sphincter pressure was carried out and a portion of about 8 cm of the intestine was removed for histological and immunohistochemical analysis and also for other biochemical studies. Finally, the animals were killed by exsanguination under deep anesthesia.¹⁸

Measurement of anal sphincter pressure

After induction of disease and treatment with mesalazine, animals were anesthetized with the aim of obtaining anal sphincter pressures. An anorectal manometry device (Proctosystem-Viotti – SP) with a balloon catheter and measurements in cm H₂O was used. The balloon catheter was introduced into the anal canal and then retracted, so as to record the sphincter pressure. Three sequential measurements were performed and then the average of the three values was obtained, in order to obtain the result of pressure.²²

Histological analysis

In histological study, a fragment of the intestine was immersed in buffered formalin. Then the fragment was embedded in paraffin blocks and sectioned on a rotary microtome (thickness, 3 μm). The sections were stained in hematoxylin–eosin (HE) for routine histologic evaluation. The slides were analyzed under a NIKON Labophot binocular microscope (200× magnification). The histological analysis was based on the presence of inflammatory infiltrate, edema, necrosis according to the damage index shown in Table 1 and adapted from Millar et al.²³

Table 1 – Index of macroscopic and microscopic changes in the colonic mucosa.

Scoring	Microscopic analysis
0	Normal
1	Slight damage and some points of inflammatory infiltrate in the mucosa
2	Damage and moderate inflammatory infiltrate in two or more areas of the mucosa
3	Damage and severe inflammatory infiltrate in the mucosa with loss of epithelium

Lipid peroxidation

Samples of bowel tissue were placed in test tubes with a mixture of trichloroacetic acid (TCA) 10% and thiobarbituric acid (TBA) 0.67%. Subsequently, the samples were heated in a water bath for 15 min and cooled on ice for approximately 5 min. TBA reacts with lipid peroxidation products forming a Schiff base; TCA is used to denature proteins present in the mixture, as well as to acidify the reaction. After cooling the samples, 1.5 mL of n-butyl alcohol was added to extract the formed pigment. The samples were placed on a stirrer for 45 s and centrifuged for 10 min at 3000 rpm. Finally, the stained product present in the supernatant was read on a spectrophotometer at a wavelength of 535 nm. The TBARS concentration obtained was expressed in nmol per milligram of protein.²⁴

Activity of antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx)

The activity of the enzyme superoxide dismutase (SOD) is defined by its ability to inhibit a detection system which reacts with superoxide radical. The SOD measurement technique is based on the inhibition of this reaction. To that end, adrenaline was used; when in an alkaline medium, this substance becomes adrenochrome, yielding O⁻ which is the enzyme substrate; thus, its definition occurs by the amount of SOD, which has the ability to inhibit by 50% the rate of oxidation of the detector adrenaline.²⁵

This enzyme catalyzes the reaction of hydroperoxide with reduced glutathione (GSH) to form oxidized glutathione (GSSG) and the product from hydroperoxide reduction.

GPx activity can be studied by measuring the rate of NADPH consumption in a system containing GSH in the presence of glutathione reductase (GR). The technique consists of determining the spectrophotometric activity of the enzyme, by measuring the rate of NADPH oxidation in a reactive mixture. GPx activity was measured in a spectrophotometer at 340 nm and expressed as nmol per minute per milligram protein (nmol/min/mg prot).²⁶

Levels of nitric oxide metabolites – nitrites and nitrates

Nitric oxide production was measured indirectly with a colorimetric quantitative test using the Griess reaction. This reaction is based on the enzymatic reduction of nitrates to nitrites in the presence of nitrate reductase and NADPH, and on the subsequent reaction of the nitrites formed (or initially present in the samples) with Griess reagent (a mixture

of sulfanilamide and naphthylethylenediamine specific for NO_2^-). The reading was performed in a microplate reader at 540 nm and the results were expressed in mmol of NO_2/NO_3 .²⁷

Immunohistochemistry of the enzyme inducible nitric oxide synthase (iNOS) and nuclear factor kappa B (NF-kB)

For immunohistochemistry, the bowel samples were incubated with 10% of rabbit serum at room temperature for blocking possible undesired reactions of the secondary antibody. Slides were incubated with rabbit polyclonal antibodies against iNOS and NF-kB (iNOS and NF-kB: Santa Cruz Biotechnology, United States) in a dilution of 1:200 overnight at 4 °C. After this period, the solution with the primary antibody was removed, the slides were washed in buffer and further incubated with the secondary antibody (biotinylated anti-rabbit IgG, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 min at room temperature. Next, the secondary antibody was removed and afterward the samples were treated with EnVision reagent and then washed three times with buffer. Nuclei were counterstained with hematoxylin. Finally, the slides were washed two times with ethanol and xylene for 10 s.

The images were photographed through a microscope equipped with a digital camera in order to capture images using the software Image-Plus (Media Cybernetics, Bethesda, USA); this done, the expression of iNOS and NF-kB was carried out by digital analysis by Adobe Photoshop® software CS3 extended, version 10.0, by counting all pixels stained by the immunohistochemistry reaction. The level of protein expression (iNOS and NF-kB) was determined by multiplying the average density of the image by the percentage of those areas positively stained by the antibodies (brown staining areas).

Statistical analysis

From the collected data, the means and standard deviations of each group were calculated using the statistical software GrapPad INSTAT, version 3.0. The test used for analysis of variance of the results was ANOVA, followed by the Student-Newman-Keuls test for parametric data. The results were considered statistically significant when a significance level of at least 5% ($p < 0.05$) was obtained.

Results

Histological analysis

Through the histological analysis, we found that the animals in CL group showed the destruction of crypts (CP) and edema of the submucosa (SB) in the animals' intestine. No histological changes were found in CO and CO + M groups. The groups treated with mesalazine – M + CL (24 and 48 h) showed preservation of CP and a decrease of submucosal edema. The results of microscopic damage indexes showed less damage in the treated groups – CL + M in both times (24 and 48 h) (Fig. 1A and B) when compared to colitis group.

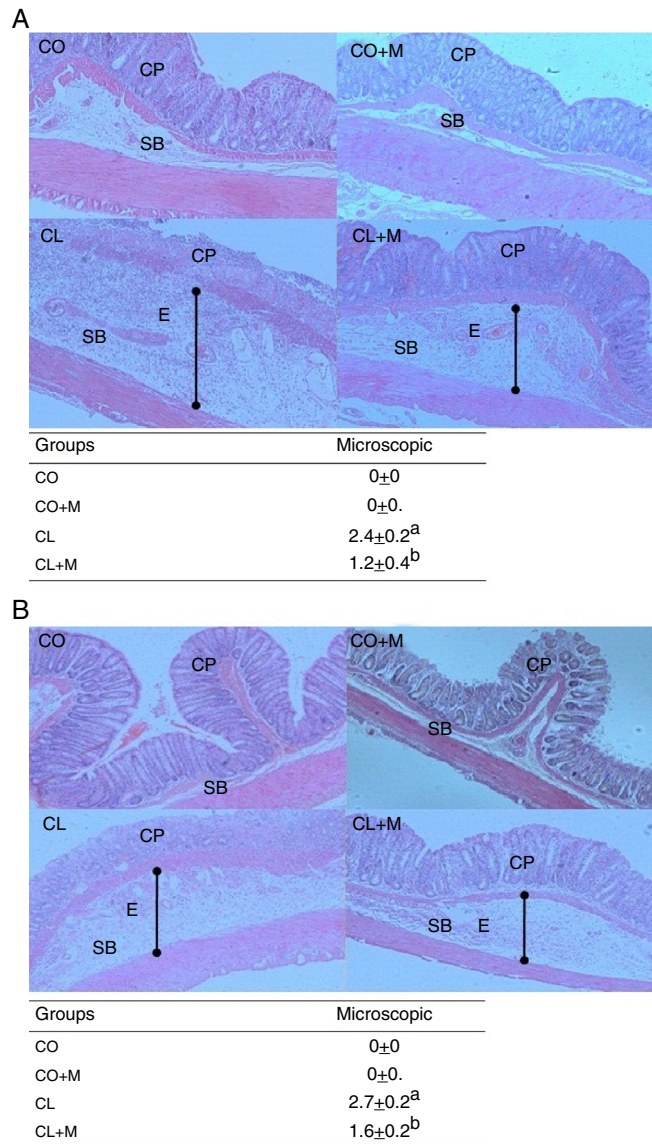


Fig. 1 – Effect of mesalazine in bowel macroscopic changes in an experimental model of colitis.

CO, control; CO + M, control + mesalazine, CL, colitis; CL + M, colitis + mesalazine; CP, crypts; E, edema; SB, submucosa.

^a Significant difference between CL and CO/CO + M.

^b Significant difference between CL + M and CL.

Anal sphincter pressure

The sphincter anal pressure results showed a significant decrease in colitis group and a significant increase in groups treated with mesalazine in both treatment times (24 and 48 h) versus colitis group ($p < 0.001$) (Fig. 2A and B).

Lipid peroxidation

The results of lipid peroxidation (LPO) using the technique of substances that react with the presence of thiobarbituric acid (TBARS) showed a significant increase in LPO in CL group versus CO and CO + M groups, and a significant reduction in LPO in

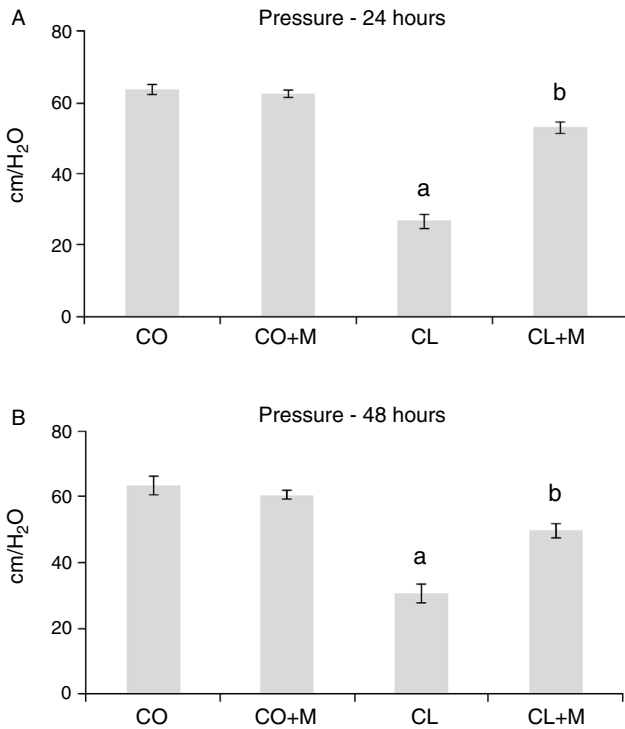


Fig. 2 – Effect of mesalazine in the levels of anal sphincter pressure in an experimental model of colitis. The results correspond to mean ± standard error (SE).

CO, control; CO + M, control + mesalazine; CL, colitis; CL + M, colitis + mesalazine.

Anal sphincter pressure at 24 and 48 h.

^a Significant difference between CL and CO/CO + M.

^b Significant difference between CL + M and CL.

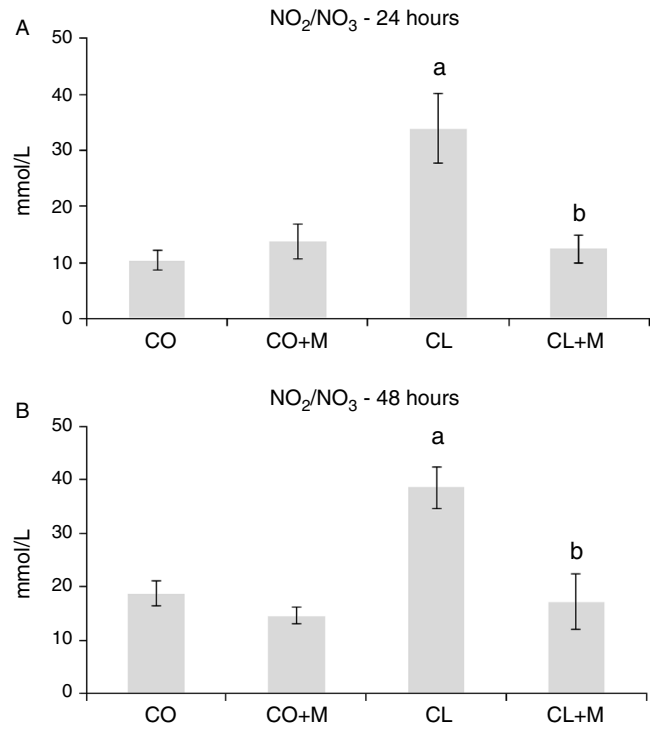


Fig. 3 – Effect of mesalazine in the levels of NO metabolites in an experimental model of colitis. The results correspond to mean ± standard error (SE).

CO, control; CO + M, control + mesalazine; CL, colitis; CL + M, colitis + mesalazine.

NO at 24 and 48 h ($p < 0.001$).

^a Significant difference between CL and CO/CO + M.

^b Significant difference between CL + M and CL.

Table 2 – Effect of mesalazine in the levels of lipid peroxidation (LPO) and activity of antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx) in an experimental model induced by acetic acid. The results correspond to mean ± standard error (SE).

Groups	24 h		
	LPO	SOD	GPx
CO	0.26 ± 0.03	4.96 ± 1.40	0.27 ± 0.03
CO+M	0.22 ± 0.03	3.66 ± 0.76	0.25 ± 0.02
CL	0.87 ± 0.05 ^a	14.35 ± 2.68 ^a	0.16 ± 0.01 ^a
CL+M	0.44 ± 0.05 ^b	10.00 ± 1.54 ^b	0.23 ± 0.01 ^b
Groups	48 h		
	LPO	SOD	GPx
CO	0.27 ± 0.05	0.57 ± 0.15	0.58 ± 0.09
CO+M	0.30 ± 0.01	0.72 ± 0.17	0.51 ± 0.06
CL	0.66 ± 0.08 ^a	2.83 ± 1.18 ^a	0.23 ± 0.03 ^a
CL+M	0.49 ± 0.05 ^b	1.05 ± 0.26 ^b	0.46 ± 0.04 ^b

LPO 24 h ($p < 0.001$); 48 h ($p < 0.05$). SOD 24 and 48 h ($p < 0.05$). GPx 24 and 48 h ($p < 0.05$).

CO, control; CO + M, control + mesalazine; CL, colitis; CL + M, colitis + mesalazine.

^a Significant difference between CL and CO/CO + M.

^b Significant difference between CL + M and CL.

groups treated with mesalazine in both treatment times (24 and 48 h) versus CL group (Table 2A – $p < 0.001$; B – $p < 0.05$).

Activity of antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx)

A significant increase in the activity of antioxidant enzyme SOD in both treatment times (24 and 48 h) was observed in the LC group versus CO and CO + M groups, and a significant reduction in CL + M group versus SC group (Table 2A and B – $p < 0.05$). A significant decrease in the activity of GPx enzyme in both treatment times (24 and 48 h) was observed in the LC group versus CO and CO + M groups, and a significant increase in CL + M group versus CL group (Table 2A; B – $p < 0.05$).

Levels of nitric oxide metabolites – nitrites and nitrates

The levels of nitric oxide metabolites (nitrites and nitrates) in both treatment times (24 and 48 h) showed a significant increase in the LC group versus CO and CO + M groups and a significant decrease in CL + M group versus CL group (Fig. 3A and B – $p < 0.01$).

Immunohistochemistry and quantification of iNOS and NF- κ B

The CL animals in both treatment times (24 and 48 h) showed a strong positive staining for iNOS and NF- κ B which was noted by the brown staining. CO and CO + M groups showed no positive staining. The treatment with mesalazine diminished the staining for iNOS and NF- κ B. Likewise, in the quantification of the expression of iNOS and NF- κ B, a significant decrease in the CL + M group was observed, compared to CL group. All images are featured in 200 \times magnification (Fig. 4A and B and Fig. 5A and B – $p < 0.001$).

Discussion

The pathophysiology of ulcerative colitis is not yet fully understood; thus, the study with experimental models can help to determine the factors involved in this disease, also allowing the study of therapeutic agents in order to achieve more effective treatments. Colitis induced by acetic acid enema have similarities with UUC in humans with respect to histological and metabolic features, promoting the development of submucosal edema, inflammatory infiltrate, colonic ulceration, destruction of crypts and depletion of goblet cells. Considering that the model evaluates acute characteristics, one can investigate the components involved in inflammation and evaluate the effectiveness of new therapies in the acute phase of the disease.^{7,28}

The histological analysis demonstrated the trophic effect of mesalazine in the colon, improving the colonic structure and decreasing the size and severity of the injury. In the treated groups (24 and 48 h) an initial injury was observed where the antioxidant action of mesalazine led to the preservation of the mucosa since its apical part still had some mucosal lesions and its basal part was intact. In their study, Song et al. demonstrated that the concomitant administration of butyrate and mesalazine in a model of experimental colitis induced by dextran sulfate sodium (DSS) improved colonic injury, the inflammatory profile of the mucosa and cecal lymph node. Normalization of neutrophil, eosinophil and activated B and T lymphocytes infiltrate was observed, highlighting the potential use of butyrate as an adjunct in the treatment of UUC.^{29,30}

In experimental trials, 5-ASA was administered in combination with NAC (N-acetylcysteine) in rats with TNBS-(2,4,6-trinitrobenzene sulfonic acid) induced colitis, and it was found that the combined treatment showed synergism versus single-agent treatment, promoting repair of inflamed mucosa.⁴

Among the factors related to UUC, one can describe the infiltration of neutrophils due to their ability to release RL and proinflammatory cytokines with the increase of LPO.¹³ On the other hand, oxidative stress has its damage minimized by antioxidant defenses of endogenous or exogenous nature. In their study, Harris et al. demonstrated that RL plays an important role in the pathogenesis assigned to LPO present in cell membranes. Biopsies performed in patients with UUC showed high LPO index versus normal subjects.³¹

In our study, we observed that mesalazine decreased LPO in treated animals (24 and 48 h), similar to the control group findings, possibly by its antioxidant action. Similar results

were found in contemporary studies that used substances with antioxidant action as glutamine, melatonin, *Boswellia serrata* and quince extract in experimental models of ulcerative colitis.^{7,13,28}

Oxidative stress has its damage minimized by non-enzymatic antioxidant defense systems, such as vitamins and/or enzymes. In our study, we observed in colitis group an increase of SOD in response to the increase of LPO due to oxidative stress, and a reduction in these parameters in both groups treated with mesalazine (24 and 48 h). GPx activity was decreased in colitis group and increased in those groups treated (24 and 48 h). Our results show that mesalazine increased enzyme activity in treated groups, confirming its action on oxidative stress. Our results corroborate the study of Arab et al. that evaluated the activity of antioxidant enzymes SOD and GPx in an experimental model of TNBS-induced colitis. After treatment with telmisartan, a significant increase was demonstrated in the activity of SOD and GPx; this outcome highlights the antioxidant effects of these treatments.¹² The implication of oxidative stress in the pathogenesis of UUC has been emphasized by several clinical and experimental studies, where the generation of RL and NO is related to intestinal lesions.^{10,11,13} In this study, the levels of anal sphincter pressure, as a physiological parameter of nitric oxide activity, were measured. The results confirm a significant decrease in anal sphincter pressure measurements in animals of the colitis group, and a significant increase in NO levels. The groups treated with mesalazine (24 and 48 h) showed an increase in anal sphincter pressure and a significant reduction in NO levels, compared to what was found in colitis group animals. Experimental animal studies indicate that nitric oxide and vasoactive intestinal polypeptide have an inhibitory action on smooth muscle, promoting relaxation of the anal sphincter, thus decreasing anal sphincter pressure levels.^{7,8,13}

Our results corroborate those of Hartmann et al. who demonstrated a significant increase in nitric oxide levels in the intestine of animals with colitis and a consequent decrease in anal sphincter pressure levels in colitis group animals, suggesting the involvement of relaxation of sphincter muscle with increasing nitric oxide levels. Fillmann et al. observed an increase in nitric oxide levels and a decrease in sphincter anal pressure levels in animals with induced colitis; and when these animals were treated with the antioxidant glutamine, these authors observed a significant decrease of nitric oxide levels and a consequent increase in sphincter anal pressure.^{7,13}

Carrier et al. found that mice subjected to colitis due to DSS showed increases in oxidative stress and in inflammatory markers as follows: TNF- α , IL-1 and NF- κ B.³² Other studies have also associated increases of NO and increases in iNOS expression in colitis models.^{8,9} In our study, we observed increased expression of iNOS and NF- κ B in colitis group animals. The group treated with mesalazine showed decreased expression of these proteins. Similar results were found in the study by Arab et al. who used telmisartan as therapy for experimental colitis and observed decreased levels of NF- κ B evaluated by immunohistochemistry, besides a diminished iNOS gene expression evaluated by PCR.¹² Davaatseren et al. used allyl isothiocyanate, an antioxidant compound found in plants, as therapy for DSS-induced colitis and reported

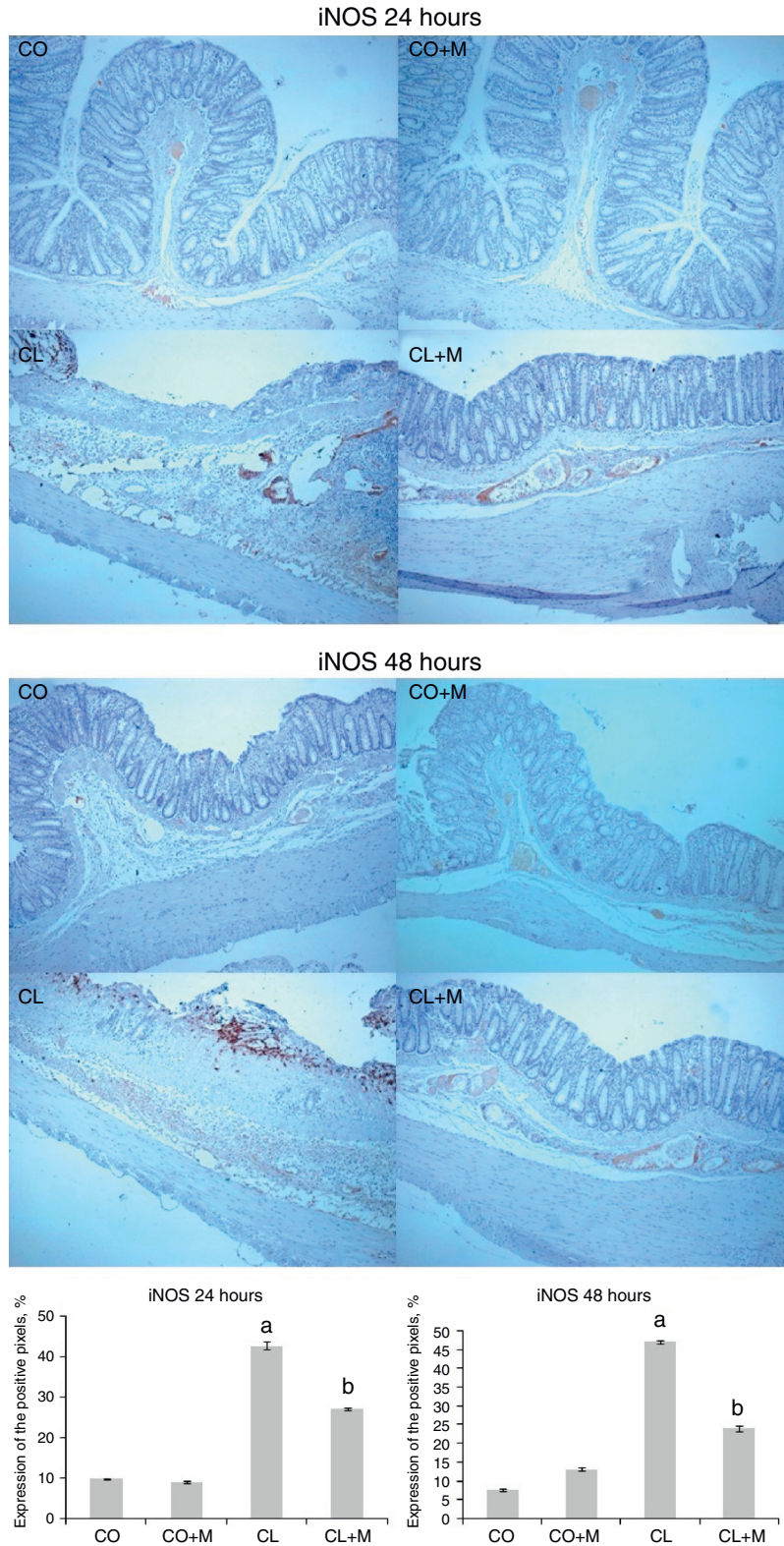


Fig. 4 – Effect of mesalazine in the expression of the enzyme inducible nitric oxide synthase (iNOS) in an experimental model of colitis. The results correspond to mean ± standard error (SE).

CO, control; CO + M, control + mesalazine; CL, colitis; CL + M, colitis + mesalazine.

iNOS at 24 and 48 h ($p < 0.001$).

^a Significant difference between CL and CO/CO + M.

^b Significant difference between CL + M and CL.

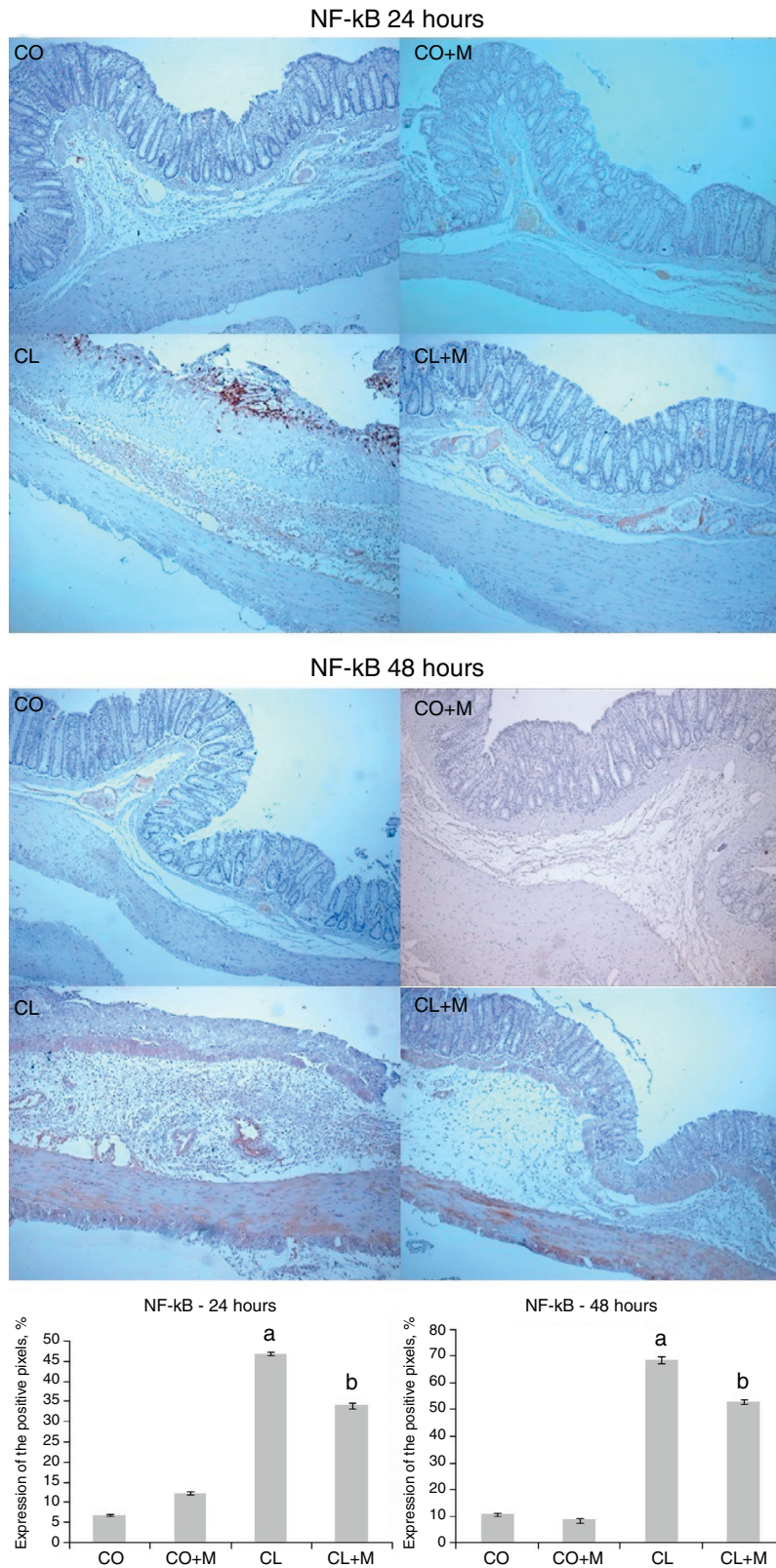


Fig. 5 – Effect of mesalazine in the expression of nuclear transcription factor kappa B (NF-kB) in an experimental model of colitis. The results correspond to mean ± standard error (SE).

NF-kB at 24 and 48 h ($p < 0.001$).

^a Significant difference between CL and CO/CO + M.

^b Significant difference between CL + M and CL.

CO, control, CO + M, control + mesalazine, CL, colitis, CL + M, colitis + mesalazine.

diminished iNOS expression in groups treated with various doses of this compound.³ Kretzmann et al. observed a decrease in the expression of p65 and p50 subunits of NF- κ B in animals treated with glutamine in the experimental model of TNBS-induced colitis.⁸

Although the pathogenesis is not yet fully understood, the treatments available have been able to intervene, improving the quality of life of patients. Therefore, it is suggested that mesalazine determines an improvement in the inflammatory bowel picture, also thanks to its antioxidant action.

Conflicts of interest

The authors declare no conflicts of interest.

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