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Black tea and theaflavins suppress various inflammatory modulators and i-NOS mediated nitric oxide synthesis during gastric ulcer healing

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Abstract

The modulation of the cyclooxygenase-independent pathway by black tea (BT) and its constituent theaflavins (TFs) during their healing action against indomethacin-induced stomach ulceration in mice was investigated. On the 3rd day of its administration, indomethacin (18 mg/kg) induced maximum stomach ulceration, which was associated with increased myeloperoxidase (MPO) activity (93.3%, $p < 0.001$), and inducible nitric oxide synthase (iNOS) expression (1.6-fold, $p < 0.001$), along with augmented levels of serum nitrite (1.5-fold, $p < 0.001$), selectins and cell adhesion molecules (CAMs), as well as reduced endothelial nitric oxide synthase (eNOS) expression (60%, $p < 0.001$). Treatment with BT (40 mg/kg) and TF (1 mg/kg) for 3 days reversed these parameters and provided excellent (78–81%) ulcer healing. However, alterations of NOS expressions and levels of selectins and CAMs were only partially responsible for the excellent healing capacity (~80%) of omeprazole (3 mg/kg × 3 days).

Keywords: *CAMs, indomethacin, iNOS, eNOS, MPO, selectins*

Introduction

Gastric ulcer is a complex pluricausal inflammatory disease and is known to develop due to the disruption of mucosal defense [1]. Although inflammation is a protective response to cellular/tissue injury, if left uncontrolled it results in destruction of normal tissues. Current evidence suggests that *Helicobacter pylori* and use of non-steroidal anti-inflammatory drugs (NSAIDs) are the major causative factors in the pathogenesis of human gastric mucosal injury. In particular, the NSAID-induced stomach ulceration is a major health concern, ranking fourth in terms of causing morbidity and mortality [2]. Currently, the use of NSAIDs accounts for ~25% of gastric ulcer cases [3]. Besides causing gastric ulceration, the NSAIDs also delay ulcer healing [4]. Although the NSAID-induced gastropathy is generally believed to be related to inhibition of prostaglandin (PG) synthesis [5], accumulated evidence suggests that multiple factors such as oxidative stress [6], neutrophil infiltration, leucocyte-endothelium interaction and cytokine imbalance [7] also play key roles in the pathogenesis of the gastric damage induced by the NSAIDs.

Neutrophils are the highest producers of reactive oxygen species (ROS) as well as nitric oxide (NO), leading to oxidative burst [8], which inflicts endothelial damage [9,10]. Since activated neutrophils produce many enzymes and free radicals that damage the gastric mucosa, these are believed to contribute to ulcer formation. The activated neutrophils produce myeloperoxidase (MPO), which possesses powerful pro-oxidative and pro-inflammatory properties. Hence,

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it is considered a useful risk marker and diagnostic tool against oxidative stress even under clinical conditions [11,12]. Because MPO is mainly produced by neutrophils, MPO activity is also considered as an index for the evaluation of neutrophil infiltration [13].

The physiologically important free radical NO, produced during arginine catabolism by nitric oxide synthase (NOS), plays dual roles in gastric mucosal defense and injury. The low concentration of NO, produced by the endothelial NOS (eNOS), one of the constitutive NOS isoforms, helps in wound healing by increasing blood flow [14] and angiogenesis [15] in the damaged gastric mucosa. However, the enhanced generation of NO by the inducible NOS (iNOS) may contribute to the pathogenesis of various gastroduodenal disorders including peptic ulcer [16]. Thus, the status of the eNOS vs iNOS expressions in the gastric tissues is very crucial for maintaining their integrity. Adhesion of neutrophilic polymorphonuclear leucocytes (PMNs) to the endothelium is a critical early step of inflammatory, immune responses and NSAID-induced gastropathy [17]. This is mediated by interaction between adhesion molecules expressed on the endothelial cell (EC) surface and their ligands on the leukocyte surface. The NSAID-induced gastric mucosal injuries are significantly reduced in neutropenic rats and by immunoneutralization of CD18, intercellular adhesion molecule 1 (ICAM-1) and P- as well as E-selectins [18,19]. The above inter-connected processes are critically governed by the imbalance of the pro- and anti-inflammatory (Th_1 vs Th_2) cytokines. However, little is known on these aspects, especially in the indomethacin-induced gastric ulceration.

Despite the present dominance of the scientifically proven therapies and availability of various synthetic anti-ulcer drugs, efficient management of the NSAIDs- and *H. pylori*-induced gastric ulceration remains a challenging medical problem. This can be attributed to the various side-effects and high cost of the existing drugs, ulcer recurrence [20], as well as microbial resistance to these drugs and emerging mutants of the microbes. For decades, doctors have recommended dietary adjustments aimed at preventing or treating symptoms of gastritis and ulceration, as diet may moderate the risk of gastritis or peptic ulcer [21]. *Camellia sinenesis* is widely grown in the tropical humid climate of South East Asia and decoction of its leaves (tea) is the most popular non-alcoholic beverage worldwide. Black tea (BT) accounts for 80% of the total tea consumption in the world. However, studies on the pharmacological properties of BT are scarce. There has been a mounting interest in exploring the possibility of using BT in its totality, and not on certain isolated fractions/constituents as a supplement among patients [22]. The prophylactic action of BT against various ulcerogens have been reported in rat models [23,24]. Very recently, we have documented impressive healing activity of BT and established its major constituent, theaflavins (TF), as the active principles. It was found that oral administration of BT (40 mg/kg) or TF (1 mg/kg) for 3 days could effectively heal the indomethacin (18 mg/kg, *p. o.*, single dose)-induced stomach ulceration in mice. The healing activity of BT and TF could be partly attributed to their antioxidant action as well as the ability to augment the COX isozymes that improved PG synthesis [25]. The aim of the present study was to understand whether BT and TF could also modulate the intrinsic COX-independent pathways of the indomethacin-mediated gastric inflammation. To this end, we examined the effect of BT and TF on pharmacological inhibition of iNOS and neutrophil infiltration, induced by indomethacin in mice. Because neutrophils are very important cells for the production of NO through iNOS [26], we also examined the effect of pharmacological inhibition of neutrophil adherence by BT and TF and the consequence in blocking the function of soluble forms of E- and P-selectins as well as ICAM-1 and vascular cell adhesion molecule 1 (VCAM-1). Further, the status of the pro- and anti-inflammatory cytokines that control the inflammatory response during wound healing was also investigated.

Materials and methods

Chemicals and reagents

Leaves of *C. sinenesis* (Brooke Bond, Red label), procured from the local market, were identified by hptlc and hplc (Jasco model PU-2080 plus chromatogram) analyses of its chemical constituents. The chemical composition has already been reported in our recent publication [25]. TF (a mixture of theaflavin-3-gallate, theaflavin-3′-gallate and theaflavin-3,3′-digallate) was procured from Sigma (St. Louis, MO). Analysis with a Bio-Whitaker QCL 1000 kits (Cambridge, MA) revealed BT and TF to be practically free of endotoxin. Indomethacin, Bradford reagent, Triton X-100, leupeptin, phenylmethylsulphonyl fluoride (PMSF), glycine, sodium dodecyl sulphate (SDS), acrylamide, bis-acrylamide, Tween 20, ethylenediaminetetraacetic acid (EDTA), 3,3′,5,5′-tetramethylbenzidine (TMB), omeprazole (Omez), Trizma base, cetyltrimethylammonium bromide (CTAB), 3,3′-diaminobenzidine (DAB) and nitrocellulose membrane were procured from Sigma Chemicals (St. Louis, MO). Other reagents used were disodium hydrogen phosphate and sodium dihydrogen phosphate (BDH, Poole, Dorset, UK), sulphuric acid, sodium chloride (Thomas Becker, Mumbai, India), horseradish peroxidase (HRPO, Sisco Research Laboratory, Mumbai, India), rabbit polyclonal iNOS and eNOS antibodies (Santacruz Biotechnology, DE), peroxidase conjugated anti-rabbit IgG antibody,

enhanced chemiluminescence detection kit (Roche, Mannheim, Germany), NOS and nitrite assay kits (Calbiochem, Darmstadt, Germany, CA) and cytokine ELISA kits (Pierce Biotechnology, Rockford, IL).

Instrumentation

The absorbance spectrophotometry was carried out at 25°C using an ELISA reader (BioTek Instrument, Winooski, VT, USA). The bands obtained from the western blots were quantified using the Gelquant software (DNR Bioimaging System, version 2.7.0, Israel). The hptlc analysis of BT was carried out with a Camag Instrument, RP-18 silica gel G plate and ethyl acetate:methanol:water = 10:1.1:1 as the solvent. The hplc analysis of TF was carried out with a Jasco chromatogram model PU-2080 plus.

Preparation of BT

Tea leaves (30 g) were soaked in pre-heated (102°C) distilled water (100 ml), allowed to stand for 5 min, and the supernatant decanted. The process was repeated twice and the combined aqueous extracts were lyophilized to get BT as a sticky dark brown solid.

Preparation of the test samples

The test samples (BT, TF and Omez) were prepared as aqueous suspensions in 2% gum acacia as the vehicle and administered to the mice orally.

Animals

Male Swiss albino mice, bred at the BARC Laboratory Animal House Facility, Mumbai, India, were procured after obtaining clearance from the BARC Animal Ethics Committee (BAEC). All the experiments were conducted with strict adherence to the ethical guidelines laid down by European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes. In addition, the ethical guidelines, laid down by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), constituted by the Animal Welfare Division, Government of India on the use of animals in scientific research was followed. The mice (6–8 weeks old, 25–30 g) were reared on a balanced laboratory diet as per National Institute of Nutrition, Hyderabad, India and given tap water *ad libitum*. They were kept at 20 ± 2°C, 65–70% humidity and 12 h day/12 h night cycles. The experiments were performed by two investigators blinded to the group and treatment of animals, which were identified by typical notches in the ear and limbs (performed at a pre-weaning stage to minimize the pain to the animals) and then randomized.

Protocol for ulceration and biochemical studies

The mice were divided into several groups (each containing five mice) and each experiment was repeated three times. Except for the normal control, ulceration in the other mice was induced by indomethacin (18 mg/kg, p. o., single dose), dissolved in distilled water and suspended in 2% gum acacia as the vehicle. For the standardization of doses, BT (10–50 mg/kg, p. o.) or TF (0.5–5.0 mg/kg, p. o.) were given to the mice once daily up to 3 days, starting the first dose 6 h after the indomethacin-administration. In the subsequent days, the test samples were given at 9 am on each day. Omez (3.0 mg/kg, p. o.) was used as the positive control. The doses of indomethacin and Omez were standardized in our earlier studies [25,27]. The normal and ulcerated control groups of mice were given the vehicle (0.2 ml) during the entire period of study. Four hours after the last dose of the test samples, the mice were sacrificed on the 3rd day under anaesthesia with thiopental, the stomachs were opened along the greater curvature, thoroughly rinsed with normal saline, and the wet weights of the tissues were recorded. Additional experiments were also carried out to assess the tissue MPO activity and iNOS expression as well as serum TNF-α level at different time points (3–24 h) after indomethacin administration. These provided information about the time of induction of these parameters in the ulcerated mice. In separate experiments, treatments were also carried out with L-NAME (10 mg/kg, once daily) and L-NIL (3 mg/kg, twice daily) alone or in conjunction with TF (at its optimized dose) for 3 days.

Ulcer healing assessment

The ulcerated portions of the stomach were sectioned after fixing in 10% formol saline solution. After 24 h of fixation followed by embedding in a paraffin block, it was cut into sections of 5 micron onto a glass slide, stained with haematoxylene-eosin and the histology examined under a light microscope. One centimetre length of each histological section was divided into three fields. The damage score (DS) [28] was assessed by scoring each field on a 0−4 scale: 0 = normal mucosa, 1 = epithelial cell damage, 2 = glandular disruption, vasocongestion or oedema in the upper mucosa, 3 = mucosal disruption, vasocongestion or oedema in the mid-lower mucosa and 4 = extensive mucosal disruption involving the full thickness of the mucosa. The overall mean value of the damage scores (DS) for each of the fields was taken as the histological ulcer index for that section.

Likewise, the inflammatory scores (IS) [29] were assigned after reviewing all slides to assess the range of inflammation as follows: 0 = normal mucosa, 1 = minimal inflammatory cells, 2 = moderate number of inflammatory cells, and 3 = large number of inflammatory cells.

Histological sections were coded to eliminate an observer bias. Data for the histological analyses are presented as the mean ± SEM from the review of a minimum of three sections per animal and five animals per group.

MPO assay

The MPO activity was assayed following a reported method [30] with slight modifications. The glandular portions of the stomach tissues were homogenized for 30 s in a 50 mM phosphate buffer (pH 6.0), containing 0.5% CTAB and 10 mM EDTA, followed by freeze–thawing three times. The homogenate was centrifuged at 12 000 × g for 20 min at 4°C. The supernatant was collected and the protein content determined. The supernatant (50 µl) was added to 80 mM phosphate buffer, pH 5.4 (250 µl), 0.03 M TMB (150 µl) and 0.3 M H_2O_2 (50 µl). After incubating the mixture at 25°C for 25 min, the reaction was terminated by adding 0.5 M H_2SO_4 (2.5 ml). The absorbance of the mixture at 450 nm was recorded using HRPO as the standard. The MPO activity is expressed as mU/mg protein.

Total NOS assay

The NOS activity in the serum was measured using a commercially available colourimetric kit following manufacturer's protocol.

Immunoblots of iNOS and eNOS

The glandular part of the stomach tissue was washed with PBS containing protease inhibitors, minced and homogenized in a lysis buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100) containing aprotinin (2 µg/ml), leupeptin (10 µg/ml) and PMSF (0.4 µM). Following centrifugation at 15 000 × g for 30 min at 4°C, the supernatant was collected and the protein concentration measured. The proteins (80 µg) were resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. The membrane was blocked for 2 h in TBST buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.02% Tween 20) containing 99% fat-free milk powder and incubated overnight at 4°C with rabbit polyclonal iNOS or eNOS antibodies. The membrane was washed for 1 h with TBST and incubated with peroxidase conjugated anti-rabbit IgG antibody (1:2500 dilution). The bands were detected using an enhanced chemiluminescence detection kit and quantified.

Immunostaining of iNOS

The immunostaining of iNOS was carried out following a reported procedure [31], with slight modification. After deparaffinization, rehydration and blocking of the endogenous peroxidase activity with 3% hydrogen peroxide in PBS, samples were exposed to protein blockers (5% normal horse serum, 1% normal goat serum in PBS) and incubated overnight at 4°C with primary antibody at the appropriate dilution. In control sections, only PBS was added omitting the antibodies. After incubation for 1 h at room temperature with peroxidase conjugated goat anti-rabbit IgG, a positive reaction was detected by exposure to DAB for 2–5 min. The slides were counterstained with Meyer's haematoxylin and visualized using a fluorescent microscope (Carl Zeiss, model Axioskop 2 mot plus).

Estimation of nitrite

Following manufacturer's instruction, the serum nitrite concentration was measured using a commercially available colourimetric kit that measures the total nitrite concentration of the sample.

Cytokines assays

The TNF-*α*, IL-6, IL-4 and IL-10 levels in the serum were estimated using commercially available ELISA kits following manufacturer's protocols.

Assays of soluble levels of selectins (sE- and sP-selectins) and CAMs (sICAM-1- and sVCAM-1)

The serum levels of sE-selectin, sP-selectin, sICAM-1 and sVCAM-1 were estimated using appropriate ELISA kits according to manufacturer's protocol.

Data analysis

All the data are presented as mean ± SEM. The parametric data, which included all the biochemical parameters, were analysed using a paired Student's *t*-test for the paired data, or one-way analysis of variance (ANOVA) followed by Dunnet's multiple comparisons post-hoc test. Non-parametric data (histology scoring) were analysed using the Kruskal–Wallis test (non-parametric ANOVA) followed by Dunn's multiple comparison post-hoc test.

Results

In our previous study, the doses of the test samples for effective ulcer healing were optimized by carrying out the treatment with different doses of BT (10–50 mg/kg) and TF (0.5–5.0 mg/kg) up to 7 days. Peak ulceration in mice was observed on the 3rd day after indomethacin (18 mg/kg, single dose) administration, and a 3-day treatment with BT (40 mg/kg),

TF (1 mg/kg) and Omez (3 mg/kg) provided optimal ulcer healing [25]. Hence, most of the present experiments were also carried out under the above conditions. We have used Omez as the positive control because of its superior anti-ulcer activity compared to other anti-ulcer drugs. Further, like the test samples, the ulcer healing activity of Omez was partially attributed to its antioxidative action [32]. Earlier, different groups have used it against indomethacin-induced gastric ulceration in animals [32,33]. The effective doses of BT and TF were reaffirmed by conducting the MPO assay using different doses of BT and TF. This also provided the status of neutrophil infiltration after ulceration and during treatment. In addition, the time course studies of the MPO activity, TNF-α level and iNOS expression were also carried out with the ulcerated mice.

Regulation of MPO activity

Our time-dependent experiments revealed increased MPO activity in the ulcerated mice at 6 h after indomethacin administration (data not shown). It reached the peak value on the 3rd day of ulceration. Compared to the normal mice, the MPO activity in the 3rd day ulcerated mice was significantly higher (93.3%). Both BT and TF reduced it dose-dependently (Figure 1). The effect of BT (40 mg/kg) was significantly better than that of its lower doses, but comparable with that of BT (50 mg/kg). Although not very effective at a lower dose (0.5 mg/kg), TF (1 mg/kg and 2.5 mg/kg) reduced the MPO activity equally as BT (40 mg/kg). Overall, treatment with BT (40 mg/kg), TF (1 mg/kg) and Omez (3 mg/kg) brought down the MPO activity by 30.6%, 34.7% and 28.2%, respectively, compared to the untreated mice. Neither BT nor TF on their own had any effect on the MPO activity in normal mice.

Compared to the ulcerated mice, those treated with L-NIL and L-NAME showed reduced MPO activity (41.7% and 28.2%, $p < 0.05$). On the other hand, compared to the TF (1 mg/kg)-treated mice, those receiving TF and L-NAME showed significantly increased MPO activity (72.3%, $p < 0.001$). However, L-NIL did not have any effect.

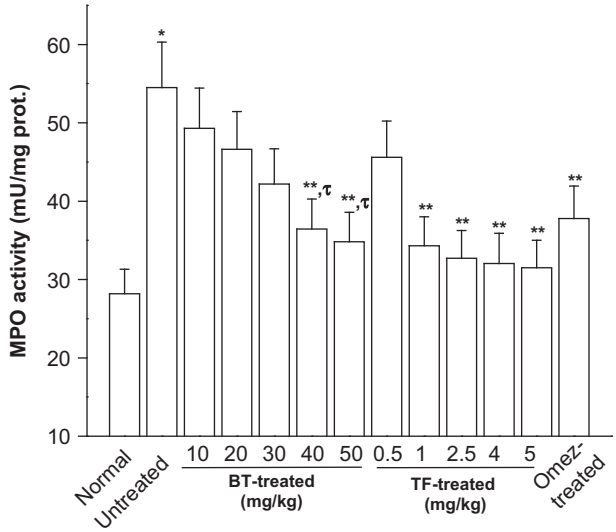

Figure 1. Effects of BT and TF in modulating mucosal MPO level in the indomethacin-induced ulcerated mice. The mice were ulcerated by indomethacin (18 mg/kg, p. o.). Treatment was carried out for 3 days with different doses of BT and TF. The supernatant of the gastric tissue homogenate was incubated with TMB and H_2O_2 in a suitable buffer and the MPO activity was assayed from the absorbance at 450 nm against HRPO as the standard. The values are mean ± SEM of three independent experiments, each with five mice per group. $^{*}p < 0.001$ compared to normal mice; $^{**}p < 0.05$, compared to untreated mice; $^{\tau}p < 0.05$, compared to BT (10–30 mg/kg) treatment.

Quantitative histology

Treatment with BT (40 mg/kg), TF (1 mg/kg) and Omez (3 mg/kg) reduced the damage score (DS) by 81.3%, 78.1% and 79.7%, respectively, compared to the ulcerated mice. Likewise, the inflammatory score (IS) was also reduced by BT (69.2%), TF (80.8%) and Omez (65.4%), compared to the untreated mice (Table I).

Regulation of the serum nitrite level

In aqueous medium, cellular NO is rapidly converted to nitrite and nitrate. However, their ratio varies substantially depending on the environment. Hence in order to investigate the effects of BT and TF on NO production in the ulcerated mice, we assayed the total nitrite concentration, after reducing the nitrate into

Table I. Comparative healing potential of the test samples on the 3rd day of ulceration.[a]

Group	DS value	DS reduction (%)	IS value	IS reduction (%)
Untreated	3.2	0	2.6	0
BT (40 mg/kg)	0.6	81.3*	0.8	69.2*
TF (1 mg/kg)	0.7	78.1*	0.5	80.8*
Omez (3 mg/kg)	0.65	79.7*	0.9	65.4*

[a]Stomach ulceration in mice was induced by oral administration of indomethacin (18 mg/kg). Treatment was carried out with BT (40 mg/kg, p. o.) and TF (1 mg/kg, p. o.) and Omez (3 mg/kg, p. o.) for 3 days. The histology was carried out 4 h after the last dose of the drugs. Each histological section (1 cm length) was divided into three fields and the mucosal damage was quantified in terms damage scores (DS) and inflammatory scores (IS) by scoring each field on a 0–4 scale as described previously [28,29]. The values are mean ± SEM of three independent experiments, each with five mice per group. $^{*}p < 0.001$ compared to ulcerated mice.

nitrite. At peak ulceration, there was a significant increase (1.5-fold) in the serum nitrite level compared to the normal mice. Treatment with BT, TF and Omez reduced it by 55.4%, 56.8% and 40.7%, respectively, compared to that in the untreated mice. BT and TF were significantly more potent than Omez (Figure 2).

Regulation of NOS activity

Compared to the normal mice, ulceration increased the serum NOS activity (Figure 3) by 4.8-fold. BT, TF and Omez suppressed it by 71.5%, 76.7% and 65.7%, respectively, compared to the untreated mice. The effect of Omez was significantly less than that of BT and TF.

Modulation of the mucosal iNOS and eNOS expressions

The Western blots of the iNOS and eNOS expressions in the gastric mucosa of the normal, ulcerated and drug (BT, TF or Omez)-treated mice are shown in Figure 4. The iNOS expression was very high in the ulcerated tissues, but much less in normal gastric tissues. Quantification of the bands revealed that stomach ulceration increased the expression of iNOS (1.6-fold), but reduced the eNOS expression (60%), compared to that in normal mice. Treatment with BT, TF and Omez reversed the changes, although the effect of Omez was much less. Immunohistochemistry of the stomach tissue of the indomethacin-administered mice (Figure 5) also confirmed significantly increased iNOS-positive cells in the gastric mucosa. The time-dependent experiments revealed that the expression of iNOS was significantly increased in the ulcerated mice at 9–12 h post-indomethacin administration (data not shown).

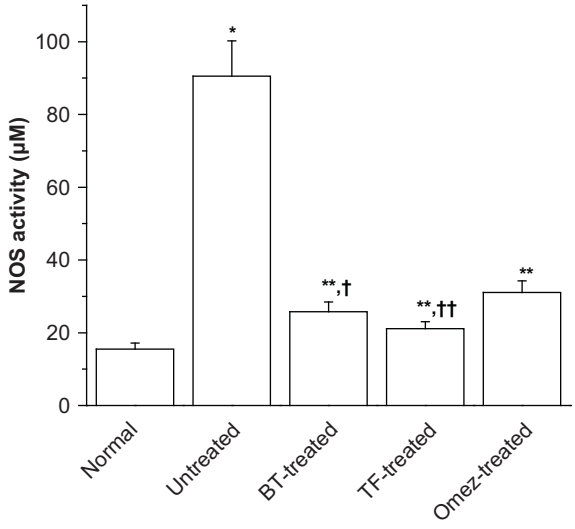

Figure 3. Effects of BT, TF and Omez in regulating serum NOS activity in the indomethacin-induced ulcerated mice. The mice were ulcerated by indomethacin (18 mg/kg, p. o.). Treatment was carried out with BT (40 mg/kg, p. o.), TF (1 mg/kg, p. o.) and Omez (3 mg/kg, p. o.) for 3 days. The NOS activity was measured using a colourimetric kit. The values are mean ± SEM of three independent experiments, each with five mice per group. $^{*}p < 0.001$ compared to normal mice; $^{**}p < 0.001$ compared to untreated mice; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, compared to Omez treatment.

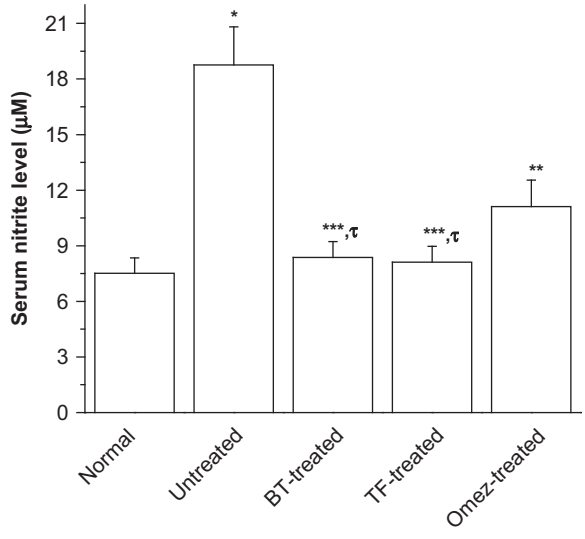

Figure 2. Effects of BT, TF and Omez in regulating serum NO level in the indomethacin-induced ulcerated mice. The mice were ulcerated by indomethacin (18 mg/kg, p. o.). Treatment was carried out with BT (40 mg/kg, p. o.), TF (1 mg/kg, p. o.) and Omez (3 mg/kg, p. o.) for 3 days. The NO level was measured using a colourimetric kit. The values are mean ± SEM of three independent experiments, each with five mice per group. $^{*}p < 0.001$ compared to normal mice; $^{**}p < 0.01$, $^{***}p < 0.001$ compared to untreated mice; $^{\tau}p < 0.05$ compared to Omez treatment.

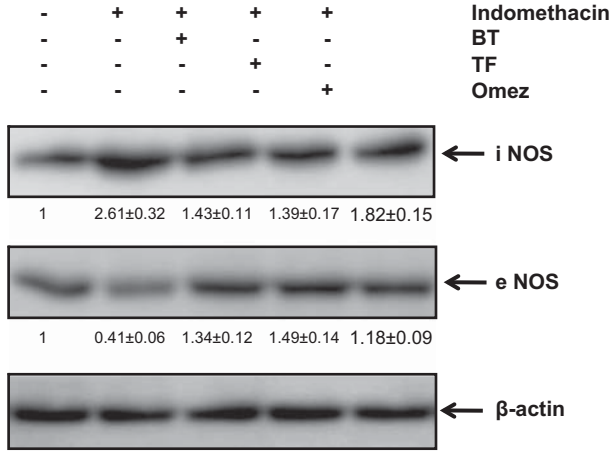

Figure 4. Immunoblots of the iNOS and eNOS expressions in the stomach tissues of normal, ulcerated and treated mice. The mice were ulcerated by indomethacin (18 mg/kg, p. o.). Treatment was carried out with BT (40 mg/kg, p. o.), TF (1 mg/kg, p. o.) and Omez (3 mg/kg, p. o.) for 3 days. The bands were quantified relative to that of β-actin bands of the corresponding lanes, using a Kodak Gelquant software. The individual bands of the ulcerated control and treatment groups were subsequently normalized, considering that of normal mice as 1. The values (arbitary unit, mean ± SEM) are the density scanning results of three independent experiments.

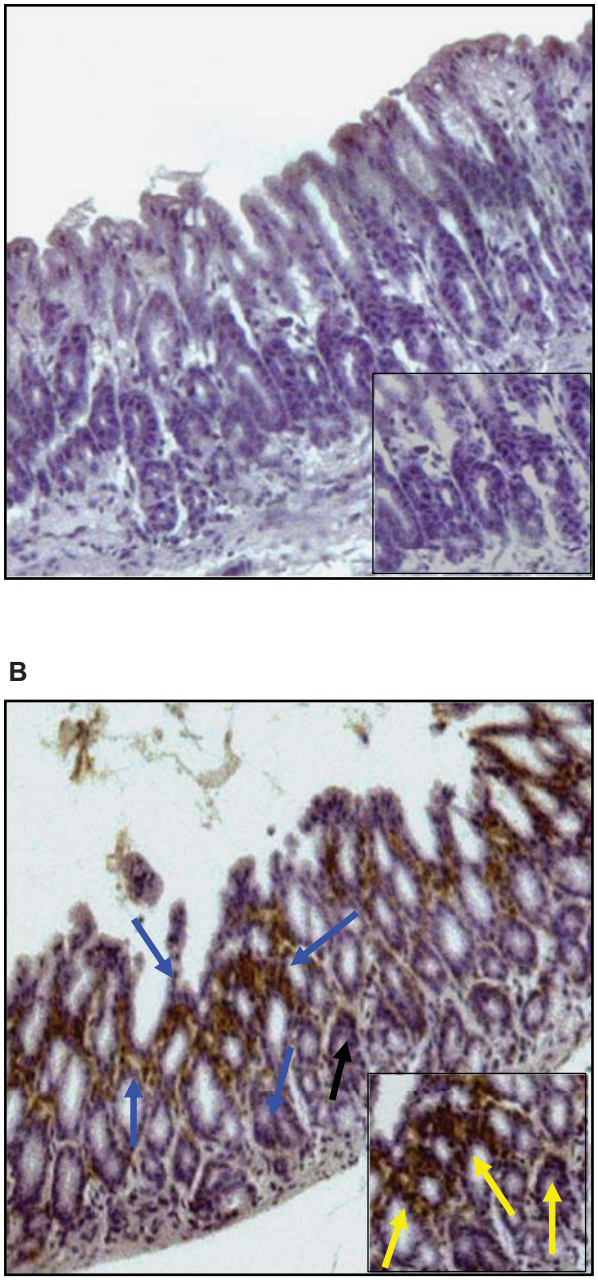

Figure 5. Increased iNOS expression in the stomach tissues of ulcerated mice, as revealed by immunohistochemistry. (A) Normal control mice; (B) ulcerated mice. Arrows indicate the iNOS-positive cells. Inset shows the localization of iNOS in indomethacin-treated mice stomach. The mice were ulcerated by indomethacin (18 mg/kg, p. o.). The immunostaining was carried out using the peroxidase conjugate. Original magnification ×400.

Modulation of the sE-selectin and sP-selectin levels

On the 3rd day after indomethacin administration, there was an increase (2-fold) in the serum sE-selectin level, compared to the normal value. Treatment with BT, TF and Omez reduced the serum sE-selectin level by 48%, 51.4% and 37.4%, respectively, compared to the ulcerated group. Ulceration also increased (1.4-fold) the serum sP-selectin level, compared to that in normal mice. Treatment with BT, TF and Omez reduced the serum sP-selectin level by 37.1%, 43.9% and 22.2%, respectively, compared to that in the untreated mice. The effect of Omez on both the parameters was significantly less than that of BT and TF. The combined results are shown in Figure.6

Modulation of the sICAM-1 and sVCAM-1 levels

The levels of serum sICAM-1 and sVCAM-1 in the ulcerated mice were augmented by 2-fold and 63.3%, compared to the normal group. The serum sICAM-1 level was reduced by 41.5%, 43.8% and 33.3%, respectively, by BT, TF and Omez. Likewise, treatment with BT, TF and Omez reduced the serum sVCAM-1 level by 33.1%, 34% and 25.5%, respectively, compared to the ulcerated group. The effect of Omez on both these parameters was significantly less than that of BT and TF. The combined results are shown in Figure 7.

Regulation of the serum Th_1 (TNF-α and IL-6) and Th_2 (IL-4 and IL-10) cytokines

Compared to the normal value, ulceration markedly increased the serum TNF-*α* and IL-6 levels equally (~2.26-fold). BT and TF suppressed the TNF-*α* level by 49.9% and 53.6%, while Omez reduced it by 20.3%, compared to that in the untreated mice (Figure 8). Likewise, BT, TF and Omez suppressed the serum IL-6 level by 60.5%, 65.9% and 30.6%, respectively, compared to that in the untreated mice. The effects of BT and TF on both the cytokines were significantly better than that of Omez. The

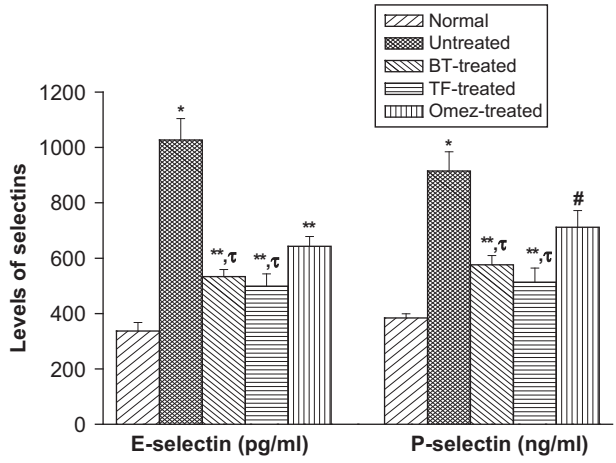

Figure 6. Effects of BT, TF and Omez in regulating serum sE- and sP-selectins in the indomethacin-induced ulcerated mice. The mice were ulcerated by indomethacin (18 mg/kg, p. o.). Treatment was carried out with BT (40 mg/kg, p. o.), TF (1 mg/kg, p. o.) and Omez (3 mg/kg, p. o.) for 3 days. The serum sE- and sP-selectins were measured by ELISA. The values are mean ± SEM of three independent experiments, each with five mice per group. $^{*}p < 0.001$ compared to normal mice, $^{\#}p < 0.05$, $^{**}p < 0.01$ compared to ulcerated mice, $^{\tau}p < 0.05$ compared to Omez treatment.

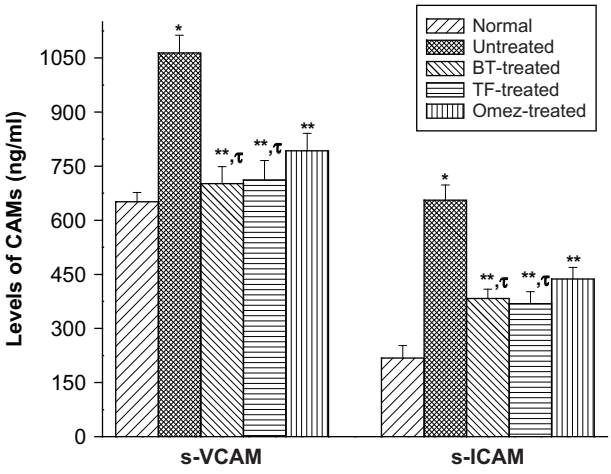

Figure 7. Ability of BT, TF and Omez in regulating serum sICAM-1 and sVCAM-1 in the indomethacin-induced ulcerated mice. The mice were ulcerated by indomethacin (18 mg/kg, p. o.). Treatment was carried out with BT (40 mg/kg, p. o.), TF (1 mg/kg, p. o.) and Omez (3 mg/kg, p. o.) for 3 days. The serum sICAM-1 and and sVCAM-1 levels were measured by ELISA. The values are mean ± SEM of three independent experiments, each with five mice per group. $^{*}p < 0.001$ compared to normal mice, $^{**}p < 0.01$ compared to ulcerated mice; $^{\tau}p < 0.05$ compared to Omez treatment.

time-dependent experiments revealed that within 3 h of its administration, indomethacin increased the serum TNF-α level considerably, compared to the normal control.

Regarding the Th_2 cytokines, the serum IL-10 and IL-4 levels in the ulcerated mice were reduced by 38.9% and 33.2%, respectively, compared to the normal mice. Treatment with BT and TF increased the serum IL-10 level appreciably by 1.1-fold and 82.6%, respectively, compared to the untreated group. Omez increased (47.8%) the cytokine, compared to the untreated group. The serum IL-4 level was restored to normalcy after treatment with BT, TF and Omez. The results are shown in Figure 9.

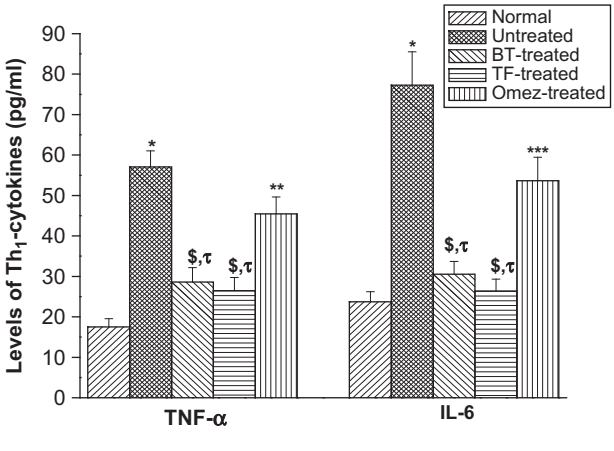

Figure 8. Modulation of the serum levels of the pro-inflammatory cytokines (TNF-α and IL-6) by BT, TF and Omez in the ulcerated mice. The mice were ulcerated by indomethacin (18 mg/kg, p. o.). Treatment was carried out with BT (40 mg/kg, p. o.), TF (1 mg/kg, p. o.) and Omez (3 mg/kg, p. o.) for 3 days. The serum cytokine levels were assayed by ELISA. The values are mean ± SEM of three independent experiments, each with five mice per group. $^{*}p < 0.001$ compared to normal mice; $^{**}p < 0.05$, $^{***}p < 0.01$, $^{\$}p < 0.001$ compared to ulcerated mice; $^{\tau}p < 0.01$ compared to Omez treatment.

Discussion

Several factors such as enzymes, cytokines and soluble mediators, liberated due to the inflammatory response, play crucial roles in the indomethacin-mediated gastric ulceration and the delayed ulcer healing [34]. Controlling these factors provides an opportunity to develop improved anti-ulcer medications, although this aspect has been largely ignored. The impressive healing capacity of BT (IC_{50} = 24.5 ± 2.79 mg/kg) and TF (IC_{50} = 0.38 ± 0.05 mg/kg) against the indomethacin-induced gastric ulceration in mice [25] encouraged us to investigate their probable modulatory effect on the COX-independent pro-inflammatory parameters [35,36]. Oxidative stress and inflammation adversely affect endothelial function and cause gastrointestinal anomalies. It is well recognized that neutrophil infiltration plays a key role in the development of injury and inflammation in a variety of tissues including gastric mucosal damage by indomethacin [37]. On the other hand, gastric mucosal NO shows biphasic behaviour, depending on its mode of generation and concentration. Therefore, these parameters were the focus of the present study.

In the previous study, we have established the healing action of BT and TF by histology. Quantification of the histological slides in terms of damage and inflammatory scores provided a better assessment of the quality of healing. BT (40 mg/kg), TF (1 mg/kg)

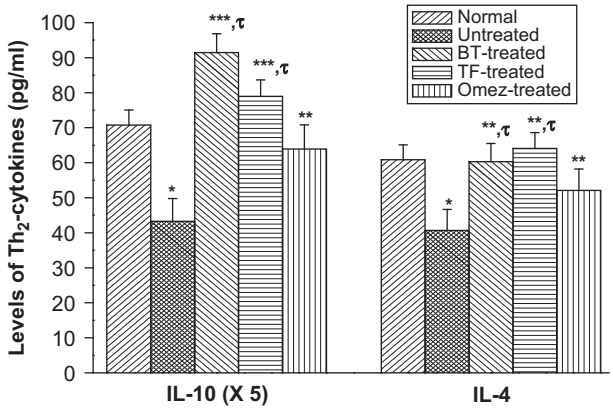

Figure 9. Modulation of the serum levels of the anti-inflammatory cytokines (IL-4 and IL-10) by BT, TF and Omez in the ulcerated mice. The mice were ulcerated by indomethacin (18 mg/kg, p. o.). Treatment was carried out with BT (40 mg/kg, p. o.), TF (1 mg/kg, p. o.) and Omez (3 mg/kg, p. o.) for 3 days. The serum cytokine levels were assayed by ELISA. The values are mean ± SEM of three independent experiments, each with five mice per group. $^{*}p < 0.01$ compared to normal mice; $^{**}p < 0.01$, $^{***}p < 0.001$ compared to ulcerated mice; $^{\tau}p < 0.05$ compared to Omez treatment.

and Omez (3 mg/kg) provided almost similar reduction (78–80%) of the damage score. Given that TF is a mixture of three types of glycosides, it is difficult to compare its effect with that of Omez on the basis of molar concentration. In consideration of the molecular weights of theaflavin (516) and Omez (345), the former appears to be more potent. This may be due to the presence of a strong antioxidant such as gallic acid in TF. However, evaluation of other factors including biodistribution and bioavailability of these drugs is required for a firm conclusion. We also checked the effect of BT and TF alone without the administration of indomethacin. None of these samples showed any adverse effect, nor did they alter any of the biochemical parameters, compared to that of the normal group.

Increased MPO activity is a widely used marker of inflammation as well as activation of neutrophils [11]. A positive correlation between MPO activity and neutrophil infiltration in intestinal inflammation models has been reported [38]. In the present study, the MPO activity in the ulcerated area of the gastric wall increased with the formation of a gastric ulcer by indomethacin, suggesting neutrophil infiltration in the gastric mucosa. Treatment with BT and TF restored the normal gastric mucosal integrity sufficiently and also reduced the MPO activity. These results suggested a close relationship between the state of the gastric inflammation and MPO activity. Earlier, pre-treatment with an antibody against neutrophils prevented the indomethacin-induced gastric ulceration [37].

Neutrophils inflict endothelial damage by generating various free radicals including NO that profoundly influences neutrophil oxidative burst [10]. It is now well-recognized that the enhanced generation of NO by the iNOS may contribute to the pathogenesis of various gastroduodenal disorders including peptic ulcer [16]. High concentrations of NO may be detrimental by promoting inflammation via mucosal swelling and epithelial damage. An increase in iNOS activity and a decrease in eNOS activity in the gastric mucosa are closely related to the development of gastric mucosal lesions. Currently we confirmed that the indomethacin-induced gastric ulceration increased the mucosal iNOS expression, but reduced the eNOS expression in mice. The elevated expression of iNOS accounted for the increased total NOS activity as well as serum nitrite level due to ulceration. Consistent with this, our results also showed that both iNOS-specific inhibitor, L-NIL, and the non-selective NOS inhibitor, L-NAME, reduced the MPO activity of the ulcerated mice. However, the protective effect of L-NIL was much more than that of L-NAME. This suggested that the iNOS-mediated NO was primarily responsible for indomethacin-induced gastric ulceration, caused by indomethacin.

The augmented neutrophil counts due to the indomethacin treatment, as observed in this study, would generate more iNOS-derived NO, and superoxide radicals. The enhanced level of NO adds to free radical load by generating secondary oxidizing and nitrating species, which might influence redox signalling events in vascular smooth muscle cells. This would affect their growth, differentiation, and apoptosis, delaying ulcer healing. Piotrowski et al. [39] showed a 12-fold increase in gastric epithelial expression of iNOS activity in the indomethacin-administered animals, compared to controls, and the increase correlated positively with the epithelium damage.

The results of our time-dependent studies revealed increased gastric MPO activity at 6 h following indomethacin administration, but the iNOS expression was increased much later. Based on these, it is tempting to propose that the increased iNOS activity may be a result of neutrophil accumulation. However, further studies are needed to clarify the sequence of events.

Treatment with BT and TF raised the eNOS/iNOS ratio to a level, favourable for efficient ulcer-healing. The reduction of the total NOS activity and nitrite level by BT and TF was primarily due to suppression of the iNOS expression. Using eNOS deficient mice, the importance of eNOS and eNOS-derived NO in regulating microvascular structure during acute inflammation was demonstrated [40]. Our results revealed that L-NAME, but not L-NIL adversely affected the healing activity of TF. Because TF itself reduced the iNOS expression, L-NIL did not show any effect on the healing efficacy of TF. However, L-NAME would negate the positive influence of TF on the eNOS expression, reducing the healing by TF. Taken together, these results suggested that the eNOS-derived NO contributed maximum to the ulcer healing property of TF, although a role for neuronal NOS-derived NO cannot be excluded. Also, the activated neutrophils, observed in the ulcerated mice, would scavenge the beneficial vasodilatory NO through NADPH oxidase or MPO catalysed reactions [41]. Thus, the ability of BT and TF to reduce the neutrophil infiltration and enhance the eNOS expression at the gastric tissues might be instrumental in their ulcer-healing action.

In contrast, despite showing less effect on modulating eNOS/iNOS expressions and NO production, Omez provided excellent healing. Factors such as control of intragastric pH [42] and stimulation of epithelial cell proliferation through increased serum gastrin level [43] are attributed to its healing property.

Leukocyte capture by and rolling on ECs, followed by more firm adhesion to the endothelium is crucial at various stages of acute inflammation. Neutrophil transendothelial migration is staged into a series of events: initial rolling (selectin mediated), activation (chemoattractant dependent), firm adhesion (integrin

mediated) and transmigration (integrin, ICAM-1 and VCAM-1 mediated). The soluble forms of adhesion molecules are good markers of an inflammatory disease [44]. The concentration of sE-selectin often correlates well with its expression on the surface of ECs. Likewise, sICAM-1 concentration is considered to reflect the expression of mICAM-1 on the ECs. Therefore, plasma concentrations of these soluble inflammatory mediators might be good markers of endothelial cell damage or activation and were assayed in the present investigation.

Our results are indicative of a prominent role of sE-selectin at the early stages of tethering and recruitment of leukocytes in gastric injury by indomethacin. Amongst the inflammatory modulators, ICAM-1 binds to CD11a/CD18 and CD11b/CD18 on leukocytes, and VCAM-1 binds to the $\alpha_4\beta_1$ integrin, located at lymphocytes, monocytes and basophils [45]. Hence, the elevated levels of sP-selectin, sICAM-1 and sVCAM-1 in the ulcerated mice would help to maintain the high MPO activity in gastric mucosa as compared to the normal group. Because P-selectin and E-selectin are critical adhesion molecules for the capture and rolling of leukocytes in the microvasculature while ICAM-1 mediates firm adhesion, the up-regulation of these CAMs by indomethacin may result in more efficient leukocyte migration into the surrounding tissues. Treatment with the test samples reduced the levels of soluble selectins and CAMs. These results demonstrate that BT and TF may interrupt the interaction of neutrophils and endothelial cells both at the early rolling and the late firm adhesion phases. Earlier, P-selectin was reported to have a more prominent role in the indomethacin-mediated ulceration [18,19]. However, we found that indomethacin raised sE-selectin more than sP-selectin and both BT and TF showed slightly better ability to reduce the former than the latter. These suggested that the test samples primarily prevented the early loose tethering of leukocytes. The relative potency of the test samples was BT~TF > Omez.

Stimulation of inflammatory cytokines is extremely important in mucosal defense. One of the most prominent modes of mediation of indomethacin-induced gastropathy is the increased expression of the pro-inflammatory cytokines [46], which also correlates with the extent of ulceration. The activation of ECs for expression of CAMs is mediated in part by inflammatory cytokines, such as TNF-α and interferon-γ (IFN-γ). During acute and chronic colitis, the sustained production of pro-inflammatory cytokines leads to the up-regulation of adhesion molecules. Our results of enhanced iNOS expression as well as the levels of soluble inflammatory modulators by indomethacin reflected a pro-inflammatory trigger. Hence, the immune response due to ulceration and its modulation by BT, TF and Omez was monitored. This enabled us to associate the inflammatory response with a better prognosis.

Indomethacin administration raised the serum levels of pro-inflammatory Th_1 cytokines (TNF-α and IL-6) and reduced the anti-inflammatory Th_2 cytokines (IL-4 and IL-10). We selected TNF-α for this study, as increased plasma level of TNF-α has been reported to increase leukocyte adherence after indomethacin administration [47]. TNF-α promotes leukocyte adhesion by enhancing the transcription-dependent expression of EC adhesion molecules that can extend and further increase the leukocyte rolling and adherence/emigration responses [45]. Likewise, IL-4 that remains under the influence of NO controls the expression of growth factors that are responsible for ulcer onset and healing. The cytokine imbalance, created by indomethacin, presumably triggered up-regulation of the adhesion molecules and augmented the iNOS/NO pathway to produce excess NO. These are likely to promote oxidative stress and result in ulceration [48].

It is becoming increasingly appreciated that amongst the Th_1 cytokines, TNF-α plays a critical role in the NSAID-induced gastric injury by modulating neutrophil infiltration. Previously TNF-α was found to be involved in the indomethacin-mediated gastric ulceration in mice and this was reduced in TNF-receptor deficient mice [16]. Hence, we also assessed the temporal profile of the serum TNF-α levels in the indomethacin group. Within 3 h of indomethacin administration, the TNF-α level was increased in the ulcerated mice, compared to the normal group. Our results were consistent with the previous report [16] and indicated that indomethacin may initially stimulate TNF-α production that activates the neutrophils to release substances, related to inflammation.

Treatment with BT and TF reversed the imbalance by reducing the Th_1 cytokines drastically, and restoring the levels of IL-4 and IL-10 to normalcy. The up-regulation of the anti-inflammatory cytokines by the tea samples is likely to inhibit the stimulatory effect of indomethacin on the release of the pro-inflammatory cytokines in blood and gastric mucosa. In the previous paper, we have reported that BT and TF augment the PGE level in the indomethacin-treated mice [25]. The increased PGE might stimulate IL-10 release, which, in turn, controls PGE and Th_1 cytokines via negative feedback.

Overall, our data demonstrates that treatment with BT and its constituent TFs can accelerate healing of indomethacin-induced gastric ulceration in mice, by a number of distinct mechanisms. It was found that the tea samples can significantly reduce the indomethacin-induced leukocyte infiltration by moderating and/ or abolishing various soluble inflammatory modulators including the adhesion molecules, NO, as well as the ratio of the Th_1/Th_2 cytokines and switching the gastric mucosal eNOS/iNOS ratio. These, along with their

ability to strengthen the mucosal defense system by augmenting antioxidants, gastric mucin, and PGE, observed previously [25], might be responsible for the excellent ulcer healing action of BT and TF.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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