Protective Effects of Alpha-Lipoic Acid Against Oxidative Injury in TNBS-induced Colitis

TNBS ile Oluşturulan Kolitte Alfa Lipoik Asitin Oksidatif Hasara Karşı Koruyucu Etkileri

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Abstract

Purpose: Alpha-Lipoic acid (ALA), which has been intensely investigated as a therapeutic agent for several diseases, is elucidated for its possible protective effect as a potent antioxidant on colonic inflammation.

Material and Methods: Following intracolonic administration of trinitrobenzene sulphonic acid, Sprague-Dawley rats were treated orally either with saline or ALA (100 mg/kg/day), for three days. On the 4th day, rats were decapitated and distal colon was removed for the macroscopic and microscopic damage scoring, for the measurement of malondialdehyde (MDA), glutathione (GSH) and collagen levels, myeloperoxidase (MPO) and Na^+- K^+-ATPase activity, luminol and lucigenin chemiluminescences (CL) and oxidant-induced DNA fragmentation. Lactate deydrogenase (LDH) activity, TNF-α, IL-1β, IL-6 and antioxidant capacity (AOC) were assayed in blood samples.

Results: Colitis caused significant increases in the colonic macroscopic and microscopic damage scores, MDA, and collagen levels, MPO activity and CL values, along with a significant decrease in tissue GSH level and Na^+- K^+-ATP ase activity. Similarly, serum cytokines as well as LDH were elevated in the vehicle-treated colitis group as compared to control group. On the other hand, ALA treatment reversed all these biochemical indices, as well as histopathological alterations induced by TNBS.

Conclusion: ALA protects the colonic tissue via its antioxidant and membrane stabilizing properties.

Key words: **Alpha lipoic acid; Colitis; Cytokines; Glutathione; Tumor necrosis factor –alpha.**

Özet

Amaç: Alfa-Lipoik asit (ALA), birçok hastalıkta terapötik ajan olarak incelenmektedir. Bu çalışmada potent bir antioksidan olan ALA'nın kolonik inflamasyonda olası protektif etkisinin incelenmesi amaçlanmıştır.

Gereç ve Yöntemler: Sprague-Dawley sıçanlara intrakolonik trinitrobenzeno sülfonik asit (TNBS), uygulamasını takiben 3 gün süreyle oral olarak serum fizyolojik ya da ALA (100 mg/kg/gün) verildi. 4. gün sonunda sıçanlar dekapite edilerek kan ve doku örnekleri alındı. Distal kolonda makroskopik ve mikroskopik hasar skorlaması yapıldı Ayrıca dokuda malondialdeit (MDA), glutatyon (GSH) ve kollagen düzeyleri ile myeloperoksidaz (MPO) ve Na^+- K^+-ATPase aktiviteleri ve luminol ve lusigenin kemiluminesans (CL) düzeyleri ve oksidan-aracılı DNA fragmentasyonu ölçüldü. Kan örneklerinde Laktat dehidrogenaz (LDH) aktivitesi, TNF-α, IL-1β, IL-6 ve antioksidan kapasite (AOC) düzeyleri değerlendirildi.

Bulgular: Kolit kolonik makroskopik ve mikroskopik hasar skorlarında, MDA ve kollagen düzeylerinde, MPO aktivitesinde anlamlı artışa, GSH düzeylerinde ve Na^+- K^+-ATP az aktivitesinde ise anlamlı olarak azalmaya neden olmaktadır. Benzer olarak kolit gruplarında serum sitokinlerinin ve LDH düzeylerinin anlamlı olarak yüksek bulunduğu görülmüştür. ALA uygulaması TNBS ile görülen biyokimyasal ve histopatolojik değişiklikleri anlamlı olarak düzeltmektedir.

Sonuç: ALA'nın antioksidan ve membrane stabilize edici etkilerinden dolayı kolon dokusunda koruyucu etki gösterdiği düşünülmektedir.

Anahtar Kelimeler: **Alfa-lipoik asit; Glutatyon; Kolit; Tümör Nekroz Faktörü – alfa; Sitokinler**.

Submitted : May 30, 2008
Revised : July 05, 2008
Accepted : December 03, 2008

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Introduction

Inflammatory bowel disease (IBD) is a chronic, debilitating disorder of the bowel in which there is targeted inflammation at one or more sites of the gastrointestinal tract (1). Results studies associated with IBD strongly suggest that inappropriate and/or excessive responses to antigens present in the normal bacterial microflora are involved in the pathogenesis of the disease (2–7). Although activation of neutrophils, macrophages, lymphocytes and mast cells is the major microbiocidal mechanism, it may ultimately give rise to mucosal distruption and ulceration (8). The infiltrated and activated neutrophils represent an important source of reactive oxygen mediators (ROMs), which lead to cellular oxidative damage by cross-linking proteins, lipids, and nucleic acids, causing cellular dysfunction and damage (9). Tumor necrosis factor (TNF-α) and interleukin-1β (IL-1β) produced by activated macrophages are key immunoregulatory cytokines that amplify the inflammatory response by activating a cascade of immune cells and their levels are often increased in both animals and patients with ulcerative colitis (10, 11). IL-1α and TNF-β stimulate production of other cytokines, arachidonic acid metabolites, and proteases by intestinal macrophages, neutrophils, smooth muscle cells, fibroblast, and epithelial cells.

Among several experimental models of IBD, trinitrobenzene sulphonic acid (TNBS)-induced colitis model, applied by intracolonic administration of diluted TNBS solutions (12-14), produces a diffuse colonic inflammation, characterized by increased leukocyte infiltration, edema and ulceration which progresses to a chronic stage and is morphologically similar to Crohn's disease (15). It can be speculated that TNBS-ethanol initiates a vicious cycle that starts when the mucosal barrier is broken down by ethanol, enabling TNBS to bind to substances of high molecular weight such as cell-surface proteins in colonic tissue (16), causing inflammation and other immunological reactions (17), which may in turn lead to the generation of ROMs and other mediators (e.g. cytokines and prostaglandins). Thus, developing new drugs that are capable of scavenging these free radicals and of stabilizing membrane structure would provide new therapeutic opportunities.

Alpha-Lipoic acid (ALA) and its reduced form dihydrolipoic acid (DHLA) have been intensely investigated as therapeutic agents for atherosclerosis, diseases of joints, AIDS, hepatic disorder and diabetic polyneuropathy. ALA and DHLA were also found to be effective against conditions in which oxidative stress has a role (18), including its possible role as a chemopreventive agent in inflammation-associated tumorigenesis (19) and ulcerogenesis (18). Accordingly, we aimed to investigate whether and to what extent ALA would provide protection against TNBS-induced colonic inflammation.

Materials and Methods

Animals. Sprague–Dawley rats of either sex (200–250 g) were kept in a room at a constant temperature 22±1 °C with 12-h light/dark cycles and fed standard pellet chow and water ad libitum. The study was approved by Marmara University School of Medicine, Animal Care and Use Committee (21.2006.mar).

Induction of colitis. After an overnight fasting, colonic inflammation was induced under light ether anesthesia by intracolonic administration of 1 ml of a 30 mg/ml TNBS solution dissolved in 40% ethanol in saline via a polyethylene catheter (PE-60) inserted into the colon with its tip positioned 8 cm from the anus . Half of the rats with colitis (n=8) were given ALA (100 mg/kg, *colitis + ALA group*) orogastrically, while the rest (n=8) was administered with the vehicle for ALA (0.9 % saline adjusted to pH 7.4 by $NaHCO_3$, *colitis group*) for 3 consecutive days. In another group of rats (n=8), 1 ml of physiological saline was administered intracolonically (*control group*).

Three days after the intracolonic administration, all rats were decapitated. Trunk blood was collected for the assessment of lactate dehydrogenase (LDH) levels, TNF-α, IL-1β, IL-6 and total antioxidant capacity (AOC). The last 8 cm of the colon was excised, opened longitudinally, and rinsed with saline solution. Then, wet weight of the distal colon was recorded (g/100 g body weight) and the mucosal lesions were scored macroscopically using the criteria outlined as follows: 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 co*lon; 6–10: *damage extending more than 2 cm along the length of colon, where the score is increased by one for each additional 1 cm damage*. Macroscopic scoring of tissue samples were performed by an observer unaware of the treatment groups.

Colonic samples were stored at -80 °C for subsequent measurement of malondialdehyde (MDA) and glutathione (GSH) levels, myeloperoxidase (MPO) and Na^+-K^+ ATPase activity and collagen content. Formation of reactive oxygen species in the tissue samples was monitored by using chemiluminescence (CL) technique with luminol and lucigenin probes. Additional tissue samples were obtained for the determination of DNA fragmentation and for histological evaluation.

Blood Assays. Serum LDH activity (21) was determined spectrophotometrically using an automated analyzer. Plasma levels of TNF-α, IL-1β and IL-6 were quantified according to the manufacturer's instructions and guidelines using enzyme-linked immunosorbent assay (ELISA) kits specific for the previously mentioned rat cytokines (Biosource International, Nivelles, Belgium). The total antioxidant capacity in plasma was measured by using colorimetric test system (ImAnOx, cataloge no.KC5200, Immunodiagnostic AG, D-64625 Bensheim), 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 to conduct the assay.

Measurement of colonic malondialdehyde and glutathione levels. Tissue samples were homogenized with ice-cold 150 mM KCl for the determination of MDA and GSH levels. The MDA levels were assayed for the products of lipid peroxidation by monitoring thiobarbituric acid reactive substance formation as described previously (22). Lipid peroxidation was expressed in terms of MDA equivalents using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} and results are expressed as nmol MDA/g tissue. GSH measurements were performed using a modification of the Ellman procedure (23). Briefly, after centrifugation at 1200 g for 10 min, 0.5 ml of supernatant was added to 2 ml of 0.3 mol/l $Na_2HPO_4.2H_2O$ solution. A 0.2 ml solution of dithiobisnitrobenzoate (0.4 mg/ml 1% sodium citrate) was added and the absorbance at 412 nm was measured immediately after mixing. GSH levels were calculated using an extinction coefficient of 1.36×10^4 M^{-1} cm^{-1}. Results are expressed in μmol GSH/g tissue.

Measurement of colonic myeloperoxidase activity. Myeloperoxidase (MPO) is an enzyme that is found predominantly in the azurophilic granules of polymorphonuclear leukocytes (PMN). Tissue MPO activity is frequently utilized to estimate tissue PMN accumulation in inflamed tissues and correlates significantly with the number of PMN determined histochemically in tissues (24). MPO activity was measured in tissues in a procedure similar to that documented by Hillegass et al (25). Tissue samples were homogenized in 50 mM potassium phosphate buffer (PB, pH 6.0), and centrifuged at 41.400 g (10 min); pellets were suspended in 50 mM PB containing 0.5 % hexadecyltrimethylammonium bromide (HETAB). After three freeze and thaw cycles, with sonication between cycles, the samples were centrifuged at 41400 g for 10 min. Aliquots (0.3 ml) were added to 2.3 ml of reaction mixture containing 50 mM PB, o-dianisidine, and 20 mM H_2O_2 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.

Measurement of Na^+- K^+ ATPase activity. The measurement of Na^+- K^+ ATPase activity is based on the measurement of inorganic phosphate that is formed from 3 mM disodium adenosine triphosphate added to the medium during the incubation period (26). The medium was incubated in a 37º C water bath for 5 min with a mixture of 100 mM NaCl, 5 mM KCl, 6 mM $MgCl_2$, 0.1 mM EDTA, 30 mM Tris HCl (pH 7.4). Following the pre-incubation period, Na_2ATP, at a final concentration of 3 mM was added to each tube and incubated at 37º C for 30 min. After the incubation, the tubes were placed in an ice bath, and the reaction was stopped. Subsequently, the level of inorganic phosphate was determined in a spectrophotometer (Shimadzu, Japan) at excitation wavelength of 690 nm. The specific activity of the enzyme was expressed as nmol Pi mg^{-1} protein h^{-1}. The protein concentration of the supernatant was measured by the Lowry method (27).

Collagen content assay. Colonic 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 (1985) (28), which is based on selective binding of the dyes Sirius Red and Fast Green FCF to collagen and non-collagenous components, respectively. Both dyes were eluted readily and simultaneously 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.

Chemiluminescence (CL) assay. To assess the contribution of reactive oxygen species in TNBS-induced colonic damage, luminol and lucigenin chemiluminescences 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 luminal, for a final concentration of 0.2 mM. Luminol detects a group of reactive species, i.e. ·OH, H_2O_2, HOCl radicals, while lucigenin is selective for O^-_2 (29, 30). 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 weights and expressed as relative light units (rlu/mg tissue) (31).

DNA fragmentation assay. Mucosal samples from colon were homogenized in 10 volumes of a lysis buffer (5 mM Tris HCL, 20 mM ethylene diamine tatraacetic acid [EDTA], 0.5 % (v/v) t-octylphenoxypolyethoxyethanol [Triton-X 100]; pH=8.0). Two separate samples of 1 mL each were taken from the sample and centrifuged at 25,000g for 30 min to separate the intact chromatin in the pellet from the fragmented DNA in the supernatant (32). The supernatant was taken out to be saved and the pellet was re-suspended in 1 mL of Tri-EDTA buffer (pH=8.0), 10 mM:1 mM, respectively. Both, the supernatant and the re-suspended pellet were assayed then for DNA content determination by diphenylamine reaction described by Burton (33).

Histopathological evaluation of colonic damage. For light microscopic analysis, samples from distal colon were fixed in 10 % buffered formalin for 48 hours, dehydrated in ascending alcohol series and embedded in paraffin wax. Approximately 5-m-thick sections were stained with hematoxylin-eosin (H&E) for general morphology. For scanning electron microscopic investigation, the samples were fixed in 4 % phosphate buffered gluteraldehyde (0.13 M and pH 7.4) for 4 hours and post-fixed with 1 % OsO_4 for one hour, dehydrated in graded alcohol series, put into amyl acetate, dried with liquid CO_2 under pressure with critical point dryer (Bio-Rad E 3000) and covered with gold particles (Bio-Rad SC502). Sections were observed under a photomicroscope (Olympus BH 2, Tokyo, Japan) or a scanning electron microscope (SEM; Jeol 1200 JSM, Tokyo, Japan) by an experienced histologist, who was unaware of the experimental groups.

Statistics. Statistical analysis was carried out using GraphPad Prism 3.0 (GraphPad Software, San Diego; CA; USA). All data were expressed as means ± SEM. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Values of $p<0.05$ were regarded as significant.

Results

As shown in Table I, increased colonic wet weight indicating tissue edema and the macroscopic lesion score of the colitis group were significantly reduced by ALA treatment ($p<0.001$). Serum LDH activity and the plasma levels of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) in the colitis group were significantly higher ($p<0.01$-0.001) than that of the control group, while treatment of ALA abolished these elevations significantly ($p<0.05$-0.01). On the other hand, AOC which was significantly decreased in the vehicle-treated colitis group ($p<0.001$), was found to be not different than the control group when the rats were treated with ALA ($p<0.05$).

Light microscopic evaluation revealed that colonic tissues of the vehicle-treated colitis group demonstrated severe degeneration with accumulation of inflammatory cells (predominantly polymorphonuclear leukocytes), along with prominent congestion of blood vessels in lamina propria accompanied with dense edema (Picture 1). In ALA-treated colitis group, the dense edema and congestion of blood vessels were regressed, but the inflammatory cells were still present in the colonic tissue. Similarly, SEM showed severe mucosal degeneration in the colitis group, while in the colonic mucosae ALA-treated colitis group, a regenerative effect was observed (Picture 1).

Table I. The colonic wet weight, serum lactate dehydrogenase (LDH) activity, TNF-α, IL-1β, and IL-6 levels and plasma total antioxidant capacity (AOC) of the control, vehicle-treated and alpha-lipoic acid (ALA)-treated colitis groups. For each group n=8.

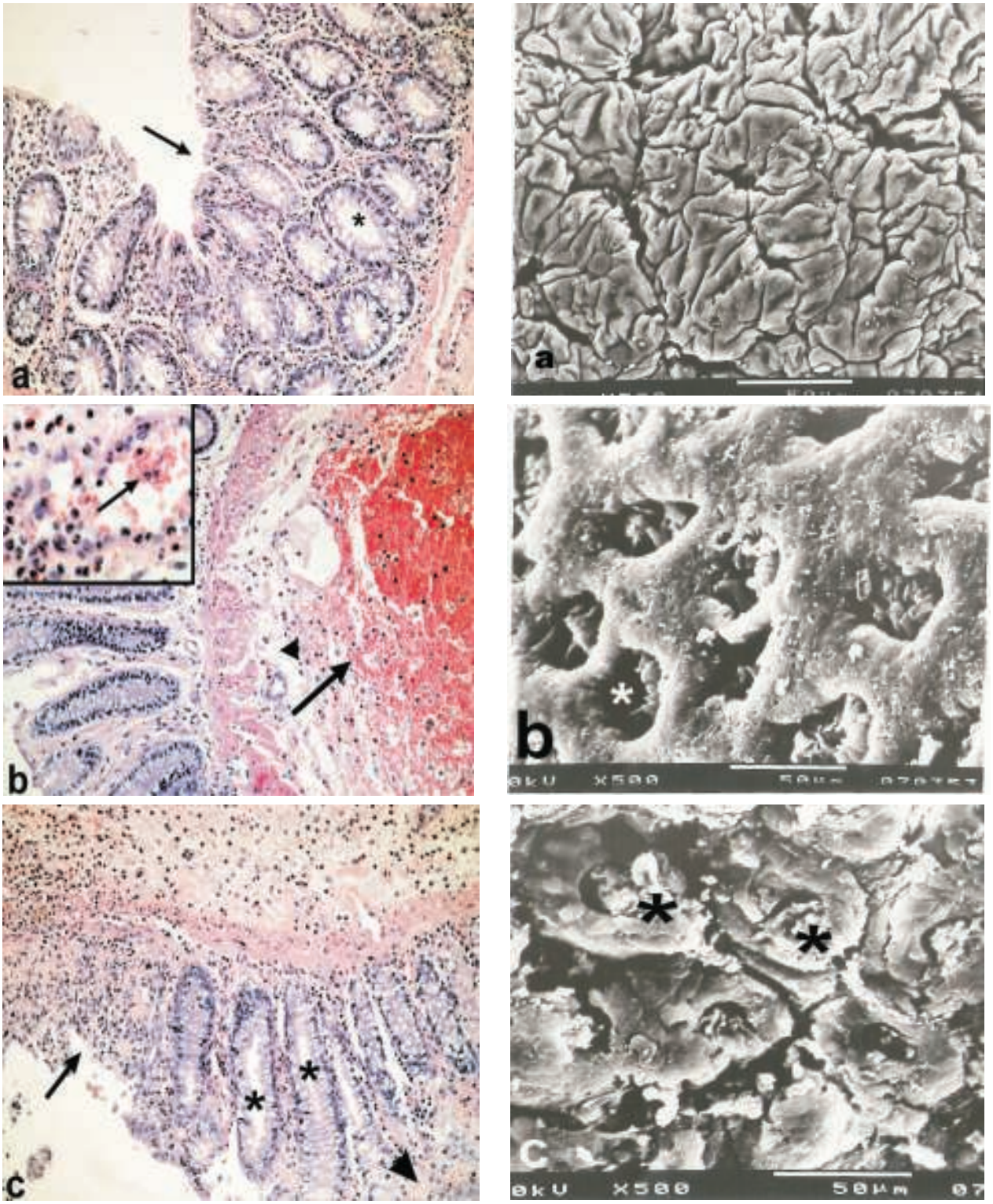
	Control	Colitis	Colitis - ALA	ANOVA (p<)
Colonic wet weight (g/100 g body weight)	0.85 ± 0.10	2.31 ± 0.17^{c}	1.47 ± 0.15^{a,f}	0.0001
Macroscopic damage score	0.1 ± 0.06	8.6 ± 0.5^{c}	3.5 ± 0.6^{c,g}	0.0001
LDH (U/I)	1555 ± 178	3116 ± 292^{c}	2183 ± 153^{d}	0,0005
TNF-α(pg/ml)	4.8 ± 0.6	13.8 ± 1.8^{c}	7.6 ± 1.1^{f}	0.0001
IL-1β (pg/ml)	31.2 ± 3,1	56.3 ± 5.8^{b}	37.5 ± 2.2^{d}	0.0001
IL-6 (pg/ml)	22.7 ± 3.8	57.0 ± 4.2^{c}	31.8 ± 4.4^{f}	0.0001
AOC (pg/ml)	352 ± 44	163 ± 21^{b}	285 ± 29^{d}	0,0030

$^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$; compared to control group; $^{d}p<0.05$, $^{f}p<0.01$, $^{g}p<0.001$; compared to untreated colitis group.

The MDA levels, measured as a major degradation product of lipid peroxidation in the colonic tissue, were found to be significantly higher in the colitis group ($p<0.001$) as compared to those of the control group, while treatment with ALA abolished these elevations ($p<0.001$; Table II). In accordance with these findings, levels of the major cellular antioxidant GSH in the vehicle-treated colitis group was depleted ($p<0.001$); however, in the ALA-treated colitis group, depleted GSH stores were partially replenished with this antioxidant ($p<0.01$; Table II). DNA fragmentation (%) in the colonic mucosa was analyzed as an indicator of cell death, including apoptosis. DNA fragmentation, which was elevated in the colonic mucosa of the non-ALA-treated colitis group ($p<0.001$), was significantly prevented by ALA treatment ($p<0.001$; Table II).

Myeloperoxidase activity, which is accepted as an indicator of neutrophil infiltration, was significantly higher in the colonic tissue of the colitis group treated with vehicle ($p<0.001$) than that of the control group (Table II). On the other hand, ALA treatment in the colitis group significantly decreased colonic MPO level ($p<0.01$) back to the levels of the control group. The activity of Na^{+}-K^{+} ATPase, indicating the functional transport capacity of the colonic cells, was found to be significantly decreased in the colitis group as compared with control group ($p<0.001$); however, ALA treatment significantly reduced the TNBS-induced decrease in colonic Na^{+}-K^{+} ATPase activity ($p<0.01$; Table II).

Chemiluminescence levels in the colonic samples detected by both luminol and lucigenin probes showed significant increases in the vehicle-treated colitis group as compared to the CL levels of the control group ($p<0.001$; Table II). On the other hand, ALA treatment in the colitis group abolished the colitis-induced increases in both lucigenin- and luminol-detected CL ($p<0.01$–0.001). The colonic collagen content, determined as an index of tissue fibrotic activity, was higher in the non-ALA treated colitis group as compared to control group ($p<0.001$), while ALA treatment has reversed this effect totally ($p<0.01$; Table II).



Picture 1. The hematoxylin and eosin-stained photomicrographs (left column; H&E; X200) and scanning electron micrographs (right column; bar: 50 μm of the colon samples, **a:** control group, regular layout of epithelium (arrow) and glands (*), **b:** vehicle-treated colitis group, severe mucosal degeneration, accumulation of polymorphonuclear leukocytes (inset), congestion of blood vessels (arrow) and dense edema (arrowhead), **c:** alpha-lipoic acid (ALA)-treated colitis group, mild edema and congestion of blood vessels (arrowhead), and accumulated inflammatory cells (arrow), regenerated glands (*).

Table 2. The malondialdehyde (MDA), glutathione (GSH) luminol CL, lucigenin CL and collagen levels, DNA fragmentation, Myeloperoxidase (MPO), Na^+-K^+-ATPase activity of the control, vehicle-treated and alpha-lipoic acid (ALA)-treated colitis groups. For each group n=8.

	Control	Colitis	Colitis - ALA	ANOVA (p<)
MDA (nmol/g)	17.2 ± 2.3	52.4 ± 4.7^{c}	24.5 ± 3.9^{f}	0.0001
GSH (μmol/g)	2.92± 0.20	1.22 ± 0.13 ^{c}	2.15 ± 0.17^{a,f}	0.0001
DNA fragmentation (%)	8.1 ±1.20	24.5 ± 2.09 ^{c}	12.7 ± 0.8^{f}	0.0001
MPO (U/g)	12.8 ± 2.2	39.1 ± 3.6 ^{c}	22.8 ± 2.4^{e}	0.0001
Na^+-K^+-ATPase (μmol/mg protein/h)	21.7 ± 1,7	4.0 ± 0.9 ^{c}	11.9 ± 1.4^{b,e}	0.0001
Luminol CL (rlu/mg)	5.4 ±0.7	12.1 ± 1.3 ^{c}	6.8 ± 0.9^{e}	0.0005
Lucigenin CL (rlu/mg)	7.4 ±0.9	17.1 ± 2.1 ^{c}	8.9 ± 1.2^{e}	0.0007
Collagen (μg/mg)	14.7 ± 2,2	40.2 ± 3.7 ^{c}	16.1 ± 1.7^{f}	0.0001

a* p<0.05, b** p<0.01, ^{c}p<0.001; compared to control group; ^{d}p<0.05, ^{e}p<0.01, ^{f}p<0.001; compared to untreated colitis group.

Discussion

As assessed by both histological and biochemical parameters, the results of the present study demonstrate that ALA treatment attenuates the severity of oxidative colonic damage along with concomitant reductions in the serum pro-inflammatory cytokines, suggesting that ALA has a potent anti-inflammatory and anti-oxidant effect on the inflamed colonic tissue.

Álpha-lipoic acid is a dithiol that is found naturally in mitochondria as the coenzyme for pyruvate dehydrogenase and α-ketoglutarate dehydrogenase (18). Both ALA and DHLA are powerful antioxidants (34) with beneficial effects in oxidative stress conditions, because of their synergistic action with other antioxidants (35). Their antioxidant functions involve: (i) quenching of reactive oxygen species; (ii) regeneration of endogenous and exogenous antioxidants involving vitamins C and E and glutathione; (iii) chelation of redox metals including Cu^{2+} and Fe^{2+}; (iv) repair of oxidized proteins (36). Furthermore, reports emphasize that lipoic acid is a potent antioxidant in various drug-induced toxicities in experimental models (37, 38) and exerts a cytoprotective effect on gastric mucosal damage in rats (39) and in inflammation-associated tumorigenesis in mice (19).

Inflammatory bowel diseases, encompassing ulcerative colitis and Crohn's disease, are idiopathic chronic inflammation in gut with diffuse inflammation of the colon and rectum, characterized by cycles of acute inflammation, ulceration and bleeding of the mucosa (40). Intracolonic TNBS-induced colitis is one of the widely used animal models of intestinal inflammation (41-43) that produces a diffuse colonic inflammation with a pathology resembling that of the IBD. This model consistently exhibits an increased expression of pro-inflammatory cytokines such as TNF-α, IL-1β, and IFNγ, and enzymes, iNOS and COX-2 (44, 45). Adam et al. (46) have demonstrated increased serum IL-2 and IL-6 levels were evident at 2 h after induction of colitis and persisted up to 14 days. Similarly, colonic administration of TNBS was shown to increase the production of serum IL-1β and colonic NF-kappa-B, which were found to be associated with increases in colonic damage score (47). In our study, the increased plasma levels of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 by colitis induction also support the notion that tissue injury induced by TNBS involves the enhanced generation of inflammatory cytokines. On the other hand, previous studies have shown that ALA inhibits the cytokine production during inflammation in various animal models, such as in sepsis, diabetes, pancreatitis (48) and in the hepatic ischemia/reperfusion injury (49). In support of these studies, in the current study, ALA treatment led to alleviation of the colonic damage in the rats, while the generation of pro-inflammatory cytokines was concomitantly depressed.

There is a great body of evidence that increased oxidative stress and impairment of the antioxidant defenses contribute to the pathogenesis of colitis. It is well-known that if the production rate of toxic oxidants exceeds the capacity of the endogenous antioxidant enzymes (e.g., superoxide dismutase, catalase and glutathione peroxidase) tissue injury is inevitable. Glutathione is an important constituent of intracellular protective mechanisms against various noxious stimuli including oxidative stress. However, reduced glutathione 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 (50). In accordance with the previous reports, our results also support the notion that depletion of tissue GSH, as observed in the TNBS-induced colonic injury, is one of the major factors that permit lipid peroxidation and subsequent tissue damage. Similar results were previously observed by the researchers (51, 52), who used the same model of colitis to determine the protective effects of other antioxidant agents. The reduction in GSH may be due to its consumption by the liberation of oxygen-derived free radicals. On the other hand, treatment with ALA for 3 days in the present study prevented the fall in colonic GSH levels. Since there is no direct evidence to imply the stimulatory effect of ALA on GSH synthesis, the maintenance of tissue GSH pools may be explained by the radical scavenging activity of ALA. However, an earlier study has demonstrated that lipoic acid treatment augmented the level of the main intracellular antioxidant glutathione in the small intesine (53, 68).

Reactive oxygen metabolites (ROM) are involved in the development of tissue injury in colitis, as well as in many inflammatory diseases (54, 55). In the current study, we investigated the free radical generation in the colonic tissue using chemiluminescence, a simple and reproducible technique for demonstrating the generation of oxidants in tissue. The luminol probe used in this technique detects H_2O_2, OH^-, hypochlorite, peroxynitrite and lipid peroxyl radicals, while lucigenin is selective for superoxide radical (29-31). Since increased CL values detected by both probes were significantly decreased with ALA treatment, it seems likely that the protective effect of ALA on the colonic tissue partly involves its direct antioxidant properties. The interaction of reactive oxygen species with biological membranes is known to produce a great variety of functional modifications. Among these, lipid peroxidation contributes to the loss of cellular functions through the inactivation of membrane enzymes and even of cytoplasmic proteins. In parallel to the CL results, the increased lipid peroxidation in the colonic tissue, as demonstrated by MDA assay, was also reversed with ALA treatment, emphasizing the antioxidant action of ALA on the deleterious consequences of ROMs in oxidative colonic injury. In accordance with our results, a previous report based on an experimental nephrotoxicity and gut inflammation model has demonstrated that lipoic acid treatment decreased lipid peroxidation and restored the transmembrane enzymes, thereby maintained the antioxidant status of the renal cells (56, 68).

Our results also indicate that instillation with TNBS impairs colonic Na^+-K^+-ATPase activity. The Na^+-K^+-ATPase, which is found exclusively in the basolateral membranes of the villi and crypt enterocytes, plays a central role in intestinal electrolyte and nutrient absorptive processes and in the pathogenesis of diarrhea (57). Since decreased Na^+-K^+-ATPase activity most likely reflects a diminished number of enzyme molecules due to a loss of Na^+-K^+-ATPase-containing mucosal cells (58), consequently, it also indicates severe mucosal inflammation and the loss of physiological function. Since ALA treatment in the present study reversed TNBS-induced increment in MDA and reduction in enzyme activity, it indicates that ALA alleviates the colonic injury and facilitates the reversal of the mucosal secretory function.

As shared by other inflammatory disorders in the gut, active lesions in the ulcerative colitis involve the migration of activated neutrophils and macrophages (59-61). It is well-known that mesengial cells and neutrophils release chemotactic substances (e.g., interleukin 8), which further promote neutrophil migration to the tissue, activate neutrophils, and increase the damage (62). The tissue-associated MPO, which is known as the index of neutrophil infiltration, plays a fundamental role in oxidant production by neutrophils (63). In our observation, elevated MPO levels in colonic tissues indicate that neutrophil accumulation contributes to the colitis-induced oxidative injury and ALA appears to have a preventive effect through the inhibition of neutrophil infiltration. Mervaala et al. (64) have shown that lipoic acid reduces angiotensin II-induced renal injury by its inhibitory action on leukocyte infiltration. The inhibitory action of ALA on neutrophils was also shown in a murine experimental autoimmune encephalomyelitis and gut inflammation model, where the anti-inflammatory effect of ALA was suggested to be partly due to the inhibition of ICAM-1 and VCAM-1, the s in the membranes of leukocytes and endothelial cells (65, 68)

It has been demonstrated that DNA is the molecule that is mostly damaged by oxygen radicals and the resultant injury (66). In the study conducted by Martin et al. (67), DNA fragmentation was significantly increased in rats with TNBS colitis. Similar to that, induction of colitis in our study resulted in increased DNA fragmentation in the colonic tissue. On the other hand, the present results also indicated that ALA treatment supports the maintenance of cellular integrity by reducing free radical generation, subsequent lipid peroxidation and DNA damage. Furthermore, as assessed by the colonic collagen content, current findings suggest that ALA 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 ALA treatment.

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

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