# Effects of esomeprazole on healing of nonsteroidal anti-inflammatory drug (NSAID)-induced gastric ulcers in the presence of a continued NSAID treatment: Characterization of molecular mechanisms

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ABSTRACT

Proton pump inhibitors promote ulcer repair in nonsteroidal anti-inflammatory drug (NSAID)-treated patients with ongoing NSAID-induced gastric toxicity, although the underlying mechanisms remain unclear. We examined the healing mechanisms of esomeprazole on NSAID-induced gastric ulcerations in the presence of a continued NSAID treatment. Ulcerations were induced in rats by oral indomethacin ($6 \mu mol/kg/day$) for 14 days. Indomethacin administration was continued, alone or combined with equivalent acid inhibitory doses of esomeprazole ($5 \mu mol/kg/day$), lansoprazole ($15 \mu mol/kg/day$) or famotidine ($20 \mu mol/kg/day$), for additional 7 days. Stomachs were then processed for: histomorphometric analysis of mucosal injury; mucosal levels of prostaglandin $E_2$ ($PGE_2$) and malondialdehyde (MDA); expression of vascular endothelial growth factor (VEGF), proliferating cell nuclear antigen (PCNA), caspase-3, and cyclooxygenase-2 (COX-2) (Western blot); expression of Ki-67 (immunohistochemistry). Indomethacin for 14 days elicited mucosal damage, reduced $PGE_2$ levels and increased MDA. After additional 7 days, indomethacin induced the following effects: further enhancement of mucosal damage and MDA content; decrease in $PGE_2$ levels; increase in COX-2 and activated caspase-3 expression; decrease in VEGF, PCNA and Ki-67 expression. In the presence of indomethacin, esomeprazole and lansoprazole were more effective than famotidine in promoting resolution of mucosal damage. Concomitantly, esomeprazole and lansoprazole, but not famotidine, restored PCNA and Ki-67 expression, and normalized MDA levels. Moreover, esomeprazole, lansoprazole and famotidine partly counteracted caspase-3 activation, without affecting VEGF expression. The healing activity of esomeprazole on indomethacin-induced gastric ulcerations can be ascribed to two mechanisms: (1) acid-dependent reduction of pro-apoptotic signalling; (2) acid-independent restoration of proliferating/repairing pathways.

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## 1. Introduction

The use of nonsteroidal anti-inflammatory drugs (NSAIDs) is associated with the occurrence of *de novo* adverse digestive events, including gastric mucosal erosions, ulcers, bleeding and perforation, as well as an increased risk of severe complications from pre-existing chronic ulcers [1]. The pathophysiology of NSAID-induced gastric injury depends, at least in part, on their ability to decrease prostaglandin production through cyclooxygenase (COX) inhibition, and partly on COX-independent mechanisms [2]. The combination of COX-dependent and COX-independent mechanisms leads to oxidative tissue injury, which seems to play a major role in the pathogenesis of NSAID-induced gastric damage [3,4]. Consistently with this view, gastric mucosal levels of malondialdehyde (MDA), a product arising from tissue oxidation, have been found to increase following NSAID administration [5]. Moreover, gastric ulcer repair is highly regulated by growth factors [6]. Among these, vascular endothelial growth factor (VEGF) promotes ulcer healing via stimulation of new microvessel formation, and indomethacin has been shown to interfere with this process through a downregulation of VEGF expression [7].

In the clinical setting, proton pump inhibitors (PPIs), including esomeprazole, have proven to be effective in the prevention of NSAID-induced gastric injury as well as in promoting the healing of NSAID-induced ulcers [8–11]. The inhibition of acid secretion accounts in part for the gastroprotective actions of PPIs, and acid-independent mechanisms can contribute to their digestive effects as well [12]. In particular, preclinical evidence suggests that, besides inhibiting acid secretion, PPIs can protect the gastric mucosa through mechanisms related to the reduction of tissue oxidative damage [12,13]. Nevertheless, while the molecular mechanisms underlying the protection afforded by PPIs in the setting of prophylactic treatments have been clarified, those implicated in the positive influence exerted by PPIs on healing of NSAID-induced gastric ulcerations remain undetermined. On the other hand, in the last decade significant advances have been pursued in understanding the molecular bases of ulcer healing and detrimental effects exerted by NSAIDs in ulcer repair [14,15].

Adverse digestive effects, occurring particularly in patients with increased risk factors, can seriously affect the quality of life of patients and may be of sufficient severity to require NSAID dose reduction or discontinuation [16,17]. However, dose reduction or discontinuation may not be feasible because of the severity of underlying diseases. Therefore, there is the need of effectively managing NSAID-associated upper gastrointestinal adverse events, so that NSAID therapy can be continued [9,18]. Although the clinical effectiveness of PPIs in promoting the healing of NSAID-induced gastric damage in the setting of a continued NSAID treatment has been proven [8,10], the mechanisms underlying their effectiveness in this condition remain to be clarified. On this basis, the present study was performed to examine the molecular mechanisms supporting the ability of esomeprazole to promote the healing of NSAID-induced gastric ulcerations in the presence of a continued NSAID treatment.

## 2. Methods

### 2.1. Animals, drug treatments and experimental design

The experiments were performed on adult male Wistar rats (250–280 g). Their care and handling were in accordance with the provision of the European Union Council Directive 86/609, recognized and adopted by the Italian Government. Gastric ulcerations were induced by daily administration of indomethacin (6 μmol/kg) via intragastric route for 14 days. Following this period of gastric injury induction, indomethacin administration was continued for additional 7 days, either alone or in combination with esomeprazole (5 μmol/kg). For comparison, experiments based on the above design were performed also with lansoprazole (15 μmol/kg) or famotidine (20 μmol/kg), a drug able to inhibit acid secretion, but devoid of acid-independent gastroprotective actions. Subgroups of animals were sacrificed after 14 days of indomethacin administration, in order to assess the presence of gastric lesions and the status of molecular markers related to mucosal damage or repair just prior the onset of treatments with antisecretory drugs.

Doses of esomeprazole, lansoprazole and famotidine with equivalent inhibitory activity on gastric acid secretion were selected by experiments on pylorus ligated rats, as reported below. The dose of indomethacin was selected on the basis of preliminary experiments. For this purpose, indomethacin was administered for 14 days at doses of 2, 6, and 18 μmol/kg/day. The results showed that the lowest dose elicited only minor ulcerations, while the highest dose led to a significant increase in mortality. By contrast, the selected dose of 6 μmol/kg/day produced a significant degree of gastric injury, without any significant increase in mortality.
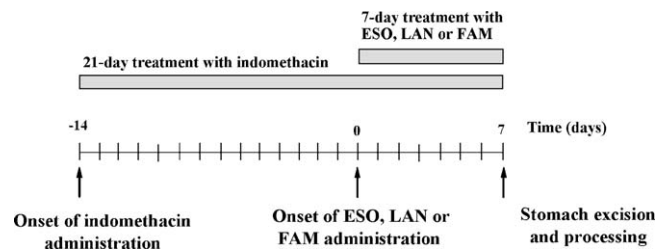


**Fig. 1.** Diagram showing the design and time-course of experimental procedures (ESO, esomeprazole; LAN, lansoprazole; FAM, famotidine).

A set of experiments was performed in order to ascertain whether the beneficial effects of the test antisecretory drugs on indomethacin-induced gastric damage resulted mainly from the prevention of *de novo* lesion development elicited by continued indomethacin administration or the decrease in severity of pre-existing lesions. In this setting, following the initial 14-day treatment with indomethacin (6 μmol/kg/day) to induce the gastric damage, indomethacin administration was stopped or continued at a lower dose (2 μmol/kg/day), devoid of significant injuring effects, in concomitance with the test antisecretory drugs for additional 7 days. The experimental design for ulcer induction and drug treatments is summarized in Fig. 1.

Additional experiments were performed to examine the possible contribution of antioxidant mechanisms in the healing of mucosal damage elicited by indomethacin. For this purpose, animals were treated with indomethacin (6 μmol/kg/day) for 14 days, followed by additional 7 days of indomethacin at the same dose plus superoxide dismutase (SOD), administered at 7 mg/kg/day by subcutaneous route. The dose of SOD was selected on the basis of its antioxidant and beneficial effects in a rat model of intestinal inflammation [19].

At the end of drug treatments, the stomachs were removed and processed for: histomorphometric evaluation of mucosal damage; assay of mucosal prostaglandin $E_2$ ($PGE_2$) and MDA levels; western blot analysis of VEGF, proliferating cell nuclear antigen (PCNA), cleaved caspase-3 and COX-2 mucosal expression; immunohistochemical analysis of Ki-67 expression.

### 2.2. Assay of gastric acid secretion in animals with pylorus ligation

The evaluation of gastric acid secretion was performed after a daily treatment with indomethacin (6 μmol/kg) for 14 days followed by indomethacin plus esomeprazole (1.5, 5 and 15 μmol/kg), lansoprazole (5, 15 and 30 μmol/kg) or famotidine (10, 20 and 40 μmol/kg) for additional 7 days. Animals were subjected to pylorus ligation for 2 h, after 22 h from the last drug dosing. Pylorus ligation was carried out as previously described [5]. In summary, during a brief anaesthesia with isoflurane, the abdomen was opened by midline laparotomy and the duodenum exteriorized. The pylorus was then ligated, the abdominal incision closed with clips and the animals were allowed to recover from anaesthesia for 10 min. Two h after pylorus ligation, the oesophageal-gastric junction was ligated and the whole stomach was excised. The gastric content was emptied, carefully collected in graduated centrifuge tubes, and centrifuged at $3000 \times g$ for 10 min. Samples with more than 0.5 ml of sediment were discarded. The level of acidity was measured by automatic potentiometric titration to pH 7.0 with 0.01 N NaOH, using a Compact titrator (Crison, Modena, Italy), and evaluated as $H^+$ output. The effects of esomeprazole, lansoprazole and famotidine on gastric acid secretion were expressed as μEqH$^+$/2 h.

### 2.3. Histomorphometric evaluation of gastric mucosal damage

The histomorphometric quantitative estimation of gastric mucosal damage was carried out as previously described [5]. Briefly, the stomach was opened along the greater curvature, gently washed with saline (154 mM NaCl), pinned upon a cork plate with the mucosal surface turned upwards, and fixed in 10% formalin buffered with phosphate for 24 h at 4 °C. Each stomach was dissected in parallel strips perpendicular to the lesser curvature at a distance of 2 mm. The strips from each stomach were sequentially placed on a glass slide and oriented with the side of each strip distal to the pylorus upwards. A solution of melted 3% agar was gently poured on the strips and quickly cooled at 4 °C to induce solidification. The agar block was then removed from the glass slide, dehydrated, and embedded in paraffin wax. Three-micrometer thick paraffin sections were cut using a microtome and stained with haematoxylin and eosin. Sections were examined by light microscopy and the length of both total and damaged mucosa was evaluated by means of a micrometric scale. The lesion index was estimated as the length fraction of damaged mucosa over the total length of mucosa, and expressed in percentage values.

### 2.4. Assay of mucosal $PGE_2$ levels

Enzyme immunoassay of $PGE_2$ in the gastric mucosa was performed using a commercial kit, as previously described [20]. Briefly, specimens of mucosa were rapidly scraped from underlying gastric tissue layers, using two glass slides kept cold on ice. The mucosa was weighed, minced by forceps, and homogenized in 1 ml of cold phosphate buffer (PBS 0.1 M, pH 7.4, containing 1 mM EDTA and 10 μM indomethacin) per gram of tissue using a polytron homogenizer (Cole Palmer Homogenizer, Vernon Hills, IL, USA). The resulting homogenate was added to an equal volume of absolute ethanol, and stirred by vortex. After 5-min incubation at room temperature, the homogenate was centrifuged at $1500 \times g$ for 10 min at 4 °C. The supernatant was treated with HCl 1 N until pH 4 was reached. Before performing the assay, samples were subjected to purification using superclean LC-18 SPE columns (Sigma Co., St. Louis, MO, USA). For this purpose, 0.5 ml of sample were added to 2 ml of ethanol and vortexed. After incubation at room temperature for 5 min, the sample was centrifuged at $3000 \times g$ for 10 min. The supernatant was then removed and applied to the LC-18 SPE column, previously activated with 5 ml of methanol followed by 5 ml of ultrapure water. The column was then washed with 5 ml of ultrapure water and 5 ml of hexane. $PGE_2$ was eluted with 5 ml of ethyl acetate containing 1% methanol. The eluted ethyl acetate fractions were collected and evaporated to dryness under nitrogen. Aliquots were used for subsequent enzyme immunoassay. $PGE_2$ concentration was expressed as nanogram per gram of wet mucosal tissue.

### 2.5. Evaluation of tissue oxidative damage

MDA concentrations in gastric mucosal tissues were determined to obtain quantitative estimates of membrane lipid peroxidation [5]. For this purpose, the gastric mucosa was excised, weighed, minced by forceps, homogenized in 2 ml of cold buffer (Tris–HCl 20 mM, pH 7.4) using a polytron homogenizer (Cole Palmer Homogenizer), and centrifuged at $1500 \times g$ for 10 min at 4 °C. Aliquots of supernatants were then used for subsequent assay procedures. Mucosal MDA concentrations were estimated using a colorimetric assay kit (Calbiochem-Novabiochem Corporation, San Diego, CA, USA). Results were expressed as nmol of MDA per mg of wet gastric tissue.

### 2.6. Western blot analysis of VEGF, PCNA, caspase-3 and COX-2

The gastric mucosa was scraped, weighed and homogenized in lysis buffer containing: HEPES 10 mmol/L, NaCl 30 mmol/L, EDTA 0.2 mmol/L, phenylmethylsulfonyl fluoride 2 mmol/L, leupeptin 10 μg/ml, aprotinin 10 μg/ml, sodium fluoride 1 mmol/L, sodium orthovanadate 1 mmol/L, glycerol 2%, $MgCl_2$ 0.3 mmol/L, and Triton-X 100 1%, using a polytron homogenizer (Cole Palmer homogenizer). Mucosal homogenates were spun by centrifugation at 20 000 r/min for 15 min at 4 °C, and the resulting supernatants were then separated from pellets and stored at −80 °C. Protein concentration was determined in each sample by the Bradford method (Protein Assay Kit, Bio-Rad, Hercules, CA, USA). To perform Western blot analysis of VEGF, PCNA, caspase-3 and COX-2, equivalent amounts of protein lysates (15 μg) were separated by electrophoresis on sodium dodecylsulfate polyacrylamide gel (12%) and transferred onto a PVDF membrane. The blots were then blocked for 2 h with 5% non-fat dried milk in TBS, and incubated overnight at room temperature with: rabbit polyclonal antibody raised against VEGF, rabbit polyclonal antibody raised against cleaved caspase-3, mouse monoclonal antibody raised against PCNA, and rabbit polyclonal antibody raised against COX-2 (dilutions 1:1000). After repeated washings with 0.1% Tween-20 in Tris-buffered saline, a peroxidase-conjugated goat anti-rabbit or anti-mouse antibody (dilution 1:10 000) was added for 1 h at room temperature. After repeated washings with 0.1% Tween-20 in Tris-buffered saline, immunoreactive bands were visualized by incubation with chemiluminescent reagents (Immobilon reagent, Millipore USA) and exposed to Kodak Image Station 440 for signal detection and densitometric image analysis. To ensure the equal loading and accuracy of changes in protein abundance, the protein levels were normalized to β-actin.

### 2.7. Immunohistochemical analysis of Ki-67

The immunohistochemical analysis of Ki-67 (a marker of cell proliferation) was performed in serial sections of gastric wall, previously fixed in 10% neutral formalin and embedded in paraffin. Sections were subjected to immunostaining for Ki-67 proliferating-cell antigen by incubation with primary monoclonal anti-mouse antibody. Sections were then incubated with biotinylated secondary anti-rabbit antibody followed by peroxidase-labeled streptavidin-HRP complex and 3,3′-diaminobenzidine tetrahydrochloride (DAB). The count of immunopositive nuclei was carried out by means of a Cell Imaging Software on five pictures (4×) randomly selected.

### 2.8. Drugs and reagents

The following drugs, antibodies and reagents were used: indomethacin, famotidine, phenylmethylsulfonyl fluoride, leupeptin, aprotinin, sodium orthovanadate, superoxide dismutase, EDTA (Sigma Chemicals Co., St. Louis, MO, USA); esomeprazole (kindly provided by AstraZeneca S.p.A., London, UK); lansoprazole (Tocris, Bristol, UK); rabbit anti-VEGF and COX-2, mouse anti-PCNA and Ki-67 antibodies, and HRP secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); rabbit anti-caspase-3 antibody (Cell Signalling Technologies, Boston, MA, USA). Other reagents were of analytical grade. Esomeprazole, lansoprazole, famotidine, and indomethacin were suspended in 1% methocel and administered by intragastric route in a volume of 0.25 ml.

### 2.9. Statistical analysis

The results are given as mean ± standard error of the mean (S.E.M.) of values obtained from 6 to 8 animals. The statistical sig-
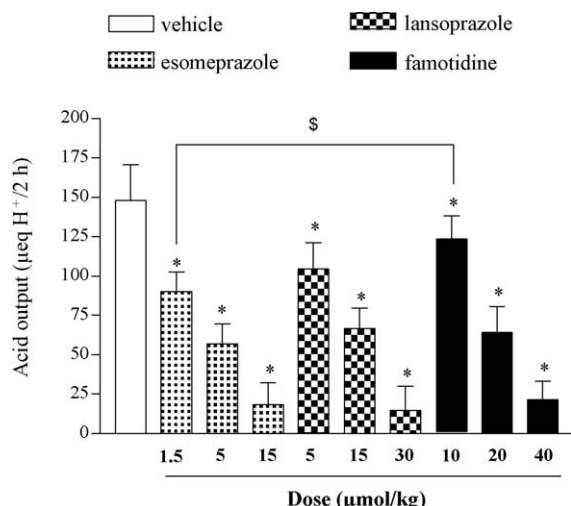
**Fig. 2.** Effects of esomeprazole (1.5, 5 and 15 μmol/kg/day), lansoprazole (5, 15 and 30 μmol/kg/day) and famotidine (10, 20 and 40 μmol/kg/day) on gastric acid secretion in animals subjected to pylorus ligation for 2 h. Animals received indomethacin for 14 days followed by indomethacin plus esomeprazole, lansoprazole or famotidine for 7 days. Pylorus ligation was carried out 22 h after the last drug administration. Each column represents the mean ± S.E.M. (vertical lines) of values obtained from 7 to 8 animals. *$P < 0.05$, significant difference vs controls (vehicle); $^\$ P < 0.05$, significant difference vs esomeprazole.

nificance of data was evaluated by one way analysis of variance (ANOVA) followed by *post hoc* analysis by Student–Newman–Keuls test, and $P$ values lower than 0.05 were considered significant. All statistical procedures were performed using GraphPad Prism 3.0 software (GraphPad, San Diego, CA, USA).

## 3. Results

### 3.1. Gastric acid secretion

A series of experiments was carried out to select doses of esomeprazole, lansoprazole and famotidine with equivalent activity in inhibiting acid secretion. After pylorus ligation for 2 h, the acid output in rats treated with drug vehicle was $147.9 \pm 22.8\,\mu EqH^+/2\,h$. Treatment with esomeprazole, lansoprazole or famotidine for 7 days dose-dependently reduced acid secretion, with maximal effects observed at 15, 30 and 40 μmol/kg/day, respectively. The acid inhibitory effects of esomeprazole, lansoprazole or famotidine were equivalent at doses of 5, 15 and 20 μmol/kg/day, respectively (Fig. 2).

### 3.2. Histomorphometric analysis of gastric damage

Administration of indomethacin (6 μmol/kg/day) for 14 days induced a significant increase in gastric mucosal damage, accounting for $5.9 \pm 0.7\%$ (Table 1). After additional 7-day treatment with indomethacin (6 μmol/kg/day) a further enhancement of total

**Table 1**
Effects of indomethacin treatment for 14 days on tested gastric parameters.

| | Vehicle | Indomethacin (6 μmol/kg/day) |
|---|---|---|
| Microscopic damage (%) | $0.33 \pm 0.11$ | $5.9 \pm 0.7^*$ |
| PGE$_2$ (ng/g) | $167.8 \pm 10.8$ | $21.5 \pm 6.4^*$ |
| MDA (nmol/mg) | $4.3 \pm 1.2$ | $8.7 \pm 1.1^*$ |
| VEGF (f.c.) | 1 | $0.73^*$ |
| PCNA (f.c.) | 1 | $0.59^*$ |
| Caspase-3 (f.c.) | 1 | $10.75^*$ |
| COX-2 (f.c.) | 1 | $1.85^*$ |

\* $P < 0.05$ vs control; f.c., fold change.

damage was detected ($9.3 \pm 1.1\%$) (Fig. 3A). Under these conditions, the administration of esomeprazole, lansoprazole or famotidine in combination with indomethacin was associated with a significant reduction of mucosal damage, with a greater efficacy of esomeprazole and lansoprazole in comparison with famotidine (Fig. 3A). When treatment with indomethacin was continued for 7 days at the lower dose of 2 μmol/kg/day, the assessment of gastric mucosal damage did not reveal any significant degree of healing or damage worsening. As for the previous set of experiments, both esomeprazole and lansoprazole and, to a minor extent, famotidine, significantly decreased gastric injury (Fig. 3B). In the setting of indomethacin discontinuation, treatment with vehicle for 7 days was associated with a moderate reduction of mucosal damage, indicating that there was some degree of spontaneous healing. In this case, the administration of esomeprazole, lansoprazole or famotidine caused a further decrease in mucosal damage, with a greater efficacy of both esomeprazole and lansoprazole, as compared with famotidine (Fig. 3C).

When animals with indomethacin-induced gastric damage were treated with SOD plus indomethacin (6 μmol/kg/day) for additional 7 days, the degree of gastric mucosal injury was significantly reduced ($5.3 \pm 1.1\%$, $P < 0.05$ vs indomethacin alone).

### 3.3. Assay of PGE$_2$

In vehicle-treated animals, gastric mucosal levels of PGE$_2$ accounted for $167.8 \pm 10.8$ ng/g. Treatment with indomethacin for 14 days was associated with a significant decrease in PGE$_2$ production (Table 1). In rats treated with indomethacin (6 μmol/kg/day) for additional 7 days there was no further decrease in PGE$_2$ levels ($25.7 \pm 6.7$ ng/g) (Fig. 4A). Under these conditions, the concomitant administration of esomeprazole, famotidine or lansoprazole for 7 days did not affect PGE$_2$ levels (Fig. 4A).

### 3.4. Assay of MDA

In rats treated with indomethacin for 14 days, gastric mucosal levels of MDA were significantly increased in comparison with vehicle-treated animals ($8.7 \pm 1.1$ vs $4.3 \pm 1.2$ nmol/mg, respectively) (Table 1). The subsequent administration of indomethacin (6 μmol/kg/day) for 7 days elicited a further increment of MDA levels (Fig. 4B). In this setting, the administration of esomeprazole, lansoprazole or famotidine in combination with indomethacin was associated with a significant reduction in MDA levels, with a greater efficacy for esomeprazole and lansoprazole in comparison with famotidine (Fig. 4B). Treatment with SOD in combination with indomethacin (6 μmol/kg/day) for 7 days was associated with a significant reduction of MDA mucosal content ($6.1 \pm 1.3$ nmol/mg, $P < 0.05$ vs indomethacin alone).

### 3.5. Western blot analysis of VEGF, PCNA, caspase-3 and COX-2

In animals treated with indomethacin for 14 days, VEGF expression was reduced ($-27\%$ vs control) (Table 1). Treatment with indomethacin (6 μmol/kg/day) for additional 7 days decreased further the expression of VEGF ($-57\%$ vs control) and, in this setting, none of test drugs were able to modify the expression of this growth factor (Fig. 5A). In animals with gastric damage induced by 14-day treatment with indomethacin, the expression of PCNA was decreased ($-41\%$ vs control) (Table 1). The continued indomethacin administration for additional 7 days did not affect further the expression of PCNA ($-48\%$ vs control). Under these conditions, the concomitant administration of esomeprazole or lansoprazole counteracted PCNA decrease, while famotidine was without significant effect (Fig. 5B). In animals treated with indomethacin
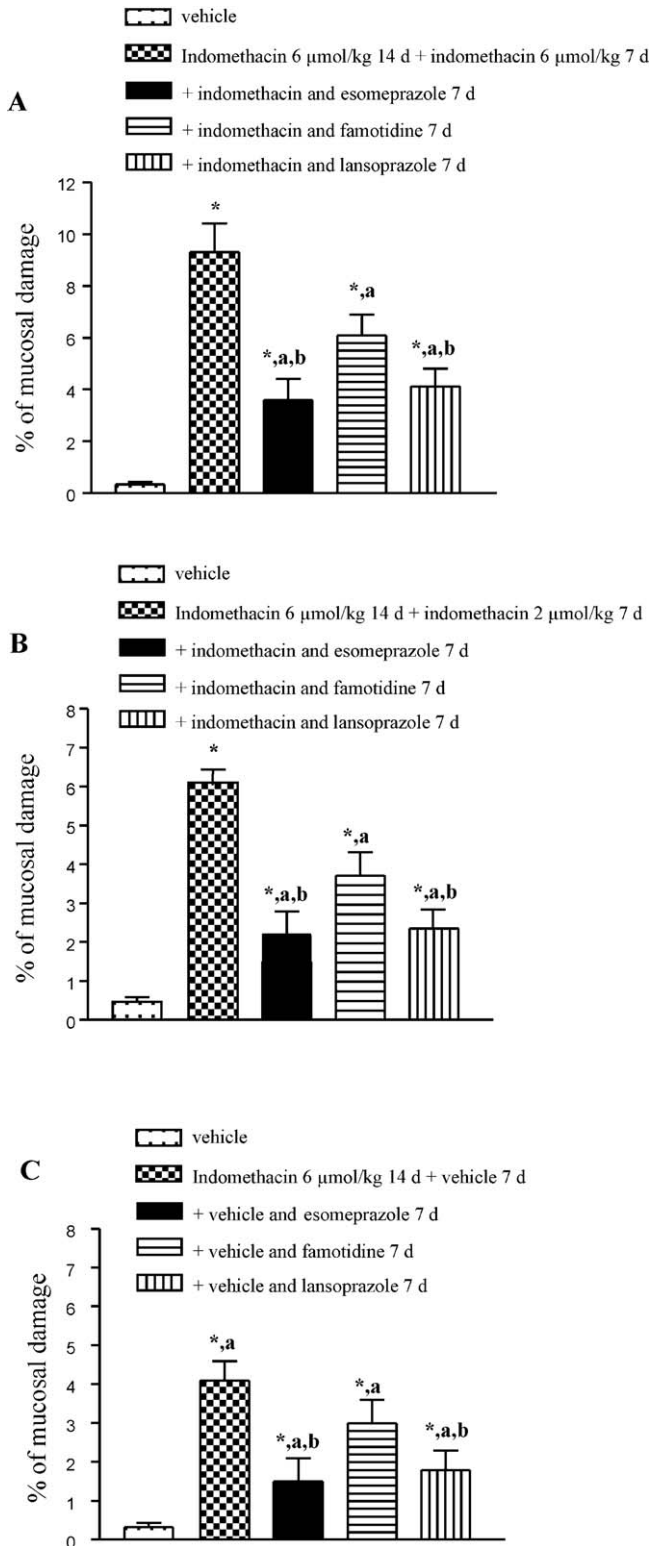
**Fig. 3.** Histomorphometric analysis of gastric mucosal damage in animals treated with vehicle, indomethacin (6 μmol/kg/day) for 14 days followed by indomethacin (6 μmol/kg/day) (A), indomethacin 2 μmol/kg/day (B), or vehicle (C), either alone or in combination with esomeprazole (5 μmol/kg/day), famotidine (20 μmol/kg/day) or lansoprazole (15 μmol/kg/day), for 7 days. Each column represents the mean ± S.E.M. (vertical lines) of values obtained from 7 to 8 animals. *$P < 0.05$, significant difference vs vehicle; [a]$P < 0.05$, significant difference vs indomethacin 14 + 7 days; [b]$P < 0.05$, significant difference vs famotidine.
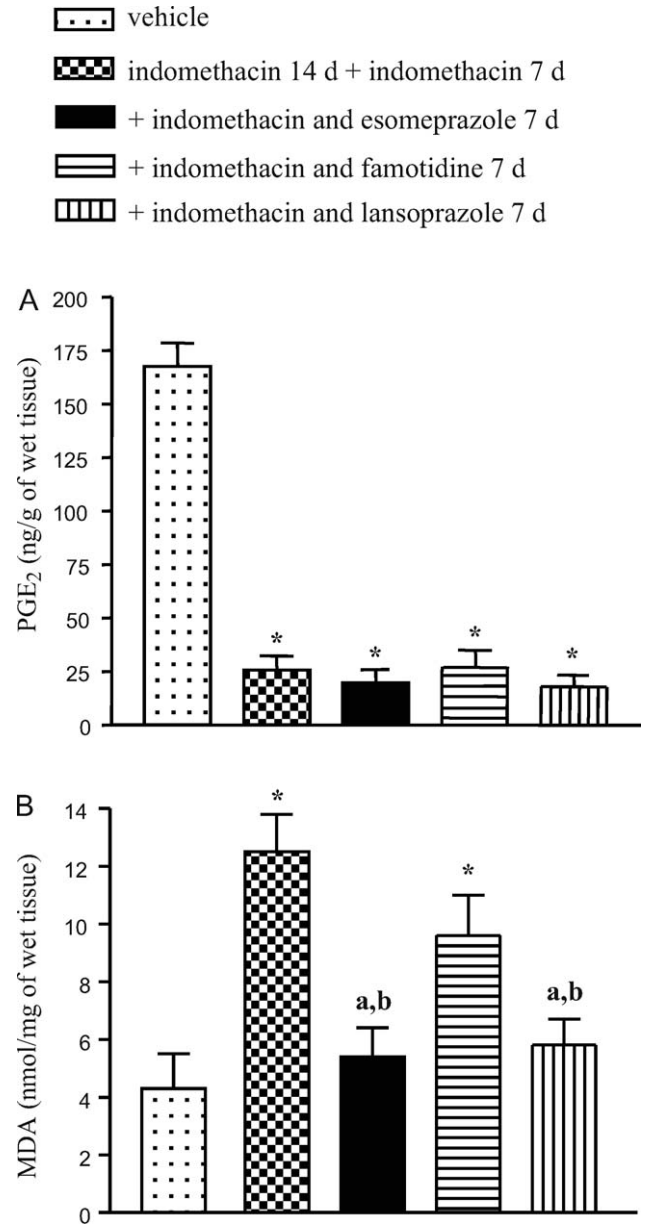


**Fig. 4.** Assay of mucosal $PGE_2$ (A) and malondialdehyde (MDA) (B) in animals treated with vehicle, indomethacin (6 μmol/kg/day) for 14 days followed by indomethacin (6 μmol/kg/day), either alone or in combination with esomeprazole (5 μmol/kg/day), famotidine (20 μmol/kg/day) or lansoprazole (15 μmol/kg/day) for 7 days. Gastric mucosal specimens were collected 24 h after the last administration. Each column represents the mean ± S.E.M. (vertical lines) of values obtained from 7 to 8 animals. *$P < 0.05$, significant difference vs vehicle; [a]$P < 0.05$, significant difference vs indomethacin 14 + 7 days; [b]$P < 0.05$ significant difference vs famotidine.

for 14 days, the expression of activated caspase-3 was enhanced (+975% vs control) (Table 1), and the continuation of NSAID administration resulted in a similar increment (+889% vs control). Under these conditions, esomeprazole, lansoprazole and famotidine partly counteracted the increase in caspase-3 expression (Fig. 5C). The expression of COX-2 was enhanced by administration of indomethacin for 14 days (+85% vs control) (Table 1), while the additional 7-day indomethacin administration did not modify the pattern of COX-2 expression (+75% vs control). Under these conditions, esomeprazole, lansoprazole or famotidine in combination with indomethacin did not exert any significant effect on COX-2 expression (Fig. 5D).
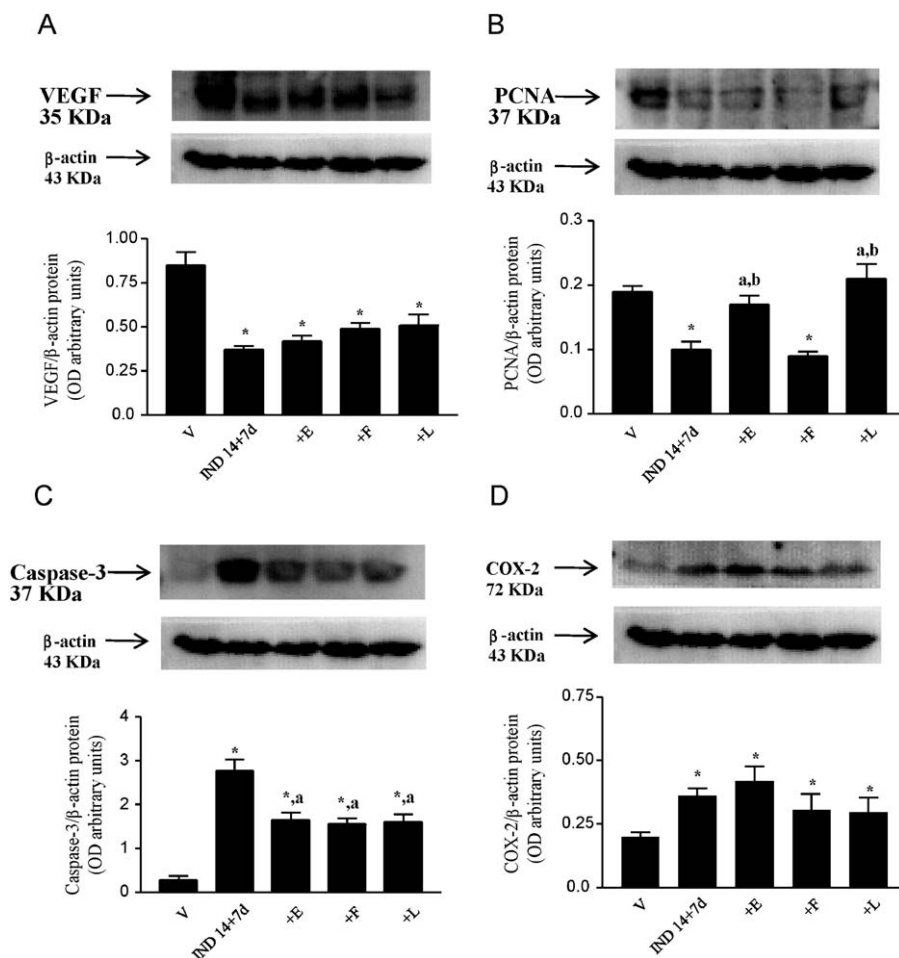
**Fig. 5.** Western blot analysis of VEGF (A), PCNA (B), caspase-3 (C), and COX-2 (D) expression in the gastric mucosa of rats treated with vehicle (V), indomethacin (6 μmol/kg/day) for 14 days followed by indomethacin (6 μmol/kg/day) for 7 days (IND 14 + 7 days), either alone or in combination with esomeprazole (+E, 5 μmol/kg/day), famotidine (+F, 20 μmol/kg/day) or lansoprazole (+L, 15 μmol/kg/day). Gastric mucosal specimens were collected 24 h after the last administration. Each column represents the mean ± S.E.M. (vertical lines) of values obtained from 6 to 8 animals. *$P < 0.05$, significant difference vs vehicle; [a]$P < 0.05$, significant difference vs indomethacin 14 + 7 days; [b]$P < 0.05$ significant difference vs famotidine.

### 3.6. Immunohistochemical analysis of Ki-67

In the gastric mucosa obtained from vehicle-treated animals, the number of immunopositive nuclei for Ki-67 accounted for $107.5 \pm 6.1$. Treatment with indomethacin for 14 days was associated with a significant reduction in Ki-67 immunopositivity ($65.2 \pm 4.7$, $P < 0.05$ vs normal animals). After additional 7 days of indomethacin administration, this value was further reduced ($48.2 \pm 8.9$, $P < 0.05$ vs indomethacin for 14 days). When animals were subjected to concomitant administration of indomethacin plus esomeprazole or lansoprazole the number of positive nuclei was significantly increased ($97.6 \pm 5.1$ and $103.3 \pm 10.8$, respectively, $P < 0.05$ vs indomethacin 14 + 7 days). By contrast, famotidine failed to modify such parameter ($62.8 \pm 9.6$). Representative pictures of immunostainings for Ki-67 in gastric sections are displayed in Fig. 6.

### 4. Discussion

The use of NSAIDs for treatment of inflammatory and painful conditions is associated with the occurrence of adverse upper digestive events, including bleeding and mucosal ulceration [21,22]. Under these circumstances, the most obvious strategy for controlling upper gastrointestinal toxicity is NSAID withdrawal, but consequent deterioration in the underlying pathological conditions

and pain recurrence may take this option undesirable [17]. Current clinical evidence suggests that PPIs, including esomeprazole, can be effective in promoting the healing of NSAID-induced gastric and duodenal ulcers even in the presence of a continued NSAID therapy [9,10]. However, the mechanisms underlying such clinical effectiveness remain unknown. The present findings indicate that, in the presence of NSAID-induced ulcerations and continued NSAID administration, esomeprazole and lansoprazole were more effective than famotidine in promoting the healing of mucosal damage, and that the two PPIs exerted similar healing effects when lansoprazole was employed at a dose three-fold higher than esomeprazole. The superiority of PPIs over famotidine is unlikely to entirely depend on the inhibition of acid secretion, since, in our study, these drugs were tested at equivalent acid inhibiting doses. In line with this proposal, our experiments demonstrated that both esomeprazole and lansoprazole, but not famotidine, counteracted tissue oxidation and reduction of mucosal cell proliferation associated with indomethacin treatment. Of note, in our experimental model, care was taken to verify whether the beneficial effects of the test antisecretory drugs, in the setting of continued indomethacin administration, resulted from healing of pre-existing lesions or prevention of *de novo* injury. For this purpose, we performed experiments in which indomethacin was stopped or administered at a lower dose, obtaining more conclusive evidence that all test drugs acted mainly by promoting ulcer healing.
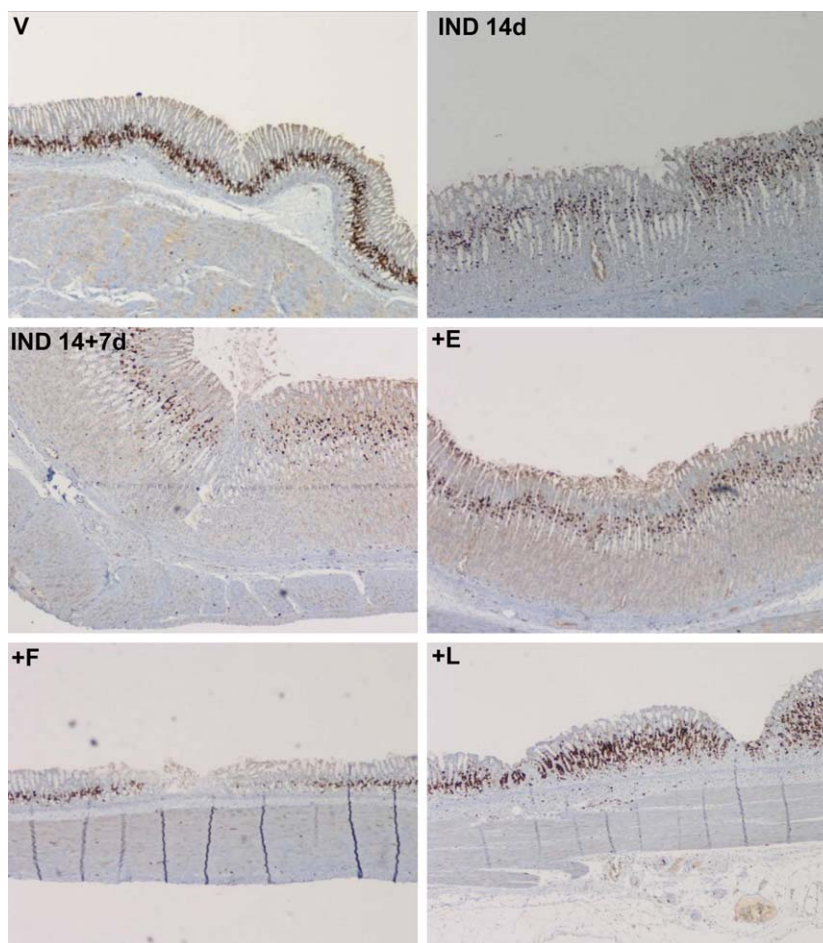
**Fig. 6.** Representative pictures showing the immunohistochemical analysis of Ki-67 in sections of gastric mucosa obtained from rats treated with vehicle (V), indomethacin (6 $\mu$mol/kg/day) for 14 days (IND 14 days) followed by indomethacin (6 $\mu$mol/kg/day) for 7 days (IND 14 + 7 days), either alone or in combination with esomeprazole (+E, 5 $\mu$mol/kg/day), famotidine (+F, 20 $\mu$mol/kg/day) or lansoprazole (+L, 15 $\mu$mol/kg/day). Gastric mucosal specimens were collected 24 h after the last drug administration (4×).

It has been established that one of the most important detrimental effects evoked by NSAIDs in the digestive system is represented by the activation of tissue oxidation [4]. In particular, acute indomethacin administration to normal rats has been shown to promote increments of gastric mucosal MDA levels [20,23]. In the present study, chronic administration of indomethacin was also associated with an increase in mucosal MDA levels, and, under these conditions, esomeprazole and lansoprazole, but not famotidine, reduced the indomethacin-induced oxidative stress. In the same setting, our experiments with the superoxide anion scavenger SOD showed that, in the presence of a continued NSAID treatment, the activation of antioxidant mechanisms contributes favourably to ulcer repair. Taken together, these findings support a significant role played by the antioxidant actions of PPIs in counteracting the healing delay maintained by continued NSAID administration. It is also worthy to mention that, according to recent reports, heme oxygenase-1 (HO-1) can protect gastric mucosal cells against various stressors, including NSAIDs. In particular, HO-1 expression was enhanced in the gastric mucosa of rats treated with indomethacin, and the inhibition of this enzyme exacerbated NSAID-induced mucosal damage, suggesting that HO-1 up-regulation contributes to the protection of gastric mucosa against injury [24]. Moreover, both omeprazole and lansoprazole were found to protect human gastric epithelial cells against oxidative stress through an increase in HO-1 expression, thus supporting the view that this enzyme pathway takes a part in the gastroprotective actions of proton pump inhibitors in the setting of NSAID-induced gastropathy [25].

The involvement of prostaglandins in the ulcer healing mechanisms activated by PPIs has been previously investigated with conflicting evidence. Some reports suggested that gastric mucosal levels of PGE$_2$ were unaffected by single-dose lansoprazole [26,27]. By contrast, lansoprazole was shown to induce an increment of gastric COX-2 expression and PGE$_2$ production after repeated administrations in rats [28]. In the present study, experiments were performed to examine the involvement of COX-2/PGE$_2$ pathway in the healing actions exerted by esomeprazole on indomethacin-induced gastric ulcerations. Our findings showed that, as expected, repeated indomethacin administration significantly decreased mucosal PGE$_2$ levels, and that the concomitant administration of esomeprazole, lansoprazole or famotidine did not affect such inhibitory effects. Moreover, in keeping with previous findings [29], indomethacin enhanced COX-2 expression, while neither famotidine nor the two PPIs did modify this expression pattern. Therefore, it is likely that esomeprazole does not influence mucosal PGE$_2$ production in the presence of NSAID treatment. In support of this contention, omeprazole was shown to prevent the development of gastric damage induced by indomethacin in rabbits, without any significant interference with the concomitant decrease in mucosal PGE$_2$ production [30].

VEGF is regarded as one of the main growth factors involved in the healing mechanisms of wounded tissues, through the stimulation of neovascularisation processes [31]. In particular, the expression of VEGF is essential for maintenance of gastrointestinal mucosal integrity, and angiogenesis is a pivotal mechanism con-

tributing to the healing of digestive ulcers [32]. Based on these considerations, we investigated the effects of indomethacin, alone or in combination with test antiulcer drugs, on the expression of VEGF in the gastric mucosa. Our findings showed that indomethacin decreased VEGF expression, and that none of the test drugs affected such expression pattern. Since VEGF expression has been reported to be stimulated by COX-2 through $PGE_2$ in cultured gastric fibroblasts [33], it is conceivable that the chronic blockade of $PGE_2$ biosynthesis by indomethacin could maintain VEGF expression in a downregulated state despite treatment with PPIs or famotidine. Is support of this view, lansoprazole was shown to enhance the expression of VEGF in the gastric ulcer margin, while this effect was prevented by its concomitant administration with indomethacin [34]. In addition, despite the inhibitory effect of indomethacin on VEGF expression, lansoprazole was still able to counteract the detrimental effects of this NSAID on ulcer healing, thus suggesting that the beneficial effects of PPIs on mucosal injury are likely to be independent from the COX-2/$PGE_2$/VEGF pathway.

In the present study, molecular markers of both cell proliferation and apoptosis were also investigated to gain insights into the effects of esomeprazole on mucosal cell turnover, as possible mechanisms contributing to its healing actions. Our results showed that chronic indomethacin was associated with a significant increase in activated caspase-3, a marker of apoptotic cell death, and that this effect was counteracted to a similar extent by esomeprazole, lansoprazole and famotidine. These findings suggest that the anti-apoptotic effects exerted by esomeprazole against indomethacin are likely to depend on the inhibition of acid secretion, since it was administered at equivalent acid-inhibitory doses with lansoprazole and famotidine. The observation that the blockade of acid secretion could result in a reduction of mucosal apoptosis is supported by the report by Nørsett et al. [35], who examined the effects of long-term inhibition of acid secretion with omeprazole on the expression of various genes in rat gastric mucosa. Their results showed that acid inhibition was associated with a downregulation in the expression of genes involved in apoptosis, such as early growth response gene (Rgrl 1) or histone deacetylase 7 (Hdac 7). Based on these findings, it is conceivable that the acid-dependent reduction in mucosal cell apoptosis takes a significant part in the beneficial effects of esomeprazole on the healing of ulcerative damage elicited by indomethacin.

Cell proliferation is known to play a major role in the healing of gastric ulcers [6]. In this respect, the present results showed that the injuring actions of indomethacin are associated with a significant reduction in the mucosal expression of PCNA and Ki-67, both regarded as reliable markers of cell proliferation and, in this setting, esomeprazole was able to reverse the inhibitory effects of this NSAID. Since the effects of esomeprazole on both PCNA and Ki-67 were mimicked by lansoprazole, but not famotidine, it can be proposed that its beneficial influence on mucosal repair depends on acid-independent mechanisms, which are likely related with its antioxidant properties. This view is supported by Jainu and Mohan [36], who demonstrated that both the antioxidant compound ascorbic acid and omeprazole enhanced the expression of growth factors, including transforming growth factor-alpha (TGF-α), in the gastric mucosa of rats treated with aspirin. Moreover, famotidine partly counteracted the indomethacin-induced delay in rat duodenal ulcer healing, without affecting mucosal PCNA expression [37]. When considering the clinical setting, these preclinical findings are consistent with the results by Tsuji et al. [38], who demonstrated that lansoprazole, but not famotidine, enhanced the expression of basic fibroblast growth factor (bFGF) in the gastric ulcer margin of patients, and that lansoprazole was more effective than famotidine in promoting ulcer healing. In addition, previous reports suggest that other growth factors are also involved in the ulcer healing effects of PPIs. For instance, Kinoshita et al.

[39] observed that the gastric levels of hepatocyte growth factor (HGF) were increased by omeprazole in rats with indomethacin-induced gastric damage. Moreover, the expression of epidermal growth factor (EGF) was found to be increased in the gastric mucosa of mice with indomethacin-induced injury, and further enhanced by omeprazole [40].

## 5. Conclusions

In conclusion, our study suggests that, in the presence of pathophysiological conditions characterized by NSAID-induced gastric ulcerations with a need to continue NSAID therapy, a co-treatment with esomeprazole can promote the healing of gastric lesions in part by inhibition of acid secretion, leading to a reduction of pro-apoptotic signalling, and partly via antioxidant mechanism, resulting in an enhancement of proliferative/repairing pathways.

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