Biochimica et Biophysica Acta 1840 (2014) 3385–3392

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

DL-trans-3,4-Dihydroxy-1-selenolane (DHS_{red}) heals indomethacin-mediated gastric ulcer in mice by modulating arginine metabolism

Saikat Chakraborty [a,1], Sudhir K. Yadav [a,1], Mahesh Subramanian [a], Michio Iwaoka [b], Subrata Chattopadhyay [a,*]

[a] Bio-Organic Division, Bhabha Atomic Research Centre, Mumbai 400 085, India
[b] Department of Chemistry School of Science, Tokai University, Kitakaname, Hiratsuka-shi, Kanagawa 259-1292, Japan

ARTICLE INFO

Article history:
Received 28 May 2014
Received in revised form 20 August 2014
Accepted 3 September 2014
Available online 16 September 2014

Keywords:
Arginase
Cytokine balance
Gastric ulcer healing
Indomethacin
NOS

ABSTRACT

Background: The importance of the arginine metabolism in gastric ulcer-healing is given relatively less attention. Hence the role of controlling this pathway by DL-trans-3,4-dihydroxy-1-selenolane (DHS_{red}) and omeprazole against indomethacin-induced stomach ulceration in mouse was investigated.
Methods: Swiss albino mice were ulcerated with indomethacin followed by treatment with the test samples, and the activities of myeloperoxidase (MPO), total nitric oxide synthase (NOS) and arginase, the expressions of inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS), and the pro-/anti-inflammatory cytokine levels were assayed. NOS-inhibitors were also used to establish the biochemical mechanism.
Results: Indomethacin induced maximum ulceration in mice on the 3rd day, associated with reduced arginase activity, eNOS expression, along with increased MPO and total NOS activities, nitric oxide (NO) generation, iNOS expression, and pro-/anti-inflammatory (Th_1/Th_2) cytokine ratio. Treatment with DHS_{red} (2.5 mg $kg^{-1} \times 3$ days) restored the cytokine balance to shift the iNOS/NO axis to the arginase/polyamine axis as revealed from the increased arginase activity and eNOS expression, and reduced iNOS expression, total NOS activity and NO level.
Conclusions: The ulcer-healing property of DHS_{red}, but not of omeprazole was due to a favorable pro-/anti-inflammatory cytokine ratio that shifted the arginine metabolism to the polyamine pathway and increased the eNOS/iNOS ratio. The healing action of omeprazole was not significantly associated with these parameters.
General significance: The shift in the arginine-metabolism from the iNOS/NO axis to the arginase/polyamine axis is guided by Th_1/Th_2 cytokines ratio and plays an important role in gastric ulcer-healing. The favourable effects of the non-toxic and water-soluble compound, DHS_{red} on these pathways and other COX-dependent and antioxidative parameters suggested it to be a promising anti-ulcer formulation for further studies.

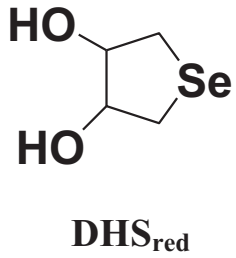
1. Introduction

Widespread use of the nonsteroidal anti-inflammatory drugs (NSAIDs) has alarmingly increased the gastrointestinal (GI) problems, and their management remains the single most serious complication of any drug therapy [1,2]. Formulation of effective and affordable anti-ulcer agents is an important goal in GI pharmacology, as the prescribed anti-ulcer drugs show various side effects and are expensive [3,4]. The trace element, selenium and some organo-selenium compounds can replenish the antioxidative defense, and are being used for treating gastric ulcers and cancers [5–7]. Very recently, a water-soluble selenium compound, DL-*trans*-3,4-dihydroxy-1-selenolane (DHS_{red}), synthesized by Iwaoka's group [8], could effectively heal the indomethacin (IND)-induced stomach ulceration in mice [9]. The healing activity of DHS_{red} was attributed partly to its antioxidant action as well as its ability to protect gastric mucin and improve prostaglandin (PG) synthesis by augmenting the cyclooxygenase (COX) isozymes. The chemical structure of DHS_{red} is shown in Fig. 1.

The stomach ulceration and delayed ulcer healing induced by the NSAIDs, including IND is often explained in terms of inhibition of the COX-dependent pathway [10]. However, the popular COX theory of IND-gastropathy is severely flawed by several heterogeneous and conflicting literature reports. This may be due to the fact that multiple COX isozymes can originate from only one gene. It is now well-accepted that IND-gastropathy is multifactorial and is promoted by neutrophil infiltration that initiates leukocyte–endothelial cell (EC) interaction via the intermediacy of various selectins and cellular adhesion molecules (CAMs) [11,12]. Nitric oxide (NO), produced from L-arginine by the nitric oxide synthases (NOSs) is an important biological regulator in many physiological and pathological processes [13]. The NOSs exist as constitutive (cNOS), and inducible (iNOS) isoforms. The low concentration of NO, produced by the constitutive endothelial

* Corresponding author. Tel.: +91 22 25592399; fax: +91 22 25505151.
E-mail address: schatt@barc.gov.in (S. Chattopadhyay).
[1] Equal contributions.

http://dx.doi.org/10.1016/j.bbagen.2014.09.004
0304-4165/



DHS_{red}

Fig. 1. Chemical structure of DHS_{red}.

NOS (eNOS) increases blood flow to help wound healing [14] by promoting angiogenesis [15,16] in the damaged gastric mucosa. However, the enhanced generation of NO by iNOS may contribute to the pathogenesis of various inflammatory processes including peptic ulcer [17,18]. Besides, arginine is also metabolized by arginase to produce the polyamines that contribute to wound healing [19,20]. Thus, the arginine-metabolizing enzymes can play important roles in ulceration and its healing. In particular, a switch from the iNOS/NO to arginase/polyamine pathway as well as an increase of the gastric eNOS/iNOS ratio may be beneficial in managing the IND-gastropathy [21,22]. All these processes are regulated by the balance between the pro- and anti-inflammatory (Th_1 *vs* Th_2) cytokines. However, scarce attention is given to the interplay of these factors in the IND-induced gastric ulceration.

The present study was conducted to examine whether the healing action of DHS_{red} against the IND-induced stomach ulceration in mice also involves regulation of the arginine metabolism through modulation of the cytokine balance. For this, we investigated the effect of DHS_{red} in elevating arginase activity as well as the eNOS/iNOS ratio in the IND group of mice. Further, the status of neutrophil infiltration, selectins, CAMs and Th_1/Th_2 cytokines in the DHS_{red}-treated ulcerated group was compared with that in the IND group. Our results clearly established that the ulcer-healing property of DHS_{red} is also governed by its ability to improve the eNOS/iNOS ratio and arginase/iNOS activities by maintaining the Th_1/Th_2 cytokine balance.

2. Materials and methods

2.1. Chemicals and reagents

DHS_{red} was synthesized and characterized as reported earlier [8]. L-Arginine, indomethacin, isonitrosopropiophenone, Bradford reagent, Triton X-100, leupeptin, phenylmethylsulfonyl fluoride (PMSF), glycine, sodium dodecyl sulfate (SDS), acrylamide, bis-acrylamide, Tween 20, ethylenediaminetetraacetic acid (EDTA), 3,3′,5,5′-tetramethylbenzidine (TMB), $MnCl_2$, urea, omeprazole (Omez), Trizma base, cetyltrimethylammonium bromide (CTAB) and nitrocellulose membrane were procured from Sigma Chemicals (St. Louis, MO). Other reagents used were disodium hydrogen phosphate and sodium dihydrogen phosphate (BDH, Pool Dorset, U.K.), sulfuric acid, hydrochloric acid, phosphoric acid, sodium chloride (Thomas Becker, Mumbai, India), horseradish peroxidase (HRPO), gum acacia (Sisco Research Laboratory, Mumbai, India), rabbit polyclonal iNOS and eNOS antibodies (Santacruz Biotechnology, Delaware), peroxidase conjugated anti-rabbit IgG antibody, enhanced chemiluminescence detection kit (Roche, Mannheim, Germany), NOS and NO assay kits (Calbiochem, California) and cytokine ELISA kits (Pierce Biotechnology, Rockford).

2.2. Instrumentation

The absorbances were measured at 25 °C using an ELISA reader (Biotech Instruments, USA).

2.3. Preparation of the drugs

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

2.4. Experimental protocol for ulceration and biochemical studies

Male Swiss albino mice, bred at BARC Laboratory Animal House Facility, Mumbai, India were procured after obtaining clearance from the BARC Animal Ethics Committee (BAEC) (Approval No. BAEC/01/11 dt. 30 March 2011). The animals were handled following International Animal Ethics Committee Guidelines, and the experiments were permitted by BAEC. 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 day/night cycle (12 h/12 h). To carry out the experiments in a blinded fashion, the animals were identified by typical notches in the ear and limbs (performed at a pre-weaning stage to minimize the pain to the animals), and randomized. The animals were deprived of food 24 h before ulcer induction, but had free access to tap water.

The mice were divided into five groups (each containing five mice), and each experiment was repeated three times. Group I mice served as normal control, while ulceration in the groups II–V mice was induced by IND (18 mg kg^{-1}, *p. o.*, single dose) dissolved in distilled water and suspended in the vehicle, gum acacia (2%). The dose of IND and the treatment regime (drug doses and period of treatment) were standardized in our earlier studies [9,23,24]. The mice of groups I and III (referred to as control) were given the daily oral dose of vehicle (gum acacia in distilled water, 0.2 ml). The group II mice (referred to as IND group) received a normal diet. The groups IV and V mice were given a daily dose of DHS_{red} (2.5 mg kg^{-1} × 3 days, *p. o.*) and Omez (3 mg kg^{-1} × 3 days, *p. o.*) respectively, starting the first dose 6 h post-IND administration. Four hours after the last dose of the treatments, the mice were sacrificed after an overdose of thiopental, the stomachs were opened along the greater curvature, and the wet weights of the tissues were recorded. The glandular portion from five animals were pooled, rinsed with an appropriate buffer, homogenized in the same buffer under cold condition and used for studying the expressions of NOSs and arginase, and myeloperoxidase (MPO) activities. The serum samples were used to assay the total NOS activity, and the nitrite and cytokines levels. In separate experiments, treatments were also carried out with L-NAME (10 mg kg^{-1}, once daily), and L-NIL (3 mg kg^{-1}, twice daily) alone or in conjunction with DHS_{red} (2.5 mg kg^{-1}, *p. o.*) for 3 days, and the MPO activity was assayed.

2.5. MPO assay

Following a reported method [25] with slight modifications, the MPO activity of the gastric tissues was determined immediately after sacrificing the animals. The entire process was carried out at 4 °C. The gastric 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 MPO activity was calculated from the absorbance of the mixture at 450 nm, using HRPO as the standard. The MPO activity is expressed as μM of H_2O_2 consumed min^{-1} mg^{-1} protein at 25 °C and pH 5.4.

2.6. Total NOS assay

The serum NOS activity was measured using a commercially available colorimetric kit following manufacturer's protocol. In this assay, the nitrite and nitrate, produced from NO is converted into nitrite and spectrophotometrically quantified using Griess reagent against KNO_3 as the standard. The NOS activity is expressed in terms of μM nitrite formed.

2.7. Nitrite assay

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

2.8. Western blots

The glandular part of the gastric mucosa, after being washed with PBS containing protease inhibitors was minced and homogenized in a lysis buffer (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% Triton X-100) containing leupeptin (2 μg ml^{-1}) 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 (40 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 (5%) and incubated overnight at 4 °C with rabbit polyclonal iNOS or eNOS antibodies (1:2000 dilution). The membrane was washed over a period of 2 h with TBST and incubated with peroxidase conjugated anti-rabbit IgG (1:2500 dilution). The bands were detected using an enhanced chemiluminescence detection kit and the intensity ratios of immunoblots to that of normal control, taken as 1 (arbitrary unit) were quantified after normalizing with respect to the loading controls. The values (arbitary unit, mean ± S.E.M.) are the density scanning results of three independent experiments.

2.9. Arginase assay

The assay was carried out following a known method [26] with minor modifications. The tissue homogenate was prepared in an ice-cold 25 mM Tris–HCl buffer (pH 7.5) and centrifugation at 12,000 ×g for 30 min at 4 °C. The reaction mixture (200 μl) containing 0.5 M L-arginine (pH 9.7), 1 mM $MnCl_2$ and the tissue extract (100 μl) was incubated for 20 min at 37.4 °C. The reaction was stopped by adding an acid mixture (800 μl, H_2SO_4–H_3PO_4–H_2O, 1:3:7) and 3% isonitrosopropiophenone followed by heating at 100 °C for 45 min, and the absorbance at 540 nm was read. The data were quantified from a calibration curve, prepared using urea (1.5–120 μg), and normalized for the tissue protein content. One unit (U) of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of urea min^{-1}.

2.10. Estimation of serum cytokine levels

The serum tumor necrosis factor-α (TNF-α) and the interleukins (IL-1β, IL-6, IL-4 and IL-10) levels were estimated using commercially available ELISA kits following manufacturer's protocols.

2.10.1. Statistical analysis

The data, expressed as the mean ± S.E.M. (n = 15) were analyzed by a paired Student's *t* test for the paired data, or one-way analysis of variance (ANOVA) followed by a Dunnet multiple comparisons post-test. A probability value of $P < 0.05$ was considered significant.

3. Results

Earlier we have found maximum ulceration in mice stomach on the 3rd day of IND (18 mg kg^{-1}, *p. o.*, single dose) administration [9]. Treatment with DHS_{red} (2.5 mg kg^{-1}) and Omez (3 mg kg^{-1}) for 3 days after ulcer induction provided optimal ulcer healing (74.7% and 67.9% respectively, $P < 0.001$), compared to the IND group. Hence, the present experiments were carried out under the same conditions. The data of the IND group was compared to that of the normal group, while those of the treated groups were compared to that of the IND group. To examine the effect of the vehicle (2% gum acacia) in ulcer healing, we kept an additional ulcerated control group, receiving the vehicle following IND administration. The data of this group was compared to that of the IND group, given a normal diet only.

3.1. Regulation of the mucosal MPO activity

The MPO activity in the IND group was increased by 1.5 fold. This was reduced by DHS_{red} and Omez by 38.9% and 31.6% respectively (Fig. 2). On its own, DHS_{red} did not change the MPO activity in the control mice. The MPO activities of the group I mice of day 1 and day 3 remained almost the same (data not shown). On the other hand, the L-NIL and L-NAME groups showed reduced MPO activity (37.2% and 28.1%). However, compared to the DHS_{red} group, those receiving DHS_{red} and L-NAME showed significantly increased (65.9%) MPO activity. However, L-NIL did not show any effect with mice receiving DHSred.

3.2. Modulation of the sE-selectin and sP-selectin levels

The serum sE-selectin and sP-selectin levels in the IND group increased to 2.7 fold and 2.0 fold respectively, compared to the normal group. Treatment with DHS_{red} reduced such increases in the serum sE-selectin and sP-selectin levels by 37.6% and 40.9% respectively. Omez reduced the parameters by 10% and 14% respectively, compared to the IND group. The results are shown in Fig. 3.

3.3. Modulation of the sICAM-1 and sVCAM-1 levels

The levels of serum sICAM-1 and sVCAM-1 in the IND group were augmented by 91.6% and 44.8% respectively. DHS_{red} and Omez reduced the serum sICAM-1 level by 41.6% and 13.7% respectively. Likewise,

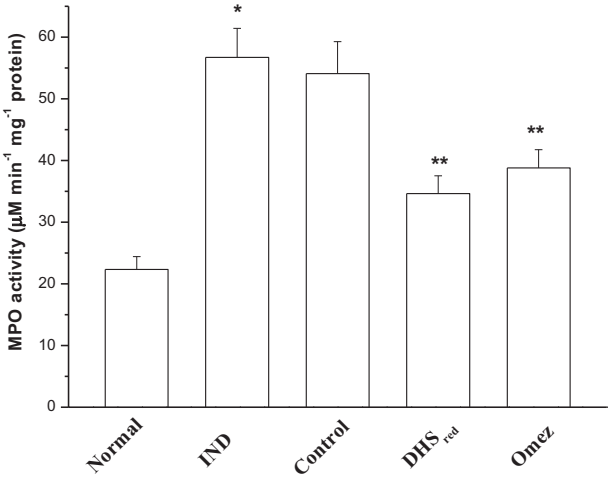


Fig. 2. Effects of DHS_{red} on mucosal MPO level in the IND-induced ulcerated mice. The mice were ulcerated with IND (18 mg kg^{-1}, *p. o.*). Treatment was carried out for 3 days with DHS_{red} (2.5 mg kg^{-1}, *p. o.*) and Omez (3 mg kg^{-1}, *p. o.*). The gastric tissue homogenate was incubated with TMB and H_2O_2 in a suitable buffer, and the MPO activity assayed from the absorbance at 450 nm using HRPO as the standard. The values are mean ± S.E.M. of three independent experiments, each with five mice per group. *$P < 0.001$ compared to the normal group; **$P < 0.01$ compared to the IND group.

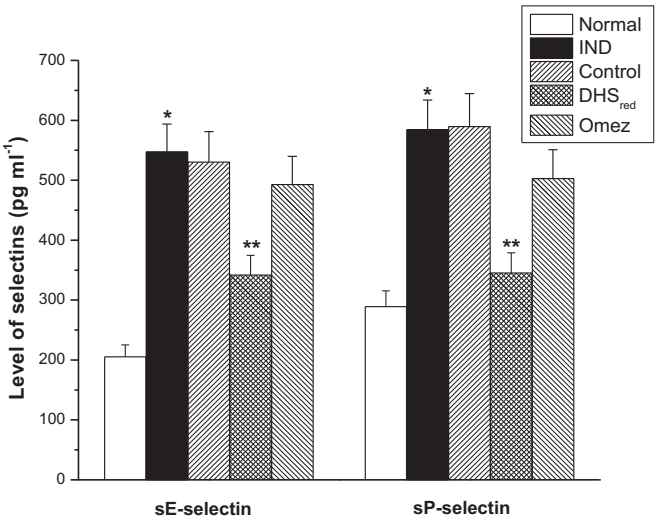


Fig. 3. Effects of DHS_{red} and Omez in regulating serum sE- and sP-selectins in the IND-induced ulcerated mice. The mice were ulcerated by IND (18 mg kg^{-1}, *p. o.*). Treatment was carried out with DHS_{red} (2.5 mg kg^{-1}, *p. o.*) and Omez (3 mg kg^{-1}, *p. o.*) for 3 days. The selectins levels were measured by ELISA. The values are mean ± S.E.M. of three independent experiments, each with five mice per group. $^{*}P < 0.001$ compared to the normal group; $^{**}P < 0.01$ compared to the IND group.

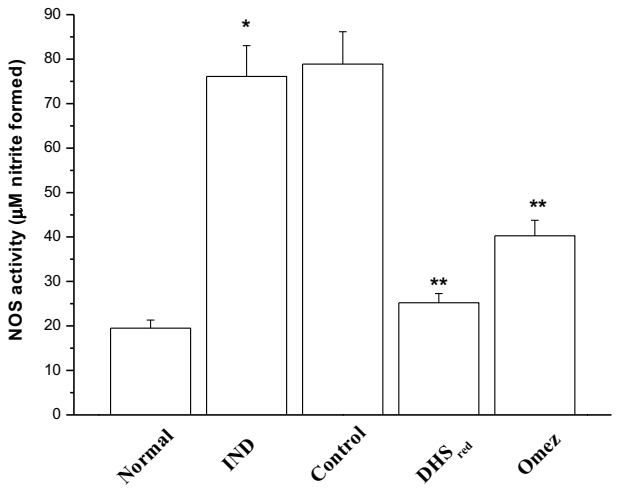


Fig. 5. Effects of DHS_{red} and Omez in regulating serum NOS activity in the IND-induced ulcerated mice. The mice were ulcerated by IND (18 mg kg^{-1}, *p. o.*). Treatment was carried out with DHS_{red} (2.5 mg kg^{-1}, *p. o.*) and Omez (3 mg kg^{-1}, *p. o.*) for 3 days. The NOS activity was measured using a colorimetric kit. The values are mean ± S.E.M. of three independent experiments, each with five mice per group. $^{*}P < 0.001$ compared to the normal group; $^{**}P < 0.01$ compared to the IND group.

treatment with DHS_{red} and Omez reduced the serum sVCAM-1 level by 27.1% and 10.5% respectively. The results are shown in Fig. 4.

3.4. Regulation of the NOS activity

A significant increase (2.9 fold) in the total NOS activity was noticed in the IND group. DHS_{red} and Omez reduced it by 66.9% and 47% respectively (Fig. 5).

3.5. Regulation of serum nitrite level

In order to investigate the effect of DHS_{red} on NO production in the ulcerated mice, we assayed the total nitrite concentration, after reducing the nitrate to nitrite. There was a significant increase (1.4 fold) in the serum nitrite level in the IND group. Treatment with DHS_{red} and Omez reduced such an increase in the parameter by 50.3% and 29.8% respectively (Fig. 6).

3.6. Modulation of mucosal eNOS and iNOS expressions

The western blots of eNOS and iNOS expressions in the gastric mucosa of the normal, IND, control and treated (DHS_{red} or Omez) groups of mice are shown in Fig. 7a. The eNOS expression was detected in both normal and ulcerated gastric tissues. In contrast, the iNOS expression was very high in the ulcerated tissues, but insignificant in normal gastric tissues. Quantification of the bands (Fig. 7b and c) revealed that stomach ulceration reduced the expression of eNOS by 59%, but increased that of iNOS by 6.7 fold. Treatment with DHS_{red} reversed the trend, increasing the eNOS expression by 3.3 fold and reducing the iNOS expression by

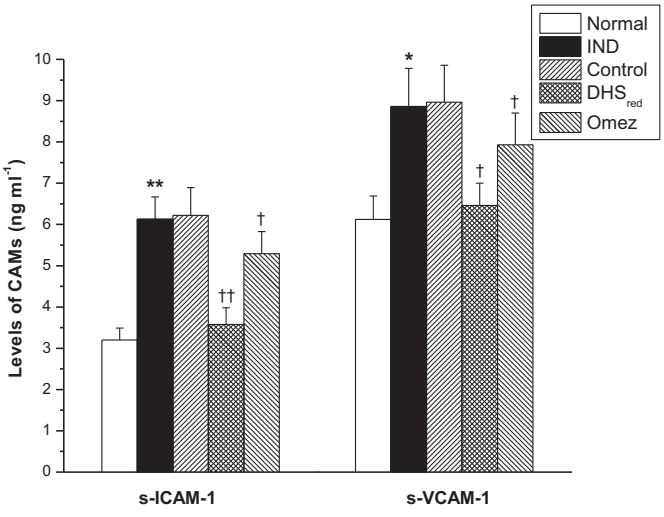


Fig. 4. Ability of DHS_{red} and Omez in regulating serum sICAM-1 and sVCAM-1 in the IND-induced ulcerated mice. The mice were ulcerated by IND (18 mg kg^{-1}, *p. o.*). Treatment was carried out with DHS_{red} (2.5 mg kg^{-1}, *p. o.*) and Omez (3 mg kg^{-1}, *p. o.*) for 3 days. The cytokines were measured by ELISA. The values are mean ± S.E.M. of three independent experiments, each with five mice per group. $^{*}P < 0.01$ $^{**}P < 0.001$ compared to the normal group; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ compared to the IND group.

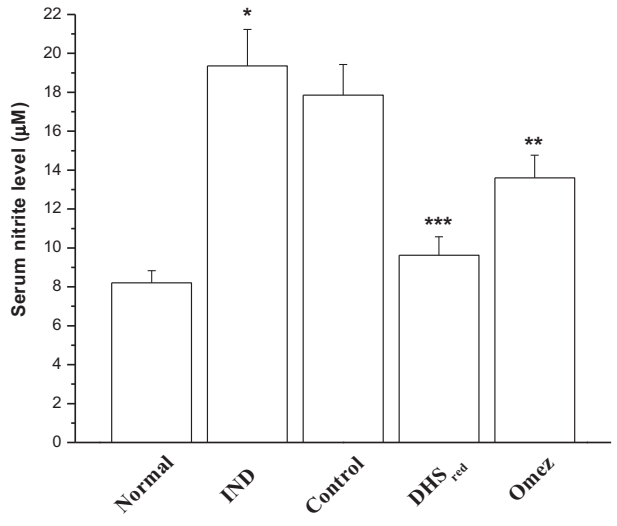


Fig. 6. Effects of DHS_{red} and Omez in regulating serum NO level in the IND-induced ulcerated mice. The mice were ulcerated by IND (18 mg kg^{-1}, *p. o.*). Treatment was carried out with DHS_{red} (2.5 mg kg^{-1}, *p. o.*) and Omez (3 mg kg^{-1}, *p. o.*) for 3 days. The NO level was measured using a colorimetric kit. The values are mean ± S.E.M. of three independent experiments, each with five mice per group. $^{*}P < 0.001$ compared to the normal group; $^{**}P < 0.05$, $^{***}P < 0.01$ compared to the IND group.

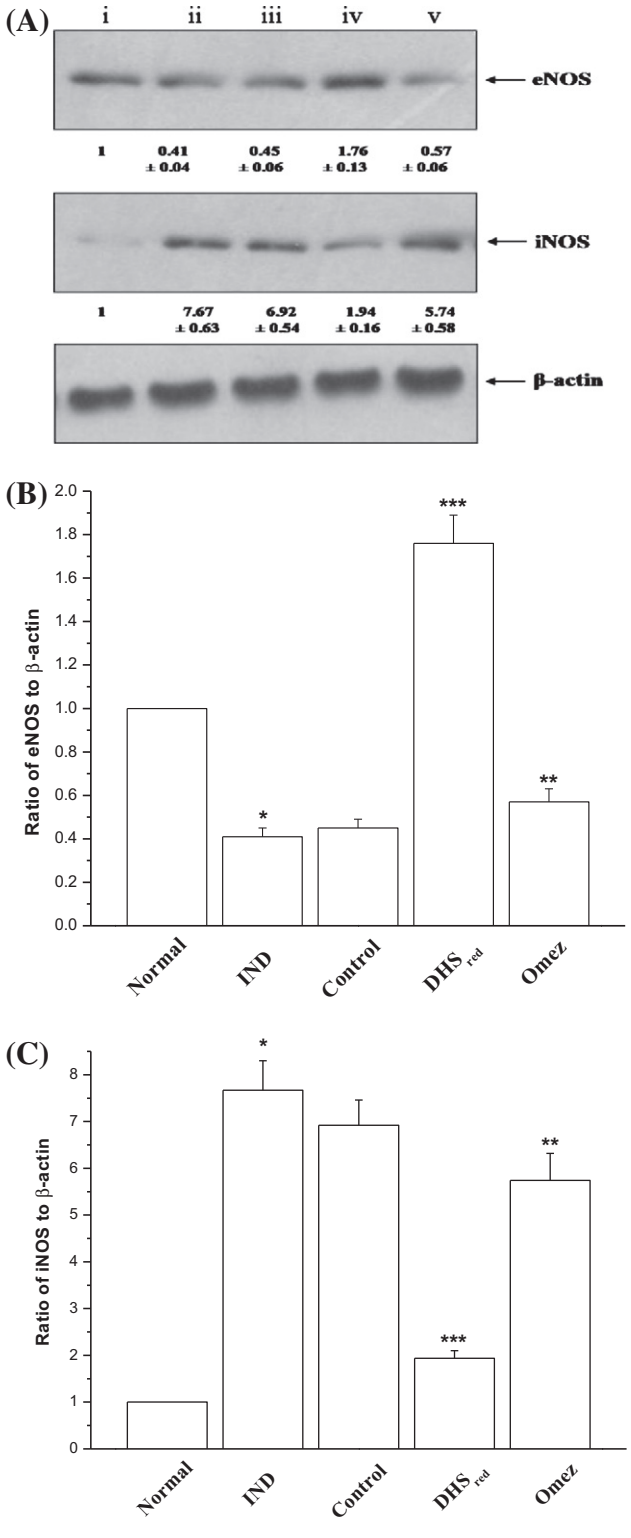


Fig. 7. Immunoblots of the eNOS and iNOS expressions in the stomach tissues (A). Groups: i — normal, ii — IND, iii — control, iv — DHS_{red}, and v — Omez. Ratios of the intensities of eNOS (B) and iNOS (C) bands to that of the respective β-actin bands as quantified from the western blot images. Ulceration in the mice was induced by IND (18 mg kg^{-1}, *p. o.*). Treatment was carried out for 3 days with DHS_{red} (2.5 mg kg^{-1}, *p. o.*) and Omez (3 mg kg^{-1}, *p. o.*). The protein bands were detected using a Kodak Gel-doc software and the intensity ratios of immunoblots to that of normal control, taken as 1 (arbitrary unit) were quantified after normalizing with respective loading controls. The values are mean ± S.E.M. of the density scanning results of three independent experiments, each with 5 mice per group. $^{*}P < 0.001$ compared to the normal group; $^{**}P < 0.05$, $^{***}P < 0.001$ compared to the IND group. Representative images of three independent similar experiments are shown.

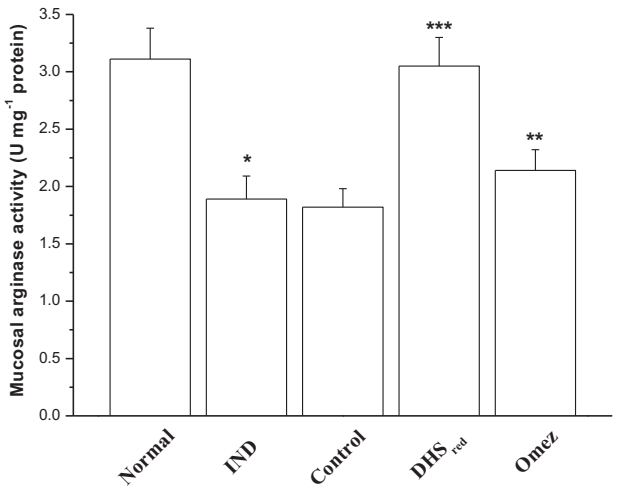


Fig. 8. Effect of DHS_{red} and Omez in regulating mucosal arginase activity in the IND-induced ulcerated mice. The mice were ulcerated by IND (18 mg kg^{-1}, *p. o.*). Treatment was carried out with DHS_{red} (2.5 mg kg^{-1}, *p. o.*) and Omez (3 mg kg^{-1}, *p. o.*) for 3 days. The arginase activity was measured using a colorimetric kit. The values are mean ± S.E.M. of three independent experiments, each with five mice per group. $^{*}P < 0.01$ compared to the normal group; $^{**}P < 0.05$, $^{***}P < 0.01$ compared to the IND group.

74.7%. In contrast, Omez increased the eNOS expression by 39% and reduced the iNOS expression by 25.2%, compared to the IND group.

3.7. Regulation of mucosal arginase activity

In the IND group, the arginase activity was depleted significantly (39.2%). Treatment with DHS_{red} brought the arginase activity to the normal level, while Omez enhanced it by 13.2% only (Fig. 8).

3.8. Regulation of serum Th_1 (IL-1β, TNF-α and IL-6) and Th_2 (IL-4 and IL-10) cytokines

The serum IL-1β and TNF-α levels were increased by 5.1 fold and 1.8 fold in the IND group. DHS_{red} and Omez suppressed such an increase in IL-1β by 61.2% and 19.4% respectively. The augmented TNF-α level of

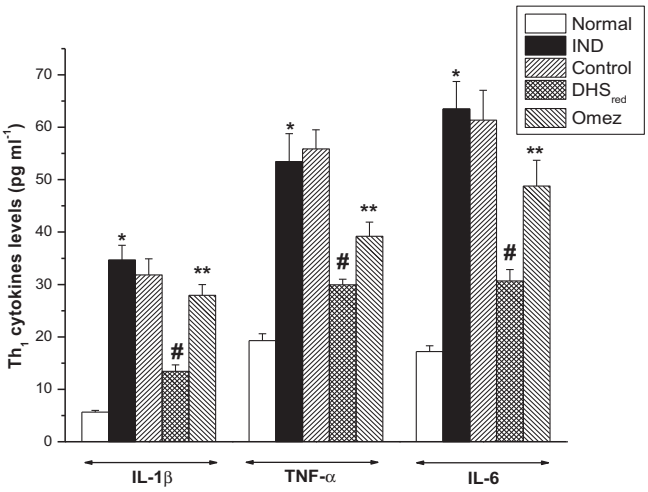


Fig. 9. Modulation of the serum pro-inflammatory cytokines IL-1β, TNF-α and IL-6 levels by DHS_{red} and Omez in the IND-induced ulcerated mice. The mice were ulcerated by IND (18 mg kg^{-1}, *p. o.*). Treatment was carried out with DHS_{red} (2.5 mg kg^{-1}, *p. o.*) and Omez (3 mg kg^{-1}, *p. o.*) for 3 days. The serum cytokine levels were assayed by ELISA. The values are mean ± S.E.M. of three independent experiments, each with five mice per group. $^{*}P < 0.001$ compared to the normal group; $^{**}P < 0.05$, $^{\#}P < 0.01$ compared to the IND group.

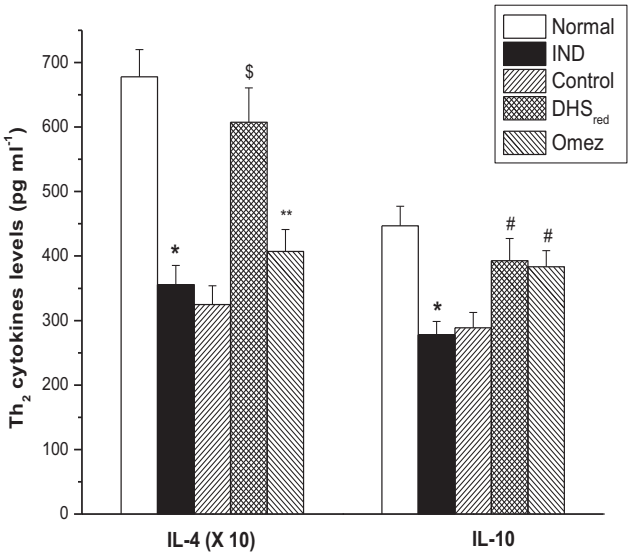


Fig. 10. Modulation of the serum anti-inflammatory cytokines IL-4 and IL-10 levels by DHS_{red} and Omez in the IND-induced ulcerated mice. The mice were ulcerated by IND (18 mg kg^{-1}, *p. o.*). Treatment was carried out with DHS_{red} (2.5 mg kg^{-1}, *p. o.*) and Omez (3 mg kg^{-1}, *p. o.*) for 3 days. The serum cytokine levels were assayed by ELISA. The values are mean ± S.E.M. of three independent experiments, each with 5 mice per group. $^{*}P < 0.01$ compared to the normal group; $^{**}P < 0.05$, $^{\#}P < 0.01$, $^{\$}P < 0.001$ compared to the IND group.

the ulcer group was also reduced by DHS_{red} (44%) and Omez (26.7%). Ulceration also increased the serum IL-6 level by 2.7 fold that was suppressed by DHS_{red} (51.7%) and Omez (23.1%) (Fig. 9). Regarding the Th_2 cytokines, the serum IL-4 and IL-10 levels in the IND group were reduced by 47.6% and 37.8% respectively. Treatment with DHS_{red} increased the serum IL-4 (70.9%) and IL-10 (41.4%) levels appreciably. Omez increased the levels of IL-4 (14.5%) and IL-10 (37.8%). The results are shown in Fig. 10.

4. Discussion

Due to the involvement of multiple factors in the IND-mediated gastropathy, it is difficult to target a specific putative mechanism as a therapeutic strategy. Several factors such as enzymes, cytokines and soluble mediators, liberated due to the inflammatory response, play crucial roles in the IND-mediated gastric ulceration and the delayed ulcer healing. Controlling these factors provides an opportunity to develop improved anti-ulcer medications, although this aspect has been largely ignored. The previously observed healing capacity of DHS_{red} against the IND-induced gastric ulceration in mice [9] encouraged us to investigate its probable modulatory effect on the COX-independent pro-inflammatory parameters [27]. Oxidative stress and inflammation adversely affect endothelial function and cause GI anomalies. Amongst the controlling factors, neutrophil infiltration plays a crucial role in the initiation and progression of gastric mucosal damage by IND [11]. On the other hand, gastric mucosal NO shows biphasic behavior, depending on its mode of generation and concentration. Therefore, these parameters were the focus of the present study. Previously, DHS_{red} (2.5 mg kg^{-1}, *p.o.*) and Omez (3 mg kg^{-1}, *p. o.*) provided optimum healing against IND (18 mg kg^{-1}, *p. o.*)-induced acute stomach ulceration in mice. Moreover, the protocols of IND-mediated ulceration in mice, and its healing by several anti-ulcer agents have also been extensively studied by our group [23,24]. Hence the present study was carried out under the same experimental conditions.

Initially we confirmed our previous histology data on the healing action of DHS_{red} by the MPO assay, which is also a reliable measure of neutrophil infiltration and inflammation even under clinical conditions [28]. Our results showed an increased MPO activity in the IND group at the time point (72 h) of maximum ulceration, suggesting significant neutrophil infiltration. Moreover, both ulcerative damage and MPO activity got reduced on the 5th day post-IND administration (data not shown). Earlier we found that the vehicle did not induce any stomach ulceration [9]. Our present MPO assay results reaffirmed this. We assessed the soluble forms of the adhesion molecules, because these are good inflammation markers [29]. Our previous results also showed an excellent correlation between the circulatory levels of these proteins and their expressions on the EC surfaces [23]. Presently IND markedly increased sE- and sP-selectins as well as ICAM-1. These may result in more efficient leukocyte migration into the surrounding tissues, because P- and E-selectins are critical for the capture and rolling of leukocytes in the microvasculature, while ICAM-1 mediates firm adhesion. In particular, the higher level of sE-selectin would be important at the early stages of inflammation, while the increased sP-selectin and sICAM-1 levels would maintain a high gastric MPO activity to sustain ulceration. Earlier P-selectin was suggested to be more crucial in IND-gastropathy [30,31].

Treatment with DHS_{red} and Omez reduced the MPO activity, and the relative efficacy was congruent with their respective ulcer-healing property. IND is known to reduce antichemotactic activity, but increase chemotactic activity in the ulcerated tissues leading to persistent neutrophils infiltration and delayed healing [32]. Thus, recession of MPO activity by DHS_{red} may reduce microcirculatory abnormalities to promote healing [33]. The associated down-regulation of the soluble selectins and CAMs by DHS_{red} (Figs. 2–4) is likely to interrupt the interactions of neutrophils and endothelial cells both at the early rolling phase (selectins-mediated), and at the late firm adhesion phase (CAM-mediated). 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. Thus, the observed reduction of the MPO activity by DHS_{red} may be because of the suppression of these CAMs. The effect of DHS_{red} on all these parameters was much more than that of Omez, and consistent with its better healing activity than Omez [9].

Neutrophils inflict endothelial damage by generating various free radicals including NO that profoundly influences oxidative burst. 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. Piotrowski et al. [34] showed a 12-fold increase in gastric epithelial iNOS expression in the IND-administered animals compared to control, and the increase correlated positively with the epithelium damage. Currently we confirmed that IND increased the mucosal iNOS expression, but reduced the eNOS expression in mice. The elevated neutrophil infiltration due to IND treatment may be responsible for the observed iNOS activation and the excessive NO generation. High concentrations of NO may promote mucosal inflammation via swelling and epithelial damage, and develop gastric mucosal lesions. 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 IND group. However, L-NIL showed a better protective effect than L-NAME. This suggested that the iNOS-mediated NO was primarily responsible for IND-gastropathy.

DHS_{red} raised the eNOS/iNOS ratio along with reduction of the total NOS activity and nitrite level favoring efficient ulcer-healing. The reduction of the total NOS activity and nitrite level by DHS_{red} was primarily due to suppression of the iNOS expression. Moreover, the observed reduction of the MPO activity by DHS_{red} was adversely affected by L-NAME, but not by L-NIL. Because, DHS_{red} itself reduced the iNOS expression, L-NIL did not show any effect on its healing activity. However, L-NAME would negate the positive influence of DHS_{red} on the eNOS expression to suppress its healing activity. This would result in MPO activation as observed in the study. Taken together, these results suggested that the eNOS-derived NO contributed maximum to the ulcer healing property of DHS_{red}, although a role for neuronal NOS-derived NO cannot be excluded. The beneficial role of eNOS and

eNOS-derived NO against acute inflammation was demonstrated earlier, using eNOS deficient mice [35].

The IND group also showed a reduced mucosal arginase activity, revealing a shift of the arginine metabolism toward the iNOS/NO pathway. Besides reducing the iNOS expression, DHS_{red} also improved the arginase activity. The switch from the iNOS to the arginase activities would increase the polyamines level and assist ulcer healing. The improved arginase activity and favorable eNOS/iNOS ratio, caused by treatment with DHS_{red} may be the key contributing factors in its efficient ulcer-healing. The healing action of Omez is attributed to its ability to control intragastric pH [36] and increase serum gastrin level that stimulates epithelial cell proliferation [37]. Consistent with this, our results revealed that the drug had marginal effects on eNOS/iNOS expressions and NO production in the IND group.

There are reports suggestive of an intense reciprocal regulation of NOS and arginase activities in vivo, and the cross-talk amongst NOS/NO and arginase/polyamine is guided by the cytokine profile of the host [21,38]. IND is a known immunomodulator and the extent of IND-induced ulceration correlates with the increase in the Th_1/Th_2 cytokine ratio [11]. Recently we found that IND upregulates TNF-α in mice gastric mucosa to induce various pro-inflammatory mediators and down-regulate the COX-dependent and angiogenic pathways. Based on these, it was proposed that TNF-α mitigation may offer a potential solution to the IND-gastropathy [23]. Hence, for a better prognosis, the immune response due to ulceration, and its modulation by DHS_{red} was monitored. IND raised the serum levels of pro-inflammatory Th_1 cytokines (IL-1β, TNF-α and IL-6) and reduced the anti-inflammatory Th_2 cytokines (IL-4 and IL-10) levels. IL-4 that remains under the influence of NO controls the expression of growth factors, responsible for ulcer onset and healing. Consistent with an earlier report, we also observed the reduction in the IL-4 level by IND [39]. The cytokine imbalance, induced by IND presumably triggered upregulation of the adhesion molecules and neutrophil infiltration, and augmented the iNOS/NO pathway to produce excess NO. These are likely to promote oxidative stress and result in sustained ulceration and delayed healing [40].

Treatment with DHS_{red}, however, reversed the cytokine imbalance by reducing the Th_1 cytokines substantially, and nearly restoring the normal levels of IL-4 and IL-10. The upregulation of the anti-inflammatory cytokines by DHS_{red} is likely to inhibit the stimulatory effect of IND in releasing the pro-inflammatory cytokines in blood. In the previous paper, we found that DHS_{red} augments the PGE level in the IND-treated mice. The increased PGE might stimulate IL-10 release, which, in turn, controls PGE and the Th_1 cytokines via a negative feedback mechanism. We selected IL-1β for this study, since depending on its concentration in different loci of the GI tract, this cytokine modulates ulcer healing via the COX-2 pathway. IL-4 and IL-10 are known to induce arginase and are often increased after trauma [21]. The enhanced IL-4 level by DHS_{red} may trigger the TGF-β–SMAD-signaling pathway to stimulate the extracellular remodeling and subsequent tissue repair. The altered arginase activity and iNOS expression during ulceration, and after DHS_{red} treatment are consistent with their respective effects in modulating the cytokines. In contrast, Omez did not alter the cytokines significantly, as reported earlier [39]. This was also reflected in its marginal effect in regulating the arginase activity.

During the entire study, the IND group of mice received a normal diet, but no vehicle. All other groups received the vehicle (2% gum acacia in water), used for the administration of the test samples as well as the normal diet. The biochemical parameters of the control and the IND groups of mice were almost identical revealing that gum acacia did not provide any healing against IND-mediated ulceration. Likewise, the data of the normal group confirmed that gum acacia also did not create stomach ulcers in the mice. Administration of DHS_{red} (2.5 mg kg^{-1}, *p. o.*) alone did not alter any of these biochemical parameters compared to the normal group (data not shown). This suggested that DHS_{red} does not have any systemic effect.

Overall, the data presented in the present study demonstrates that treatment with the organo-selenium compound, DHS_{red} can accelerate healing of IND-induced gastric ulceration in mice, by a number of distinct mechanisms. It was found that DHS_{red} can significantly reduce the IND-induced neutrophil infiltration by moderating various soluble inflammatory modulators such as the adhesion molecules, NO as well as the Th_1/Th_2 cytokine profiles, altering the gastric mucosal eNOS/iNOS ratio and switching the iNOS/NO pathway to the arginase/polyamine pathway. These, along with its ability to strengthen mucosal defense system by augmenting antioxidant status, gastric mucin and PGE, as disclosed in our previous studies might contribute to its ulcer healing action.

References

[1] S.H. Roth, Nonsteroidal anti-inflammatory drug gastropathy: we started it, why don't we stop it? J. Rheumatol. 32 (2005) 1189–1191.
[2] J.L. Wallace, NSAID gastropathy and enteropathy: distinct pathogenesis likely necessitates distinct prevention strategies, Br. J. Pharmacol. 165 (2012) 67–74.
[3] S.E. Miederer, Will anti-ulcer drugs soon differ only in their side effects? Fortschr. Med. 104 (1986) 918–920.
[4] F.K.L. Chan, Primer: managing NSAID-induced ulcer complications—balancing gastrointestinal and cardiovascular risks, Nat. Clin. Pract. Gastroenterol. Hepatol. 3 (2006) 563–573.
[5] K. Fiskin, A. Ozkan, A.G. Ayhan, Investigation of protective effects of selenium and vitamin E against DNA oxidation, membrane damage and alteration of COMT metabolism in smoke-exposed male mice (*Mus musculus*, Balb/c), Acta Biol. Hung. 57 (2006) 403–413.
[6] W.P. Banner, J.J. De Cosse, Q.H. Tan, M.S. Zedeck, Selective distribution of selenium in colon parallels its antitumor activity, Carcinogenesis 5 (1984) 1543–1546.
[7] I.L. Slootmans, A. Dirk, V. Berghe, E.V. Dyck, P. Piot, Susceptibility of 40 *Haemophilus ducreyi* strains to 34 antimicrobial products, Antimicrob. Agents Chemother. 24 (1983) 564–567.
[8] M. Iwaoka, T. Takahashi, S. Tomoda, Synthesis and structural characterization of water-soluble selenium reagents for the redox control of protein disulfide bonds, Heteroat. Chem. 12 (2001) 293–299.
[9] S. Chakraborty, S.K. Yadav, M. Subramanian, K.I. Priyadarsini, M. Iwaoka, S. Chattopadhyay, DL-*trans*-3,4-Dihydroxy-1-selenolane (DHS red) accelerates healing of indomethacin-induced stomach ulceration in mice, Free Radic. Res. 46 (2012) 1378–1386.
[10] F. Halter, A. Schamassman, B.M. Peskar, A.S. Tarnawski, Cyclooxygenase-2 implications on maintenance of gastric mucosal integrity and ulcer healing: controversies and perspectives, Gut 49 (2001) 443–453.
[11] J.L. Wallace, C.M. Keenan, D.N. Granger, Gastric ulceration induced by nonsteroidal anti-inflammatory drugs is a neutrophil-dependent process, Am. J. Physiol. 259 (1990) G462–G467.
[12] M.H.L.P. Souza, J.M.S.C. Mota, R.B. Oliveira, F.Q. Cunha, Gastric damage induced by different doses of indomethacin in rats is variably affected by inhibiting iNOS or leukocyte infiltration, Inflamm. Res. 57 (2008) 28–33.
[13] C. Bogdan, Nitric oxide and the immune response, Nat. Immunol. 2 (2001) 907–916.
[14] Y.C. Hou, A. Janczuk, P.G. Wang, Current trends in the development of nitric oxide donors, Curr. Pharm. Des. 5 (1999) 417–441.
[15] L. Ma, J.L. Wallace, Endothelial nitric oxide synthase modulates gastric ulcer healing in rats, Am. J. Physiol. Gastrointest. Liver Physiol. 279 (2000) G341–G346.
[16] M. Ziche, L. Morbidelli, E. Masini, S. Amerini, H.J. Granger, C.A. Maggi, Nitric oxide mediates angiogenesis in vivo and endothelial cell growth and migration in vitro promoted by substance P, J. Clin. Invest. 94 (1994) 2036–2044.
[17] M. Jaiswal, N.F. LaRusso, G.J. Gores, Nitric oxide in gastrointestinal epithelial cell carcinogenesis: linking inflammation to oncogenesis, Am. J. Physiol. Gastrointest. Liver Physiol. 281 (2001) G626–G634.
[18] M.H.L.P. Souza, H.P. Lemos, R.B. Oliveira, F.Q. Cunha, Gastric damage and granulocyte infiltration induced by indomethacin in tumour necrosis factor receptor 1 (TNF-R1) or inducible nitric oxide synthase (iNOS) deficient mice, Gut 53 (2004) 791–796.
[19] T.D. Warner, F. Giuliano, I. Vojnovic, A. Bukasa, J.A. Mitchell, J.R. Vane, Nonsteroidal drug selectivities for cyclooxygenase-1 rather than cyclooxygenase-2 are associated with human gastrointestinal toxicity: a full in vitro analysis, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 7563–7568.
[20] J. Satriano, Interregulation of nitric oxide and polyamines, Amino Acids 26 (2004) 321–329.
[21] J.D. Shearer, J.R. Richards, C.D. Mills, M.D. Caldwell, Differential regulation of macrophage arginine metabolism: a proposed role in wound healing, Am. J. Physiol. 272 (1997) E181.
[22] M. Modollel, I.M. Corraliza, F. Link, E.K. Soler, Reciprocal regulation of the nitric oxide synthase/arginase balance in mouse bone marrow derived macrophages by Th_1 and Th_2 cytokines, Eur. J. Immunol. 25 (1995) 1101–1104.
[23] S.K. Yadav, B. Adhikary, S. Chand, B. Maity, S.K. Bandyopadhyay, S. Chattopadhyay, Molecular mechanism of indomethacin-induced gastropathy, Free Radic. Biol. Med. 52 (2012) 1175–1187.
[24] S.K. Yadav, B. Adhikary, S.K. Bandyopadhyay, S. Chattopadhyay, Inhibition of TNF-α as well as NF-κB and JNK pathways accounts for the healing action of the natural

phenolic, allylpyrocatechol against indomethacin gastropathy, Biochim. Biophys. Acta (Gen.) 1830 (2013) 3776–3786.
[25] K. Suzuki, H. Ota, S. Sasagawa, T. Sakatani, T. Fujikura, Assay method for myeloperoxidase in human polymorphonuclear leukocytes, Anal. Biochem. 132 (1983) 345–352.
[26] M. Del Ara Rangel, R.A. Gozalez-Polo, A. Caro, E. del-Amo, L. Palomo, E.G.H. Soler, J.M. Fuentes, Diagnostic performance of arginase activity in colorectal cancer, Clin. Exp. Med. 2 (2002) 53–57.
[27] I. Tegeder, J. Pfeilschifter, G. Geisslinger, Cyclooxygenase-independent actions of cyclooxygenase inhibitors, FASEB J. 15 (2001) 2057–2072.
[28] K. Nishida, Y. Ohta, T. Kobayashi, I. Ishiguro, Involvement of the xanthine–xanthine oxidase system and neutrophils in the development of acute gastric mucosal lesions in rats with water immersion restraint stress, Digestion 58 (1997) 340–351.
[29] B. Glowinska, M. Urban, J. Peczynska, B. Florys, Soluble adhesion molecules (sICAM-1, sVCAM-1) and selectins (sE selectin, sP selectin, sL selectin) levels in children and adolescents with obesity, hypertension, and diabetes, Metabolism 54 (2005) 1020–1026.
[30] J.L. Wallace, K.E. Arfors, G.W. McKnight, A monoclonal antibody against the CD18 leukocyte adhesion molecule prevents indomethacin-induced gastric damage in the rabbit, Gastroenterology 100 (1991) 878–883.
[31] J.L. Wallace, W. McKnight, M. Miyasaka, T. Tamatani, J. Paulson, D.C. Anderson, D.N. Granger, P. Kubes, Role of endothelial adhesion molecules in NSAID-induced gastric mucosal injury, Am. J. Physiol. 265 (1993) G993–G998 (Gastrointest Liver Physiol 28).
[32] H. Fujita, S. Takahashi, S. Okabe, Mechanism by which indomethacin delays the healing of acetic acid-induced ulcer in rats: role of neutrophils antichemotactic and chemotactic activities, J. Physiol. Pharmacol. 49 (1998) 71–82.
[33] Y. Tsukimi, C. Nozue, S. Okabe, Effects of teminoprazole, omeprazole and sucralfate on indomethacin-induced delayed healing of kissing gastric ulcers in rats, J. Gastroenterol. Hepatol. 11 (1996) 335–340.
[34] J. Piotrowski, A. Slomiany, B.L. Slomiany, Activation of apoptotic caspase-3 and nitric oxide synthase-2 in gastric mucosal injury induced by indomethacin, Scand. J. Gastroenterol. 34 (1999) 129–134.
[35] J.C. Luo, V.Y. Shin, E.S.L. Liu, W.H.L. So, Y.N. Ye, F.Y. Chang, C.H. Cho, Non-ulcerogenic dose of dexamethasone delays gastric ulcer healing in rats, J. Pharmacol. Exp. Ther. 307 (2003) 692–698.
[36] J.L. Goldstein, J.F. Johanson, C.J. Hawkey, L.J. Suchower, K.A. Brown, Clinical trial: healing of NSAID-associated gastric ulcers in patients continuing NSAID therapy — A randomized study comparing ranitidine with esomeprazole, Aliment. Pharmacol. Ther. 26 (2007) 1101–1111.
[37] Y. Takeuchi, S. Kitano, T. Bandoh, T. Matsumoto, D. Baatar, S. Kai, Acceleration of gastric ulcer healing by omeprazole in portal hypertensive rats. Is its action mediated by gastrin release and the stimulation of epithelial proliferation? Eur. Surg. Res. 35 (2003) 75–80.
[38] J. Satriano, Agmatine: at the crossroads of the arginine pathways, Ann. N.Y. Acad. Sci. 1009 (2003) 34–43.
[39] B.L. Slomiany, J. Piotrowski, A. Slomiany, Role of endothelin-1 and constitutive nitric oxide synthase in gastric mucosal resistance to indomethacin injury: effect of antiulcer agents, Scand. J. Gastroenterol. 34 (1999) 459–464.
[40] S. Chatterjee, S. Premachandran, R.S. Bagewadikar, S. Bhattacharya, S. Chattopadhyay, T.B. Poduval, Arginine metabolic pathways determine its therapeutic benefit in experimental heat stroke: role of Th 1/Th 2 cytokine balance, Nitric Oxide 15 (2006) 408–416.