

Alendronate induces gastric damage by reducing nitric oxide synthase expression and NO/cGMP/K_{ATP} signaling pathway

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ABSTRACT

Chronic use of alendronate has been linked to gastrointestinal tract problems. Our objective was to evaluate the role of the NO/cGMP/K_{ATP} signaling pathway and nitric oxide synthase expression in alendronate-induced gastric damage. Rats were either treated with the NO donor, sodium nitroprusside (SNP; 1, 3, and 10 mg/kg), or the NO synthase (NOS) substrate, L-arginine (L-Arg; 50, 100, and 200 mg/kg). Some rats were pretreated with either ODQ (a guanylate cyclase inhibitor; 10 mg/kg) or glibenclamide (K_{ATP} channels blocker; 10 mg/kg). In other experiments, rats were pretreated with L-NAME (non-selective NOS inhibitor; 10 mg/kg), 1400W (selective inducible NOS [iNOS] inhibitor; 10 mg/kg), or L-NIO (a selective endothelial NOS [eNOS] inhibitor; 30 mg/kg). After 1 h, the rats were treated with alendronate (30 mg/kg) by gavage for 4 days. SNP and L-Arg prevented alendronate-induced gastric damage in a dose-dependent manner. Alendronate reduced nitrite/nitrate levels, an effect that was reversed with SNP or L-Arg treatment. Pretreatment with ODQ or glibenclamide reversed the protective effects of SNP and L-Arg. L-NAME, 1400W, or L-NIO aggravated the severity of alendronate-induced lesions. In addition, alendronate reduced the expression of iNOS and eNOS in the gastric mucosa. Gastric ulcerogenic responses induced by alendronate were mediated by a decrease in NO derived from both eNOS and iNOS. In addition, our findings support the hypothesis that activation of the NO/cGMP/KATP pathway is of primary importance for protection against alendronate-induced gastric damage.

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1. Introduction

Bisphosphonates are the class of drugs most commonly used for the control, prevention, and treatment of metabolic bone diseases, including osteoporosis [1]. Alendronate is a frequently used bisphosphonate, due to dosing convenience and benefits related to the prevention of fractures. However, recent studies have shown that chronic alendronate use may be associated with side effects primarily involving the gastrointestinal tract, including gastric ulcer and erosive esophagitis [2,3].

Recently, studies have demonstrated that gaseous mediators, such as carbon monoxide (CO) [4] and hydrogen sulfide (H₂S) [5], contribute to the protection and maintenance of gastric mucosal

integrity against alendronate-induced gastric damage. Similarly, nitric oxide (NO) may protect the gastrointestinal mucosa from damage caused by chemical agents, such as ethanol [6] and NSAIDs [7]. However, few studies have demonstrated the involvement of NO in the pathogenesis of alendronate-induced gastropathy.

NO plays an important role in regulating various cellular functions in the cardiovascular, immune, and neuronal systems, as well as in the gastrointestinal tract. NO is synthesized in endothelial cells by the nitric oxide synthase (NOS) family of enzymes; NOS enzymes convert the amino acid, L-arginine, into L-citrulline and NO [8]. Constitutive NOS (cNOS), which is expressed in normal conditions, comprises the 2 isoforms endothelial NOS (eNOS) and neuronal NOS (nNOS), that play important roles in the maintenance of mucosal integrity [9,10]. A third isoform, which is known as inducible NOS (iNOS), is not constitutively expressed. Rather, it is stimulated by factors such as cytokines or microbial products [11].

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In this study, we have investigated the role of the NO/cGMP/ K_{ATP} signaling pathway and nitric oxide synthase expression in alendronate-induced gastric damage in rats.

2. Materials and methods

2.1. Animals

Female Wistar rats (100–140 g) were obtained from the Department of Physiology and Pharmacology, Federal University of Ceará. The animals were deprived of food for 18–24 h before the experiment, but had free access to water. All animal treatments and surgical procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD) and were approved by the local ethics committee (Protocol No. 0067/10).

2.2. Drugs and solutions

Sodium nitroprusside (SNP), L-arginine (L-Arg), 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), glibenclamide, N^G-nitro-L-arginine methyl ester (L-NAME), 1400W and alendronate were purchased from Sigma Aldrich (St. Louis, MO). L-N^G-iminoethyl-L-ornithine (L-NIO) was purchased from Cayman Chemical (Ann Arbor, MI). ODQ was dissolved 0.01% DMSO. Glibenclamide was dissolved in 0.01 N NaOH containing 4% glucose. Alendronate was dissolved in saline and adjusted to pH 7.0 by adding NaOH or HCl [12]. All other drugs were dissolved in saline.

2.3. Effect of sodium nitroprusside or L-arginine on alendronate-induced gastric damage

The animals were pretreated with saline, SNP (a nitric oxide donor: 1, 3, and 10 mg/kg, po) or L-Arg (a substrate of NOS; 50, 100 and 200 mg/kg, ip). After 30 min, alendronate (30 mg/kg, pH 7.0, po) was administered. The control group received only saline. All drugs were administered once daily for 4 days [4]. On the last day of treatment, 4 h after alendronate administration, the animals were killed and their stomachs removed. Gastric damage was measured using Image J® software. A sample was fixed in 10% formalin immediately after its removal for subsequent histopathological assessment. Other samples were then weighed, frozen, and stored at –80 °C until assayed for NO₃/NO₂ production [13], glutathione (GSH) levels [14], malondialdehyde (MDA) concentration [15], myeloperoxidase (MPO) activity [16] and cytokine levels [17].

2.4. Histological evaluation of gastric damage

For histological evaluation, the stomach samples were fixed in 10% formalin solution for 24 h. After fixation, the samples were transferred to a solution of 70% alcohol. The material was then embedded in paraffin and sectioned; 4-μm-thick sections were deparaffinized, stained with hematoxylin and eosin (H&E), and then examined under a light microscope by an experienced pathologist without knowledge of the treatments (Soares, PMG). The specimens were assessed according to the criteria as previously described [18].

2.5. Measurement of nitrite plus nitrate production

Homogenate of gastric tissue was incubated in a microplate with nitrate reductase for 12 h to convert NO₃ in NO₂. Nitric oxide production was determined by measuring nitrite concentrations in an ELISA plate reader at 540 nm using the Griess method [13].

Results were expressed as micromoles of nitrite (μmol) using the internal standard curve.

2.6. Role of soluble guanylate cyclase and ATP-Sensitive K⁺ channels on gastroprotective effect of sodium nitroprusside or L-arginine

The animals were pretreated with ODQ (guanylate cyclase inhibitor: 10 mg/kg, ip) or glibenclamide (a drug that blocks ATP-Sensitive K⁺ Channels: 10 mg/kg, ip). One hour after, received SNP (a nitric oxide donor: 10 mg/kg, po) or L-Arg (a substrate of NOS; 200 mg/kg, ip). After 30 min, the animals received alendronate (30 mg/kg, pH 7.0, po). The control group received only saline. All drugs were administered once daily for 4 days. On the last day of treatment, 4 h after alendronate administration, the animals were killed, their stomachs removed, gastric damage and biochemists analysis were determined as described above.

2.7. Role of nitric oxide synthase on alendronate-induced gastric damage

The animals were initially treated with L-NAME (a non-selective inhibitor of NOS; 3 mg/kg, ip), 1400W (a selective inhibitor of iNOS; 10 mg/kg, ip) or L-NIO (a selective inhibitor of eNOS; 30 mg/kg, sc) [6,19]. After 1 h, the animals received alendronate (30 mg/kg, pH 7.0, po). All drugs were administered once daily for 4 days [4]. On the last day of treatment, 4 h after alendronate administration, the animals were killed and their stomachs removed and gastric damage and biochemists analysis were determined as described above. Samples were removed for immunohistochemistry analysis and western blot for iNOS and eNOS as described below.

2.8. Immunohistochemistry for eNOS and iNOS

The samples from the stomach of rats undergoing alendronate-induced gastric damage were assessed for the expression of eNOS and iNOS by employing an immunohistochemical technique. The slides mounted from the paraffin blocks were deparaffinized and then hydrated. Endogenous peroxidase activity was blocked with 1% H₂O₂ diluted in methanol. Then, the slides were washed in phosphate-buffered saline (PBS). Next, the slides were incubated with primary antibody (1:400, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C. After washing, the slides were incubated with biotinylated secondary antibody, diluted in PBS plus bovine serum albumin (PBS-BSA). Negative control sections were processed simultaneously as described above, but without adding an antibody. Finally, the tissue was stained for antigen–antibody complexes using a peroxidase detection system and then viewed under a microscope.

2.9. Western blot for iNOS and eNOS

Briefly, the tissues were homogenized in 0.2 ml of lysis buffer containing protease inhibitors. Total protein (50 μg protein/well) was resolved on 10% (iNOS) or 12.5% (eNOS) sodium dodecyl sulfate–polyacrylamide gel (SDS–PAGE) and transferred to a nitrocellulose membrane (Hybond-ECL, Amersham Pharmacia Biotech, Amersham, UK). The membranes were blocked with 5% skimmed milk/Tris-buffered saline with 0.1% Tween 20 (TBS-T) for 2 h at 4 °C followed by an overnight incubation period at 4 °C with the primary antibodies (rabbit polyclonal anti-iNOS, 1:500; anti-eNOS, 1:800 or anti-β-actin, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA). The blots were washed, followed by incubation with horseradish peroxidase-conjugated secondary antibody (donkey anti-rabbit immunoglobulin G, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at 4 °C. The membranes were washed,

incubated with electrogenerated chemiluminescence (Amersham Pharmacia Biotech), and exposed to Hyperfilm ECL (Amersham Pharmacia Biotech) to observe the marked proteins. Densitometry analyses were performed by Image J® software. Data were expressed as the relative density of iNOS/β-actin and eNOS/β-actin bands.

2.10. Glutathione (GSH) levels

The samples were homogenized in 0.02 M EDTA (1 ml/100 mg of tissue). Aliquots (400 µl) of the tissue homogenate were mixed with 320 µl of distilled water and 80 µl of 50% (w/v) trichloroacetic acid in glass tubes and centrifuged at 3000 rpm for 15 min. Next, 400 µl of each supernatant was mixed with 800 µl of Tris buffer (0.4 M, pH 8.9) and 20 µl of 0.01 M DTNB. Subsequently, the samples were stirred for 3 min and read on a spectrophotometer at 412 nm [14]. The results are expressed as micrograms of GSH per gram of tissue (µg/g).

2.11. Malondialdehyde (MDA) concentration

The samples were homogenized in cold 1.15% KCl (1 ml/100 mg of tissue). Briefly, 250 µl of each homogenate was added 1% phosphoric acid (H_3PO_4) and 0.6% *tert*-butyl alcohol (aqueous solution). Then, this mixture was stirred and heated in a boiling water bath for 45 min. The mixture was then cooled immediately in an ice water bath followed by the addition of 4 ml of *n*-butanol. This mixture was shaken and the butanol layer was separated by centrifugation at 1200 rpm for 15 min. Optical density was determined to be 535 and 520 nm, and the optical density difference between the two determinations was calculated as the *tert*-butyl alcohol value [15]. Results are expressed as nanomoles per gram of tissue (nmol/g).

2.12. Myeloperoxidase (MPO) activity

Briefly, tissue was homogenized in potassium buffer with 0.5% of hexadecitrimetilamônio (HTAB) (1 ml/100 mg of tissue). Then, homogenate was centrifuged at 4500 rpm for 20 min. MPO activity in the resuspended pellet was assayed by measuring the change in absorbance at 450 nm using o-dianisidine dihydrochloride and 1%

hydrogen peroxide [16]. The results were expressed as the MPO units per mg of tissue (UMPO/mg of tissue).

2.13. Cytokine measurements

Briefly, microtiter plates were coated overnight at 4 °C with an polyclonal anti-rat TNF-α or IL-1β (4 µg/ml, DuoSet ELISA Development kit R&D Systems). After blocking the plates, the samples and standards were added at various dilutions in duplicate and incubated at 4 °C for 24 h. The plates were washed with buffer (0.01 M phosphate, 0.05 M NaCl, 0.1% Tween 20, pH 7.2). After washing the plates, biotinylated sheep polyclonal anti-TNF-α or anti-IL-1β (diluted 1:1000 with assay buffer containing 1% bovine serum albumin [BSA]) was added to the wells. After further incubation at room temperature for 1 h, the plates were washed and 50 µl of avidin-conjugated horseradish peroxidase diluted 1:5000 was added to the wells. The color reagent o-phenylene-diamine (OPD; 40 µg/well) was added 15 min later and the plates were incubated in the dark at 37 °C for 15–20 min. The enzyme reaction was stopped with H_2SO_4 and absorbance was measured at 490 nm [17]. Values are expressed as pictograms of cytokines per milliliter (pg/ml).

2.14. Statistical analysis

Data were described as either means ± SEM or median, as appropriate. Analysis of Variance (ANOVA) followed by Student-Newman-Keuls test was used to compare means and Kruskal-Wallis nonparametric test, followed by Dunńs tests to compare medians; $P < 0.05$ was defined as statistically significant.

3. Results

3.1. Effect of SNP or L-arginine on alendronate-induced gastric damage

In the present study, we observed that pretreatment with either the NO donor, sodium nitroprusside (SNP; Fig. 1A) or the NOS substrate, L-arginine (L-Arg; Fig. 1B) prevented alendronate-induced macroscopic gastric damage in a dose-dependent manner. Furthermore, the results of microscopic analysis indicated that alendronate administration induced alterations in the gastric region characterized by epithelial cell loss, inflammatory cell, edema,

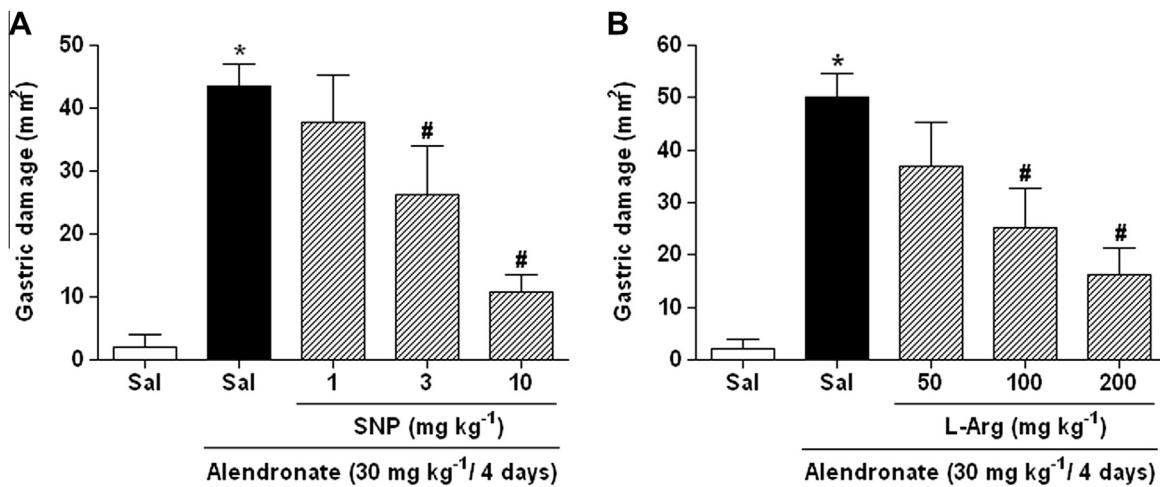


Fig. 1. Effect of (A) sodium nitroprusside (SNP) and (B) L-arginine (L-Arg) pretreatment on alendronate-induced gastric damage. Rats were treated with either saline (control), SNP, or L-Arg 30 min before the administration of alendronate (30 mg/kg). The control group received only saline. All drugs were administered once daily for 4 days. Macroscopic gastric lesions were determined 4 h after alendronate administration on the fourth day. Data are expressed as the mean ± S.E.M. of 5–6 rats per group. * $P < 0.05$ vs. saline group, # $P < 0.05$ vs. alendronate group, one-way ANOVA and Newman-Keuls test.

and intense hemorrhage (Fig. 2 and Table 1). These changes were significantly prevented in rats pretreated with either SNP (Fig. 2D) or L-Arg (Fig. 2E).

3.2. Nitrite/nitrate production

As shown in Fig. 3, administration of alendronate to gastric tissue significantly reduced the generation of nitrite and nitrate metabolites of NO compared to controls. Pretreatment with SNP or L-Arg reversed this effect of alendronate; nitrite and nitrate levels were raised to values similar to those observed in the control group.

3.3. Role of soluble guanylate cyclase and ATP-sensitive K^+ channels on protective effect of SNP or L-arginine

To assess the contribution of soluble guanylate cyclase and K_{ATP} channels to the protective effects of SNP and L-Arg, rats were

pretreated with either ODQ, a guanylate cyclase inhibitor, or glibenclamide, a K_{ATP} channel blocker. Fig. 4 shows that pretreatment with ODQ or glibenclamide significantly ($P < 0.05$) reversed the positive effect that SNP (Fig. 4A) and L-Arg (Fig. 4B) had on alendronate-induced gastric macroscopic damage.

3.4. Role of nitric oxide synthase on alendronate-induced gastric damage

The role of NOS in alendronate-induced gastric damage was also evaluated and the results are shown in Fig. 5. Pretreatment with L-NAME, 1400W, or L-NIO markedly aggravated the severity of alendronate-induced gastric macroscopic damage.

The expression of iNOS (Fig. 6A) and eNOS (Fig. 6B) was evident from immunohistochemical analysis of the gastric mucosa of control rats. However, lower staining levels of both iNOS and eNOS were observed in rats treated with alendronate. The results of protein expression experiments by western blotting were in

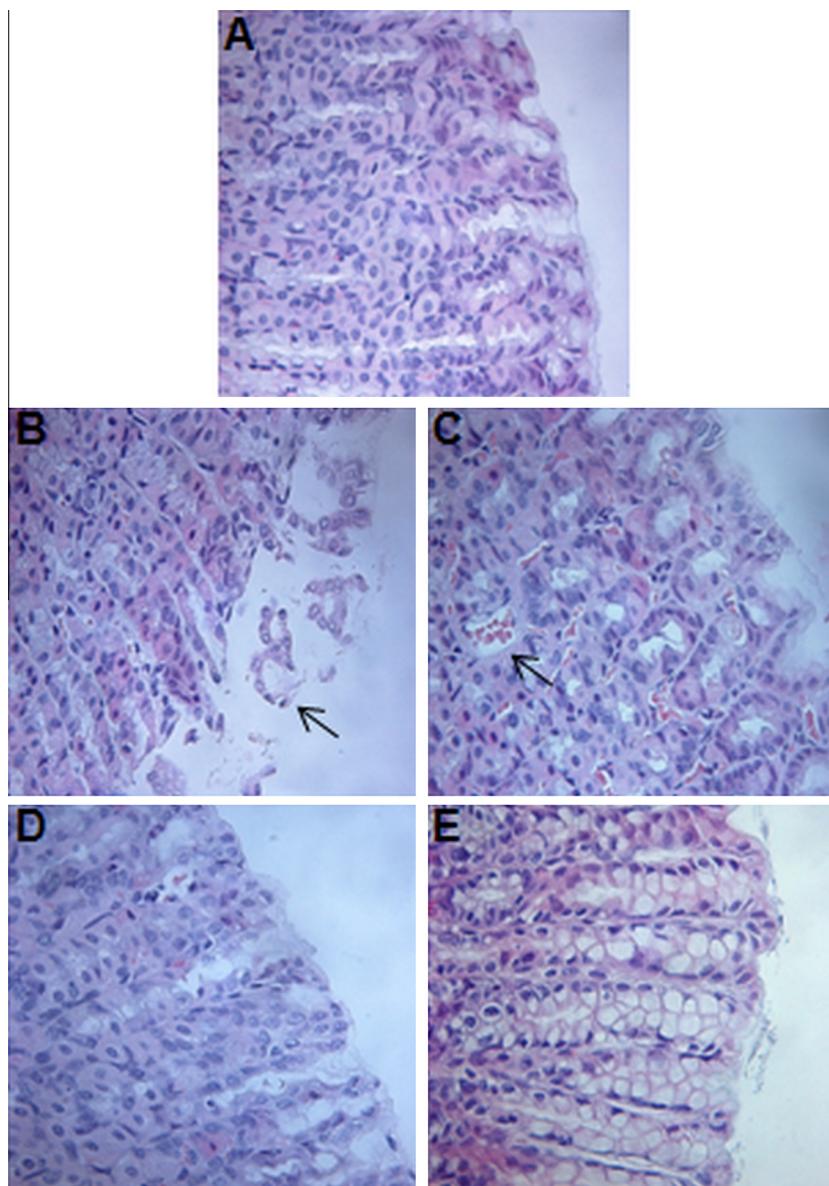


Fig. 2. Histopathological changes in the gastric mucosa (40 \times magnification). (A) Control (saline) group showing gastric mucosal integrity. Alendronate (30 mg/kg, po) group showing lesions in the superficial gastric glandular region with (B) epithelial cell loss, (C) and bleeding. (D) Treatment with sodium nitroprusside (SNP; 10 mg/kg, po) + alendronate (30 mg/kg po), and with (E) L-Arginine (L-Arg; 200 mg/kg, ip) + alendronate (30 mg/kg po), showing reduction in alendronate-induced microscopic lesions.

Table 1

Effect of sodium nitroprusside or L-arginine in alendronate-induced gastric microscopic damage.

Experimental group	Hemorrhagic damage (score 0–4)	Edema (score 0–4)	Epithelial cell loss (score 0–3)	Inflammatory cells (score 0–3)	Total (score 0–14)
Saline	0 (0–1)	0	0 (0–1)	0	0
Saline + alendronate	3 (1–3) ^a	3 (2–3) ^a	3 (2–3) ^a	2 (2–3) ^a	11 (2–3) ^a
SNP + alendronate	2 (0–2)	0 (0–1) ^b	0 (0–1) ^b	0 (0–1) ^b	2 (0–2) ^b
L-Arg + Alendronate	1 (0–2) ^b	1 (0–1) ^b	0 (0–1) ^b	1 (0–1) ^b	3 (0–1) ^b

Data shown are medians with minimal and maximal scores shown in parentheses. Kruskal–Wallis nonparametric test, followed by Dunn's test was used for multiple comparisons for histological assessment.

^a $P < 0.05$, when compared with saline group.

^b $P < 0.05$, when compared with alendronate group.

agreement with immunohistochemical findings; protein expression levels of iNOS (Fig. 7A) and eNOS (Fig. 7B) were significantly ($P < 0.05$) reduced after treatment with alendronate.

3.5. GSH, MDA, MPO and cytokine levels

Alendronate significantly reduced GSH levels, increased MDA concentrations, and increased MPO activity in gastric tissue when

compared to control, as shown in Table 2. Pretreatment with SNP or L-Arg significantly increased GSH levels, reduced MDA concentrations, and reduced MPO levels ($P < 0.05$) in rats that received alendronate. Furthermore, we observed that administration of either ODQ or glibenclamide reversed the gastroprotective effects of SNP or L-Arg, as evidenced by changes in GSH, MDA and MPO. Similarly, L-NAME combined with L-Arg treatment significantly reduced GSH levels, increased MDA concentrations, and increased MPO activity compared to rats treated with L-Arg alone. In contrast, pretreatment with L-NAME, 1400W, and L-NIO had no effect on GSH levels, MDA concentration, or MPO activity in the gastric mucosa of rats treated with alendronate alone (Table 2).

Treatment with either SNP or L-Arg significantly ($P < 0.05$) reduced TNF- α levels in rats with alendronate-induced gastric damage. In addition, SNP treatment led to a reduction in IL-1 β levels (Fig. 8). The observed changes in TNF- α were significantly reversed with L-NAME pretreatment, but not with L-Arg pretreatment. L-NAME pretreatment had no impact on IL-1 β levels.

4. Discussion

In the present study, we show that SNP and L-Arg, acting via NO/cGMP/K_{ATP}-dependent mechanisms, protect the gastric mucosa against alendronate-induced damage. In addition, we demonstrated that alendronate reduced NO generation by modulating NOS expression.

Consistent with previous studies [4,20,21], we observed that when given as a single injection for 4 days to fasting rats, alendronate damaged the gastric mucosa causing lesions to appear in the antrum and extend further to develop into ulcers with a white cap.

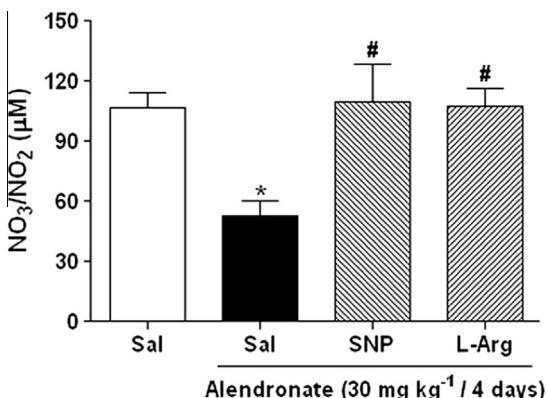


Fig. 3. Nitric oxide levels (NO₃/NO₂) in rats pretreated with sodium nitroprusside (SNP) and L-arginine (L-Arg) in rats with alendronate-induced gastric damage. Rats were pretreated with either saline, SNP (10 mg/kg, po), or L-Arg (200 mg/kg, ip) 30 min before alendronate (30 mg/kg) administration. The control group received only saline. All drugs were administered once daily for 4 days. Data are expressed as the mean \pm S.E.M. of 5–6 rats per group. * $P < 0.05$ vs. saline group, # $P < 0.05$ vs. alendronate group, one-way ANOVA and Newman–Keuls test.

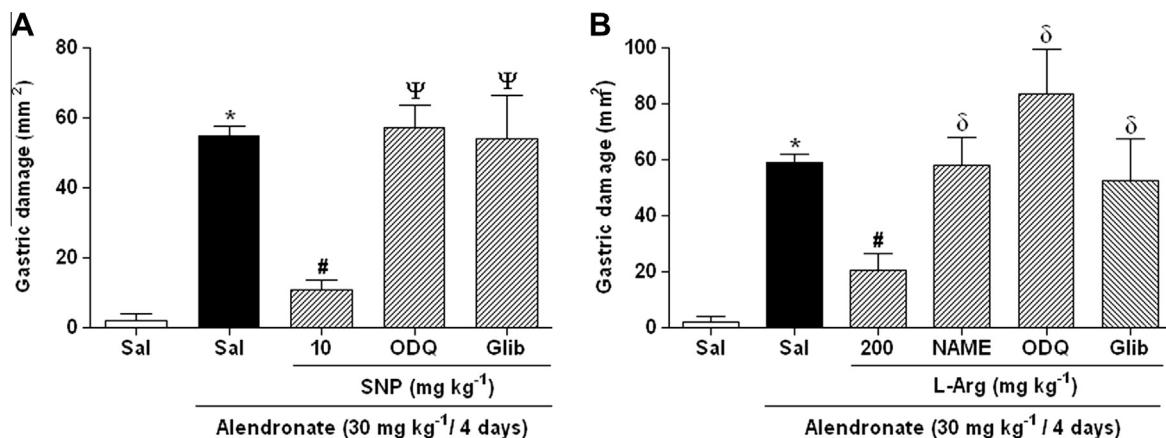


Fig. 4. Role of soluble guanylate cyclase (sGC) and ATP-sensitive K⁺ channels (K_{ATP}) in the gastroprotective effect of sodium nitroprusside (SNP) and L-arginine (L-Arg) in a rat model of alendronate-induced gastric damage. Rats were pretreated with either saline, L-NAME (3 mg/kg, ip), ODQ (10 mg/kg, ip) or glibenclamide (5 mg/kg, ip). Either SNP (10 mg/kg, po) or L-Arg (200 mg/kg, ip) was administered after 1 h, followed by alendronate (30 mg/kg, po) injection 30 min later. The control group received only saline. Data are expressed as mean \pm S.E.M. of 5–6 rats per group. * $P < 0.05$ vs. saline group, # $P < 0.05$ vs. alendronate group, Ψ $P < 0.05$ vs. SNP + alendronate group, δ $P < 0.05$ vs. L-Arg + alendronate group, one-way ANOVA and Newman–Keuls test.

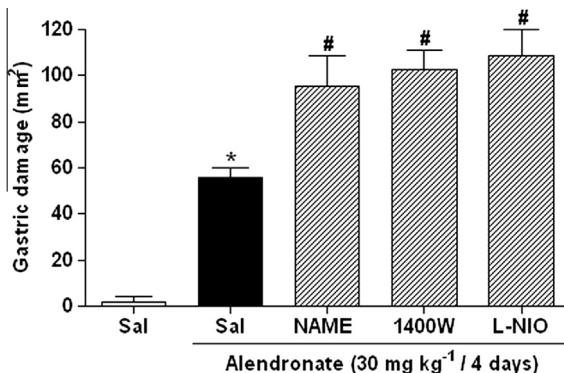


Fig. 5. Role of nitric oxide synthase (NOS) in alendronate-induced gastric damage. Rats were treated with either saline, L-NAME (3 mg/kg, ip), 1400 W (10 mg/kg, ip), or L-NIO (30 mg/kg, sc). Alendronate (30 mg/kg) was administered after 1 h. The control group received only saline. All drugs were administered once daily for 4 days. Data are expressed as the means \pm S.E.M. of 5–6 rats per group. * P < 0.05 vs. saline group, # P < 0.05 vs. alendronate group, one-way ANOVA and Newman–Keuls test.

In contrast, lesions that formed in the corpus healed. A previous study demonstrated that the white cap covering the damaged mucosa is composed mainly of inflammatory cells and fibrin-like substances [22]. Histologically, we observed that gastric ulcers caused by alendronate administration induced severe edema, hemorrhagic damage, epithelial cell loss, and infiltration of inflammatory cells in the submucosa. Other studies have reported that when alendronate is applied to the gastric mucosa it causes a decrease in transmucosal potential difference of the stomach, which is suggestive of a disruption to surface epithelial cells due to a direct action [12]. These toxic effects of alendronate in the stomach have been linked to a direct effect of this agent on the mucosal surface [22,23].

Several studies have shown that bisphosphonates also interfere with cell migration at the site of inflammation, and this effect is

associated with increased production of proinflammatory cytokines, IL-1 β , TNF- α , and IL-6, and oxidative stress [24,25]. In the present study, increased MDA and MPO levels, increased accumulation of TNF- α and IL-1 β , and decreased GSH levels by alendronate in the gastric mucosa suggest that neutrophil infiltration and proinflammatory cytokines (TNF- α and IL-1 β) contribute to oxidative gastric damage. Activated neutrophils are also a potential source of oxygen metabolites that can contribute to gastric mucosal injury [26]. It has been suggested that oxygen-derived free radicals may contribute to alendronate-induced gastric mucosal lesions [20,21].

NO plays a critical role in mucosal integrity, either by direct action, or by modulating the effects of other substances [27] involved in important physiological functions, such as regulation of mucosal blood flow and mucus generation [28]. In the present study, we demonstrated that both the NO donor, SNP, and the NOS substrate, L-Arg protected the gastric mucosa against alendronate-induced macroscopic damage. Histological observations further validated macroscopic findings that SNP and L-Arg prevent gastric damage induced by subchronic treatment with oral alendronate. Furthermore, SNP and L-Arg inhibited alendronate-induced elevations in MDA, MPO, TNF- α , and IL-1 β levels, and alendronate-induced reductions in GSH. Together, these data suggest that the gastroprotective effects of SNP and L-Arg may be dependent on their inhibitory effects on neutrophil infiltration and neutrophil-associated TNF- α and IL-1 β responses. Thus, the mechanism through which NO exerts its gastroprotective effect appears to involve a reduction of lipid peroxidation induced by alendronate in the gastric mucosa. Therefore, the effects of NO may result in a decreased redox state in alendronate-induced gastropathy.

It is well known that NO modulates the activity of mucosal immunocytes, as well as modulating leukocyte–endothelial interactions. NO inhibits recruitment of neutrophils into the gastrointestinal tract mucosa, and inhibitors of NO synthesis enhance leukocyte recruitment [23,29]. The anti-inflammatory properties of NO include inhibiting the production of important pro-inflammatory molecules [29].

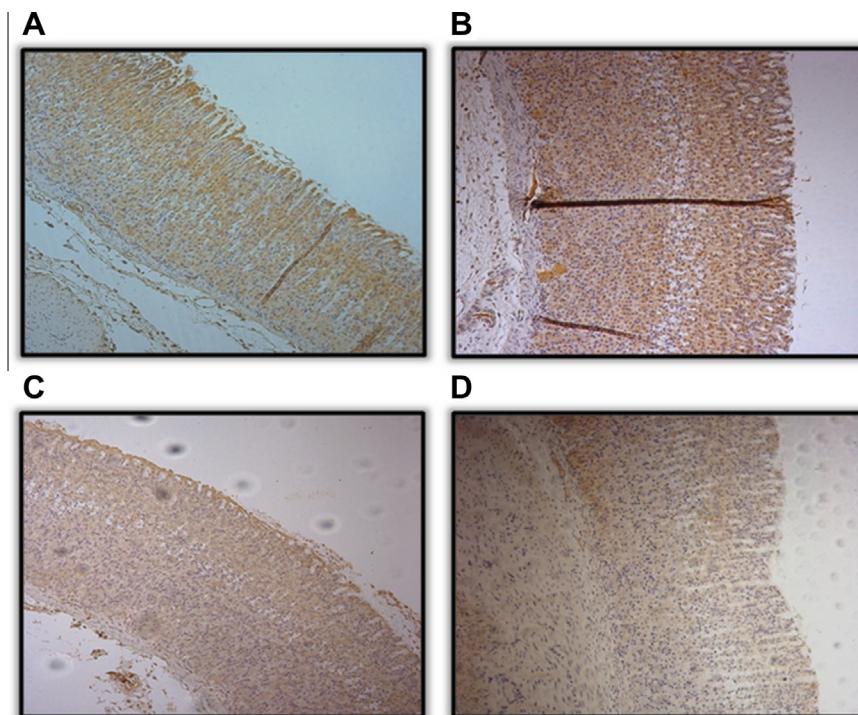


Fig. 6. Photomicrographs of gastric mucosa at 100 \times magnification. (A) iNOS and (C) eNOS immunoreactivity detected in normal gastric tissue. Decreased (B) iNOS and (D) eNOS immunoreactivity in gastric mucosal tissue after alendronate was administered once daily for 4 days.

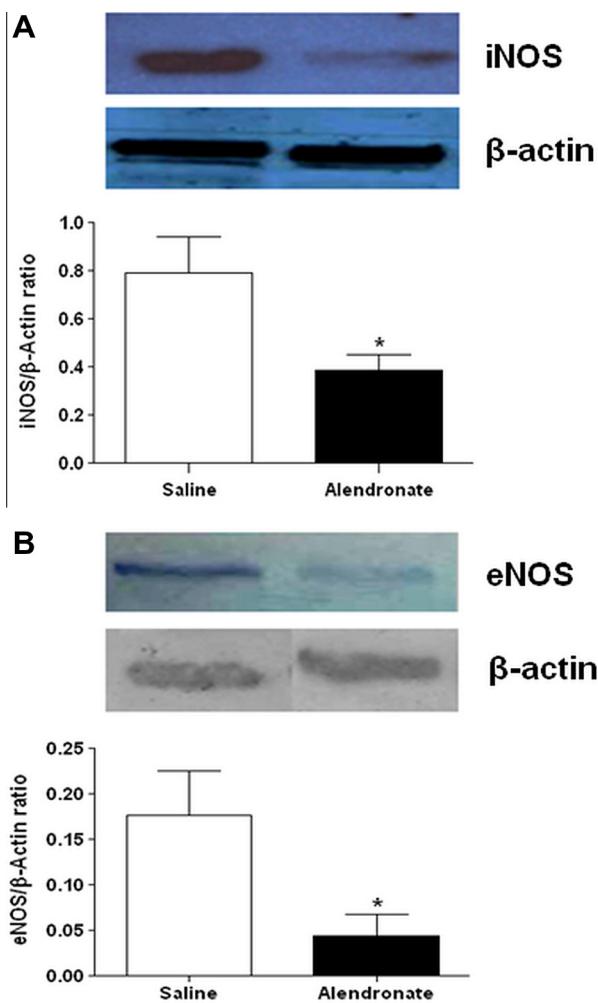


Fig. 7. Protein expression of (A) iNOS and (B) eNOS determined by Western blot analysis of tissue from rats with alendronate-induced gastric damage. The results are reported as the relative density of iNOS/β-actin and eNOS/β-actin bands. * $P < 0.05$ vs. saline group, one-way ANOVA and Newman–Keuls test.

Studies have also documented that NO donors and L-Arg promote gastroprotection [6] and accelerate the healing of experimental gastric ulcers [30]. Therefore, it can be inferred that NO

synthesis plays an essential role in gastric protection against alendronate. To demonstrate this, we measured the nitrite and nitrate levels in the gastric mucosa as an estimation of NO production. The inorganic anions, nitrate (NO_3^-) and nitrite (NO_2^-), are products of endogenous NO metabolism. NO generated by NOS enzymes is oxidized in the blood and tissues to form nitrate and nitrite [8,28]. We observed that alendronate significantly reduced nitrite and nitrate levels in gastric tissue, and that pretreatment with either SNP or L-Arg reversed these effects; raising nitrite and nitrate levels to values similar to those observed in the controls.

Several actions of NO are mediated by activation of the intracellular second messenger, cyclic GMP (cGMP). Levels of cGMP are increased in response to activation of soluble guanylate cyclase by NO. In addition, it is well known that NO and cyclic GMP can activate different types of K_{ATP} channels [31,32] and that the activation of the NO/cGMP/ K_{ATP} pathway has gastroprotective effects [6]. We investigated whether the cGMP/ K_{ATP} pathway participated in the gastroprotective effects exhibited by NO in rats with alendronate-induced gastric damage. Using pharmacological approaches, we demonstrated that inhibition of soluble guanylate cyclase by ODQ, and blockade of K_{ATP} channels with glibenclamide, reversed the protective effects of SNP and L-Arg against alendronate-induced damage, and reversed the deleterious changes in GSH, MDA, and MPO levels in the gastric mucosa. Likewise, pretreatment with the non-selective NOS inhibitor, L-NAME, abolished the effects of L-Arg. Thus, our results indicate that the NO/cGMP/ K_{ATP} pathway is of primary importance in the protection of the gastric mucosa.

The three enzymatic sources of NO, nNOS, eNOS, and iNOS, have been characterized in the gastrointestinal tract. Several studies have demonstrated that NO plays a dual role in the ulcerogenic response of the gastrointestinal mucosa depending on the NOS isoform involved; a protective effect of NO is derived from nNOS and eNOS, and the inhibition of these enzymes can result in disturbances in GI motility, blood flow, secretion, and gastric ulcers [8,23]. In contrast, iNOS, which produces large amounts of NO under certain pathological conditions, is thought to contribute to mucosal injury and dysfunction [23]. However, there is little data to support this theory. Several other studies have suggested the possibility that excessive endogenous NO production, although having potentially detrimental hypotensive effects, serves an essential beneficial role that has yet to be elucidated [33–38]. Nishio et al. (2006) also demonstrated that endogenous NO derived from both constitutive and inducible forms of NOS contributes to

Table 2
GSH, MDA, and MPO levels in alendronate-induced gastric damage.

Experimental group	GSH ($\mu\text{g/g}$ tissue)	MDA (nmol/g tissue)	MPO (U/mg tissue)
Saline	446.4 \pm 21.4	26.6 \pm 4.4	3.9 \pm 0.9
Saline + alendronate	160.9 \pm 8.7 ^a	124.1 \pm 4.2 ^a	36.0 \pm 5.9 ^a
SNP + alendronate	471.6 \pm 45.2 ^b	85.6 \pm 3.6 ^b	15.1 \pm 1.7 ^b
ODQ + SNP + alendronate	220.2 \pm 30.3 ^c	98.6 \pm 10.6	30.9 \pm 1.6 ^c
Glibenclamide + SNP + alendronate	239.4 \pm 33.6 ^c	95.4 \pm 9.4	32.6 \pm 6.6 ^c
L-Arg + alendronate	318.7 \pm 16.8 ^b	56.4 \pm 6.0 ^b	16.3 \pm 2.4 ^b
L-NAME + L-Arg + alendronate	204.3 \pm 16.9 ^d	97.2 \pm 10.6 ^d	37.4 \pm 4.5 ^d
ODQ + L-Arg + alendronate	190.9 \pm 32.0 ^d	102.2 \pm 19.9 ^d	45.4 \pm 3.8 ^d
Glibenclamide + L-Arg + alendronate	223.7 \pm 8.1 ^d	113.3 \pm 11.7 ^d	28.8 \pm 2.8 ^d
L-NAME + alendronate	192.6 \pm 28.4	99.9 \pm 7.3	40.6 \pm 4.2
1400W + alendronate	178.5 \pm 32.5	125.8 \pm 27.5	37.8 \pm 5.1
L-NIO + alendronate	174.4 \pm 23.0	107.1 \pm 20.1	45.2 \pm 4.4

Results are expressed as the means \pm S.E.M. of 5–6 rats per group.

^a $P < 0.05$, when compared with saline group.

^b $P < 0.05$, when compared with alendronate group.

^c $P < 0.05$, when compared with SNP + alendronate group.

^d $P < 0.05$, when compared with L-Arg + alendronate group.

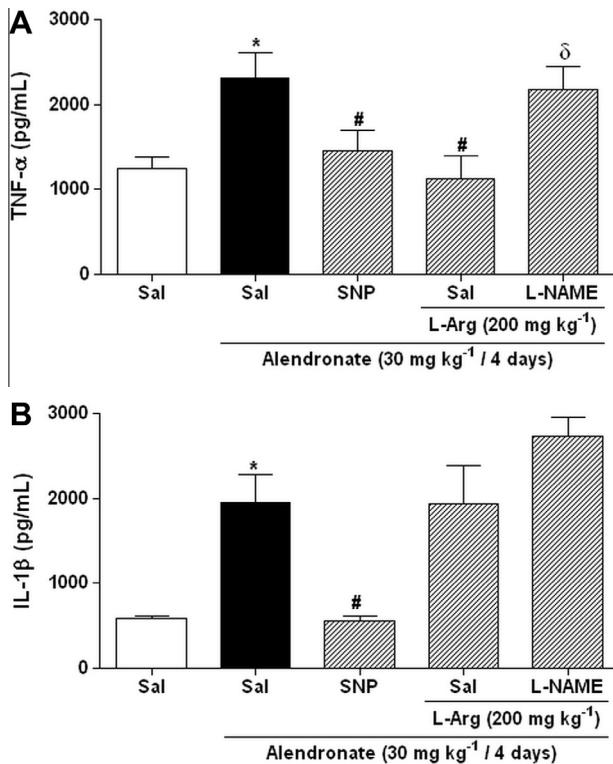


Fig. 8. Levels of cytokines TNF- α and IL-1 β in rats with alendronate-induced gastric damage. Rats were pretreated with either sodium nitroprusside (SNP; 10 mg/kg, po) or L-arginine (L-Arg; 200 mg/kg, ip). A third group received L-NAME (10 mg/kg, ip) + L-Arg (200 mg/kg, ip). Alendronate (30 mg/kg) was administered after 1 h. The control group received only saline. All drugs were administered once daily for 4 days. Data are expressed as mean \pm S.E.M. of 5–6 rats per group. * P < 0.05 vs. saline group, # P < 0.05 vs. alendronate group, δ P < 0.05 vs. L-Arg + alendronate group, one-way ANOVA and Newman-Keuls test.

mucosal defense against iodoacetamide-induced gastric damage, partly by reducing acid secretion and maintaining mucosal integrity [39].

The roles played by different isoforms of NOS during the development of alendronate-induced ulcers are not well understood. In our study, we demonstrated that NO produced by eNOS and iNOS are essential for the promotion of gastroprotection against alendronate-induced damage. We showed that the administration of a nonselective inhibitor of NOS (L-NAME), a selective inhibitor of iNOS (1400W), or a selective inhibitor of eNOS (L-NIO), aggravated the severity of the lesions induced by alendronate, but did not alter the biochemical parameters analyzed (MPO, GSH and MDA). Since subchronic administration of alendronate diminished NO levels in the gastric mucosa, we believe that regardless of source, increased levels of NO would be beneficial. Indeed, eNOS and iNOS may provide an endogenous mechanism to increase local NO levels, minimize gastric dysfunction, and increase gastric mucosal defense. We also demonstrated using immunohistochemistry that alendronate reduced the expression of iNOS and eNOS in the gastric mucosa. This finding was consistent with the observed reduction in protein expression levels of both iNOS and eNOS after treatment with alendronate. These findings strongly suggest that the increased gastric ulcerogenic response induced by alendronate is mediated by a decrease in NO derived from eNOS and iNOS.

Other possible protective mechanism of NO may be a decrease in gastric acid secretion and gastric acidity. In fact, it seems consensus that NO, either generated endogenously or administered exogenously, produced in low or large quantity, inhibits acid secretion under basal and stimulated conditions in rats [40], rabbits [41] and isolated human gastric glands [42]. The literature also show

that alendronate increase the gastric acid secretion and gastric acidity in rats [43] and this condition is essential to increase the irritating effect of alendronate in gastric mucosa, since at very low pH conditions ($\text{pH} < 2$) alendronate is known to be more harmful. Thus, the absorption profile and consequent gastric damage of alendronate may differ when NO-donors or NO-inhibitors are used.

Since NO produced by eNOS is generally considered to be important in maintaining mucosal integrity [8,10], it is reasonable to assume that alendronate-induced lesions are aggravated by L-NAME and L-NIO. However, we show that the involvement of iNOS/NO in gastric mucosal defense against irritation induced by subchronic administration of alendronate further suggests that iNOS is also responsible for the production of NO under such conditions. Moreover, it was recently reported that NO generated by iNOS contributes to mucosal protection during the late phase of adaptative cytoprotection, while increasing tissue injury in the chronic phase [37]. Indeed, the suppression of NO synthesis by cNOS and/or iNOS inhibition during the late phase may render the gastric mucosa more susceptible to injury [44], whereas administration of NO donors can protect the stomach from injury [45].

5. Conclusions

In summary, eNOS- and iNOS-derived NO prevents alendronate-induced gastric damage by activation of soluble guanylate cyclase and K_{ATP} channels. Furthermore, it decreases direct oxidative damage, and causes inhibition of neutrophil infiltration. Although there are many mechanisms through which this effect can occur, our data support the hypothesis that activation of the NO/cGMP/ K_{ATP} pathway is of primary importance. These observations also raise the possibility that NO-releasing agents could be used to improve resistance to gastric mucosa injury.

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References

- [1] C.M. Brandão, M.G. Lima, A.L. Silva, G.D. Silva, A.A. Guerra, F.A. Acúrcio, Treatment of postmenopausal osteoporosis in women: a systematic review, *Cad. Saúde Pública* 24 (2008) 592–606.
- [2] P.D. Papapetrou, Biphosphonate-associated adverse events, *Hormones* 8 (2009) 96–110.
- [3] K. Amagase, A. Inaba, T. Senta, Y. Ishikawa, K. Nukui, T. Murakami, K. Takeuchi, Gastric ulcerogenic and healing impairment effects of risedronate, a nitrogen-containing bisphosphonate in rats. Comparison with alendronate and minodronate, *J. Physiol. Pharmacol.* 62 (2011) 609–618.
- [4] N.R. Costa, R.O. Silva, L.A. Nicolau, L.T. Lucetti, A.P. Santana, K.S. Aragão, P.M.G. Soares, R.A. Ribeiro, M.H.L.P. Souza, A.L. Barbosa, J.V. Medeiros, Role of soluble guanylate cyclase activation in the gastroprotective effect of the HO-1/CO pathway against alendronate-induced gastric damage in rats, *Eur. J. Pharmacol.* 700 (2013) 51–59.
- [5] L.A.D. Nicolau, R.O. Silva, S.R.B. Damasceno, N.S. Carvalho, N.R.D. Costa, K.S. Aragão, A.L.R. Barbosa, P.M.G. Soares, M.H.L.P. Souza, J.V.R. Medeiros, The hydrogen sulfide donor, Lawesson's reagent, prevents alendronate-induced gastric damage in rats, *Braz. J. Med. Biol. Res.* 00 (2013) 1–7.
- [6] J.V. Medeiros, G.G. Gadelha, S.J. Lima, J.A. Garcia, P.M. Soares, A.A. Santos, G.A. Brito, R.A. Ribeiro, M.H. Souza, Role of the NO/cGMP/ K_{ATP} pathway in the protective effects of sildenafil against ethanol-induced gastric damage in rats, *Br. J. Pharmacol.* 153 (2008) 721–727.
- [7] M.D. Khattab, M.Z. Gad, D. Abdallah, Protective role of nitric oxide in indomethacin-induced gastric ulceration by a mechanism independent of gastric acid secretion, *Pharmacol. Res.* 43 (2001) 463–467.
- [8] S. Moncada, R.M.J. Palmer, E.A. Higgs, Nitric oxide: physiology, pathology and pharmacology, *Pharmacol. Rev.* 43 (1991) 109–142.

[9] C. Nathan, Nitric oxide as a secretory product of mammalian cells, *FASEB J.* 6 (1992) 3051–3064.

[10] J. Lopez-Belmonte, B.J.R. Whittle, S. Moncada, The action of nitric oxide donors in the prevention or induction of injury to the rat gastric mucosa, *Br. J. Pharmacol.* 108 (1993) 73–78.

[11] A.K. Nussler, T.R. Billiar, Inflammation, immunoregulation, and inducible nitric oxide synthase, *J. Leukoc. Biol.* 54 (1993) 171–178.

[12] K. Kanatsu, E. Aihara, M. Okayama, S. Kato, K. Takeuchi, Mucosal irritative and healing impairment action of risedronate in rat stomachs: comparison with alendronate, *J. Gastroenterol. Hepatol.* 19 (2004) 512–520.

[13] L.C. Green, D.A. Wagner, J. Glogowski, P.L. Skipper, J.S. Wishnok, S.R. Tannenbaum, Analysis of nitrate, nitrite, and [¹⁵N] nitrate in biological fluids, *Anal. Biochem.* 126 (1982) 131–138.

[14] J. Sedlak, R.H. Lindsay, Estimation of total, protein-bound, and nonprotein sulphydryl groups in tissue with Ellman's reagent, *Anal. Biochem.* 25 (1968) 192–205.

[15] M. Mihara, M. Uchiyama, Determination of malonaldehyde precursor in tissues by thiobarbituric acid test, *Anal. Biochem.* 86 (1978) 271–278.

[16] P.P. Bradley, R.D. Christensen, G. Rothstein, Cellular and extracellular myeloperoxidase in pyogenic inflammation, *Blood* 60 (1982) 618–622.

[17] F.Q. Cunha, M.A. Boukili, J.I. Motta, B.B. Vargaftig, S.H. Ferreira, Blockade by fenspiride of endotoxin-induced neutrophil migration in the rat, *Eur. J. Pharmacol.* 238 (1993) 47–52.

[18] L. Laine, W.M. Weinstein, Histology of alcoholic hemorrhagic "gastritis": a prospective evaluation, *Gastroenterology* 94 (1988) 1254–1262.

[19] S. Kato, F. Ohkawa, Y. Ito, K. Amagase, K. Takeuchi, Role of endothelial nitric oxide synthase in aggravation of indomethacin-induced gastric damage in adjuvant arthritic rats, *J. Physiol. Pharmacol.* 60 (2009) 147–155.

[20] G. Sener, K. Paskaloglu, C. Kapucu, S. Cetinel, G. Contuk, G. Ayanoglu-Dulger, Octreotide ameliorates alendronate-induced gastric injury, *Peptides* 25 (2004) 115–121.

[21] G. Sener, C. Kapucu, S. Cetinel, E. Cikler, R.G. Ayanoglu-Dulge, Gastroprotective effect of leukotriene receptor blocker montelukast in alendronate-induced lesions of the rat gastric mucosa, *Prostaglandins Leukot. Essent. Fatty Acids* 72 (2005) 1–11.

[22] Y. Ohashi, E. Aihara, K. Amagase, K. Takeuchi, Induction of antral ulcers by alendronate, a nitrogen-containing bisphosphonate, in rat stomachs, *Gastroenterology* 134 (2009). A–134.

[23] J.L. Wallace, L. Ma, Inflammatory mediators in gastrointestinal defense and injury, *Exp. Biol. Med.* 226 (2001) 1003–1015.

[24] K. Yamaguchi, K. Motegi, Y. Iwakura, Y. Endo, Involvement of interleukin-1 in the inflammatory actions of aminobisphosphonates in mice, *Br. J. Pharmacol.* 130 (2000) 1646–1654.

[25] D. Thiebaud, A. Sauty, P. Burckhard, P. Leuenberger, L. Sitzler, J.R. Green, A. Kandra, J. Zieschang, P. Ibarra de Palacios, An in vitro and in vivo study of cytokines in the acute-phase response associated with bisphosphonates, *Calcif. Tissue Int.* 61 (1997) 386–392.

[26] G.W. Sullivan, I.J. Sarembock, J. Linden, The role of inflammation in vascular diseases, *J. Leukoc. Biol.* 67 (2000) 591–602.

[27] S. Calatayud, D. Barrachina, J.V. Esplugues, Nitric oxide: relation to integrity, injury, and healing of the gastric mucosa, *Microsc. Res. Tech.* 53 (2001) 325–335.

[28] J.O. Lundberg, E. Weitzberg, M.T. Gladwin, The nitrate–nitrite–nitric oxide pathway in physiology and therapeutics, *Nat. Rev. Drug Discov.* 7 (2008) 156–167.

[29] D.M. McCafferty, E. Sihota, M. Muscara, J.L. Wallace, K.A. Sharkey, P. Kubes, Spontaneously developing chronic colitis in IL-10/iNOS double-deficient mice, *Am. J. Physiol. Gastrointest. Liver Physiol.* 279 (2000) 90–99.

[30] S.N. Elliott, W. McKnight, G. Cirino, J.L. Wallace, A nitric oxide-releasing nonsteroidal anti-inflammatory drug accelerates gastric ulcer healing in rats, *Gastroenterology* 109 (1995) 524–530.

[31] S.L. Archer, J.M. Huang, V. Hampl, D.P. Nelson, P.J. Shultz, E.K. Weir, Nitric oxide and cGMP cause vasorelaxation by activation of a charybdotoxin-sensitive K channel by cGMP-dependent protein kinase, *Proc. Natl. Acad. Sci.* 91 (1994) 7583–7587.

[32] V.M. Bolotina, S. Najibi, J.J. Palacino, P.J. Pagano, R.A. Cohen, Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle, *Nature* 368 (1994) 850–853.

[33] B.L. Tepperman, B.D. Soper, Nitric oxide synthase induction and cytoprotection of rat gastric mucosa from injury by ethanol, *Can. J. Physiol. Pharmacol.* 72 (1994) 1308–1312.

[34] S. Mariotto, M. Menegazzi, A. Carcereri de Prati, L. Cuzzolin, A. Adami, H. Suzuki, G. Benoni, Protective effect of NO on gastric lesions and inhibition of expression of gastric inducible NOS by flurbiprofen and its nitro-derivative, nitroflurbiprofen, *Br. J. Pharmacol.* 116 (1995) 1713–1714.

[35] H. Yu, E.F. Sato, Y. Minamiyama, T. Arakawa, K. Kobayashi, M. Inoue, Effect of nitric oxide on stress-induced gastric mucosal injury in the rat, *Digestion* 58 (1997) 11–318.

[36] Y. Li, C.H. Cho, The ulcer healing effect of protamine sulphate in rat stomach, *Aliment. Pharmacol. Ther.* 13 (1999) 1351–1362.

[37] H. Yamamoto, A. Tanaka, T. Kunikata, T. Hirata, S. Kato, K. Takeuchi, Inducible types of cyclooxygenase and nitric oxide synthase in adaptive cytoprotection in rat stomachs, *J. Physiol. (Paris)* 93 (1999) 405–412.

[38] S. Kato, A. Tanaka, A. Konaka, T. Kunikata, K. Takeuchi, Changes in gastric mucosal ulcerogenic responses in rats with adjuvant arthritis: role of nitric oxide, *Aliment. Pharmacol. Ther.* 13 (1999) 833–840.

[39] H. Nishio, Y. Hayashi, S. Terashima, K. Takeuchi, Role of endogenous nitric oxide in mucosal defense of inflamed rat stomach following iodoacetamide treatment, *Life Sci.* 79 (2006) 1523–1530.

[40] S. Kato, M. Kitamura, R.P. Korolikiewicz, K. Takeuchi, Role of nitric oxide in regulation of gastric acid secretion in rats: effects of NO donors and NO synthase inhibitor, *Br. J. Pharmacol.* 123 (1998) 839–846.

[41] H. Kim, K.H. Kim, Effects of a nitric oxide donor and nitric oxide synthase inhibitors on acid secretion of isolated rabbit gastric glands, *Pharmacology* 53 (1996) 331–339.

[42] A. Berg, S. Redeen, A.C. Ericson, S.E. Sjöstrand, Nitric oxide – an endogenous inhibitor of gastric acid secretion in isolated human gastric glands, *BMC Gastroenterol.* 4 (2004) 1–9.

[43] G. Sener, O. Sehirli, S. Cetinel, S. Midillioglu, N. Gedik, G. Ayanoglu-Dülger, Protective effect of taurine against alendronate-induced gastric damage in rats, *Fundam. Clin. Pharmacol.* 19 (2004) 93–100.

[44] P. Kubes, S. Kanwar, X.F. Niu, J. Gaboury, Nitric oxide synthesis inhibition induces leukocyte adhesion via superoxide and mast cells, *FASEB J.* 7 (1993) 1293–1299.

[45] M. Sueimatsu, T. Tamatani, F.A. Delano, M. Miyasaka, M. Forrest, H. Suzuki, G.W. Schmid-Schönbein, Microvascular oxidative stress preceding leukocyte activation elicited by in vivo nitric oxide suppression, *Am. J. Physiol.* 266 (1994) 2410–2415.