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Effects of gallic acid in a rat model of inflammatory bowel disease induced by trinitrobenzenesulfonic acid

V. P. Marinov[1], M. A. Tzaneva[2], M. D. Zhelyazkova-Savova[1], S. Gancheva[1], S. V. Valcheva-Kuzmanova[1]*

[1]*Department of Pharmacology and Clinical Pharmacology and Therapeutics, Medical University Prof. Dr. Paraskev Stoyanov, Varna, Bulgaria*

[2]*Department of Preclinical and Clinical Sciences, Medical University Prof. Dr. Paraskev Stoyanov, Varna, Bulgaria*

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Inflammatory bowel disease is a group of chronic disorders of the colon and small intestine. Trinitrobenzenesulfonic acid (TNBS)-induced experimental colitis is a commonly used model to investigate its pathogenesis. Gallic acid (GA) is a naturally occurring phenolic acid, possessing promising antioxidant and anti-inflammatory properties.

The aim of the study was to evaluate the effects of GA in a TNBS-induced rat colitis model.

Male Wistar rats were divided in 5 experimental groups: control and TNBS, receiving distilled water, and three groups treated respectively with GA at doses of 20, 40, and 80 mg/kg orally for 8 days. The treatment started 24 hours after the induction of colitis that was achieved by rectal administration of TNBS to all groups except for the control one. The body weight and stool consistency were monitored. The severity of colitis was evaluated by macroscopic and histopathological examination. Oxidative stress was assessed in rat serum.

The results showed that GA decreased the weight loss and diarrhea severity. The macroscopic signs of TNBS-induced colitis were ameliorated. The necrotic area, colon weight, adhesions to adjacent organs, and the wall thickening did not differ significantly in GA groups compared to the control. The histopathological results showed some improvement in all GA groups regarding the epithelium injury and inflammatory cell infiltration score. The markers of oxidative stress were reduced in GA-treated groups.

In conclusion, GA decreased the TNBS-induced damage in the experimental model of colitis. The beneficial effect of GA might be related to its antioxidant, anti-inflammatory and astringent properties.

Key words: Inflammatory bowel disease, TNBS-induced colitis, gallic acid, rat model

INTRODUCTION

Inflammatory bowel disease (IBD) is a group of inflammatory diseases of the gastrointestinal tract with a chronic and relapsing occurrence of the symptoms. The two major forms are ulcerative colitis (UC) and Crohn's disease (CD). The exact risk factors and the etiopathological processes involved are not yet completely elucidated. Genetic contributions have been identified epidemiologically [1]. It is accepted nowadays that in IBD environmental factors trigger an abnormal immune response against the gut microbial flora in a genetically susceptible host [2]. Free radicals, produced during the inflammatory response, play an important role in the pathogenesis of the disease.

Different chemically induced or genetically engineered animal models have been developed to study IBD. Chemically induced models of intestinal inflammation are most commonly used. One of them uses trinitrobenzenesulfonic acid (TNBS) which induces severe colonic inflammation when administered intrarectally in rodents. TNBS binds to high molecular weight tissue proteins, renders them immunogenic, leading to acute Th1 inflammation [3]. The resulting colitis presents clinical and histopathological findings that resemble those seen in CD [4, 5].

Polyphenols are some of the most widely distributed compounds synthesized in plants, possessing strong antioxidant and other biological activities [6]. There are numerous reports showing beneficial properties of polyphenols in inflammatory conditions including IBD models [7]. Gallic acid (GA) is a naturally occurring phenolic acid, possessing promising antioxidant and anti-inflammatory properties.

The aim of this study was to investigate the effects of GA in a rat model of TNBS-induced colitis.

EXPERIMENTAL

Experimental substances

2,4,6-trinitrobenzenesulfonic acid (TNBS) and gallic acid (GA) were purchased from Sigma-Aldrich (Germany).

Animals

The study was carried out on 60 male Wistar rats (weight 250-350 g). The animals were housed in plastic cages in a well-ventilated room at a temperature of 22 ± 1 °C and on a 12/12 light/dark

* To whom all correspondence should be sent:
E-mail: stefkavk@yahoo.com

cycle. Rats were deprived of food for 24 h before the induction of colitis. Throughout the rest of the experiment, the animals had free access to food and drinking water. The animals were divided in 5 experimental groups, each of 12 rats: Control, TNBS, TNBS+GA20, TNBS+GA40 and TNBS+GA80.

All procedures concerning animal treatment and experimentation were conducted in compliance with the national laws and policies, in conformity with the international guidelines (EU Directive 2010/63/EU for animal experiments).

Induction of colitis

Colitis was induced according to the procedure described by Morris et al. [8]. The animals were anesthetized with thiopental intraperitoneally (50 mg/kg, dissolved in saline to a volume of 2 ml/kg). TNBS (10 mg dissolved in 0.25 ml of 50% ethanol) was inserted in the colon by a soft cannula (external diameter 1.5 mm) at a depth of 8 cm from the anus. Control rats received 0.25 ml of 50% ethanol intrarectally. The animals were kept in a head-down position for 10 min to prevent the leakage of fluid from the anus.

Oral treatment

The oral treatment with GA or the solvent (distilled water) was given by an orogastric cannula from the 2nd day (24 h after the induction of colitis) and lasted until the 9th day of the experiment. Groups Control and TNBS received distilled water (10 ml/kg). Groups TNBS+GA20, TNBS+GA40 and TNBS+GA80 were treated with GA at doses of 20 mg/kg, 40 mg/kg, 80 mg/kg, respectively, dissolved in distilled water to a total volume of 10 ml/kg. According to previous studies, GA treatment is not associated with toxicity even if used in doses as high as 1000 mg/kg [9]. The doses of GA used in this experiment were similar to those used by other authors for evaluation of its anti-inflammatory effect [10,11].

Body weight

The initial body weight of the animals was measured 24 hours before the colitis induction. The subsequent body weights were measured on the 2nd, 3rd, 7th and 10th day after the induction of colitis. The weight gain or reduction was also calculated.

Diarrhea score

During the first three days after the induction of colitis, the degree of diarrhea and the presence of blood in the stools were evaluated daily. Regarding the consistency of the stools, wet and pasty stools were scored one point and semiliquid or watery diarrhea – two points [12]. Presence of blood in the stools was marked as one point and severe rectal bleeding – as two points.

Macroscopic assessment of colitis

The animals were anesthetized with diethyl ether and sacrificed on the 10th experimental day. Blood for biochemical tests was collected from the sublingual veins. A laparotomy was performed for macroscopic evaluation. Adhesions of the colon to adjacent organs and sings of obstruction were evaluated. The large intestine was removed from the anus to the caecum, then opened longitudinally and cleaned. The length (cm) and the weight (g) of the organ were recorded and the dimensions of the necrotic area were measured (mm). Thickening of the colon wall was also evaluated [13]. The following scores were used: Adhesions: 0 = No adhesions, 1 = Difficult dissection, 2 = Visible adhesions, 3 = "Wrapped" intestine; Obstruction: 0 = No obstruction, 1 = Need for gentle manual cleaning, 2 = Fecal impaction; Wall thickening: 0 = Similar to uninflamed intestine, 1 = Thicker than normal (1–2 mm), 2 = Much thicker than normal (> 2 mm).

Histopathological assessment of colitis

Samples of colon wall from the area of injury (or a corresponding site, if no detectable alterations) were taken and fixed in 10% neutral-buffered formaldehyde solution. Fixed tissues were embedded in paraffin blocks, cut into sections and stained with hematoxylin and eosin (H&E) for light microscopy histopathological investigation.

To evaluate microscopic lesions in the large intesine, scoring criteria were used according to Elli et al. [14]. The scores for epithelium and glands were as follows: 0 = normal, 1 = focal destruction of epithelial surface and/or glands, 2 = zonal destruction of epithelial surface and/or zonal crypt loss, 3 = diffuse mucosal ulceration involving submucosa and/or diffuse crypt loss. The following scores for inflammatory cell infiltration were used: 0 = absence of infiltrate, 1 = subepithelial and in the lamina propria, 2 = infiltrate reaching the muscularis mucosae, 3 = severe and diffuse infiltrate reaching the submucosa and/or involving the muscularis propria.

Thiobarbituric acid reactive substances (TBARS) assay

Blood, collected from the sublingual veins before the sacrifice of the experimental animals, was centrifuged at 2000 rpm for 10 min to obtain serum.

Thiobarbituric acid reactive substances (TBARS), as end products of lipid peroxidation, served as markers of oxidative stress. TBARS were determined in rat serum spectrophotometrically according to the method of Ohkawa et al. [15]. The method measures quantitatively the colored product from the reaction of thiobarbituric acid with lipid peroxides at 532 nm. TBARS concentration was determined in nmol/ml serum. Malondialdehyde, the major lipid peroxide obtained in the process of peroxidation of membrane polyunsaturated fatty acids, was used as a standard.

Statistical analysis

Results are presented as means ± S.E.M. The data were analyzed by one-way ANOVA, followed by Dunnett's multiple comparison test. A level of $p < 0.05$ was considered significant. All analyses were performed using GraphPad Prism statistical software, version 5.00.

RESULTS

Body weight changes and diarrhea severity

The average body weight of all groups, measured on the 1st and 3rd day after colitis induction, was reduced. On the 7th experimental day, there was a reduction of the body weight of all TNBS-treated groups, while control animals showed a small increase in body weight. The weight loss was significant in TNBS group compared to the control ($p < 0.05$) and was not significant in the groups treated with GA (Fig. 1A).

The animals showed signs of diarrhea, more prominent in the TNBS-treated groups, up to 3 days after the induction of colitis. Increased frequency of defecation, pasty or semiliquid consistency of stools and rectal bleeding appeared in most of the animals. On the 1st day after the induction of colitis, statistically significant impairment was observed in all TNBS-treated groups compared to the control group (Fig. 1B). On the 2nd day after colitis induction, the diarrhea score was higher than the control in all TNBS-treated groups, except for the group treated with the highest GA dose (Fig. 1C). On the 3rd day after colitis induction, statistically significant differences in the diarrhea score, compared to control, were observed only in the TNBS group. All the groups treated with GA showed an improvement. The diarrhea scores of GA-treated groups did not differ significantly from the control score. The diarrhea score of TNBS+GA80 group was significantly lower ($p < 0.05$) than that of TNBS group (Fig. 1D).

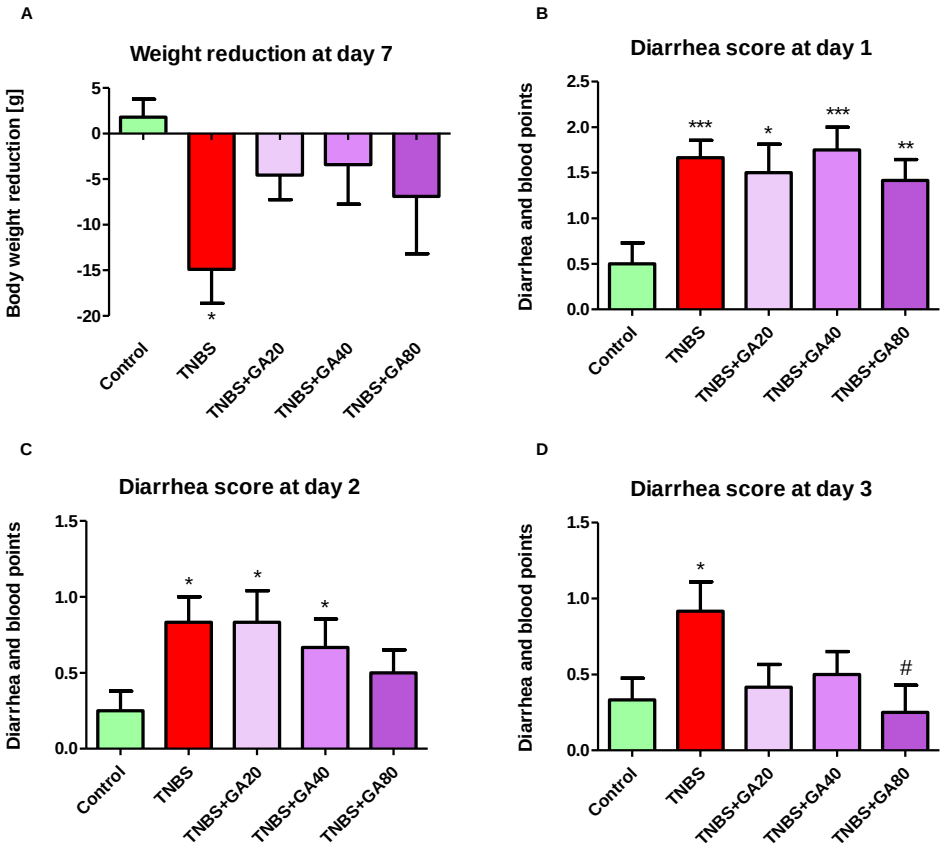

Fig. 1. Effect of gallic acid (GA) at doses of 20, 40, 80 mg/kg on weight changes (A) and diarrhea score on the 1st (B), 2nd (C), and 3rd (D) day after the induction of colitis in a model of 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. Control; #$p < 0.05$ vs TNBS.

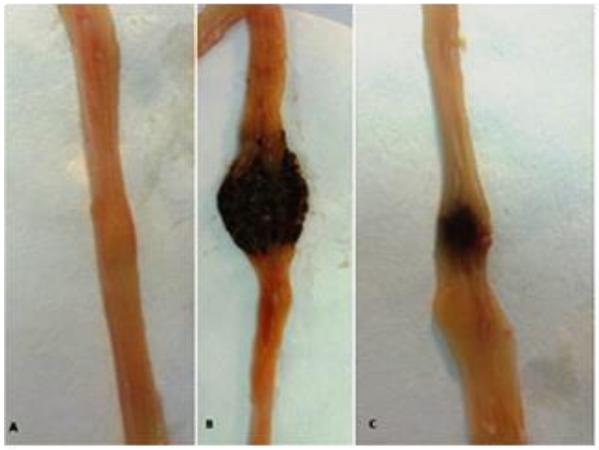

Fig. 2. Macroscopic appearance of rat colons in a model of 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis: A. Control; B. TNBS; C. TNBS+GA80.

Macroscopic assessment of colitis

The typical macroscopic appearances of the colon of rats belonging to groups Control, TNBS and TNBS+GA80 are shown on Fig. 2. The rats form the control group showed similar to normal macroscopic appearance of the colon (Fig. 2A). In the TNBS group, there were hemorrhagic ulcerations, covered with a fibrinoid necrotic matter at the site of TNBS application (Fig. 2B).

The results from the macroscopic evaluation of colitis severity are presented on Fig. 3. In the TNBS group, the area of necrosis was extensive with a mean value of 3.5 ± 1.0 cm^2 (Fig. 3A). As a result of the inflammation, the weight of the colon at the site of injury was increased (Fig. 3C), as was the ratio between the colon weight and length (Fig. 3D). The colon wall was thicker than normal (Fig. 3E).The adherence of the colon to adjacent organs was pronounced (Fig. 3F). All macroscopic features used for evaluation of colitis severity, excluding colon length and wall thickening, were significantly different from those of the control group ($p < 0.05$).

Treatment of animals with GA improved the macroscopic signs of colitis. The necrotic area, the colon weight and length, the thickening of the colon wall, and the adhesion scores of GA-treated groups did not differ significantly from those of the control animals (Fig. 3).

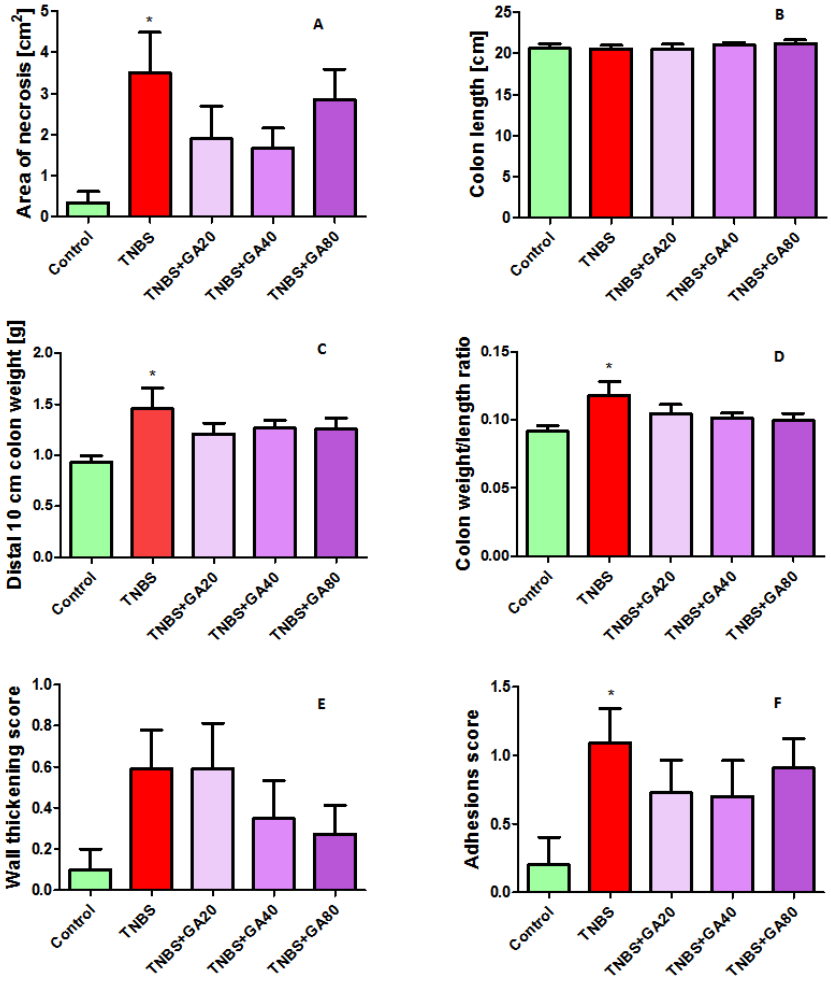

Fig. 3. Effect of gallic acid (GA) at doses of 20, 40 and 80 ml/kg on macroscopic indices of colonic damage in a rat model of 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis; *$p < 0.05$ vs. Control.

Histopathological examination and microscopic scoring for colitis evaluation

The histological examination of colon samples is presented on Fig. 4. The colon samples of control rats showed a normal microscopic appearance (Fig. 4A). TNBS caused a variable degree of alterations on the colon wall – from focal and zonal destructions of the epithelial surface to diffuse ulcerations involving the submucosa. Inflammatory cell infiltration varied from subepithelial or in lamina propria to reaching the submucosa and muscularis propria. The epithelium injury and inflammatory cell infiltration were most severe in the TNBS group (Fig. 4B). The changes were less pronounced in GA-treated animals (Fig. 4C).

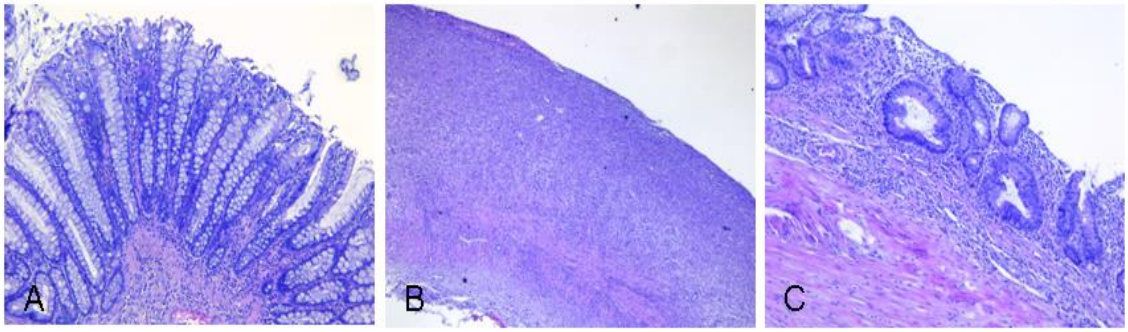

Fig. 4. Microscopic appearance of colons in a rat model of 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis: A. Control – Normal intestinal wall; B. TNBS – Diffuse destruction and inflammatory cell infiltration of intestinal wall; C. TNBS+GA40 – Zonal destruction of epithelial surface and inflammatory cell infiltration involving the muscularis mucosae. H & E staining; magnification x 100

The microscoping scoring of colonic damage is shown on Fig. 5. GA only slightly attenuated the microscopic signs of colonic damage. The epithelium destruction score (Fig. 5A) and the cell infiltration score (Fig. 5B) were highest in the TNBS group. The lowest epithelium injury was observed in TNBS+GA20 rats (Fig. 5A), and the lowest cell infiltration score was found in TNBS+GA40 and TNBS+GA80 groups (Fig. 5B) but the decrease was not significant.

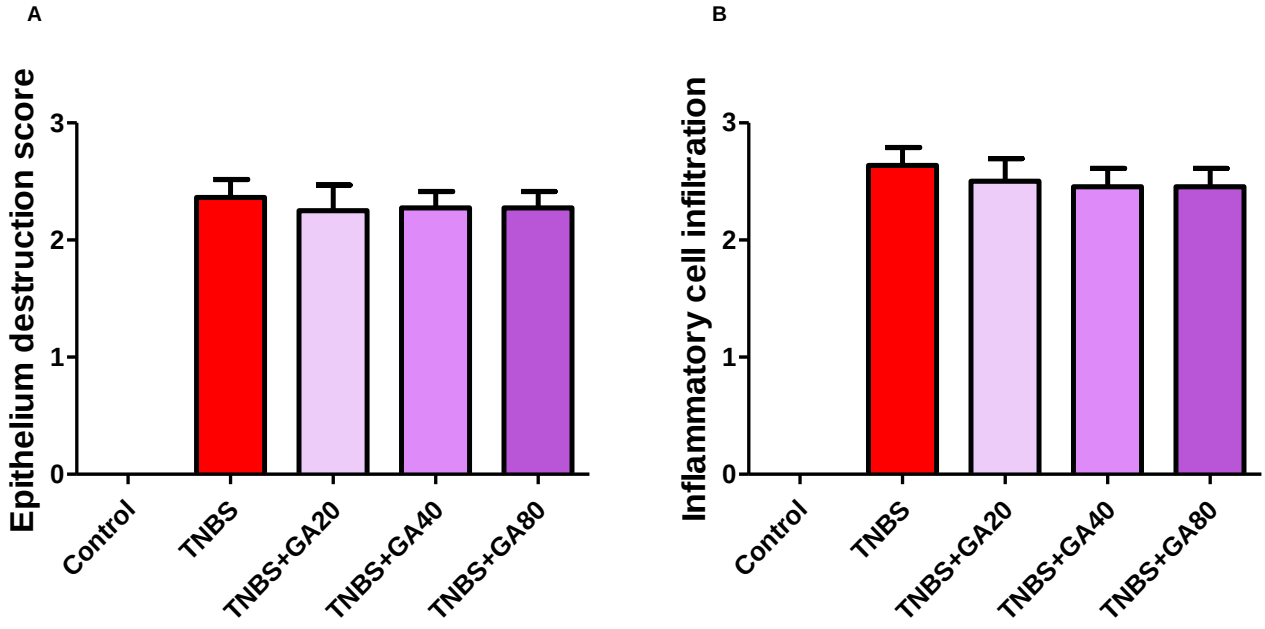

Fig. 5. Effect of gallic acid (GA) at doses of 20, 40 and 80 ml/kg on microscopic scoring of colonic damage in a rat model of 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis.

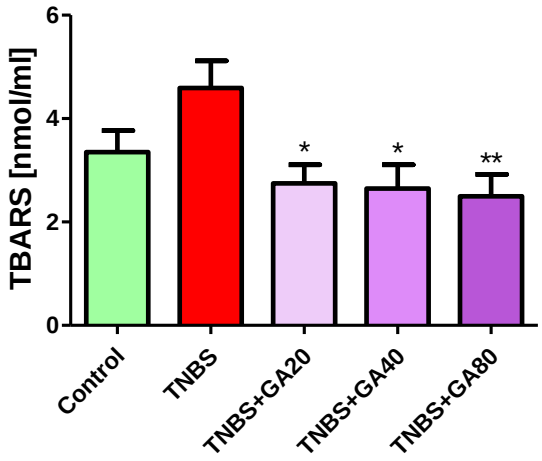

Fig. 6. Effect of gallic acid (GA) at doses of 20, 40 and 80 ml/kg on serum thiobarbituric acid reactive substances (TBARS) in a rat model of 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis; *p < 0.05, **p < 0.01 vs. Control

Thiobarbituric acid reactive substances (TBARS)

TNBS caused an increase of TBARS. The serum TBARS concentrations were significantly lower in all groups treated with GA, compared to the TNBS group (Fig. 6).

DISCUSSION

The current investigation was undertaken in order to study the effects of GA on the severity of TNBS-induced experimental rat colitis. This model of colitis was chosen because of its significant macroscopic, histologic and immunologic similarities with IBD in humans, especially CD [16]. TNBS in combination with ethanol, applied rectally, causes initially an acute and thereafter, a chronic colitis. Ethanol, as a breaker of the mucosal barrier, makes possible the interaction of TNBS with the tissue proteins of the colon and the resulting immune response [17]. In the current experiment, TNBS caused severe ulcerative colitis, associated with a decrease in animal weight and a change in number of functional, macroscopic and microscopic indices.

In IBD, due to the inflammation present, free radicals like reactive oxygen species (ROS) and reactive nitrogen species are formed. The main leukocyte enzymes involved in the synthesis of ROS are myeloperoxidase (MPO) and nitric oxide synthase, catalyzing the formation hypochlorous acid (HOCl) and nitric oxide (NO), respectively [18]. These molecules are crucial for the bactericidal leucocyte action during the inflammatory response, but they also readily interact with cellular proteins, lipids and nucleic acids. As a result, cellular and tissue damage is caused and other highly reactive, harmful molecules, such as lipid peroxides, are formed.

GA, similarly to other phenolic acids, exhibits a strong MPO inhibitory activity [19]. It has been found to act as a free radical scavenger having the highest antioxidant capacity among various other phenolic acids (chlorogenic, protocatechuic and vanillic acid) [20]. The activity was about 40% higher than the next most active compound – chlorogenic acid. The three hydroxyl groups in the molecule of GA, not only contribute significantly to the antioxidant activity, but also provide a greater stability of the molecule, because of the hydrogen bonds between these groups [20]. In this experiment, GA reduced the serum concentrations of TBARS, markers of oxidative stress. Thus, the antioxidant properties of GA probably play an important role in the observed beneficial effects.

Gallic acid has also shown pronounced anti-inflammatory properties, studied in a murine macrophage cell line. It inhibited prostaglandin E2 production after lipopolysaccharide stimulation, without exerting cytotoxic effects at the same time [21]. The anti-inflammatory activity of GA has been reported also in a model of zymosan-induced acute paw swelling in mice. In vitro studies on the mode of action of GA revealed that this molecule interfered with the functioning of polymorphonuclear leukocytes. Structure-activity relationship analysis showed that the o-dihydroxy group of GA was important for its activity in vitro [22]. Scavenging of superoxide anions, and inhibition of MPO synthesis and activity probably also contribute to the inhibition of the inflammatory process.

GA possesses some astringent properties [23] which might contribute to the decrease of the diarrhea score observed in this experiment. The astringent action causes coagulation of the proteins on the surface of the intestinal mucosa. If mucosal ulcerations are present, the proteins on their surface are also coagulated. The final result is formation of a protective, insoluble protein layer, reduction of hemorrhage and secretions, and protection of epithelial cells underneath, that speeds up the regeneration process.

All these activities probably act together and may explain the beneficial effects of GA in TNBS-induced rat colitis, demonstrated in our experiment.

In conclusion, GA decreased the TNBS-induced damage in the experimental model of colitis. In addition to the local macroscopic and histopathological improvement of colitis, GA reduced the markers of oxidative stress in the rat serum. The effect of GA in this experiment might be

related to its antioxidant, anti-inflammatory, and astringent properties.

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