Int Surg 2015;**100:**1364–1374
DOI: 10.9738/INTSURG-D-15-00118.1

Methylene Blue Inhibits the Inflammatory Process of the Acetic Acid-Induced Colitis in Rat Colonic Mucosa

Soykan Dinc[1], Muzaffer Caydere[2], Giray Akgul[3], Erdinc Yenidogan[4], Semra Hucumenoglu[2], Mohanraj Rajesh[5]

[1]*Department of General Surgery and* [2]*Department of Pathology, Ankara Training and Research Hospital, Cebeci, Ankara, Turkey*

[3]*Department of General Surgery, Beypazari City Hospital, Beypazari, Ankara, Turkey*

[4]*Department of General Surgery, University of Gazi Osman Pasa, Tokat, Turkey*

[5]*Department of Pharmacology, College of Medicine and Health Sciences, United Arab Emirates University, Al Ain, United Arab Emirates*

Inflammatory bowel disease is a serious health problem. Although it has been widely investigated, treatment of inflammatory bowel diseases currently remains a challenging clinical problem. Overproduction of nitric oxide has been demonstrated to cause tissue damage and inflammation. In this study, the effect of methylene blue (MB), a well-known inhibitor of nitric oxide synthesis, was investigated in acetic acid (AA)-induced colitis model in Sprague-Dawley rats. Eighty male rats were randomized into 4 groups (control, control MB, colitis, colitis + MB). AA was applied to groups 3 and 4. MB was added into groups 2 and 4. Three days later, animals were killed and the 8 cm distal colonic segment was resected, and the specimens were examined using macroscopical, histological, and biochemical methods. The results of the macroscopic and microscopic examination showed that in group 4 the mucosal damage and inflammation score was significantly lower than group 3. Increased intestinal permeability in acetic acid-administered group was significantly reversed by MB application. Myeloperoxidase activity and malondialdehyde levels increased significantly, while superoxide dismutase and catalase activities were suppressed after AA-administration. These biochemical parameters were reversed in MB-treated group. Administration of acetic acid resulted in increased levels of tumor

Corresponding author: Soykan Dinc, MD, Department of General Surgery, Ankara Training and Research Hospital, 06340, Cebeci, Ankara, Turkey. Tel.: +905065828255; Fax: +903123633396; E-mail: soykanege@hotmail.com

necrosis factor-α, interleukin-1β, interleukin-6, total nitrite/nitrate levels, and nitric oxide synthase activity. These biochemical alterations were also significantly reversed by MB application. In conclusion, our results indicate that MB decreases the level of nitric oxide and decreases inflammation in acetic acid-induced colitis.

Key words: Colitis – Methylene blue

Ulcerative colitis is a debilitating bowel disease that continues to be a serious health problem in the world.[1] Complications of this disease cause life-threatening clinical conditions that can necessitate surgical treatments. The medical treatment for ulcerative colitis is limited due to its complicated pathology.[2] Immunologic deficits, genetic background, stress, medical drug side effects, and irregular diet are reported as etiologic factors. Currently, the most frequently used drugs for ulcerative colitis are sulfasalazine and its derivatives, corticosteroids and immuno-suppressants.[3–5] However, treatments with these agents are not complete, and drug-induced side effects may cause major complications.[6]

Nitric oxide (NO) is a pleiotropic free radical messenger molecule that is synthesized from L-arginine by NO synthase (NOS). There is a large body of evidence indicating that the inducible form of NOS is up-regulated in various forms of mucosal inflammation.[7,8] Furthermore, multiple detection strategies have demonstrated that inducible NOS expression, enzymatic activity, and NO production are increased in human inflammatory bowel disease tissues.[9,10] There is also evidence that the level of NOS-derived NO correlates well with disease activity in ulcerative colitis.[10] NO has also been shown to be involved in the pathogenesis of inflammatory disorders of the joint and lungs.[10] Therefore, NO inhibitors can be utilized as therapeutic agents in the management of inflammatory diseases.

Methylene blue (MB), a heterocyclic phenothiazine-based compound, is a well-known inhibitor of NOS and guanylate cyclase.[11,12] MB has been used for therapeutic purposes for more than a century.[13–15] In clinics, it has been used for the treatment of methemoglobinemia, surgical localization of parathyroid glands, localization of fistula, and cyanate antidote for many decades.[16–18] Currently, it has been found to improve the hypotension associated with various clinical states such as septic shock, cardiac shock, and vasoplegia, without major side effects.[16,19,20] On the basis of earlier results indicating the inhibitory effects of MB on the inflammatory process, we investigated the effects of MB on the inflammatory process on an experimental colitis model in rats.

Materials and Methods

Male Sprague-Dawley rats (weighing between 250 and 300 g) were used after a 1-week acclimation period (temperature, 23–28°C; humidity, 60%). The animals were housed for 12 to 24 hours under light/dark cycles at a constant temperature of 21°C to 22°C and fed a standard rat chow and water *ad libitum*. All rats fasted 24 hours prior to the experimental procedures. All rats were weighed and the weight was recorded before the experimental procedures. The procedures and animal protocols followed in this study were in accordance with the guide for the care and use of laboratory animals (National Institutes of Health Publication No: 86-23, revised 1985) and approved by the animal ethic committee of Ankara Training and Research Hospital.

Eighty rats were randomized into 4 groups: group 1, control group (n = 20); group 2, control MB-treated group (n = 20); group 3, acetic acid (AA)-induced colitis group (n = 20); group 4, colitis and intraperitoneal MB-treated group (n = 20).

Induction of colitis and treatment

Colitis was induced in rats of groups 3 and 4 by intraperitoneal administration of 2 mL of 5% AA in 0.9% NaCl. After ether anesthesia, a soft 6-Fr pediatric catheter was introduced into the anus for 8 cm, and AA was carefully administered. Before the removal of the catheter, 2 mL air was applied to spread AA into the colon. This method has been shown to cause histopathologic changes similar to those observed in human ulcerative colitis.[21,22] The rats in the control group (group 1) were subjected to the same procedure, except that the AA was substituted with isotonic saline. The control-MB group (group 2) was treated the same as in group 1, except that 2 mL of 1% (vol/vol) MB was applied 5 min after intrarectal administration of isotonic saline.

In the MB treatment group, 5 minutes after AA application, 2 mL of 1% (vol/vol) MB was admin-

istered via the intraperitoneal route, and the AA treatment group received 2 mL intraperitoneal saline (group 4). On the fourth day, the animals were sacrificed by cervical decapitation, and the abdomen was opened by a midline incision. The distal 8 cm of the colon was removed, isolated from surrounding tissues, opened along the antimesenteric border, and rinsed with saline solution, and the length and weight were measured [in order to determine the colon weight/colon length ratio (milligrams per centimeter) used as an indirect marker of inflammation].

The colon samples were examined macroscopically using the grading scale by Morris *et al*.[23] According to this scale, a score of 0 = no damage, 1 = localized hyperemia but no ulcers, 2 = linear ulcers with no significant inflammation, 3 = linear ulcer with inflammation at 1 site, 4 = ulceration and inflammation at 2 or more sites, and 5 = ulceration and inflammation at 2 or more sites or 1 major site of inflammation and ulceration extending more than 1 cm along the length of the colon. Ten animals from each group were randomly chosen for histologic examination, and the other 10 samples were preserved for biochemical examinations. For biochemistry analysis, tissues were kept at –80°C for storage or kept at –25°C and evaluated immediately for biochemical parameters. For histologic examination, tissues were fixed in 10% formalin. For biochemical measurements, the colonic samples were cleared of fecal content. Cardiac blood was drawn in a sterile fashion to determine serum total protein, albumin, and NOS levels.

The dose of AA was selected on the basis of preliminary experiments showing remarkable colonic damage associated with high reproducibility and low mortality for the intrarectal application of 2 mL of 5% AA. The time point of damage evaluation (*i.e.*, 3 days after AA administration) was chosen because maximal AA-induced inflammation has been reported in mice after 3 days.[21,22] The dose of MB was selected on the basis of earlier experiments indicating the effectiveness of this concentration of MB without any toxicity.[24–26]

Histologic evaluations

After washing the mucosa with saline solution, mucosal injury was assessed macroscopically using the grading scale as described before. After macroscopic examination, a segment of the distal colon was fixed in 10% formalin solution. For each animal, 2 tissue samples (20 mm) were obtained and stained by hematoxylin-eosin. The tissue sections were examined by 2 pathologists. Formalin-fixed colonic samples were embedded in paraffin, and sections were stained with hematoxylin-eosin. The degree of inflammation of the colon was graded semiquantitatively from 0 to 11 according to previously established criteria.[27] The semiquantitative scores were as follows: 1 (0–3) referred to loss of mucosal architecture, 2 (0–3) referred to cellular infiltration, 3 (0–3) referred to muscle thickening, 4 (0–1) referred to crypt abscess formation, and 5 (0–1) referred to goblet cell depletion.

Intestinal permeability measurements

Intestinal permeability was examined using a fluorescein isothiocyanate (FITC)-labeled dextran method, as described previously.[28] Two days after AA administration, rats were gavaged with 600 mg/kg body weight FITC-conjugated dextran (molecular mass, 3–5 kd). One day later, blood was collected by cardiac puncture, and the serum was immediately analyzed for FITC-derived fluorescence using a fluorescent microplate reader with excitation-emission wavelengths of 485 to 520 nm (LS55Luminescence Spectrometer, PerkinElmer Instruments, Waltham, Massachusetts). Preliminary experiments showed that FITC-dextran was stable after 24 hours from its preparation. Serial-diluted FITC-dextran was used to generate a standard curve. Intestinal permeability was expressed as nanomolars of FITC found in the serum.

Measurement of myeloperoxidase activity

Myeloperoxidase (MPO) is an enzyme that is present predominantly in the azurophilic granules of polymorphonuclear leukocytes. Activity of MPO is commonly utilized to estimate tissue polymorphonuclear leukocyte accumulation in inflamed tissues and correlates significantly with the number of polymorphonuclear leukocytes determined histochemically in tissues. MPO activity was assessed according to the method described previously.[29] In brief, full-thickness colon tissues were homogenized in a solution containing 0.5% (wt/vol) hexadecyltrimethyl ammonium bromide dissolved in 10 mM sodium phosphate buffer (pH 7.4) in an ice bath using a polytron homogenizer (50 mg tissue/mL). The homogenates were centrifuged for 30 minutes at 20,000*g* at 4°C. An aliquot of the supernatant was allowed to react with a solution of tetramethyl benzidine (1.6 mM) and 0.1 mM hydrogen peroxide. The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was defined as the quantity of

enzyme degrading 1 μmol peroxide/min at 37°C and was expressed in units per milligram wet tissue.

Measurement of malondialdehyde levels

Malondialdehyde (MDA) can be detected in quantifiable amounts with the thiobarbituric acid reactive species (TBARS) assay. The TBARS assay kit was used to measure MDA levels (Zeptometrix, Buffalo, New York). Briefly, full-thickness colonic tissue was homogenized in ice-cold 10% trichloroacetic acid (TCA) solution and then centrifuged. The supernatant portion was mixed with an equal volume of TBARS (0.67%) and heated at 90°C for 15 minutes. The TBARS were measured in nanomoles per milligram protein according to absorbance at 532 nm.

Measurements of superoxide dismutase and catalase activities

Superoxide dismutase (SOD) activity was measured according to the method described previously.[30] This method uses xanthine and xanthine oxidase to generate superoxide radicals that react with *p*-iodonitrotetrazolium violet to form a red formazan dye, which was measured spectroscopically at 505 nm. The colon tissue samples (0.5 g) were homogenized with 5 volumes per weight of 0.05 M phosphate buffer, pH 7.0, containing 1% (g/mL) Triton X-100 in a glass–glass homogenizer. The extract was centrifuged for 20 minutes at 10,000*g* (4°C). The supernatant was used in the assay for SOD. SOD activity was measured using the Ransod kit (catalog number SD 125, Randox Laboratories, Crumlin, County Antrium, Northern Ireland). One unit of SOD activity was defined as the amount of enzyme that causes 50% inhibition of the rate of reduction of 2-(4-iodophenyl)-3-(nitrophenol)-5-phenyltetrazolium chloride under the conditions of assay (at 37°C and pH 7.0). The amount of total protein of colon was determined using the method of Lowry *et al*.[31] Enzyme activities were expressed as units per milligram protein. The activity of catalase in colonic tissue samples was measured using a catalase assay kit (Cayman Chemical Company, Ann Arbor, Michigan). Enzyme activity was expressed as nanomoles per minute per milligram tissue.

Measurements of nitrite and nitrate levels

Total nitrite and nitrate content (NOx), both stable end products of NO, were measured by spectrophotometric methods with a nitric oxide assay kit (Calbiochem, San Diego, California). Full-thickness colon samples were pooled, weighed, and homogenized in phosphate-buffered saline at pH 7.4 (10 volumes) with a polytron (PT 1020, 350D, Lucerne, Switzerland). The homogenate was centrifuged at 10,000*g* for 20 minutes, and supernatant was filtered through 10 k of molecular weight cutoff Ultrafree-MC (Millipore, Bedford, Massachusetts). Filtrates were incubated with nitrate reductase for 3 hours and assayed for nitrite contents with Griess reagents, and absorbance was measured at 540 nm.

Measurements of NOS activity

NOS activity was determined by measuring the conversion of $[^3H]$L-arginine to $[^3H]$L-citrulline with an NOS assay kit (Calbiochem). Full-thickness colon samples were homogenized in 20 volumes of 25 mM Tris-HCl, 1 mM EDTA, and 1 mM ethylene glycol tetraacetic acid, pH 7.4. The homogenates were centrifuged at 10,000*g* for 5 minutes, and at 4°C, the supernatant was collected. Briefly, 5 μL of supernatant for each sample was incubated with a buffer containing cofactors required for NOS enzyme activity (25 mM Tris-HCl, 1 mM nicotinamide adenine dinucleotide phosphate (reduced form), 3 μM tetrahydrobiopterin, 1 μM flavin adenine dinucleotide, 1 μM flavin adenine mononucleotide, 0.8 mM $CaCl_2$, and 0.05 μC [^{3}H]ethylene glycol tetraacetic-arginine (specific activity, 60 Ci/mmol) for 60 minutes at 35°C. The reaction was stopped with a stop buffer (400 μL) composed of 25 mM Tris-HCl and 2.5 mM EDTA, pH 5.5, and 100 μL equilibrated resin was added. Samples were transferred to spin cups and centrifuged at 1200*g* for 30 seconds. The radioactivity in elutes was quantified in a liquid scintillation counter. NOS activity was determined from the difference between $[^3H]$L-citrulline produced from control samples and containing 1 mM L-NAME, a competitive inhibitor of NOS. Protein content in homogenates was determined with a protein assay kit based on the Lowry method (Bio-Rad, Hercules, California), and NOS activity was expressed as pmol of $[^3H]$L-citrulline formed per minute per milligram protein.

Measurement of cytokines

Full-thickness colon samples were homogenized in 20 volumes of 25 mM Tris-HCl, 1 mM EDTA, and 1 mM ethylene glycol tetraacetic, pH 7.4. The homogenates were centrifuged at 10,000*g* for 5 minutes, and at 4°C, the supernatant was used for the measurement of cytokines. The levels of tumor necrosis factor-α,

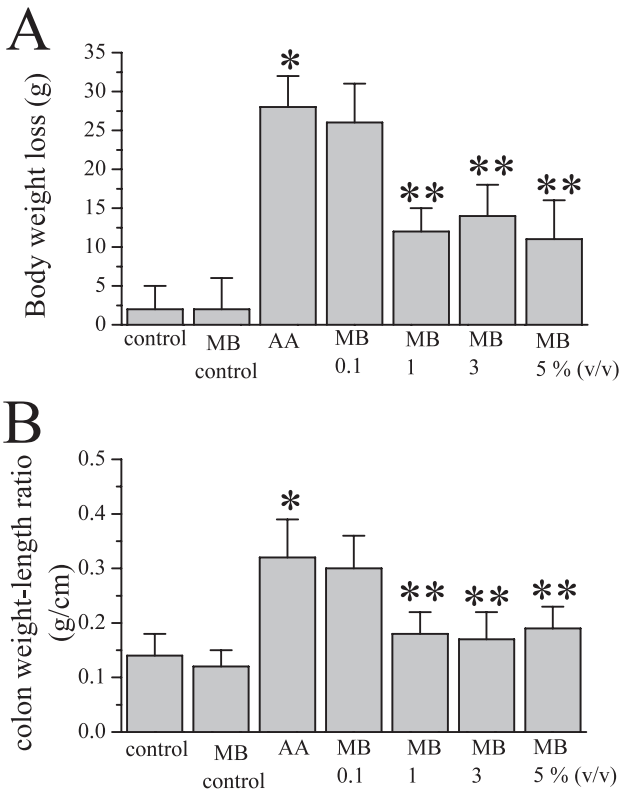


Fig. 1 The effects of MB treatment on body weight and colon length-weight ratio in AA-induced colitis. (A) The effect of MB treatment on body weight loss of AA-induced colitis. (B) The effect of MB treatment colon length-weight ratio of AA-induced colitis. *Significant difference (ANOVA, $P < 0.05$) between control and AA-administered group. **Significant difference (ANOVA, $P < 0.05$) between AA-administered group and MB-treated group.

interleukin (IL)-1β, and IL-6 in the colon samples were measured using a commercially available enzyme-linked immunosorbent assay kit (R&D Systems Inc, Biosource, Minneapolis, Minnesota). Briefly, cytokine molecules of the specimen bind to monoclonal antibody–precoated wells and react with an enzyme-conjugated secondary antibody. The change in the color produced on addition of the chromogen substrate is proportional to the amount of the cytokine.

Statistical analysis

Data were expressed as mean ± SE. Statistical analysis was carried out using 1-way analysis of variance (ANOVA) followed by Newman-Keuls post hoc test. $P \leq 0.05$ was considered statistically significant. All statistics tests were conducted using GraphPad Prism (version 5) software (GraphPad Software, Inc, La Jolla, California).

Results

Macroscopic and histologic examinations of the colon

All the animals survived during the experimental procedures. Compared with the control groups, there was a significant reduction of weight measured on day 4 of the experiments in the AA-administered group. The weight loss in MB-treated animals was significantly less than the AA-administered group (Fig. 1A). In line with this finding, the food intake of the animals in the AA-administered group was significantly less than the control group (8.8 ± 1.9 g/d in controls versus 5.1 ± 0.8 g/d in the AA-administered group; n = 20; ANOVA, $P < 0.05$). Compared with the AA-administered group, reduction in the food consumption of the animals was significantly reversed in the MB-treated group (7.9 ± 1.2 g/d). Administration of AA caused a significant increase in colon weight/colon length ratio, a simple and reliable marker of intestinal inflammation/damage.[32] Administration of MB significantly reduced the effects of AA on colon weight/colon length ratio (Fig. 1B).

Macroscopic examination was conducted to assess the inflammatory damage induced by AA administration. The mean values of the macroscopic examination and scoring of the groups are presented in Table 1. The results between the control groups and the colitis group are statistically different (n = 10; ANOVA, $P < 0.05$). The difference between the results of the colitis group and MB-treated colitis group was also significant (n = 10; ANOVA, $P < 0.05$). The results of histologic examination are presented in Table 1. Histologic evaluations of colonic mucosa of healthy control animals showed a normal appearance with

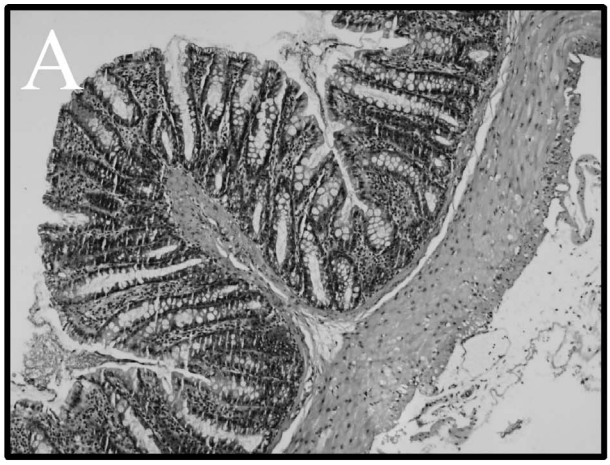
Table 1 Results of histologic and macroscopic scoring of colon specimens of the groups

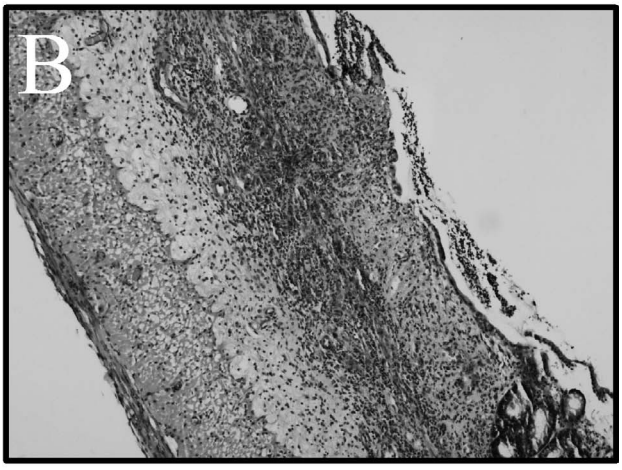
	Group 1 (control group)	Group 2 (control MB group)	Group 3 (AA-administered group)	Group 3 (AA + MB administered group)
Histologic	0.50 ± 0.53	0.40	7.54 ± 2.16*	4.8 ± 1.77**
Macroscopic	0.40 ± 0.46	0.40	3.62 ± 1.77*	1.78 ± 0.79**

The mean and standard deviations of the groups are presented in the table.

*$P < 0.05$ between control and AA-administered group.

**$P < 0.05$ between AA-administered group and MB-treated group.





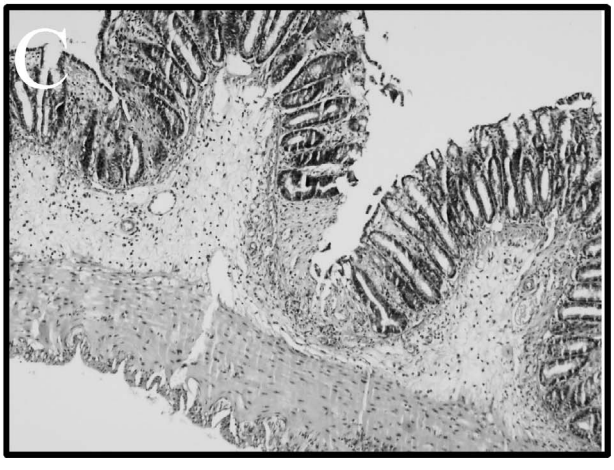


Fig. 2 Histopathologic examinations of the effect of MB treatment on AA-induced colitis. (A) Hematoxylin-eosin–stained photomicrograph showing normal intact mucosa and colon crypts. (B) Hematoxylin-eosin–stained photomicrograph showing acetic acid–induced colitis with massive necrotic destruction of epithelium, loss of the crypts, dilated blood vessels, areas of hemorrhages, inflammatory edema, and focal inflammatory cell infiltrate. (C) Hematoxylin-eosin–stained photomicrograph in acetic acid–induced colitis treated with MB. Treatment with MB attenuated the extent and severity of the histologic signs of cell damage, with more goblet cells formation in the mucosal epithelium with no apparent edema (×200).

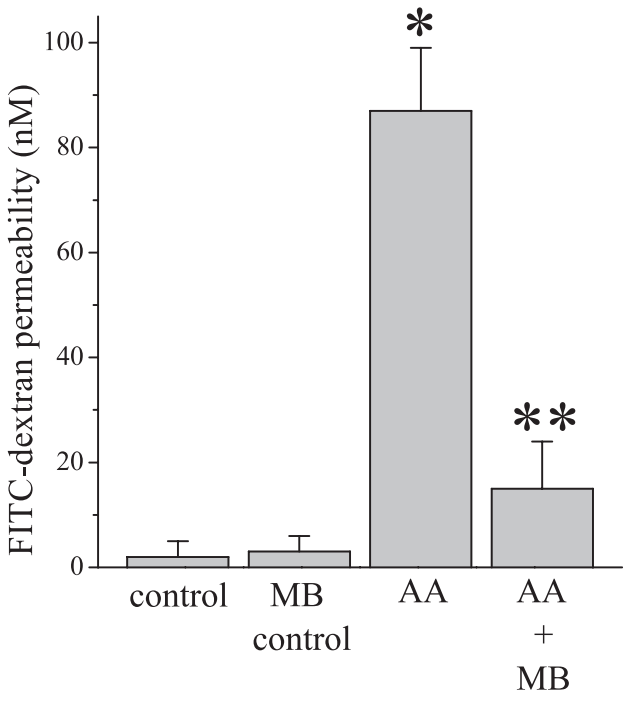


Fig. 3 The effect of MB on colon permeability. The effect was evaluated as permeability to FITC-dextran (see "Materials and Methods" section). *Significant difference (ANOVA, $P < 0.05$) between control and AA-administered group. **Significant difference (ANOVA, $P < 0.05$) between AA-administered group and MB-treated group.

intact epithelium (Fig. 2A). In the AA-administered group, colons showed tissue injury, which was mainly characterized by necrosis involving the full thickness of the mucosa, infiltrations of granulocytes into the mucosa/submucosa, and edema of the submucosa (Fig. 2B). Application of MB (2 mL, 1% vol/vol) reduced the signs of colon injury. In the colon of MB-treated animals, the glands were regenerating, the edema in the submucosa was reduced, and the erosion area was superficial (Fig. 2C).

Effect of MB on intestinal permeability

FITC-conjugated dextran presence was not detected in the serum of healthy control animals, which is suggestive of intestinal membrane integrity (Fig. 3).

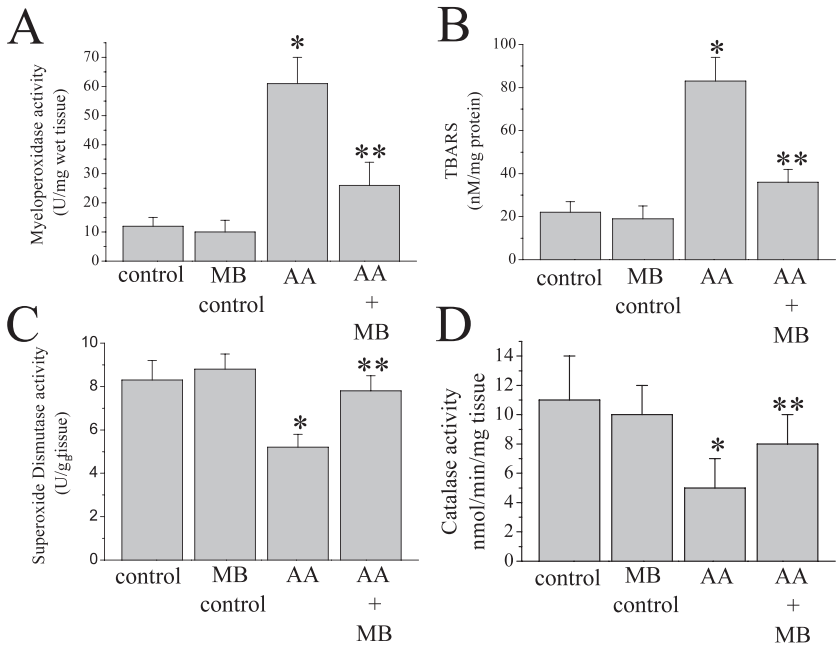


Fig. 4 The effect of MB treatment on the activities of myeloperoxidase, superoxide dismutase, catalase enzymes, and malondialdehyde levels in AA-induced colitis. (A) The effect of MB on myeloperoxidase activity in AA-induced colitis. (B) The effect of MB on malondialdehyde levels on AA-induced colitis. (C) The effect of MB on superoxide dismutase activity in AA-induced colitis. (D) The effect of MB on catalase activity in AA-induced colitis. *Significant difference (ANOVA, $P < 0.05$) between control and AA-administered group. **Significant difference (ANOVA, $P < 0.05$) between AA-administered group and MB-treated group.

The results for groups 1, 2, 3, and 4 were 2.02 ± 0.36, 2.81 ± 0.31, 83.15 ± 15.03, and 18.36 ± 5.11 nM, respectively. In group 3 (AA) the amount of FITC-conjugated dextran was significantly higher than group 1 (control; $P < 0.0001$). The results for group 4 (AA + MB) were significantly lower than group 3 (AA; $P < 0.0001$).

Effect of MB on MPO activity and MDA level

The levels of MPO activity in groups 1, 2, 3, and 4 were 12.11 ± 2.02, 10.45 ± 1.75, 60.95 ± 14.31, and 25 ± 7.02 U/mg wet tissue, respectively. The amount of MPO in group 3 (AA) was significantly higher than the control group ($P < 0.0001$), and the result for group 4 (AA + MB) was significantly lower than the AA group (Fig. 4A).

In our experiments, MDA levels were measured by the TBARS assay. The TBARS levels of groups 1, 2, 3, and 4 were 20.72 ± 6.19, 19.8 ± 4.22, 80.84 ± 11.09, and 35.30 ± 12.92 nM/mg protein, respectively. The differences between groups 1 (control) and 3 (AA) are also significant ($P < 0.0001$), and the results of group 4 (AA + MB) are significantly lower than group 3 (AA; $P < 0.0001$) (Fig. 4B).

Effect of MB on SOD and catalase activity

The effects of MB treatment were also investigated regarding the activity of antioxidant enzymes such as SOD and catalase. Administration of AA produced a significant decrease in SOD activity ($P < 0.001$) (Fig. 4C). The mean values for groups 1, 2, 3, and 4 were 8.24 ± 1.63, 8.76 ± 1.07, 5.17 ± 0.92, and 7.8 ± 1.51 U/g tissue, respectively. MB application counteracted AA-induced reduction in SOD activity ($P < 0.001$). Similarly, the catalase activity was significantly reduced in the AA-administered group. The decreased activity of catalase was significantly reversed in the MB-applied colitis group ($P < 0.001$; Fig. 4D). The catalase levels of groups 1, 2, 3, and 4 were 11.74 ± 3.46, 9.94 ± 1.26, 4.75 ± 0.65, and 7.70 ± 1.35 nmol/min/mg tissue, respectively.

Effects of MB on NO and NOS

The results of the NO levels for groups 1, 2, 3, and 4 were 38.16 ± 6.23, 36.16 ± 4.2, 70.63 ± 10.9, and 43.23 ± 10.23 μM, respectively. The mean value of group 3 (AA) was significantly higher than the control group (group 2). The mean value of the MB-treated colitis group (group 4) is significantly lower than the AA colitis group (group 3; $P < 0.0001$) (Fig. 5A).

The results of NOS activity in groups 1, 2, 3, and 4 were 6.88 ±1.21, 6.11 ± 1.39, 17.23 ± 4.10, and 7.11 ± 1.82 pmol $[^3H]$ citrulline/mg protein/min, respectively. The differences between groups 1 and 3 is significant ($P < 0.0001$). The differences between groups 3 and 4 were also significant ($P < 0.0001$) The mean value of the MB-treated colitis group was lower than the AA colitis group (Fig. 5B).

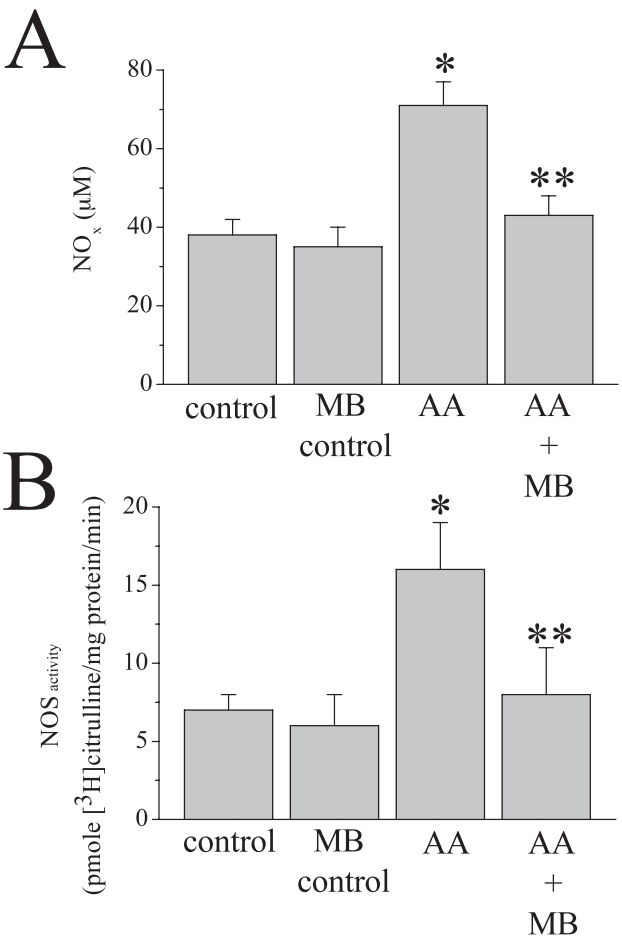


Fig. 5 Effects of MB treatment on total nitrite and nitrate levels and NOS activity measured in AA-induced colon inflammation. (A) The effect of MB on total nitrite and nitrate levels in AA-induced colon inflammation. (B) The effect of MB on NOS activity in AA-induced colon inflammation. *Significant difference (ANOVA, $P < 0.05$) between control and AA-administered group. **Significant difference (ANOVA, $P < 0.05$) between AA-administered group and MB-treated group.

Effect of MB on proinflammatory cytokines

Colonic damage by AA administration was also evident with increased levels of proinflammatory cytokines; tumor necrosis factor-α, IL-1β, and IL-6. The values of tumor necrosis factor-α in groups 1, 2, 3, and 4 were 6.02 ± 1.14, 5.06 ± 0.85, 16.04 ± 3.27, and 7.14 ± 1.61 pg/mg tissue, respectively. The mean values of IL-6 levels of groups 1 to 4 were 12.08 ± 0.82, 10.20 ± 3.98, 28.10 ± 8.68, and 14.13 ± 4.87 pg/mg tissue, respectively. The third proinflammatory cytokine IL-1β's mean values for groups 1, 2, 3, and 4 were 7.82 ± 1.56, 7.01 ± 1.31, 25.06 ± 7.04, and 12.95 ± 2.4 pg/mg tissue, respectively. The

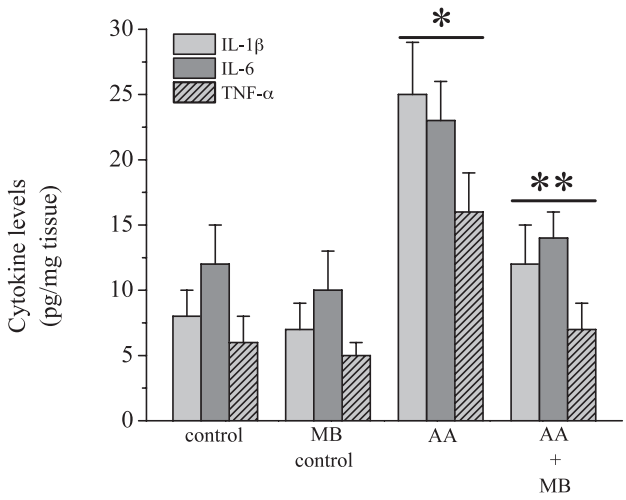


Fig. 6 Effect of MB treatment on tissue cytokine levels in AA-induced colon inflammation. Bars indicate the means and standard errors. *Significant difference (ANOVA, $P < 0.05$) between control and AA-administered group. **Significant difference (ANOVA, $P < 0.05$) between AA-administered group and MB-treated group.

differences between the groups of all cytokines results are almost parallel. The mean values of the cytokine levels of group 3 (AA) are higher than the control group, and the mean values of cytokine levels of group 4 (AA + MB) are lower than group 3 (AA). The difference in these comparisons is also seen in Fig. 6.

Discussion

Results of the present study indicate for the first time that intraperitoneal application of MB effectively reduces the inflammatory process in an AA-induced colitis model. The findings of this study show that oxidative damage contributes to the development of colon injury in an acute model of ulcerative colitis induced by colonic infusion with AA, a reproducible and simple model that shares many features with human colitis.[21,22] Furthermore, we documented that MB counteracts AA-induced colitis by decreasing the inflammatory cytokines and the levels of oxidative stress.

Several factors such as neutrophil infiltration and the overproduction of proinflammatory mediators, including cytokines, and reactive oxygen nitrogen species have been implicated in the pathogenesis of ulcerative colitis.[1,2,4] These proinflammatory mediators ultimately lead to the disruption and the ulceration of the mucosa.[33,34] Several antioxidants

were investigated in experimental models of inflammatory bowel disease.[35] However, interventional studies have failed to reciprocate the preclinical findings.[35] MB has been shown to act as an electron sink for xanthine oxidase and direct scavenge free radicals.[36] MDA is one of the lipid peroxidation products, and its accumulation is considered an index for oxidative insult in the tissue milieu.[37] Here we observed significantly increased malondialdehyde content in the colon tissues of AA-treated animals, which was inhibited to an extent upon MB treatment. Previous studies have suggested that the balance between oxidant and antioxidant represents a key step in driving colon inflammation.[38] Accordingly, in our present study, we found that reduced SOD and catalase activities in the AA-treated colon tissues and MB treatment significantly augmented these endogenous antioxidant enzymes. Collectively, these findings suggest that MB effectively confers protection against AA-induced colitis via counteracting oxidative stress.

Routinely, tissue MPO activities were used as a bona fide marker to access the leukocyte infiltration to the injured tissue.[39] Previous studies have documented that MB was effective in arresting leukocyte (polymorphonuclear cells) infiltration in a murine model of colitis.[40] The profound MPO attenuating potential of MB supports the notion that leukocyte-derived reactive oxygen species and inflammatory mediators are critical in the adhesion of these inflammatory cells to the inflamed colon mucosal epithelium. NO is a highly reactive gaseous mediator released from cellular components of the gastrointestinal tract (GIT), and its production and diffusion are often increased upon various inflammatory stress.[41] In fact, increased NO levels were reported in subjects with inflammatory bowel disease, and its inhibition by supplementation of N (G)-monomethyl-L-arginine (L-NMMA), an inhibitor of NOS, significantly improved the colonic motility.[42] The principle mechanism by which MB inhibits NOS is by inhibition of guanylate cyclase, a rheostat that governs the prenylation of the NOS enzyme that regulates its catalytic activity.[33] Several studies have reported altered levels of NO with GIT diseases, and it can play a bidirectional role in the disease process.[41] A recent study has documented that MB directly inhibits NO synthesis upon inflammatory stress in colon cancer cells, via inhibiting nuclear factor κB.[43] Our findings of profound increases in NO levels, which coincided with enhanced NOS activities in AA-treated rats, are consistent with the aforementioned activity of MB.[43]

Intestinal epithelial hyperpermeability plays key role in the pathogenesis of gut mucosal barrier dysfunction.[44] Oxidative stress–driven inflammatory cytokine production has been identified as a pivotal player that promotes intestinal epithelial hyperpermeability, which perpetuates the inflammatory reaction in the GIT.[44] A previous report[45] suggested that MB significantly inhibits adhesion molecules and proinflammatory cytokine expression, and our results also support this observation, since MB significantly inhibited the expression of proinflammatory cytokines and prevented the leakiness of the intestinal epithelium.

In summary, our present findings suggests that MB inhibits the AA-induced colon tissue injury via attenuation of oxidative stress and inflammatory pathways. Owing to the apt pharmacologic properties of MB, it could be perused for further development for clinical utility in treating inflammatory bowel disease and perhaps other chronic inflammatory diseases affecting the GIT.

References

1. Actis GC, Pellicano R, Rosina F. Inflammatory bowel diseases: current problems and future tasks. *World J Gastrointest Pharmacol Ther* 2014;**5**(3):169–174
2. Domènech E, Mañosa M, Cabré E. An overview of the natural history of inflammatory bowel diseases. Dig Dis 2014;**32**(4): 320–327
3. Kawalec P, Mikrut A, Łopuch S. Systematic review of the effectiveness of biological therapy for active moderate to severe ulcerative colitis. *J Gastroenterol Hepatol* 2014;**29**(6):1159–1170
4. Kedia S, Ahuja V, Tandon R. Management of acute severe ulcerative colitis. *World J Gastrointest Pathophysiol* 2014;**5**(4): 579–588
5. Bryant RV, Brain O, Travis SP. Conventional drug therapy for inflammatory bowel disease. *Scand J Gastroenterol* 2015;**50**(1): 90–112
6. Rosenberg LN, Peppercorn MA. Efficacy and safety of drugs for ulcerative colitis. *Expert Opin Drug Saf* 2010;**9**(4):573–592
7. Kolios G, Valatas V, Ward SG. Nitric oxide in inflammatory bowel disease: a universal messenger in an unsolved puzzle. *Immunology* 2004;**113**(4):427–437
8. Kim YJ, Kim EH, Hahm KB. Oxidative stress in inflammation-based gastrointestinal tract diseases: challenges and opportunities. *J Gastroenterol Hepatol* 2012;**27**(6):1004–1010
9. Goodman JE, Hofseth LJ, Hussain SP, Harris CC. Nitric oxide and p53 in cancer-prone chronic inflammation and oxyradical overload disease. *Environ Mol Mutagen* 2004;**44**(1):3–9
10. Cross RK, Wilson KT. Nitric oxide in inflammatory bowel disease. *Inflamm Bowel Dis* 2003;**9**(3):179–189

11. Ignarro LJ, Kadowitz PJ. The pharmacological and physiological role of cyclic GMP in vascular smooth muscle relaxation. *Annu Rev Pharmacol Toxicol* 1985;**25**:171–191
12. Wiklund L, Basu S, Miclescu A, Wiklund P, Ronquist G, Sharma HS. Neuro- and cardioprotective effects of blockade of nitric oxide action by administration of methylene blue. *Ann N Y Acad Sci* 2007;**1122**:231–244.
13. Oz M, Lorke DE, Petroianu GA. Methylene blue and Alzheimer's disease. *Biochem Pharmacol* 2009;**78**(8):927–932
14. Oz M, Lorke DE, Hasan M, Petroianu GA. Cellular and molecular actions of Methylene Blue in the nervous system. *Med Res Rev* 2011;**31**(1):93–117
15. Evora PR, Evora PM, Celotto AC, Rodrigues AJ, Joviliano EE. Cardiovascular therapeutics targets on the NO-sGC-cGMP signaling pathway: a critical overview. *Curr Drug Targets* 2012; **13**(9):1207–1214
16. Wainwright M, Crossley KB. Methylene Blue—a therapeutic dye for all seasons? *J Chemother* 2002;**14**(5):431–443
17. Bradberry SM. Occupational methaemoglobinaemia. Mechanisms of production, features, diagnosis and management including the use of methylene blue. *Toxicol Rev* 2003;**22**(1):13–27
18. Patel HP, Chadwick DR, Harrison BJ, Balasubramanian SP. Systematic review of intravenous methylene blue in parathyroid surgery. *Br J Surg* 2012;**99**(10):1352
19. Jang DH, Nelson LS, Hoffman RS. Methylene blue for distributive shock: a potential new use of an old antidote. *J Med Toxicol* 20139(3):242–249
20. Lo JC, Darracq MA, Clark RF. A review of methylene blue treatment for cardiovascular collapse. *J Emerg Med* 2014;**46**(5): 670–679
21. Kim HS, Berstad A. Experimental colitis in animal models. *Scand J Gastroenterol* 1992;**27**(7):529–537
22. Kawada M, Arihiro A, Mizoguchi E. Insights from advances in research of chemically induced experimental models of human inflammatory bowel disease. *World J Gastroenterol* 2007;**13**(42):5581–5593
23. Morris GP, Beck PL, Herridge MS, Depew WT, Szewczuk MR, Wallace JL. Hapten-induced model of chronic inflammation and ulceration in the rat colon. *Gastroenterology* 1989;**96**(3):795–803
24. Galili Y, Ben-Abraham R, Rabau M, Klausner J, Kluger Y. Reduction of surgery-induced peritoneal adhesions by methylene blue. *Am J Surg* 1998;**175**(1):30–32
25. Kluger Y, Weinbroum A, Ben-Abraham R, Galili Y, Klausner J, Rabau M. Reduction in formation of peritoneal adhesions by methylene blue in rats: a dose response study. *Eur J Surg* 2000; **166**(7):568–571
26. Dinc S, Ozaslan C, Kuru B, Karaca S, Ustun H, Alagol H, *et al*. Methylene blue prevents surgery-induced peritoneal adhesions but impairs the early phase of anastomotic wound healing. *Can J Surg* 2006;**49**(5):321–328
27. Appleyard CB, Wallace JL. Reactivation of hapten-induced colitis and its prevention by anti-inflammatory drugs. *Am J Physiol* 1995;**269**(1 Pt 1):G119–G125
28. Osanai M, Nishikiori N, Murata M, Chiba H, Kojima T, Sawada N. Cellular retinoic acid bioavailability determines epithelial integrity: role of retinoic acid receptor alpha agonists in colitis. *Mol Pharmacol* 2007;**71**(1):250–258
29. Mullane KM, Kremer R, Smith B. Myeloperoxidase activity as a quantitative assessment of neutrophil infiltration into ischemic myocardium. *J Pharmacol Methods* 1985;**14**(3):157–167
30. Fridovich I. Superoxide radical: an endogenous toxicant. *Annu Rev Pharmacol Toxicol* 1983;**23**:239–257
31. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; **193**:265–275
32. Galvez J, Garrido M, Merlos M, Torres MI, Zarzuelo A. Intestinal anti-inflammatory activity of UR-12746, a novel 5-ASA conjugate, on acute and chronic experimental colitis in the rat. *Br J Pharmacol* 2000;**130**(8):1949–1959
33. Mayer B, Brunner F, Schmidt K. Inhibition of nitric oxide synthesis by methylene blue. *Biochem Pharmacol* 1993;**45**(2): 367–374
34. Kankuri E, Hamalainen M, Hukkanen M, Salmenpera P, Kivilaakso E, Vapaatalo H, *et al*. Suppression of pro-inflammatory cytokine release by selective inhibition of inducible nitric oxide synthase in mucosal explants from patients with ulcerative colitis. *Scand J Gastroenterol* 2003;**38**(2): 186–192
35. Sung MK, Park MY. Nutritional modulators of ulcerative colitis: clinical efficacies and mechanistic view. *World J Gastroenterol* 2013;**19**(7):994–1004
36. Gentschew L, Ferguson LR. Role of nutrition and microbiota in susceptibility to inflammatory bowel diseases. *Mol Nutr Food Res* 2012;**56**(4):524–535
37. Kelner MJ, Bagnell R, Hale B, Alexander NM. Methylene blue competes with paraquat for reduction by flavo-enzymes resulting in decreased superoxide production in the presence of heme proteins. *Arch Biochem Biophys* 1988;**262**(2):422–426
38. Piechota-Polanczyk A, Fichna J. The role of oxidative stress in pathogenesis and treatment of inflammatory bowel diseases. *Naunyn Schmiedebergs Arch Pharmacol* 2014;**387**(7):605–620
39. Minaiyan M, Hajhashemi V, Rabbani M, Fattahian E, Mahzouni P. Evaluation of anti-colitic effect of fluvoxamine against acetic acid-induced colitis in normal and reserpinized depressed rats. *Eur J Pharmacol* 2015;**746**:293–300
40. Wu XF, Ouyang ZJ, Feng LL, Chen G, Guo WJ, Shen Y, *et al*. Suppression of NF-κB signaling and NLRP3 inflammasome activation in macrophages is responsible for the amelioration of experimental murine colitis by the natural compound fraxinellone. *Toxicol Appl Pharmacol* 2014;**281**(1):146–156
41. Roediger WE. Nitric oxide from dysbiotic bacterial respiration of nitrate in the pathogenesis and as a target for therapy of ulcerative colitis. *Aliment Pharmacol Ther* 2008;**27**(7):531–541

42. Corsetti M, Vos R, Gevers A, Demedts I, Janssens J, Tack J. Influence of nitric oxide synthase inhibition on the motility and sensitivity of distal colon in man. *Neurogastroenterol Motil* 2013;**25**(4):e256–e262
43. Huang C, Tong L, Lu X, Wang J, Yao W, Jiang B, Zhang W. Methylene blue attenuates iNOS induction through suppression of transcriptional factor binding amid iNOS mRNA transcription. *J Cell Biochem* 2015;**116**(8):1730–1740
44. Fink MP. Intestinal epithelial hyperpermeability: update on the pathogenesis of gut mucosal barrier dysfunction in critical illness. *Curr Opin Crit Care* 2003;**9**(2):143–151
45. Werner I, Guo F, Stock UA, Lupinski M, Meybohm P, Moritz A, Beiras-Fernandez A. Methylene blue modulates adhesion molecule expression on microvascular endothelial cells. *Inflamm Res* 2014;**63**(8):649–656