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Green tea polyphenol extract attenuates colon injury induced by experimental colitis

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Abstract

Inflammatory bowel disease (IBD) is characterised by oxidative and nitrosative stress, leukocyte infiltration, and up-regulation of intercellular adhesion molecule 1 (ICAM-1) expression in the colon. The aim of the present study was to examine the effects of green tea extract in rats subjected to experimental colitis induced by intracolonic instillation of dinitrobenzene sulphonic acid (DNBS). At 4 days after DNBS administration the rats were sacrificed. Treatment with green tea extract significantly attenuated diarrhoea and loss of body weight. This was associated with a remarkable amelioration of the disruption of the colonic architecture, significant reduction of colonic myeloperoxidase (MPO) and tumor necrosis factor-α (TNF-α) production. Green tea extract also reduced the appearance of nitrotyrosine immunoreactivity in the colon and reduced the up-regulation of ICAM-1.

Keywords: *Colitis, green tea extract, free radical, inflammatory infiltration and ICAM-1*

Abbreviations: *IBD, Inflammatory bowel disease;* (O_2^-)*,, superoxide; NO, nitric oxide; OH, hydroxyl radicals; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; PARS, poly(ADP-Ribose)synthetase; EGCG, eigallocatechin-3-gallate; DNBS, dinitrobenzene sulphonic acid; GTE, green tea extract; MPO, myeloperoxidase; CDAI, Crohn's disease activity index; PMNs, polymorphonuclear cells; ICAM-1, intercellular adhesion molecule-1; HO-1, heme-oxygenase-1*

Introduction

A growing body of data indicates that oxygen derived free radicals such as superoxide (O_2^-), nitric oxide (NO) and hydroxyl radicals (OH) have a role in mediating intestinal damage in inflammatory bowel disease. Several studies suggest that peripheral blood monocytes [1] and isolated intestinal macrophages from patients with inflammatory bowel disease produce increased amounts of free radicals. Also, high numbers of peripheral neutrophils, which are capable of producing large amounts of oxygen derived free radicals, migrate into the intestinal wall of such patients [2]. The pro-inflammatory roles of reactive oxygen species (ROS) are well known: recruitment of neutrophils at sites of inflammation, formation of chemotactic factors, depolymerization of hyaluronic acid and collagen, lipid peroxidation, release of cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) [3]. Moreover, ROS can also cause DNA damage resulting in the activation of the nuclear enzyme poly(ADP-ribose)synthetase (PARS) and poly (ADP-ribose) synthase-driven cell death. Interventions which reduce the generation or

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the effects of ROS exert beneficial effects in a variety of models of inflammation including the DNBS-induced colitis used here [3].

Green tea as well as black tea contains various polyphenolic compounds (flavan-3ols) generally known as catechins. Epigallocatechin-3-gallate (EGCG) is a major catechin present in green tea. Pharmacological functions of catechins have been studied, including protection against coronary heart disease [4], various types of cancer and inflammatory diseases [5]. Recently, various studies have clearly demonstrated that consumption of green and black tea significantly increased human plasma antioxidant capacity *in vivo*. Moreover, recently it has been demonstrated that green tea catechins possess potent antioxidative properties on low density lipoproteins *in vitro* and *ex vivo* [6]. Therefore, it has been also demonstrated that EGCG reduced the neutrophil chemotaxis *in vitro* [7] as well as possesses antiplatelet activities. In addition the protective effects of tea catechins were evaluated by the attenuation of NO formation from inducible NO synthase, protein kinase C [8], activation of extracellular mitogen-activated protein kinases [9], and STAT-1 [10]. Furthermore, recently Varilek and colleagues [11] have clearly demonstrated that green tea extract attenuated inflammation in IL-2 deficient mice and suggest a role for green tea extract in treating chronic inflammatory diseases such as inflammatory bowel disease.

The objectives of the present study were to address whether green tea extract exerted protection on the chronic inflammatory response (colitis) caused by injection of dinitrobenzene sulphonic acid (DNBS) in the rat. To verify that green tea extract exerts this beneficial therapeutic effect by interfering with neutrophils infiltration and release of pro-inflammatory mediators (e.g. ROS, TNF-α), we have investigated the effects of green tea extract on (i) the degree of colonic injury, (ii) the rise in MPO activity in the mucosa, (iii) the production of TNF-α in the colon levels, (iv) the increase in staining (by immunohistochemistry) for nitrotyrosine, as well as (v) the increased expression of ICAM-1 and (vi) the release of HO-1 caused by DNBS in the colon.

Materials and methods

Animals

Male Sprague-Dawley rats (300–350 g; Charles River; Milan; Italy) were housed in a controlled environment and provided with standard rodent chow and water. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purpose (D.M. 116192) as well as with the EEC regulations (O.J. of E.C. L 358/1 12/18/1986).

Green tea extract

Green tea extract (GTE) was a kind gift of Indena (Milano, Italy), and it was defined by the producer as a caffeine-free extract from green tea leaves. The content of GTE was investigated by HPLC and is characterized by a high content (75 ± 5%) of polyphenols, the major constituent being epigallocatechin-3-gallate at 62% and the minor ones being epicatechin-3-gallate, epigallocatechin and epicatechin (Figure 1; Patent Appl. WO 96/28178). GTE used here to reduce DNBS-induced colitis have previously been reported to reduce the tissue injury caused by ischemia-reperfusion in the heart [12].

Induction of experimental colitis

Colitis was induced using a technique of acid-induced colonic inflammation as described previously [13]. On day 0, in fasted rats lightly anaesthetised with isoflurane, a 3.5 F catheter was inserted into the colon via the anus until approximately the splenic

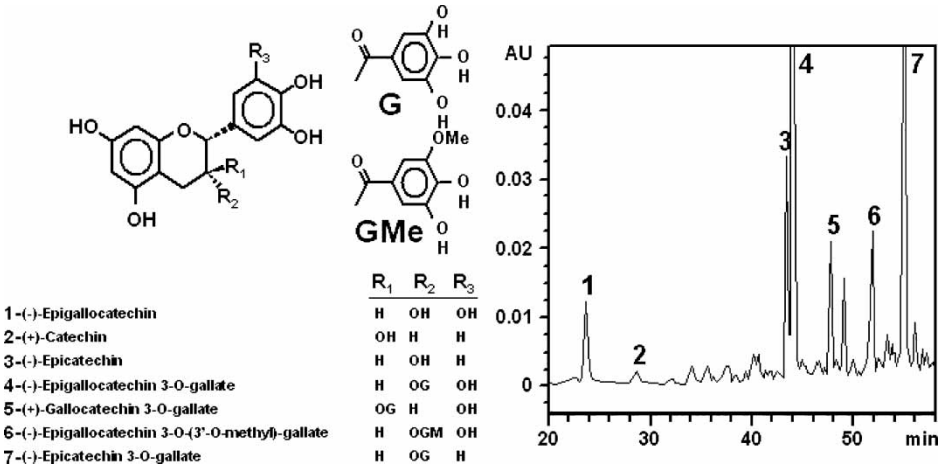

Figure 1. Polyphenol constituents and HPLC profile of Green tea extract

flexure (8 cm from the anus). 2,4,6-dinitrobenzene sulphonic acid (DNBS; 25 mg/rat $n = 10$) was dissolved in 50% ethanol (total volume, 0.8 ml). Thereafter, the animals were kept for 15 min in a Trendelenburg position to avoid reflux. Ten animals (sham-colitis) received an enema with vehicle alone (50% ethanol, 0.8 ml). After colitis and sham-colitis induction, the animals were observed for 4 days. On day 4, the animals were weighed and anaesthetised with chloral hydrate (400 mg/kg, intraperitoneally). The abdomen was opened by a midline incision. The colon was removed, freed from surrounding tissues, opened along the antimesenteric border, rinsed, weighed, and processed for histology and immunohistochemistry. GTE (50 mg/kg, n = 10) or vehicle (saline solution $n = 10$) was given daily as an intraperitoneal bolus starting 24 h after DNBS administration.

Evaluation of colonic damage

On day 4, the animals were weighed and anaesthetized with chloral hydrate, and the abdomen was opened by a midline incision. The colon was removed, freed from surrounding tissues, opened along the antimesenteric border, rinsed, weighed and processed for histology and immunohistochemistry. Colon damage (macroscopic damage score) was evaluated and scored by two independent observers as described previously [13,14] according to the following criteria: 0, no damage; 1, localized hyperaemia without ulcers; 2, linear ulcers with no significant inflammation; 3, linear ulcers with inflammation at one site; 4, two or more major sites of inflammation and ulceration extending >1 cm along the length of the colon and 5–8, one point is added for each centimetre of ulceration beyond an initial 2 cm.

Light microscopy

After fixation for 1 week at room temperature in Dietrich solution (14.25% ethanol, 1.85% formaldehyde, 1% acetic acid), samples were dehydrated in graded ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, New Jersey). Thereafter, 7-μm sections were deparaffinized with xylene, stained with haematoxylin-eosin and trichromic van Giesson's stain, and observed in a Dialux 22 Leitz (Wetziar, Germany) microscope. In order to have a quantitative estimation of colon damage, section ($n = 6$ for each animals) was scored by 2 independent observers blinded to the experimental protocol. The following morphological criteria were considered: Score 0, no damage; score 1 (mild), focal epithelial oedema and necrosis; score 2 (moderate), diffuse swelling and necrosis of the villi; score 3 (severe), necrosis with presence of neutrophil infiltrate in the submucosa; score 4 (highly severe), widespread necrosis with massive neutrophil infiltrate and hemorrhage.

Myeloperoxidase activity

Myeloperoxidase (MPO) accumulation was determined as previously described [15]. At 4 days after intra-colonic injection of DNBS, the colon was removed and weighed. The colon was homogenized in a solution containing 0.5% hexa-decyl-trimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 min at 20,000*g* at 4°C. An aliquot of the supernatant was then allowed to react with a solution of tetra-methyl-benzidine (1.6 mM) and 0.1 mM H_2O_2. The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1 μmol of peroxide per min at 37°C and was expressed in milliunits per gram weight of wet tissue.

Measurement of TNF-α

Portions of terminal colon, collected at 4 days after intra-colonic injection of DNBS, were homogenized as previously described [16] in PBS containing 2 mmol/l of phenyl-methyl sulfonyl fluoride (Sigma Chemical Co.) and tissue levels of TNF-α were evaluated. The assay was carried out by using a colorimetric, commercial kit (R&D System, Milan, Italy) according to the manufacturer instructions. All TNF-α determinations were performed in duplicate serial dilutions.

Localization of nitrotyrosine, ICAM-1, nitrotyrosine and HO-I by immunohistochemistry

Four days after the administration of DNBS, the tissues were fixed in 10% PBS-buffered formaldehyde and 8 μm sections were prepared from paraffin embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% H_2O_2 in 60% methanol for 30 min. The sections were permeabilized with 0.1% Triton X-100 in PBS for 20 min. Non-specific adsorption was minimized by incubating the section in 2% normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with avidin and biotin (DBA, Milan, Italy). Sections were incubated overnight with anti-nitrotyrosine rabbit polyclonal antibody (1:500 in PBS), with anti-ICAM-1 polyclonal antibody (CD54) (1:500 in PBS, v/v) (DBA, Milan, Italy), with anti-HO-I polyclonal antibody (1:100 in PBS, v/v) (Santa Cruz, DBA, Milan, Italy). Specific labeling was detected with a biotin-conjugated goat anti-rabbit, donkey anti-goat or goat anti-mouse IgG and avidin-biotin peroxidase complex (DBA, Milan, Italy). To verify the binding specificity for ICAM-1 or HO-I some sections were also incubated with primary antibody only (no secondary antibody) or with secondary antibody only (no primary antibody).

In these situations, no positive staining was found in the sections indicating that the immunoreactions were positive in all the experiments carried out. In order to confirm that the immunoreactions for the nitrotyrosine were specific some sections were also incubated with the primary antibody (anti-nitrotyrosine) in the presence of excess nitrotyrosine (10 mM) to verify the binding specificity.

Immunocytochemistry photographs ($N = 5$) were assessed by densitometry by using Optilab Graftek software on a Macintosh personal computer.

Reagents

Biotin blocking kit, biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex were obtained from Vector Laboratories (DBA, Milan, Italy). Primary anti-nitrotyrosine antibody was purchased from Upstate Biotech (DBA, Milan, Italy). Primary ICAM-1 (CD54) for immunoistochemistry was purchases by Pharmingen (DBA, Milan, Italy). Reagents and secondary and nonspecific IgG antibody for immunohistochemical analysis were from Vector Laboratories InC (DBA, Milan, Italy). All other reagents and compounds used were obtained from Sigma Chemical Company (Milan, Italy).

Statistical analysis

All values in the figures and text are expressed as mean ± SEM of *n* observations, where *n* represents the number of animals studied. In the experiments involving histology or immunohistochemistry, the figures shown are representative of at least 3 experiments performed on different experimental days. Data sets were examined by one- and two-way analysis of variance and individual group means were then compared with Student's unpaired *t*-test. Non-parametric data were analyzed with the Fisher's exact test. A *P*-value less than 0.05 was considered significant.

Results

The degree of colitis (histology and general assessment)

Four days after intra-colonic administration of DNBS, the colon appeared flaccid and filled with liquid stool. The macroscopic inspection of cecum, colon and rectum showed presence of mucosal congestion, erosion and hemorrhagic ulcerations (Figure 2b and d). The histopathological features included a transmural necrosis and oedema and a diffuse leukocyte cellular infiltrate in the submucosa of colon section from DNBS-treated rats (Figure 3b and d). The observed inflammatory changes of the large intestine were associated with an increase in the weight of the colon (Figure 4A). The treatment with GTE significantly reduced the extent and severity of the histological signs of colon injury (Figures 2c, d and 3c, d) as well as the colon weight (Figure 4A). Four days after colitis induced by DNBS treatment, all rats had diarrhea and a significant reduction in body weight (compared with the control groups of rats) (Figure 4B). GTE treatment resulted in a significant reduction of lost of body weight induced by DNBs-administration in rats (Figure 4B). No histological alteration was observed in the colon tissue from vehicle-treated rats (Figures 2a, d and 3a, d).

Production of TNF-α after DNBS administration

To test whether the treatment with GTE may modulate the inflammatory process through the regulation of the secretion of others cytokines, we analyzed the colon levels of pro-inflammatory cytokines TNF-α. A substantial increase of TNF-α formation was found in colon samples collected from vehicle-treated rats at 4 days after DNBS administration (Figure 4C). In contrast a significant inhibition of TNF-α levels was observed in the colon tissues collected from of DNBS-treated rats, which have been treated with GTE (Figure 4C).

ICAM-1 expression and polymorphonuclear (PMN) infiltration

The colitis caused by DNBS was also characterized by an increase in MPO activity, an indicator of the neutrophils accumulation in the colon (Figure 4D). This finding is consistent with the observation made with light microscopy that the colon of vehicle-treated DBNS-rats contained a large number of neutrophils. On the contrary, GTE treatment significantly reduced the degree of PMN infiltration (determined as increase in MPO activity) in inflamed colon (Figure 4D). To further elucidate the effect of GTE treatment on neutrophils accumulation in inflamed colon, we evaluated the intestinal expression of ICAM-1. Tissue sections obtained from sham-treated rats with anti-ICAM-1 antibody showed a specific staining along the vessels, demonstrating that ICAM-1 is expressed constitutively in endothelial cells (Figures 5a and 6). After DNBS administration, the staining intensity substantially increased in the vessels of the lamina propria and submucosa. Immunohistochemical staining for ICAM-1 was also present in epithelial cells of injured colon and in infiltrated inflammatory cells in damaged tissues from DNBS-treated rats (Figures 5b and 6). Section from GTE-treated rats did not reveal any up-regulation of the constitutive ICAM-1, which was normally expressed in the endothelium along the vascular wall (Figures 5c and 6).

Nitrotyrosine formation

To determine the localization of peroxynitrite formation and/or other nitrogen derivatives produced during

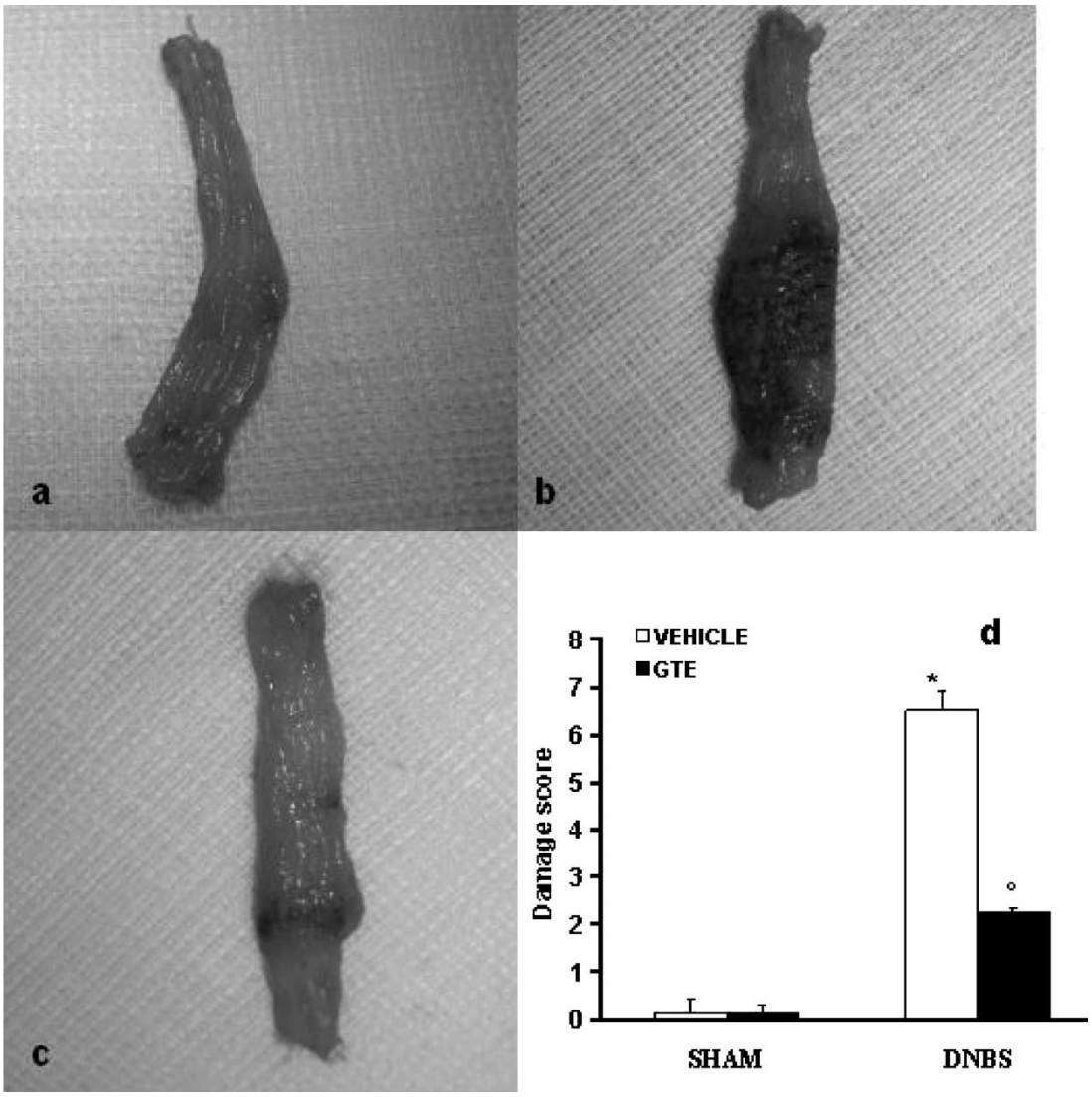

Figure 2. Effect of GTE treatment on clinical expression of DNBS-induced colitis and macroscopic damage score. Colon tissues from sham-treated rats (a), from DNBS-treated rats at 4 days post DNBS administration (b) and from DNBS-treated rats which have received GTE treatment (c). The macroscopic damage score (d) was made by two independent observers. Figure is representative of all the animals in each group. Data are means ± SEM of 10 rats for each group. $^{*}P < 0.01$ vs. SHAM; $^{\circ}P < 0.01$ vs. DNBS.

colitis, nitrotyrosine, a specific marker of nitrosative stress, was measured by immunohistochemical analysis in the distal colon. Sections of colon from sham-administered rats did not stain for nitrotyrosine (Figures 5d and 6). Colon sections obtained from vehicle-treated DNBS-treated rats exhibited positive staining for nitrotyrosine (Figures 5e and 6) localized in inflammatory cells and in disrupted epithelial cells. Section from GTE-treated rats shown significantly less positive staining for nitrotyrosine (Figures 5f and 6).

The expression of heme-oxygenase-1 (HO-1)

To elucidate whether GTE causes the expression of the protective enzyme HO-1, we have investigated the effects of GTE on the colon expression of HO-1. When compared to colon tissue section from DNBS-treated rats (Figures 5h and 6), which had not been treated with GTE, the GTE treatment induced a significant increase in the colon expression of HO-1 (Figures 5i and 6).

Discussion

The present findings demonstrate that GTE attenuates DNBS-induced colitis in the rats, an established model for human Crohn's disease. In particular we have demonstrated that GTE treatment inhibits: (i) the degree of diarrhoea and weight loss, (ii) the degree of colonic injury, (iii) the infiltration of the colon PMNs, (iv) the positive staining (immunohistochemistry) for nitrotyrosine, as well as (v) the increased expression of ICAM-1 and (vi) TNF-α production caused by DNBS in the colon. What, then, is the

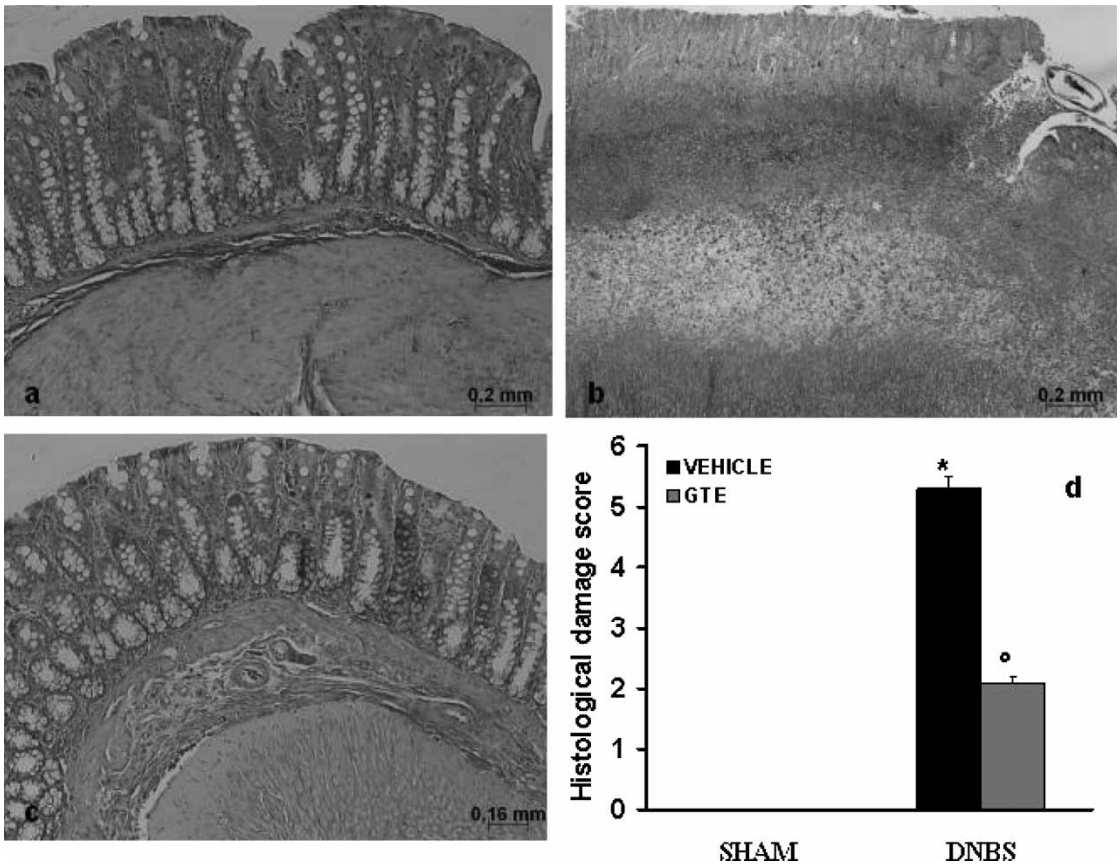

Figure 3. Effect of GTE treatment on colon injury and histological score. No histological alteration was observed in the colon section from sham-treated rats (a). Mucosal injury was produced after DNBS administration characterized by absence of epithelium and a massive mucosal and submucosal infiltration of inflammatory cells (b). Treatment with GTE (c) corrected the disturbances in morphology associated with DNBS administration. The histological score (d) was made by two independent observers. Figure is representative of at least 3 experiments performed on different experimental days. Data are means ± SEM of 10 rats for each group. $^{\star}P < 0.01$ *vs.* SHAM; $^{\circ}P < 0.01$ *vs.* DNBS.

mechanism by which GTE inhibits the colon inflammation caused by injection of DNBS?

Reactive oxygen and nitrogen species play a key role in inflammatory bowel disease [17]. These species are cytotoxic agents, inducing lipid peroxidation and other cellular oxidative stress by cross linking proteins, lipids and nucleic acids, which then cause cellular dysfunction, damage and eventually death. Evidence consistent with damage by reactive radical species is provided by the increase in lipid peroxides in rectal biopsy specimens from patients with ulcerative colitis [17]. In the present study, we found that the mucosal damage induced by intracolonic administration of DNBS was associated with a significant presence of nitrotyrosine formation. Recent evidence indicates that nitration of tyrosine can result from a number of chemical actions, and can be considered as a global marker of nitrosative stress. Nitrotyrosine can be formed from the reaction of nitrite with hypochlorous acid or the reaction of nitrite with myeloperoxidase and hydrogen peroxide [18]. These data are consistent with previous findings that immunohistochemical staining for nitrotyrosine was localized on epithelial cells in rat colitis and in active Crohn's lesions in humans [19]. The pathogenic role of nitrogen derived species such as peroxynitrite in inflammatory bowel disease is further supported by the fact that intracolonic administration of exogenous peroxynitrite induces a severe colonic inflammation which mimics the features of both ulcerative colitis and Crohn's disease [20].

In the present study we observed that epithelial disruption was significant less in rats treated with GTE. Indeed, GTE treatment prevented the formation of tissue nitrotyrosine staining in DNBS-treated animals. Furthermore, GTE-treated rats are more resistant to DNBS-induced colitis with a significant resolution of the macroscopic and histological signs of the inflammatory process. The antioxidant properties of GTE have attracted considerable attention for the prevention of oxidative stress-related diseases such as ischemic diseases [21].

TNF-α is clearly involved in the pathogenesis of colitis since this cytokine is present in colon tissues and can be detected immunohistochemically in the inflamed tissues [22]. Direct evidence that TNF-α plays a role in the pathogenesis of experimental colitis

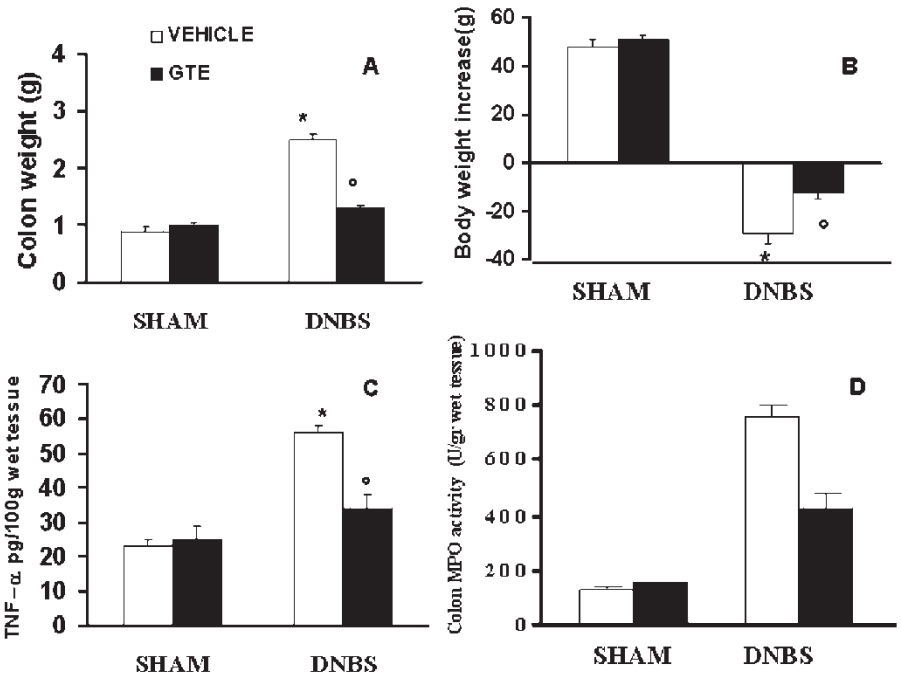

Figure 4. Effect of GTE treatment on colon weight (A), body weight loss (B), colon levels of TNF-α (C) and polymorphonuclear leukocyte infiltration (D) after DNBS intracolonic administration. 4 days after DNBS administration a significant increase in the weight of the colon, a significant reduction in body weight increase, a significant increase of TNF-α production in the colon and a significant increase of myeloperoxidase (MPO) activity were observed. Treatment with GTE significantly reduced the increase of colon weight, the body weight lost, the increase of colon TNF-α production as well as the increase of MPO activity. Data are means ± SEM of 10 rats for each group. *$P < 0.01$ vs. SHAM; °$P < 0.01$ vs. DNBS.

has been obtained in animal models in which blocking of the action of this cytokines has been shown to delay the onset of experimental colitis, suppress inflammation, and ameliorate colon destruction that corresponds to the anti-inflammatory response. A role for TNF-α in human disease came from recent studies using Infliximab [23], a chimeric anti-TNF antibody, and CDP571, a humanized monoclonal antibody to TNF-α and membrane bound TNF without fixing complement or mediating antibody-dependent cellular cytotoxicity [23]. In both cases, significant reduction in Crohn's disease activity index (CDAI) as well as attenuation of attenuated histopathology and endoscopic inflammation in Crohn's disease patients was observed.

Since ROS regulate cytokine release (by mechanisms yet to be defined) we postulate that inhibition of TNF-α in the colon plays an important role in the overall beneficial effects observed with GTE.

Neutrophils play a crucial role in the development and full manifestation of gastrointestinal inflammation, as they represent a major source of free radicals in the inflamed colonic mucosa [24]. The interactions of PMN with the endothelium are regulated by various adhesion molecules. In particular, the firm adherence involves the interaction of β_2 integrins (i.e. CD11/CD18) on the PMN surface and intercellular adhesion molecule-1 (ICAM-1) on the endothelial cell surface.

In the present study, we have observed that ICAM-1 was expressed in endothelial and epithelial cells, and neutrophils in the distal colon in DNBS treated rats. This was associated with a significant reduction of ICAM-1 expression in endothelial and epithelial cells in the distal colon in GTE-treated rats. Our data confirm that ROS formation contribute to the regulation of neutrophil infiltration and is consistent with previously published data in models of acute inflammation including the model of DNBS-induced colitis [25].

A variety of environmental stresses (including inflammation) lead to the expression of heme-oxygenase-1 (HO-1), which results in the formation of the antioxidant bilirubin as well as carbon monoxide. There is substantial evidence that this up-regulation of HO-1 protects many tissues and organs against subsequent injury [26]. We report here that GTE causes the up-regulation of the expression of the protective protein HO-1 in colon. These findings suggest that the up-regulation of the expression of HO-1 caused by GTE may contribute to the protective effects of this extract *in vivo*.

Taken together, the data presented in the present study and in another recent report [20] demonstrates that GTE treatment shows a remarkable recovery of the mucosal morphology. Therefore, a major finding of this study was that beneficial effect observed in GTE-treated rats was associated with a reduction in

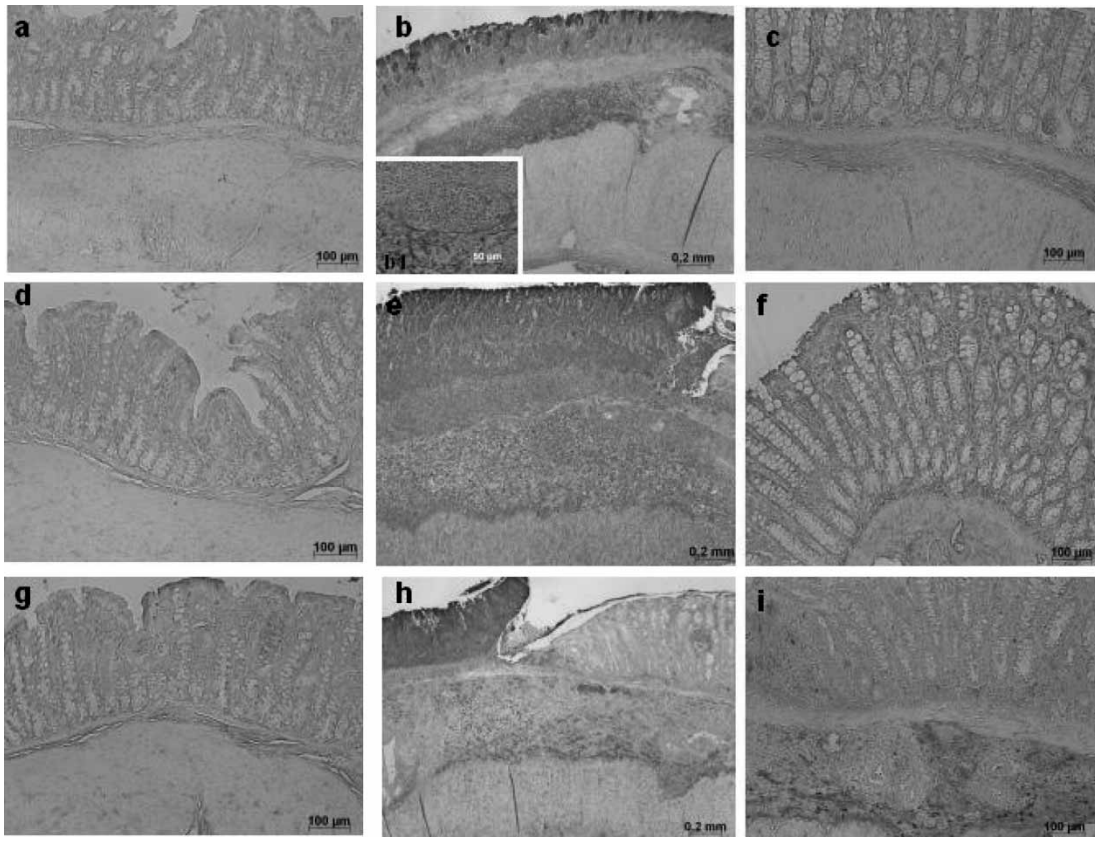

Figure 5. Immunohistochemical localization of ICAM-I, nitrotyrosine and HO-I in the colon. Immunohistochemical analysis for ICAM-1 (b see inset b1), for nitrotyrosine (e) and for HO-I (h) show positive staining in the injured area from DNBS-treated rats. The intensity of the positive staining for ICAM-1 (c) for nitrotyrosine (f) was markedly reduced in tissue section obtained from DNBS-treated rats which have been treated with GTE. On the contrary, the intensity of the positive staining for HO-1 (i) was markedly enhanced in tissue section obtained from DNBS-treated rats which have been treated with GTE. No positive staining for ICAM-1 (a), for nitrotyrosine (d) and for HO-I (g) was observed in the tissue section from sham-treated rats. Figure is representative of at least 3 experiments performed on different experimental days.

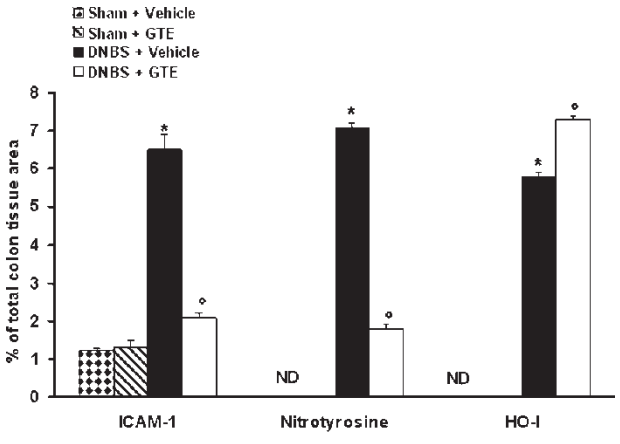

Figure 6. Typical densitometry evaluation densitometry analysis of immunocytochemistry photographs ($n = 5$) for ICAM-1, nitrotyrosine and HO-I from colon was assessed. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). Data are expressed as % of total tissue area. ND: Not detectable. $^{*}P < 0.01$ vs. SHAM; $^{\circ}P < 0.01$ vs. DNBS.

oxidative and nitrosative damage after DNBS administration as well as with the inhibition of inflammatory cell infiltration.

In particular GTE treatment, reduced (i) the nitration of proteins (ii) the formation of the pro-inflammatory cytokines, (iii) the expression of the adhesion molecule ICAM-1 and enhanced the HO-I formation. Finally, we speculate that GTE and related compounds may be useful in the therapy of conditions associated with inflammation.

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