

ORIGINAL RESEARCH

The effect of betulinic acid on TNBS-induced experimental colitis

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ABSTRACT: In this study we have investigated the possible protective effect of betulinic acid (BA) on colonic inflammation in rats. Colitis was induced in Sprague-Dawley rats of both sexes by intracolonic administration of 1 ml trinitrobenzene sulphonic acid (TNBS). Colitis-induced rats received orogastrically either betulinic acid (50 mg/kg/day) or vehicle (0.05% DMSO) for 3 days. At the 72nd hour of colitis induction, the rats were decapitated and trunk blood was collected for the measurement of TNF- α , IL-1 β , lactate dehydrogenase (LDH) levels and total antioxidant capacity (AOC). The distal 8 cm of colon were scored macroscopically, and the degree of oxidant damage was evaluated by malondialdehyde (MDA) and glutathione (GSH) levels, myeloperoxidase activity (MPO), collagen content and by histological analysis. Generation of oxidants was evaluated by tissue luminol and lucigenin chemiluminences (CL). Colitis caused significant increases in the colonic CL values, macroscopic damage scores, MDA, MPO and collagen levels, along with a significant decrease in tissue GSH level. Similarly, serum TNF- α , IL-1 β , as well as LDH were elevated and AOC was reduced in the vehicle-treated colitis group as compared to control group. On the other hand, betulinic acid treatment reversed all these biochemical indices, as well as histopathological alterations induced by TNBS, suggesting that betulinic acid protects the colonic tissue via its radical scavenging and antioxidant activities.

KEY WORDS: betulinic acid; colitis; oxidative damage; inflammation; trinitrobenzene sulphonic acid

INTRODUCTION

Inflammatory bowel diseases (IBD) are idiopathic chronic inflammation in gut with diffuse inflammation of the colon and rectum (1). Although the precise etiology of IBD is not known, many factors have been implicated; including neutrophil infiltration and overproduction of proinflammatory mediators such as cytokines, arachidonate metabolites and reactive oxygen mediators (2). Medication of IBD includes glucocorticoids, 5-aminosalicylic acid and immunosuppressive agents (3). However due to adverse effects of steroids, reduced effectiveness of 5-aminosalicylic acid in severe IBD or serious complications

of immunosuppressive agents, their usage in IBD is limited and novel agents useful for the treatment of IBD are being developed.

Different parts of the plants are used in the traditional system of medicine for the treatment of various human ailments (4). Plant-derived biological compounds with antioxidant properties may contribute to the protection of cells and tissues against deleterious effects of reactive oxygen species (ROS) (6,7). Betulinic acid is a naturally occurring pentacyclic triterpene. It has several botanical sources, but can also be chemically derived from betulin, a substance found in abun-

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dance in the outer bark of white birch trees (*Betula alba*) (7). Betulinic acid has been found to selectively kill human melanoma cells while leaving healthy cells alive and also several betulinic acid derivatives are potent and highly selective inhibitors of HIV-1 (8). Since triterpenoids have a similarity to steroidal compounds, their effects have often been attributed to a mechanism related to antiinflammatory action. In our previous study we have demonstrated that betulinic acid attenuates ischemia/reperfusion-induced oxidant responses and improved renal function by regulating apoptotic function of leukocytes and inhibiting neutrophil infiltration (9).

On the basis of this background, using biochemical and histological examination, we aimed to study the putative protective effects of betulinic acid on the colonic tissue in a rat model of colitis.

MATERIALS AND METHODS

Animals

Adult Sprague-Dawley rats (250–300, both sexes) were kept in a light- and temperature-controlled room with 12:12-h light-dark cycles, where the temperature (22 ± 0.5 °C) and relative humidity (65–70 %) were kept constant. The animals were fed a standard pellet and food was withdrawn overnight before colitis induction. Access to water was allowed ad libitum. Experiments were approved by the Marmara University Animal Care and Use Committee.

Induction of colitis and drug administration

Animals were fasted for 18 h before the induction of colitis. Under light ether anesthesia, a polyethylene catheter (PE-60) was inserted into the colon with its tip positioned 8 cm from the anus. To induce colitis, a single solution of 1 ml of a 30 mg/ml trinitrobenzene sulphonic acid (TNBS) solution, dissolved in 40 % ethanol in saline was instilled. The rats in the control group were subjected to the same procedure with the exception that an equal volume of isotonic saline was substituted for TNBS. Betulinic acid [(3beta)-3-hydroxylup-20(29)-en-28-oic acid; Sigma-Aldrich, St. Louis, MO, USA,] was dissolved in 0.05% DMSO as a vehicle. Betulinic acid (50 mg/kg; i.p.; colitis+BA group) or vehicle were given orally 5 min after induction of colitis and the treatment was continued for the following 3 days. Similarly, control rats were also treated with either betulinic acid or vehicle. Each group consists of 8 rats. At the 72nd hour of experiment, rats were decapitated and trunk blood was collected for the assessment of lactate dehydrogenase (LDH) activity, as a marker of tissue injury, and the levels of the pro-inflammatory cytokines, TNF- α and IL-1 β and antioxidant capacity (AOC). Distal 8 cm of the colon obtained from each animal were initially examined for recording macroscopic damage scores and tissue wet weight index (WWI), and then stored at -80 °C until the determination of malondialdehyde (MDA), glutathione (GSH) levels, myeloperoxidase activity (MPO), collagen content and luminol and lucigenin chemiluminescence (CL). For the histological analysis, extra 1-square cm samples were obtained from each animal at 8 cm from anus to be fixed in formaldehyde.

Assessment of colitis severity

The distal 8 cm of the colons were opened longitudinally down their mesenteric borders, cleansed of luminal contents, gently rinsed in saline and dried on filter paper. The severity of colitis

was assessed using macroscopic and microscopic damage scoring, WWI and tissue collagen content.

Three days after the induction of colitis, all rats were decapitated. The last 8 cm of the colon was excised, opened longitudinally, and rinsed with saline solution. The mucosal lesions were scored macroscopically using the criteria outlined in Table 1 (10). The scoring of colonic damage was performed by an observer who was unaware of the treatments received by the rats. After scoring, tissue weights were recorded, corrected for body weight and expressed as tissue WWI (g/100 g body weight).

TABLE 1. Criteria for macroscopic scoring of colonic lesions

Score	Appearance
0	No damage
1	Localized hyperemia, no ulcers
2	Ulceration without hyperemia or bowel wall thickening
3	Ulceration with inflammation at one site
4	Two or more sites of ulceration/inflammation
5	Major sites of damage extending more than 1 cm along the length of colon
6–10	If damage extends more than 2 cm along the length of colon, the score is increased by one for each additional 1 cm

Biochemical analysis

Measurement of serum lactate dehydrogenase (LDH) activity, and cytokine levels

Lactate dehydrogenase (LDH) activity, an indicator of tissue damage, was determined spectrophotometrically using an automated analyzer (Bayer Opera biochemical analyzer, Germany) (11). Plasma levels of TNF- α , and IL-1 β were quantified using enzyme-linked immunosorbent assay (ELISA) kits specific for the rat cytokines according to the manufacturer's instructions and guidelines (Biosource Europe S. A., Nivelles, Belgium). The total AOC in plasma was measured by using colorimetric test system (ImAnOx, catalogue no.KC5200, Immunodiagnostic AG, D-64625 Bensheim, Germany), according to the instructions provided by the manufacturer. These particular assay kits were selected because of their high degree of sensitivity, specificity, inter- and intraassay precision and small amount of plasma sample required conducting the assay.

Chemiluminescence (CL) assay

To assess the contribution of reactive oxygen species in ethanol-induced tissue damage, luminol and lucigenin chemiluminences were measured as indicators of radical formation. Measurements were made at room temperature using Junior LB 9509 luminometer (EG&G Berthold, Germany). Specimens were put into vials containing PBS-HEPES buffer (0.5 M PBS containing 20 mM HEPES, pH 7.2). ROS were quantitated after the addition of enhancers, lucigenin or luminol, for a final concentration of 0.2 mM. Counts were obtained at 1 min intervals and the results were given as the area under curve (AUC) for a counting period of 5 min. Counts was corrected for wet tissue weight and expressed as relative light units (rlu/mg tissue) (12).

Tissue malondialdehyde (MDA) and glutathione (GSH) assays

Colonic samples were homogenized in ice-cold 150 mM KCl for the determination of MDA and GSH levels. The MDA lev-

els were assayed for products of lipid peroxidation (13). Results were expressed as nmol MDA g⁻¹ tissue. GSH was determined by the spectrophotometric method using Ellman's reagent (14) and the results were expressed as µmol GSH g⁻¹ tissue.

Tissue myeloperoxidase (MPO) activity

The activity of tissue-associated myeloperoxidase (MPO), a natural constituent of primary granules of neutrophils, was determined in the colonic samples according to the method of Hillegass et al. (15). Since a direct relationship between the tissue MPO activity and the number of neutrophils was previously shown (16), MPO activity was regarded as an indication of neutrophil accumulation. All reagents for MPO assay were obtained from Sigma. The tissue samples (0.2–0.3 g) were homogenized in 10 volumes of ice-cold potassium phosphate buffer (50 mM K₂HPO₄, pH 6.0) containing hexadecyltrimethylammonium bromide (HETAB; 0.5%, w/v). Tissue samples were homogenized in 50 mM potassium phosphate buffer (PB, pH 6.0), and centrifuged at 41,400 g (10 minutes); pellets were suspended in 50mMPB containing 0.5 % hexadecyltrimethylammonium bromide. After three freeze and thaw cycles, with sonication between cycles, the samples were centrifuged at 41,400 g for 10 minutes. Aliquots (0.3 ml) were added to 2.3 ml of reaction mixture containing 50 mM PB, o-dianisidine, and 20 mM H₂O₂ solution. One unit of enzyme activity was defined as the amount of MPO present that caused a change in absorbance measured at 460 nm for 3 min. MPO activity was expressed as U/g tissue.

Tissue collagen was measured as a free radical-induced fibrosis marker. Tissue samples were cut with a razor blade, immediately fixed in 10% formalin then samples were embedded in paraffin, and sections, approximately 15 µm thick were obtained. The evaluation of collagen content was based on the method published by Lopez De Leon and Rojkind (17), which is based on selective binding of the dyes Sirius Red and Fast Green FCF to collagen and noncollagenous components, respectively. Both dyes were eluted readily and simultaneously by using 0.1 N NaOH-methanol (1:1, v/v). Finally, the absorbances at 540 and 605 nm were used to determine the amount of collagen and protein, respectively.

Histological evaluation

Full-thickness colon samples were collected for histological examination and fixed in 10% neutral buffered formalin solution. After fixation, tissue samples were dehydrated in

graded ethanol series, cleared in toluene and embedded in paraffin. Tissue sections (5 microns thick) were stained by routine hematoxylin and eosin (H&E) stain for general morphological evaluation and Alcian Blue stain for mucin demonstration. Stained sections were examined and photographed under an Olympus BX51 photomicroscope (Tokyo, Japan).

Statistical analysis

All data are expressed as mean ± SEM. Statistical analysis was carried out using InStat statistical package (GraphPad Software, San Diego, CA, USA). Following the assurance of normal distribution of data, groups of data were compared with one-way analysis of variance (ANOVA) followed by Tukey-Kramer post hoc test for multiple comparisons. Values of p<0.05 were regarded as significant.

RESULTS

Severity of colonic injury

When compared with the colonic tissue of the control group, TNBS administered rats showed increased tissue WWI and the macroscopic damage score (p<0.001). On the other hand, betulinic acid treatment reduced both parameters significantly (p<0.05- 0.01), however they were still higher than control (p<0.001; Figure 1).

Biochemical parameters in the plasma

As shown in Figure 2, plasma cytokines, TNF-α and IL-1β, and lactate dehydrogenase (LDH) activity were significantly elevated in the vehicle-treated colitis group (p<0.001), while AOC was decreased. On the other hand, betulinic acid treatment decreased the plasma TNF-α, IL-1β levels and LDH activity and increased AOC levels (p<0.05-0.01).

Luminol and lucigenin chemiluminescence (CL) levels

Luminol and lucigenin CL levels in the vehicle-treated colitis group were increased dramatically (p<0.01-0.001) as compared to those in the control group, while erdosteine treatment following colitis induction prevented radical formation (p<0.01-0.001; Figure 3).

Colonic malondialdehyde (MDA) and glutathione (GSH) levels, myeloperoxidase (MPO) activity and collagen content

Colitis induction with TNBS followed by vehicle treatment significantly increased MDA level and decreased GSH level in the colonic tissue (p<0.001; Figure 4), as compared to control

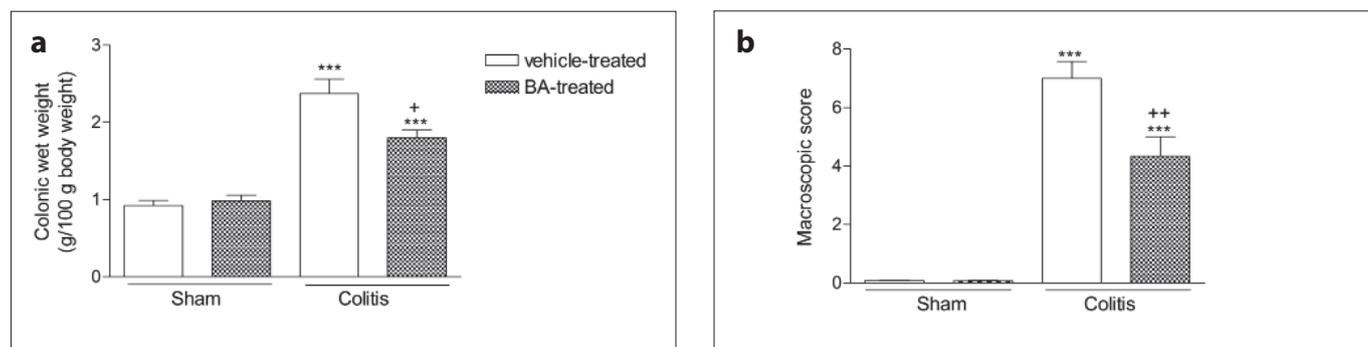


FIGURE 1. a) Macroscopic scores, **b)** wet weight indices (WWI) in the colonic tissues of vehicle- or betulinic acid (BA) treated control and colitis groups. (n=8 per group). ***p<0.001 compared to control group. +p<0.05, ++p<0.01, +++p<0.001 compared to vehicle-treated colitis groups.

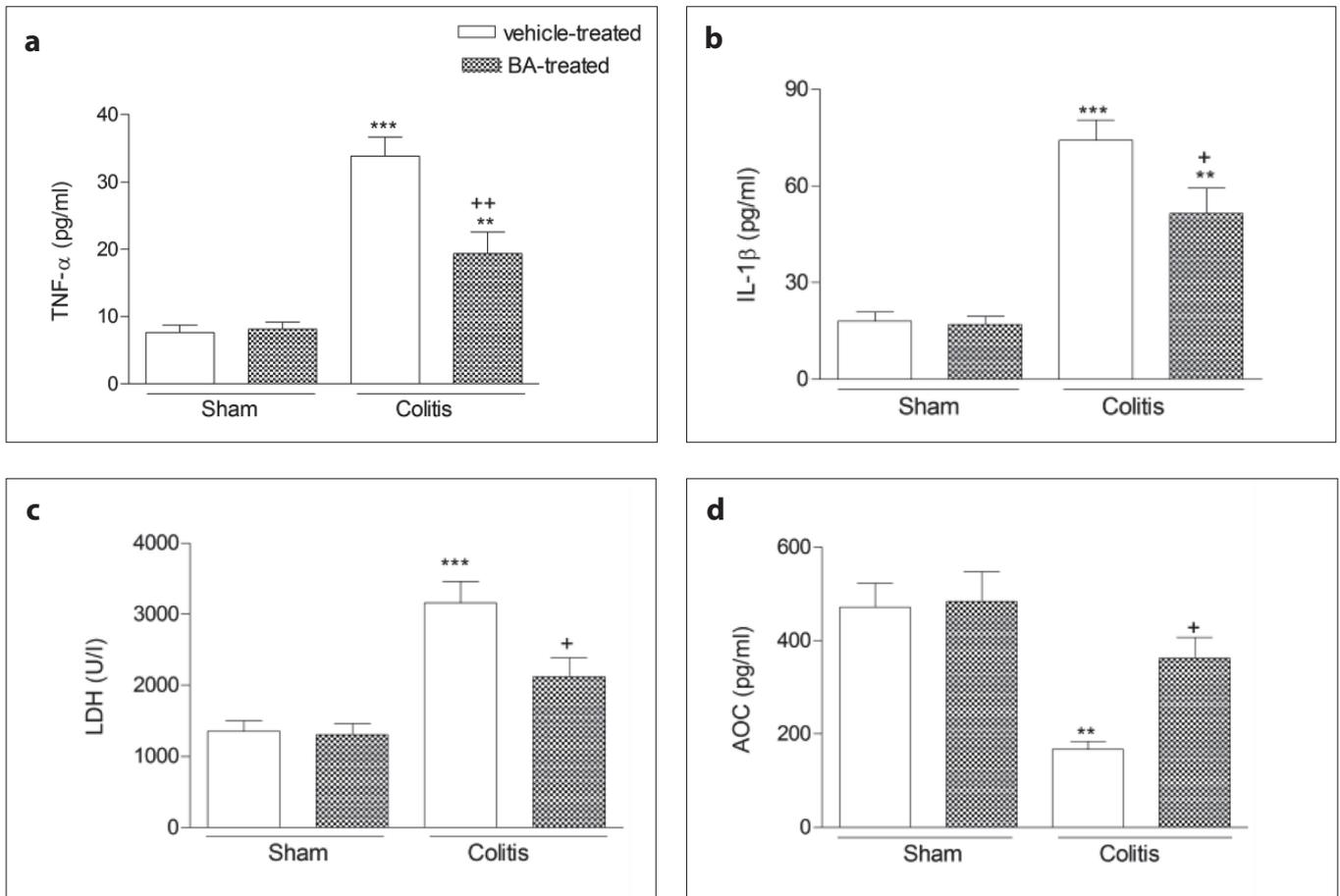


FIGURE 2. Plasma **a)** Tumor necrosis factor-alpha (TNF- α), **b)** interleukin 1-beta (IL-1 β), **c)** lactate dehydrogenase (LDH) levels and **d)** antioxidant capacity (AOC) in the vehicle- or betulinic acid (BA) treated control and colitis groups. (n=8 per group). **p<0.01, ***p<0.001 compared to control group. +p<0.05, ++p<0.01, compared to vehicle-treated colitis groups.

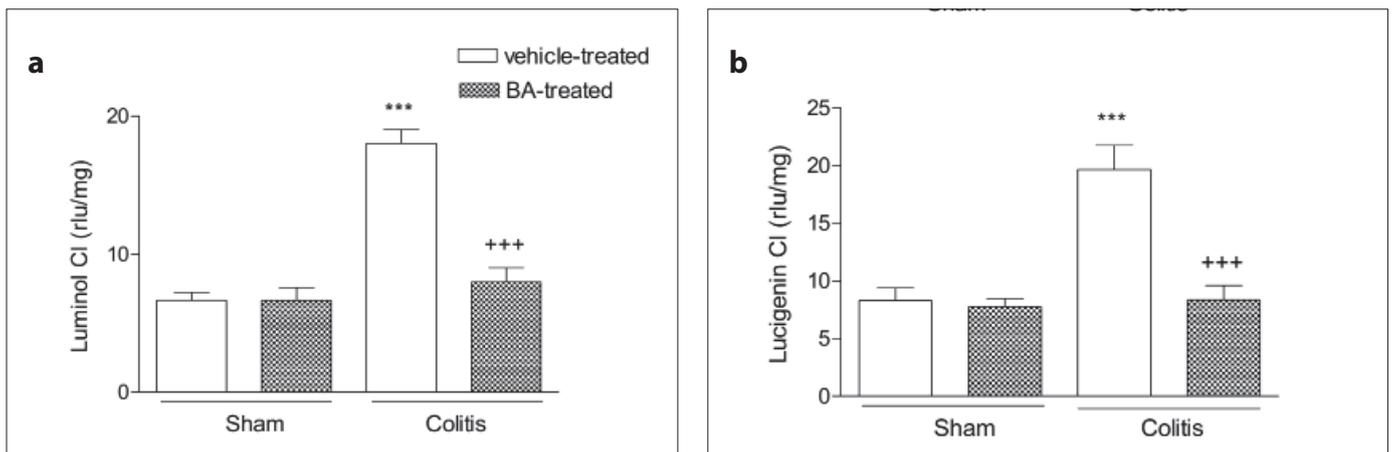


FIGURE 3. a) Luminol, b) lucigenin chemiluminescence (CL) levels in the colonic tissues of vehicle- or betulinic acid (BA) treated control and colitis groups. (n=8 per group). ***p<0.001 compared to control group. +++p<0.001 compared to vehicle-treated colitis groups.

group; while betulinic acid treatment abolished colitis-induced elevation in MDA level and decrease in GSH level (p<0.01).

Intracolonic instillation of TNBS, as assessed by elevated MPO activity in the colonic tissues of the vehicle-treated group, caused a significant increase in neutrophil infiltration when

compared to the control groups (p<0.001; Figure 5a). On the other hand, betulinic acid treated colitis group the colonic MPO activity back to the control level (p<0.001).

As an indicator of enhanced tissue fibrotic activity caused by oxidative stress, the collagen content in the colonic tissue of

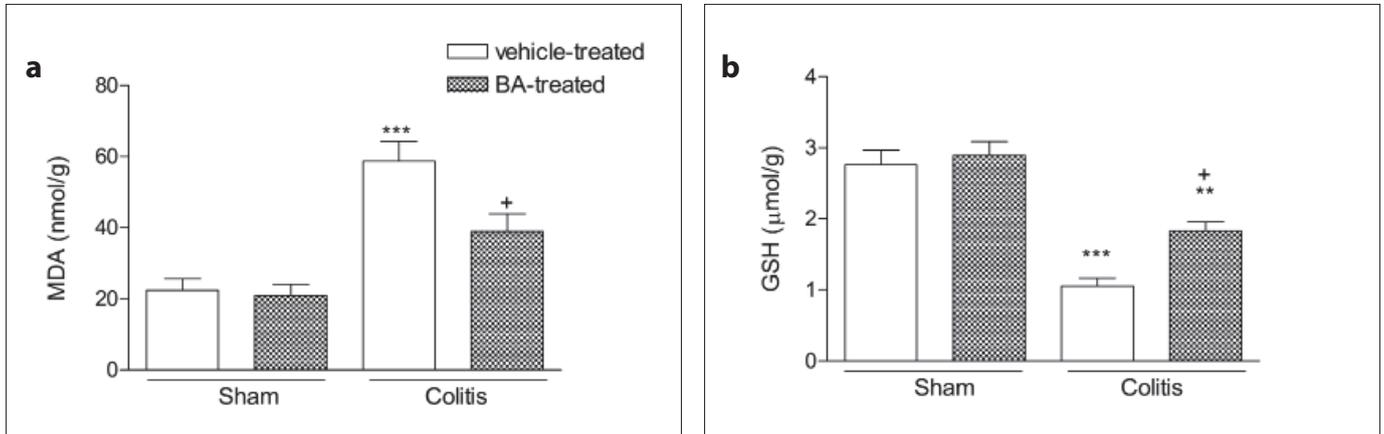


FIGURE 4. a) Malondialdehyde (MDA) and b) glutathione (GSH) levels in the colonic tissues of vehicle- or betulinic acid (BA) treated control and colitis groups. (n=8 per group). **p<0.01, ***p<0.001 compared to control group. +p<0.05, compared to vehicle-treated colitis groups.

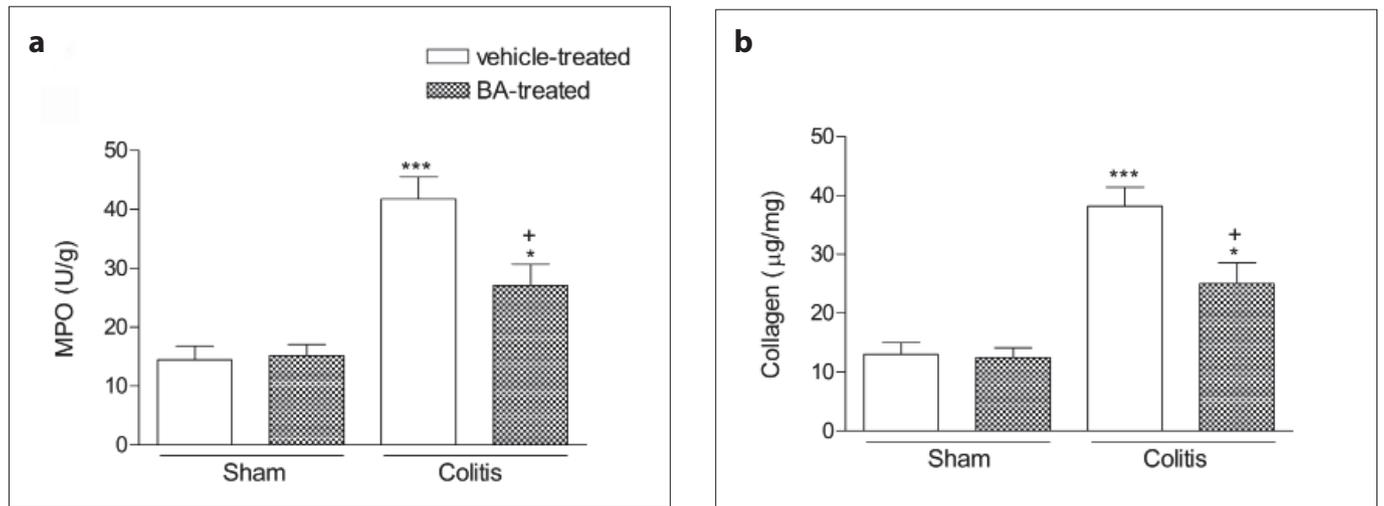


FIGURE 5. a) Myeloperoxidase (MPO) activity and b) collagen contents in the colonic tissues of vehicle- or betulinic acid (BA) treated control and colitis groups. (n=8 per group). *p<0.05, ***p<0.001 compared to control group. +p<0.05, compared to vehicle-treated colitis groups.

vehicle-treated colitis group was markedly increased (p <0.001) with respect to control groups, while betulinic acid treatment prevented the increase fibrotic activity significantly (p<0.001; Figure 5b).

Light microscopic evaluation of stained sections both in the vehicle- or BA-treated control groups revealed regular colon mucosa with surface epithelium (Figure 6a, b). In the vehicle-treated colitis group, severe damage of mucosa with epithelial degeneration, necrosis, submucosal edema and inflammatory cell infiltration were observed (Figure 6c). However, the BA-treated colitis group showed healed colonic epithelium with mild inflammatory cell infiltration (Figure 6d).

DISCUSSION

As confirmed through macroscopic scores and biochemical data, the results of the present study demonstrate that treatment with betulinic acid markedly reduced the severity of TNBS-induced colitis. Furthermore, all parameters indicated

the presence of inflammation and oxidative injury in inflamed colonic tissue, while betulinic acid showed a potent anti-inflammatory and antioxidant effect.

Proinflammatory cytokines play a key role in the pathophysiology of IBD (18), and anti-TNF-α antibodies are therapeutically used in some severe forms of IBD (19). Similarly, in the study of Genc et al (6) plasma cytokines were increased significantly following TNBS administration. In agreement with these reports in our study TNBS caused significant increase in both TNF- α and IL-1 β levels. Furthermore LDH levels were significantly increased and AOC was decreased demonstrating generalized tissue damage and oxidative stress respectively. On the other hand betulinic acid treatment decreased both cytokines and LDH levels. In our previous study we have demonstrated that following renal ischemia reperfusion injury betulinic acid treatment significantly reduced LDH and TNF-α levels (9).

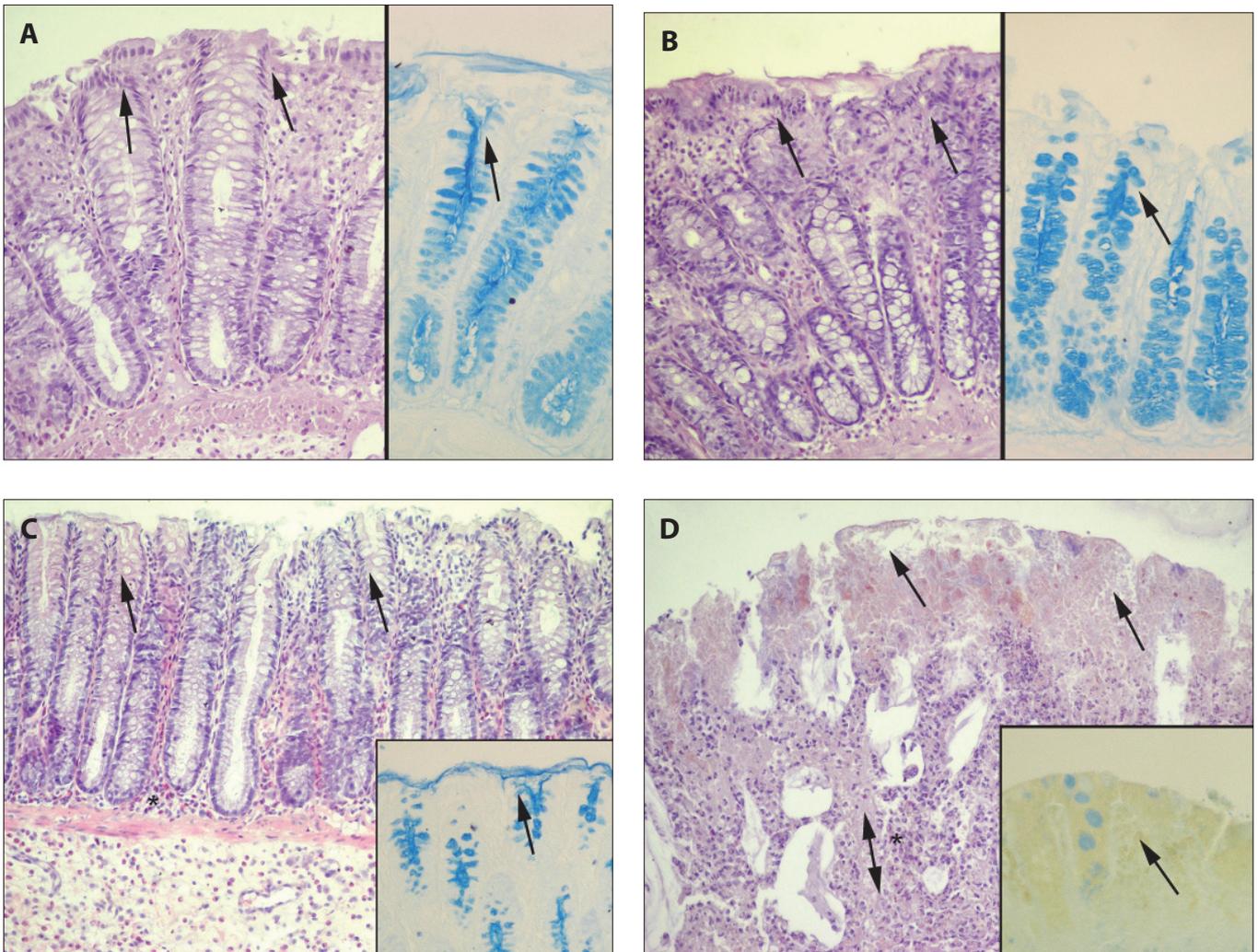


FIGURE 6. Micrographs illustrating the histological appearances of colonic tissues in different experimental groups. Control group (a), regular colon morphology (arrows); inset, normal colonic epithelium (arrow), Alcian blue staining; BA group (b), normal colon morphology (arrows); inset, normal colonic epithelium (arrow), Alcian blue staining; Colitis DMSO group (c), severe damage of mucosa with epithelial degeneration (arrows), necrosis, severe submucosal edema (double-headed arrow) and inflammatory cell infiltration (*); inset, necrotic colonic epithelium (arrow), Alcian blue staining; Colitis Betulinic acid group (d), mucosa with no localized epithelial degeneration (arrows), submucosal edema healing with mild inflammatory cell infiltration (*); inset, healed colonic epithelium (arrow), Alcian blue staining. H&E staining, original magnifications, $\times 200$, insets Alcian blue staining, original magnifications, $\times 200$.

Toxic oxidants can cause damage if the rate of their production exceeds the capacity of the endogenous antioxidant enzymes (e.g. superoxide dismutase, catalase and glutathione peroxidase). Thus, increased oxidative stress and impairment of the antioxidant defenses by the deleterious effect of ROS contribute to the pathogenesis of colitis. There is substantial evidence that excessive production of ROS by inflamed mucosa contributes significantly to development of tissue injury in ulcerative colitis (20-22). In accordance with these previous findings, in the present study, increased MDA levels as an index of lipid peroxidation with a concomitant decrease in GSH content in the colonic tissue demonstrates the involvement of ROS-induced damage in the pathogenesis of colitis. Since the quantitation of ROS is highly difficult due to their reactive nature and short lives, we used a simple but a reproducible technique for demonstrating the generation of oxidants in tissues. In this study we used two CL probes, luminol and lucigenin, which were differ in selectivity. Luminol detects H_2O_2 , $\cdot OH$, hy-

pochlorite, peroxyxynitrite and lipid peroxy radicals, whereas lucigenin is particularly sensitive to superoxide radicals (12). In the present study, the luminol and lucigenin-enhanced CL data revealed that TNBS-induced tissue injury involve toxic oxygen metabolites. Furthermore betulinic acid treatment attenuated the increases in tissue luminol- and lucigenin-enhanced CL, and prevented elevations in tissue MDA. Thus it seems likely that betulinic acid ameliorates TNBS-induced oxidative injury, in part, by scavenging the reactive oxygen radicals.

GSH is an important constituent of intracellular protective mechanisms against various noxious stimuli, including oxidative stress. However, reduced GSH as the main component of endogenous non protein sulfhydryl pool, is known to be a major low-molecular-weight scavenger of free radicals in the cytoplasm (23). It was reported that tissue GSH levels and the activities of GSH reductase and GSH peroxidase, which are

critical constituents of GSH-redox cycle, were significantly reduced owing to oxidative stress, permitting enhanced free radical-induced tissue damage (24). In accordance with previous reports, our results also show that depletion of tissue GSH, is one of the major factors that permit lipid peroxidation and subsequent tissue damage. The decrease in colonic GSH levels may be due to its consumption during TNBS-induced oxidative stress. Furthermore, prevention of colonic GSH depletion by betulinic acid may be responsible for the maintenance of this antioxidant in protecting colonic tissue against oxidative stress.

It is well known that administration of an enema containing the contact-sensitizing allergen TNBS in ethanol causes an acute inflammation, which progresses to a chronic stage and is morphologically similar to Crohn's disease (25). In the present study, histologic analysis revealed that intracolonic TNBS causes severe damage of mucosa with epithelial degeneration, necrosis, severe submucosal edema and inflammatory cell infiltration. As known, accumulation and activation of neutrophils induce tissue injury through the production of ROS and release of various cytotoxic proteins (e.g. proteases, MPO and lactoferrin) into the extracellular fluid. Myeloperoxidase activity, an indirect marker of neutrophil infiltration (16), has been shown to be significantly increased in colonic tissues of rats with colitis (26, 27). Similarly in the present study increased MPO activity in colonic tissues suggest that neutrophil accumulation in this tissue contributes to tissue damage. On the other hand, in the betulinic acid treated colitis groups, MPO activities were decreased. The effects of betulinic acid on MPO activity were previously studied by Ekşioğlu-Demiralp et al (9) where the authors demonstrated that ischemia reperfusion-

induced elevation of MPO in renal tissues was reduced by betulinic acid treatment. Furthermore, as assessed by the colonic collagen content, our findings suggest that betulinic acid may have an additional protective effect by inhibiting the production and deposition of extracellular matrix components that result in tissue fibrosis. All of the above mentioned results are further supported by our histological data, which reveal that the severity of colonic injury is ameliorated by betulinic acid treatment.

In conclusion, betulinic acid, by preventing free radical damaging cascades and oxidant radical release, supports the maintenance of colonic integrity against chronic inflammatory processes. Furthermore, betulinic acid augments the level of the main intracellular antioxidant glutathione in the colon and the total antioxidant capacity in plasma. On the basis of these data, we recommend investigation of the effects of betulinic acid supplementation in further experimental and clinical studies to confirm whether betulinic acid may provide an important contribution to the treatment of inflammatory bowel disease.

DECLARATION OF INTEREST

The study was supported by Marmara University Scientific Research Projects Commission (SAG-D-060308-0038). The authors report no conflicts of interest.

The authors alone are responsible for the content and writing of the paper.

Betulinik asitin TNBS ile oluşturulan deneysel kolit üzerine etkileri

ÖZET: Bu çalışmada betulinik asitin sıçanlarda kolondaki inflamasyon üzerinde muhtemel koruyucu etkileri araştırıldı. Her iki türden Sprague-Dawley sıçanlarda 1 ml trinitrobenzen sülfonik asit (TNBS)'in intrakolonik uygulaması ile kolit oluşturuldu. Kolit oluşturulan sıçanlara oral gavaj ile taşıyıcı (0.05% DMSO) veya betulinik asit (50 mg/kg/gün) 3 gün süreyle uygulandı. 72 saat sonra dekapite edilen hayvanlardan kan örnekleri alınarak TNF- α , IL-1 β , laktat dehidrojenaz (LDH) düzeyleri ve antioksidan kapasite (AOK) tayinleri yapıldı. Kolon dokusunun 8 cmlik distal kısmı makroskopik olarak skorlandı ve dokuda oksidatif hasar malondialdehit (MDA) ve glutatyon (GSH) düzeyleri, myeloperoksidaz (MPO) aktivitesi, kollagen içeriği incelemeleri ve histolojik analizler yapıldı. Oksidan türevlerin oluşumu luminol ve lusigenin kemiluminesans (KL) ile değerlendirildi. Kolit oluşumu kolon dokusunda KL değerlerini, makroskopik skoru, MDA, MPO ve kollagen düzeylerini artırırken GSH düzeyleri anlamlı derecede azaldı. Benzer şekilde serum TNF- α , IL-1 β , ve LDH artarken AOK ise azaldı. Buna karşılık betulinik asit uygulaması TNBS uygulamasının neden olduğu tüm biyokimyasal ve histopatolojik değişimleri geri çevirdi. Bu bulgular betulinik asitin kolonda radikal süpürücü ve antioksidan etkileri ile koruyucu olduğunu düşündürmektedir.

ANAHTAR SÖZCÜKLER: betulinik asit; kolit; oksidatif hasar; inflamasyon; trinitrobenzen sülfonik asit

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