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ANTI-INFLAMMATORY EFFECTS OF NESFATIN-1 ON ACETIC ACID-INDUCED GASTRIC ULCER IN RATS: INVOLVEMENT OF CYCLO-OXYGENASE PATHWAY

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In order to elucidate the contribution of cycloxygenase (COX) enzymes in the anti-oxidant and anti-inflammatory mechanisms of nesfatin-1, which improves the healing process of chronic gastric ulcers, either acetic acid (80%; ulcer groups; n = 40) or saline (control groups; n = 40) was applied to the serosal surface of male Sprague Dawley rats' stomachs for 1 min. Both the control and ulcer groups were treated daily with either i.p. saline or nesfatin-1 (0.3 μg/kg; for 3 days). Nesfatin-1-treatment was preceded with i.p. saline, COX-2 inhibitor NS-398 (2 mg/kg), COX-1 inhibitor ketorolac (3 mg/kg) or non-selective COX inhibitor indomethacin (5 mg/kg) for 3 days. The rats were decapitated at the end of the third day, and their trunk blood was collected for the measurements of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-10 using ELISA. The induction of ulcers resulted in increased macroscopic scores, along with elevated gastric malondialdehyde, luminol- and lucigenin-enhanced chemiluminescence levels and myeloperoxidase activity. On the other hand, nesfatin-1 treatment abolished these elevations. Depleted glutathione, superoxide dismutase and catalase activity levels in the saline-treated ulcer group were preserved in the nesfatin-1-treated ulcer group. Increased levels of serum TNF-α, IL-1β, IL-10 in the saline-treated ulcer group, as compared to control group, were significantly decreased in the nesfatin-1-treated ulcer group. The inhibition of COX-1, and/or COX-2 reversed most of the alterations induced with nesfatin-1, but COX-2-blockade was consistently more effective to abolish all nesfatin-1-induced changes. Our results suggest that nesfatin-1 ameliorates ulcer-induced inflammatory response through the modulation of oxidant-antioxidant balance. As selective pharmacological inhibition of COX-1 or COX-2 suppresses the antioxidant/anti-inflammatory effects of nesfatin-1, it appears that nesfatin-1 decreases inflammatory mediators and neutrophil migration by a COX-dependent mechanism, especially by a COX-2- dependent mechanism, during the ulcer healing stage.

Key words: *gastric ulcer, nesfatin-1, cyclooxygenase, prostaglandins, myeloperoxidase, reactive oxygen metabolites, superoxide dismutase*

INTRODUCTION

Peptic ulcer is a frequently diagnosed worldwide health problem (1), caused by the dominance of aggressive factors over protective factors. Gastric mucosal integrity is protected by the presence of tight junctions between epithelial cells, intact microcirculation, bicarbonate secretion, and the availability of prostaglandins and nitric oxide. On the other hand, the aggressive factors that oppose are acid, pepsin, bile acids, *Helicobacter pylori*, and exposure to gastrotoxic agents like ethanol and non-steroidal anti-inflammatory drugs (NSAIDs) (2). NSAIDs exert their injurious effects mainly through the inhibition of cyclooxygenases (COX), while a topical mechanism independent of COX involves diffusion of acidic NSAIDs. The topical mechanism independent of COX damages mucosal cells, disturbs microcirculation and activates mast cells for the release of various pro-inflammatory mediators (3, 4). In an intact stomach, the constitutively produced COX-1 enzyme is responsible for the production of most of the prostaglandins, which regulate mucosal blood flow, mucus and bicarbonate secretions. Simultaneously, the inducible COX-2 isoform facilitates ulcer healing by its impact on proliferation, angiogenesis and reconstruction of the damaged mucosa (5, 6). However, it was suggested that both of the COX isoforms contribute to prostaglandin production during the healing of chronic gastric ulcers, by maintaining mucosal integrity and decreasing acid secretion (7, 8).

Nesfatin-1, an 82-amino-acid polypeptide (9), was initially identified as a satiety molecule, which centrally integrates feeding and metabolic functions (10-12). Peripheral inflammatory stimuli were postulated to activate the nesfatinergic neurons in the hypothalamic and brain stem areas (13), while exogenous administration of nesfatin-1 has exerted anti-apoptotic and anti-inflammatory effects on oxidative tissue damage (14, 15). In addition to its extensive distribution in the hypothalamus and brain

stem (16, 17), nesfatin-1 was also expressed in several peripheral organs, including the stomach (18, 19). We have recently demonstrated the anti-inflammatory and anti-oxidant effects of nesfatin-1 on NSAID-induced acute gastric injury (20), while Szlachcic *et al*. (21) have indicated that the healing-promoting effect of nesfatin-1 in chronic gastric ulcers operates through its stimulatory action on gastric blood flow and mucosal restoration. In addition, nesfatin-1-induced protection in stress-induced acute gastric injury was attenuated by the suppression of COX-1 and COX-2 activity, along with an increased gastric blood flow, augmented gastrin release and depressed gastric acid secretion (22, 23). However, the involvement of oxidant-antioxidant balance in COX-dependent nesfatin-1-induced gastroprotection has not been investigated yet. Thus, the aim of the present study conducted in an acetic acid-induced gastric ulcer model was to elucidate whether anti-oxidant and anti-inflammatory mechanisms of nesfatin-1 involves the activation of COX enzymes.

MATERIALS AND METHODS

Animals

Male Sprague Dawley rats (250 – 300 g, 12 weeks old, n = 80), supplied by the Marmara University (MU) Animal Center (DEHAMER), were habituated before experiments. The rats were housed in relative humidity (65 – 70 %) and temperature-controlled room (22 ± 2°C) with standardized light/dark (12h/12 h) cycles. The rats were fed with standard rat pellets and had free access to water. All experimental protocols were approved by the MU Animal Care and Use Committee.

Experimental design

In half of the randomly divided rats, a gastric ulcer was induced by serosally applied acetic acid (24), while saline was applied in the other half of rats (*Fig. 1*). Immediately after the serosal applications, groups were treated intraperitoneally (i.p.) with either saline or nesfatin-1 (0.3 µg/kg/day; Cat.No: 003-22B; Phoenix Pharmaceuticals, Inc., Burlingame, CA) for 3 days. Fifteen minutes before nesfatin-1 injections, the rats were i.p. administered with COX-2-selective inhibitor NS-398 (2 mg/kg/day; Sigma), COX-1-preferring inhibitor ketorolac (3 mg/kg/day; Sigma), non-selective COX inhibitor indomethacin (5 mg/kg/day; Sigma) or saline for 3 days. Each of the treatment groups consisted of 8 rats. The rationale for the dose of nesfatin-1 was based on our previous dose-response experiments (20), while the doses of the COX inhibitors were selected from previous reports (7, 22, 25).

Induction of acetic acid-induced gastric ulcer

As it was described previously by Okabe and Pfeiffer (26), a gastric ulcer was induced by serosal application of acetic acid. It was previously demonstrated that gastric mucosal ulcers in this model became chronic within 2 – 3 days, and healed completely within 2 – 3 weeks without perforation or penetration to the surrounding organs (26). After an overnight fasting and under anesthesia (ketamine, 100 mg/kg and chlorpromazine, 0.75 mg/kg), the stomach was gently exteriorized through a small incision. The barrel of a 3-ml syringe was placed on the serosal surface of the stomach, and its content (0.5 ml of acetic acid, 80%, vol/vol) was emptied on the corpus area (60 mm^2). After a 1-min contact, the area exposed to acid was gently rinsed with saline and the incision was closed. In the control rats, similar surgical procedures were performed, but a saline-containing syringe was placed on the serosal surface of the stomach. On the 3rd day and 4 hours after the last injections, the rats were decapitated and their trunk blood was collected for the measurement of serum tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-10 levels. The stomachs were dissected along the greater curvature, and rinsed with saline for macroscopical analysis. Lengths of hemorrhagic lesions in the glandular mucosae were calculated per stomach and expressed as the macroscopic ulcer index (mm). Gastric tissue samples were analyzed for the

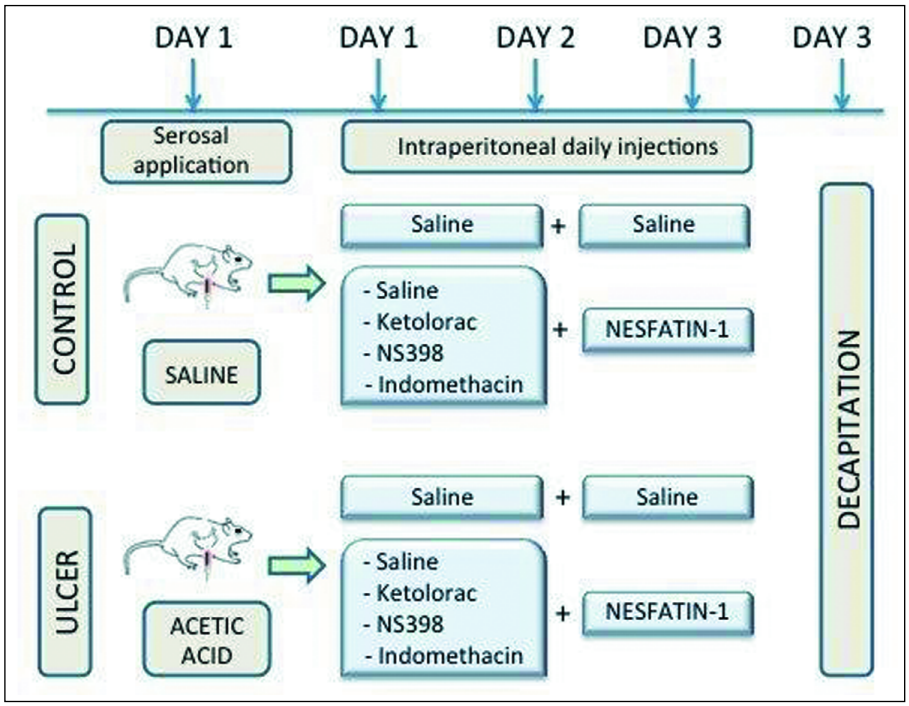

Fig. 1. Diagram illustrating the experimental groups and the timings of ulcer induction and treatments. Injections of saline, nesfatin-1 (0.3 µg/kg/day), COX-2 inhibitor NS-398 (2 mg/kg/day), COX-1 inhibitor ketorolac (3 mg/kg/day) or non-selective COX inhibitor indomethacin (5 mg/kg/day) were continued for 3 days.

levels of COX-1, COX-2, prostaglandin E_2 (PGE_2), prostacyclin (PGI_2), malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) and myeloperoxidase activity (MPO), while luminol and lucigenin-enhanced chemiluminescence were performed to measure reactive oxygen metabolites (ROM). In addition, immunoreactivity of nuclear factor kappa B (NF-κB), COX-1, COX-2, PGE_2 and PGI_2 were evaluated in gastric samples, and histological analyses of the microscopic lesions were performed.

Measurement of serum TNF-α, IL-1β and IL-10 levels and gastric levels of COX-1, COX-2, PGE_2 and PGI_2

Blood samples were centrifuged at 4°C at 3000 g for 15 min, and serum samples were collected. According to the instructions of the manufacturer, serum TNF-α (Cat.No:201-11-0765), IL-1β (Cat.No:201-11-0120) and IL-10 (Cat.No: 201-11-0109) levels were measured by enzyme-linked immunosorbent assay (ELISA) kits (SunRed, PRC). Gastric tissue samples were homogenized in 10 volumes of ice-cold 0.9% isotonic sodium chloride solution. The levels of COX-1 (Cat.No: 201-11-0909), COX-2 (Cat.No:201-11-0297), PGE_2 (Cat.No: 201-11-0505) and PGI_2 (Cat.No: 201-11-0503) were detected in the supernatants of tissue homogenates by ELISA kits, by following the instructions of the manufacturer (SunRed, PRC).

Measurement of gastric myeloperoxidase activity

MPO activity, which is verified to correlate with the histochemically determined neutrophil count, is commonly used for the estimation of tissue neutrophil recruitment to inflamed tissues (27). For the assay of MPO activity, as described by Bradley *et al*. (27), gastric tissue samples were homogenized in 10 volumes of ice-cold potassium phosphate buffer (50 mM K_2HPO_4, pH 6.0) containing hexadecyltrimethylammonium bromide (HETAB; 0.5%, w/v). Homogenized samples were centrifuged at 12,000 g for 10 min at 4°C, and the supernatants were discarded. The pellet was then re-homogenized with an equivalent volume of 50 mM K_2HPO_4 containing 0.5% (w/v) HETAB and 10 mm EDTA (Sigma Chemical Co., St. Louis, MO, USA). MPO activity was assessed by measuring the H_2O_2-dependent oxidation of o-dianisidine.2HCl. One unit of enzyme activity was defined as the amount of MPO present per gram of tissue weight, that caused a change in absorbance of 1.0 min^{-1} at 460 nm and 37°C. MPO activity was expressed as U/g tissue.

Measurement of gastric malondialdehyde and glutathione levels

Samples of gastric tissues were homogenized (Ultra Turrax) in 10 volumes of ice-cold 10% trichloroacetic acid and centrifuged at 3000 g for 15 min at 4°C. The supernatant was removed and recentrifuged at 10,000 g for 8 min for determination of MDA and GSH levels. The MDA levels were assayed as byproducts of lipid peroxidation by measuring the formation of thiobarbituric acid-reactive substances as previously described (28). Lipid peroxide levels were expressed in terms of MDA equivalents using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}. GSH measurements were performed using a modified Ellman procedure, and expressed in nmol GSH/g tissue (29).

Measurement of gastric superoxide dismutase and catalase activities

The SOD converts superoxide radical into hydrogen peroxide and molecular oxygen, while the catalase converts hydrogen peroxide into water. In this way, two toxic radicals are converted to water and oxygen. To measure antioxidant SOD activity in gastric tissue samples, the method previously described by Mylroie *et al*. was used (30). Briefly, the SOD activity measurement depends on the oxidation of dianisidine photosensitized by riboflavin. Measurements were performed in cuvettes containing 2.8 ml 50 mM KH_2PO_4 (pH = 7.8) with 0.1 mM EDTA, 0.1 mM 0.39 mM riboflavin in 10 mM KH_2PO_4 (pH 7.5), 0.1 ml of 6 mM o-dianisidin.2 HCl in deionized water, and tissue extract (50, 100 µl). Then, the components in cuvettes were illuminated with fluorescent tubes (Sylvania GRO-LUX F18W/GRO, Erlangen, Germany) at 37°C. Absorbance was measured at 460 nm with a Schimadzu UV-02 model spectrophotometer at 0 and 8 min of illumination, and the net absorbance was calculated. A standard curve was prepared routinely with bovine SOD (Sigma Chemical Co, St Louis, MO, USA) as reference, and the SOD activity of supernatant was calculated in the form of U/mg protein (31). The method for the measurement of CAT activity is based on the catalytic activity of the enzyme, that catalyzes the decomposition reaction of H_2O_2 to give H_2O and O_2 (32). Briefly, the absorbance of the tissue samples containing 0.4 ml homogenate and 0.2 ml H_2O_2 was read at 240 nm and 20°C, against a blank containing 0.2 ml phosphate buffer and 0.4 ml homogenate for about 1 minute. Catalase measurements were expressed as U/ml/min.protein.

Measurement of gastric luminol- and lucigenin-enhanced chemiluminescence levels

For the direct noninvasive measurement of reactive oxygen metabolites (ROM), chemiluminescence (CL) assay was performed. Luminol and lucigenin probes were used as enhancers to overcome the potential variability and low intensity of native CL. Lucigenin probe is more specific for superoxide radical, while luminol detects OH, H_2O_2, and HOCl radicals (20). In the presence of luminol or lucigenin (0.2 mM each), luminescence of the gastric samples were recorded using Mini Lumat LB 9509 luminometer (EG&G Berthold, Germany) at room temperature. At 1 minute intervals, all counts were obtained for 5 minutes and the results were expressed as area under the curve (AUC) of relative light unit (rlu) per mg tissue. The calculation was based on the integration of the curve by linear approximation (33).

Immunohistochemical assays

Five-µm-thick sections obtained from paraffin blocks were incubated in 3% H_2O_2 to block endogenous peroxidase activity. Antigen retrieval was achieved by applying sodium citrate buffer. After rinsing in phosphate buffer and incubating in blocking solution, the sections were incubated in anti-NF-κB (Santa Cruz, sc-109, rabbit polyclonal, 1:50), anti-COX-1 (Abcam, ab109025, rabbit monoclonal, 1:100), anti-COX2 (Abcam, ab52237, rabbit monoclonal, 1:100), anti-PGE_2 (Abcam, ab2318, rabbit polyclonal, 1:100) or anti-PGI_2 synthase (Santa Cruz, sc-20933, rabbit polyclonal, 1:50) antibodies overnight at 4°C. After rinsing in phosphate buffer, biotinylated secondary antibody, streptavidin peroxidase (Invitrogen 859043, Histostain Plus Broad Spectrum) and 3'-3'-diaminobenzidine (DAB, Invitrogen 002020, DAB Plus Substrate Kit) were applied. Negative control stainings were applied by omitting the primary antibody. Mayer's hematoxylin was used for counterstaining. Sections were examined and photographed by an Olympus DP72 CCD camera attached BX51 light microscope. Staining intensity was scored using a semi-quantitative method (0: No staining, 1: mild staining, 2: moderate staining, 3: intense staining).

Histological analysis

Gastric tissues were fixed in 4% paraformaldehyde, and then processed routinely for light microscopy. After dehydrating in graded series of ethanol, the fixed gastric tissues were cleared in xylene, incubated in liquid paraffin overnight at 60°C, and were embedded in paraffin. Five-µm-thick sections obtained by a rotary microtome were stained with hematoxylin and eosin, and then they were examined and photographed by an Olympus DP72 CCD camera attached BX51 light microscope. A semi-quantitative method, where 0: none; 1: mild; 2: moderate; 3: severe, was applied for scoring the damage in the gastric mucosa and submucosa, by modifying the previously described criteria: desquamation of surface epithelium (0 – 3); degeneration of glandular cells (0 – 3); submucosal edema (0 – 3); and inflammatory cell infiltration (0 – 3) with a maximum score of 12 (34).

Statistical analysis

The results are expressed as the mean ± S.E.M. One-way analysis of variance (ANOVA) and Tukey-Kramer multiple comparison tests were used for statistical analysis (GraphPad Prism 6.0, San Diego, CA, USA). Values of $P < 0.05$ were considered as significant.

RESULTS

Macroscopic ulcer index

When examined on the 3rd day, serosal application of acetic acid had resulted in extensive gastric lesions in the rats treated with saline ($P < 0.01$, *Fig. 2A*), as compared to serosally saline-applied and saline-treated control group with no ulcers. In all the serosally saline-applied control groups that have received either of the treatments (nesfatin-1 plus saline, nesfatin-1 plus ketorolac, nesfatin-1 plus NS-398 or nesfatin-1 plus indomethacin), no ulcers were observed (data not shown). Treatment with nesfatin-1 significantly ($P < 0.05$) reduced the macroscopic ulcer index. Nesfatin-1-induced reduction in macroscopic ulcer index was reversed significantly by indomethacin ($P < 0.05$), while the selective COX inhibitor NS-398 did not alter the inhibitory effect of nesfatin-1 on mucosal ulceration. On the other hand, macroscopic index in ketolorac-treated ulcer group was not different than that of the saline-treated ulcer group.

Serum levels of tumor necrosis factor-α, interleukin-1β and interleukin-10

On the 3rd day of gastric ulcer induction, serum levels of the pro-inflammatory TNF-α and IL-1β and the anti-inflammatory

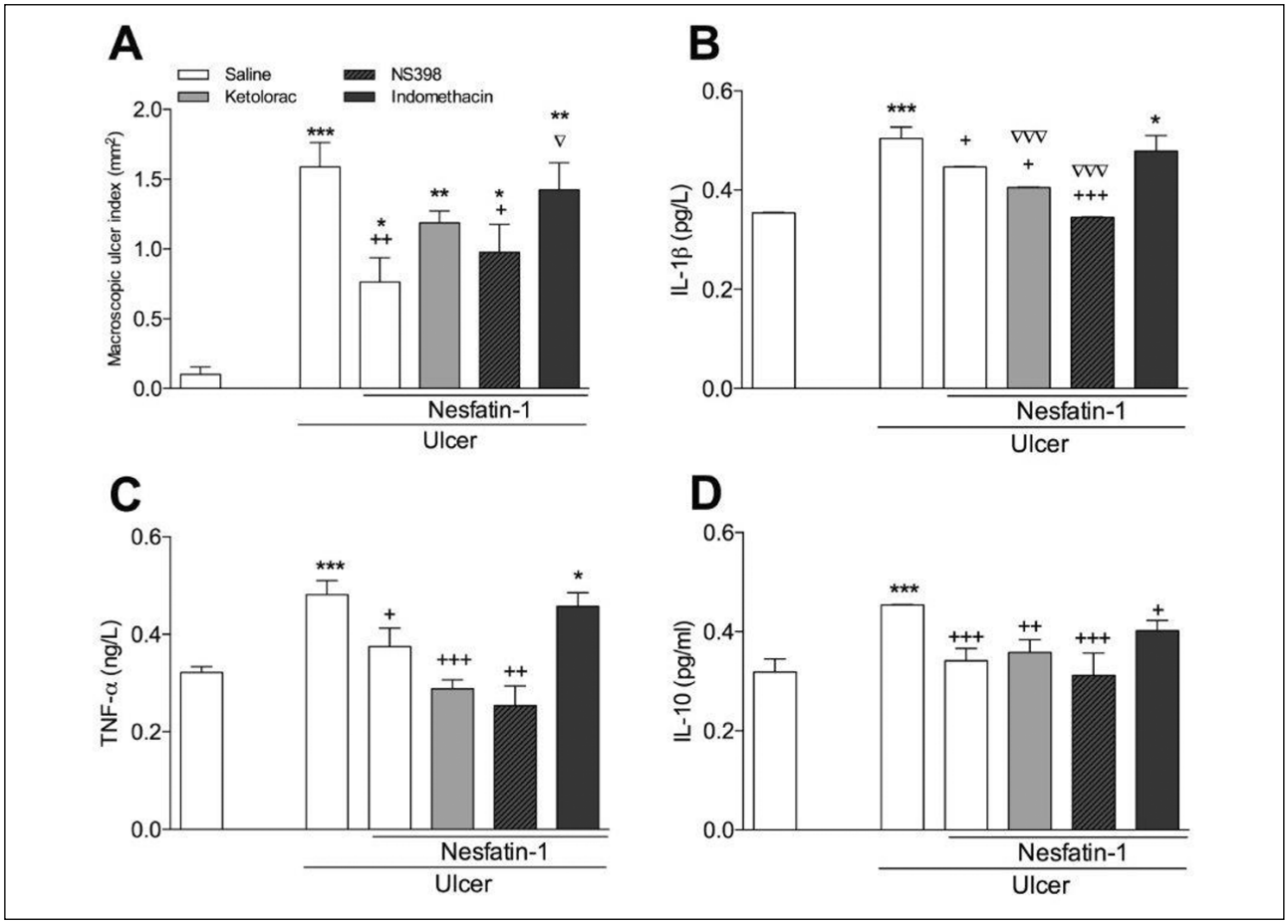

Fig. 2. (*A*) Macroscopic score (mm²); (*B*) Interleukin-1β (IL-1β); (*C*) Tumor necrosis factor-α (TNF-α); (*D*) IL-10. Levels in the control and ulcer groups treated for 3 days with saline, nesfatin-1 (0.3 µg/kg/day), COX-2 inhibitor NS-398 (2 mg/kg/day), COX-1 inhibitor ketorolac (3 mg/kg/day) or non-selective COX inhibitor indomethacin (5 mg/kg/day). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus saline-treated control group; $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$ versus saline-treated ulcer group. $^{\nabla}P < 0.05$, $^{\nabla\nabla\nabla}P < 0.001$ versus saline plus nesfatin-1-treated ulcer group. For each group n = 8. The results are expressed as the mean ± S.E.M. One-way analysis of variance (ANOVA) and Tukey-Kramer multiple comparison tests were used.

IL-10 levels were found to be elevated ($P < 0.001$, *Fig. 2B-2D*), while nesfatin-1 treatment significantly ($P < 0.05$ and $P < 0.001$) suppressed these elevations. Cytokine levels suppressed by nesfatin-1 were not reversed by either of the selective COX inhibitors. Meanwhile, the serum levels of TNF-α and IL-1β in rats pretreated with the non-selective COX inhibitor indomethacin were not different than those of the saline-treated ulcer group.

Oxidative parameters: myeloperoxidase activity, malondialdehyde and reactive oxygen metabolites levels in gastric tissues

The induction of an ulcer significantly increased the MPO activity and MDA levels ($P < 0.001$, *Fig. 3A-3B*) with respect to the control group, while nesfatin-1 treatment inhibited ulcer-induced increases in both MPO activity and MDA level ($P < 0.001$). On the other hand, selective COX-2 inhibitor NS-398 significantly reduced the inhibitory effect of nesfatin-1 on MPO activity and MDA level ($P < 0.05$ and $P < 0.001$), while the non-selective COX inhibitor indomethacin also decreased the inhibitory effect of nesfatin-1 on lipid peroxidation.

Gastric luminol and lucigenin chemiluminescence levels revealed that ROM generation was significantly increased in the saline-treated ulcer group ($P < 0.001$, *Fig. 3C-3D*), while nesfatin-1 treatment abolished these elevations ($P < 0.01$). None of the COX-inhibitors altered a nesfatin-1-induced decrease in lucigenin-enhanced chemiluminesence, which is specific for superoxide radical. However, both specific COX inhibitors reversed the suppressive effect of nesfatin-1 on luminol-enhanced chemiluminescence, which is sensitive to OH, H_2O_2, and HOCl radicals ($P < 0.05 – 0.01$).

Antioxidants: levels of glutathione, superoxide dismutase activity and catalase activity in gastric tissues

The induction of an ulcer depleted the levels of GSH, as well as SOD and CAT activities in the gastric tissues of saline-treated rats ($P < 0.001$, *Fig. 4*), while nesfatin-1 treatment replenished the levels of these antioxidants ($P < 0.01 – 0.001$). In the ulcer groups, blockade by any of the three COX inhibitors significantly reversed the replenishment of SOD and CAT activity levels achieved by nesfatin-1 treatment ($P < 0.05 – 0.001$), while nesfatin-1-induced replenishment of gastric GSH levels was reversed by NS-398 and indomethacin ($P < 0.001$ and $P < 0.05$).

Gastric levels of cyclooxygese-1, cyclooxygenase-2, prostacyclin and prostaglandin E_2

Enzyme levels of COX-1 and COX-2, as well as PGE_2 and PGI_2 levels, were elevated in the ulcer groups which were either

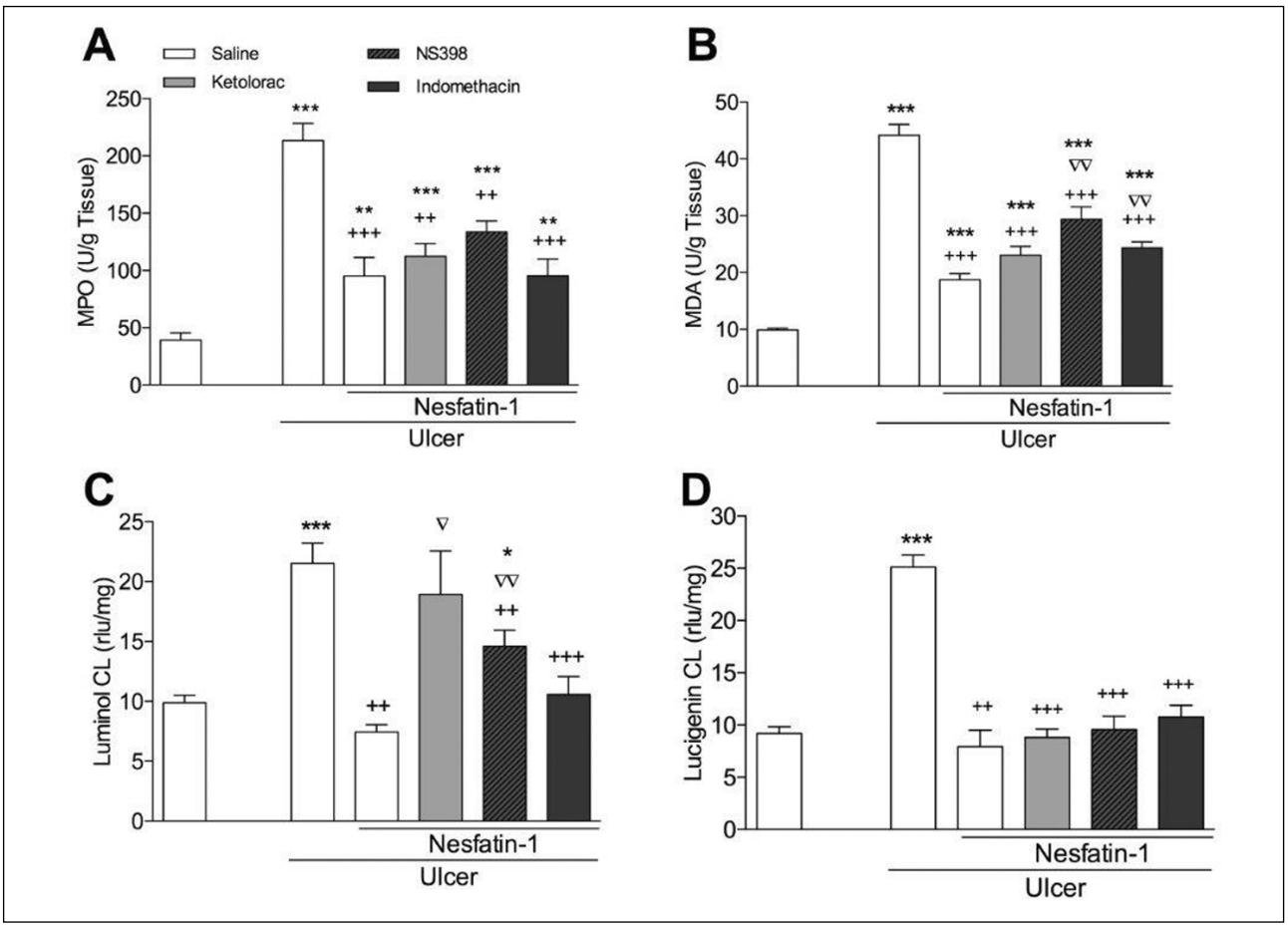

Fig. 3. (*A*) Myeloperoxidase (MPO); (*B*) Malondialdehyde (MDA); (*C*) Luminol-enhanced chemiluminesance; (*D*) Lucigenin-enhanced chemiluminesance. Levels in the control and ulcer groups treated for 3 days with saline, nesfatin-1 (0.3 μg/kg/day), COX-2 inhibitor NS-398 (2 mg/kg/day), COX-1 inhibitor ketorolac (3 mg/kg/day) or non-selective COX inhibitor indomethacin (5 mg/kg/day). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus saline-treated control group, $^{++}P < 0.01$, $^{+++}P < 0.001$ versus saline-treated ulcer group, $^{\nabla}P < 0.05$, $^{\nabla\nabla}P < 0.01$ versus saline plus nesfatin-1-treated ulcer group. For each group n = 8. The results are expressed as the mean ± S.E.M. One-way analysis of variance (ANOVA) and Tukey-Kramer multiple comparison tests were used.

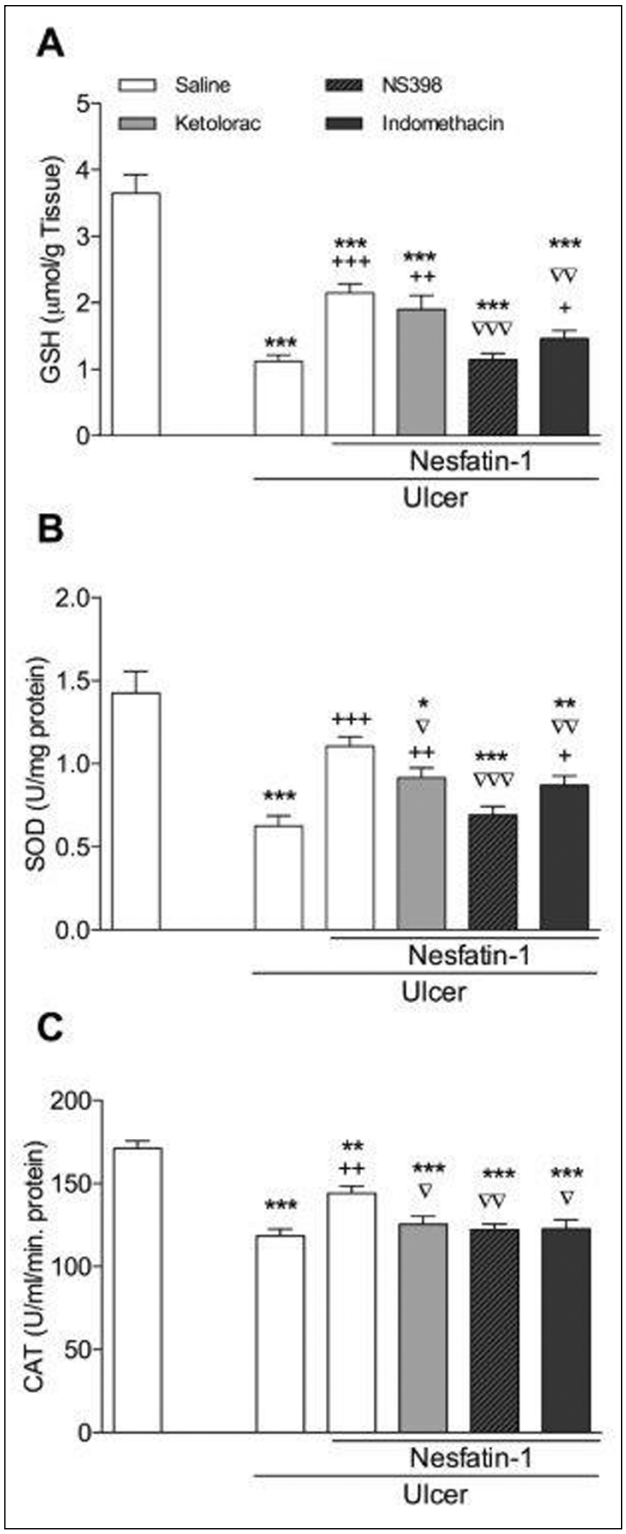

Fig. 4. (*A*) Glutathione (GSH); (*B*) Superoxide dismutase (SOD); (*C*) Catalase (CAT). Levels in the control and ulcer groups treated for 3 days with saline, nesfatin-1 (0.3 µg/kg/day), COX-2 inhibitor NS-398 (2 mg/kg/day), COX-1 inhibitor ketorolac (3 mg/kg/day) or non-selective COX inhibitor indomethacin (5 mg/kg/day). *P < 0.05, **P < 0.01, ***P < 0.001 versus saline-treated control group, $^{+}$P < 0.05, $^{++}$P < 0.01, $^{+++}$P < 0.001 versus saline-treated ulcer group, $^{\nabla}$P < 0.05, $^{\nabla\nabla}$P < 0.01, $^{\nabla\nabla\nabla}$P < 0.001 versus saline plus nesfatin-1-treated ulcer group. For each group n = 8. The results are expressed as the mean ± S.E.M. One-way analysis of variance (ANOVA) and Tukey-Kramer multiple comparison tests were used.

treated with saline or any of the COX inhibitors (P < 0.05 – 0.001; *Table 1*). However, the ulcer-induced increase in gastric COX-1 level was not observed when the rats were treated with nesfatin-1. Except for the reduced COX-1 level detected in the indomethacin plus nesfatin-1-treated group, nesfatin-1 treatment with or without the blockade of COX inhibitors did not alter the levels of enzymes or prostaglandins in the control groups.

Histopathological findings

Hematoxylin and eosin stained paraffin sections revealed normal surface epithelium and gastric glands in the control groups (*Fig. 5A*). Desquamation of surface epithelium, severe degeneration of the gastric glands, submucosal edema, and inflammatory cell infiltration in the mucosa and submucosa were observed in the ulcer groups (*Fig. 5B*). In the nesfatin-1-treated ulcer group (*Fig. 5C*), regeneration of the gastric mucosa with normal surface epithelium and gastric glands was evident with mild degeneration in some gastric gland cells and decreased inflammatory cell infiltration, while submucosal edema was still present. In the nesfatin-1 plus ketorolac- or indomethacin-treated ulcer groups (*Fig. 5D* and *5F*), surface epithelium and most of the gastric glands were in normal appearance with mild degeneration in some glandular cells, decreased submucosal edema and inflammatory cell infiltration. Similarly, in the ulcer group that received NS-398 in conjunction with nesfatin-1 (*Fig. 5E*), there was mucosal healing with mild desquamation of surface epithelium and mild degeneration of gastric glands, but submucosal edema and inflammatory cell infiltration were severe. Multiple comparison tests revealed that gastric injury score was significantly higher in the saline-treated ulcer group as compared to the control group (P < 0.0001; *Fig. 6A*). Damage scores of the gastric mucosa and submucosa were significantly decreased in the ulcer groups treated with nesfatin-1 alone or in combination with ketorolac or indomethacin (P < 0.01). Although the average score in the NS-398 plus nesfatin-1-treated ulcer group was lower compared to the saline-treated ulcer group, the score was not significantly different.

Immunohistochemistry of NF-κB

In contrast to absence of NF-κB immunoreactivity in the control group (*Fig. 5G*), gastric glands showed an intense NF-κB immunoreactivity in the saline-treated ulcer group (P < 0.01, *Figs. 5H* and *6B*). On the other hand, a mild NF-κB immunoreactivity was observed in the gastric glands of nesfatin-1-treated ulcer group, with a moderate reaction in some surface epithelial and gastric gland cells (P < 0.01; *Figs. 5I*, and *6B*). Similarly, mild immunoreactivity was evident in the gastric glands of the ulcer groups treated with nesfatin-1, along with any of the COX inhibitors (P < 0.05; *Figs. 5J-5L* and *6B*).

Immunohistochemistry of cyclooxygenase-1 and cyclooxygenase-2

When compared to mild COX-1 immunoreactivity (arrow) in the gastric glands of the control group (*Fig. 7A*), immunoreactivity was significantly higher (P < 0.05; *Fig. 6C*) and more intense in the severely degenerated gastric glands of the saline-treated ulcer group (*Fig. 7B*). Gastric glands of the nesfatin-1-treated ulcer group demonstrated mild immunoreactivity with a significantly lower score (P < 0.01; *Fig. 6C* and *7C*). Higher and moderate COX-1 immunoreactivity was observed in the gastric glands of the ulcer groups treated with nesfatin-1, along with any of the COX inhibitors (*Fig. 7D-7F*).

In the saline-treated ulcer group, glandular immunoreactivity was more intense (P < 0.05; *Figs. 6D* and *7H*) than the non-ulcer

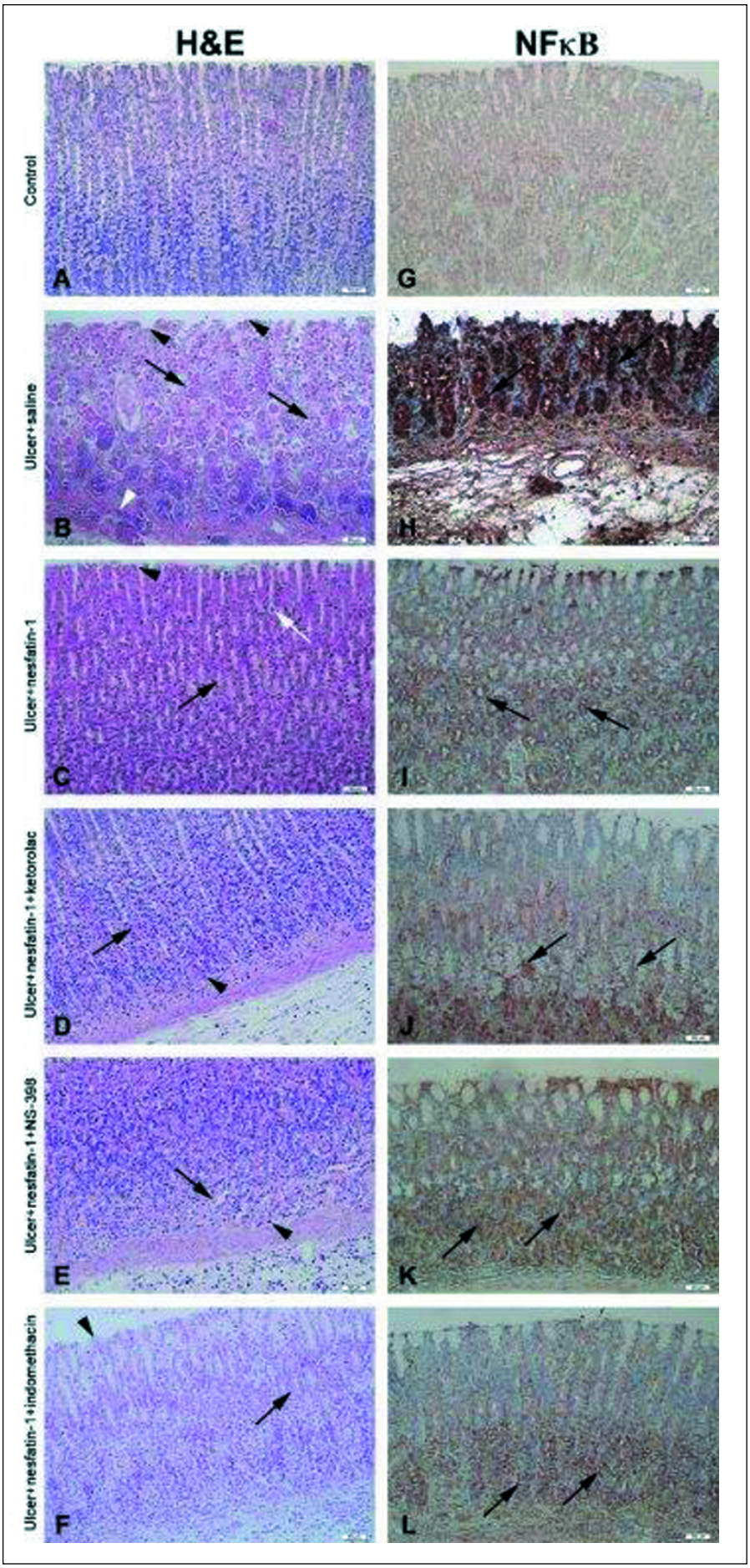

Fig. 5. Hematoxylin and eosin staining (*A-F*). (*A*) Normal epithelial and gastric gland structures in fundic region of the stomach of the control group. (*B*) Loss of surface epithelium (arrowhead) and severe damage of the gastric glands (arrow) in the saline-treated ulcer group; white arrowhead: inflammatory cell infiltration. (*C*) Normal epithelium (arrowhead) and gastric glands (arrow) in the saline plus nesfatin-1 treated ulcer group; white arrow: degeneration in parietal cells. (*D*) Normal gastric glands (arrow) in the nesfatin-1 plus ketorolac treated ulcer group; arrowhead: inflammatory cell infiltration. (*E*) Damage in the gastric glands (arrow) in the nesfatin-1 plus NS398-treated ulcer group; arrowhead: inflammatory cell infiltration. (*F*) Normal epithelial (arrowhead) and gastric gland (arrow) structures in the nesfatin-1 plus indomethacin-treated ulcer group. NF-κB immunohistochemistry (*G-L*). Absent NF-κB immunoreactivity in the control group (*G*). Intense NF-κB immunoreactivity in the gastric glands (arrow) of the saline-treated ulcer group (*H*). Mild NF-κB immunoreactivity (arrow) in the gastric glands of the ulcer groups treated with saline plus nesfatin-1 (*I*), nesfatin-1 plus ketorolac (*J*), nesfatin-1 plus NS398 (*K*), and nesfatin-1 plus indomethacin (*L*). *A-L*: × 200; bar: 50 µm.

Table 1. COX-1, COX-2, PGI_2 and PGE_2 levels in the gastric tissues of ulcer groups and the control group.

Experimental groups		Gastric tissue levels (ng/ml)			
		COX-1	**COX-2**	**PGI_2**	**PGE_2**
	control + saline	0.17 ± 0.01	0.12 ± 0.02	0.16 ± 0.01	0.16 ± 0.01
ULCER	saline + saline	0.23 ± 0.02 *	0.19 ± 0.02 *	0.23 ± 0.02*	0.26 ± 0.02 ***
	saline + nesfatin-1	0.19 ± 0.02	0.19 ± 0.01 ***	0.21 ± 0.01*	0.22 ± 0.01 *
	ketorolac + nesfatin-1	0.21 ± 0.01 *	0.15 ± 0.01 *	0.21 ± 0.01*	0.18 ± 0.02 **,+
	NS-398 + nesfatin-1	0.22 ± 0.01 **	0.21 ± 0.02**	0.23 ± 0.02 **	0.25 ± 0.02 **
	indomethacin + nesfatin-1	0.21 ± 0.01 **	0.16 ± 0.01 **	0.23 ± 0.02 *	0.24 ± 0.02*

*P < 0.05, **P < 0.01, ***P < 0.001, compared to non-ulcer control group. +P < 0.05, compared to saline + saline-treated ulcer group.

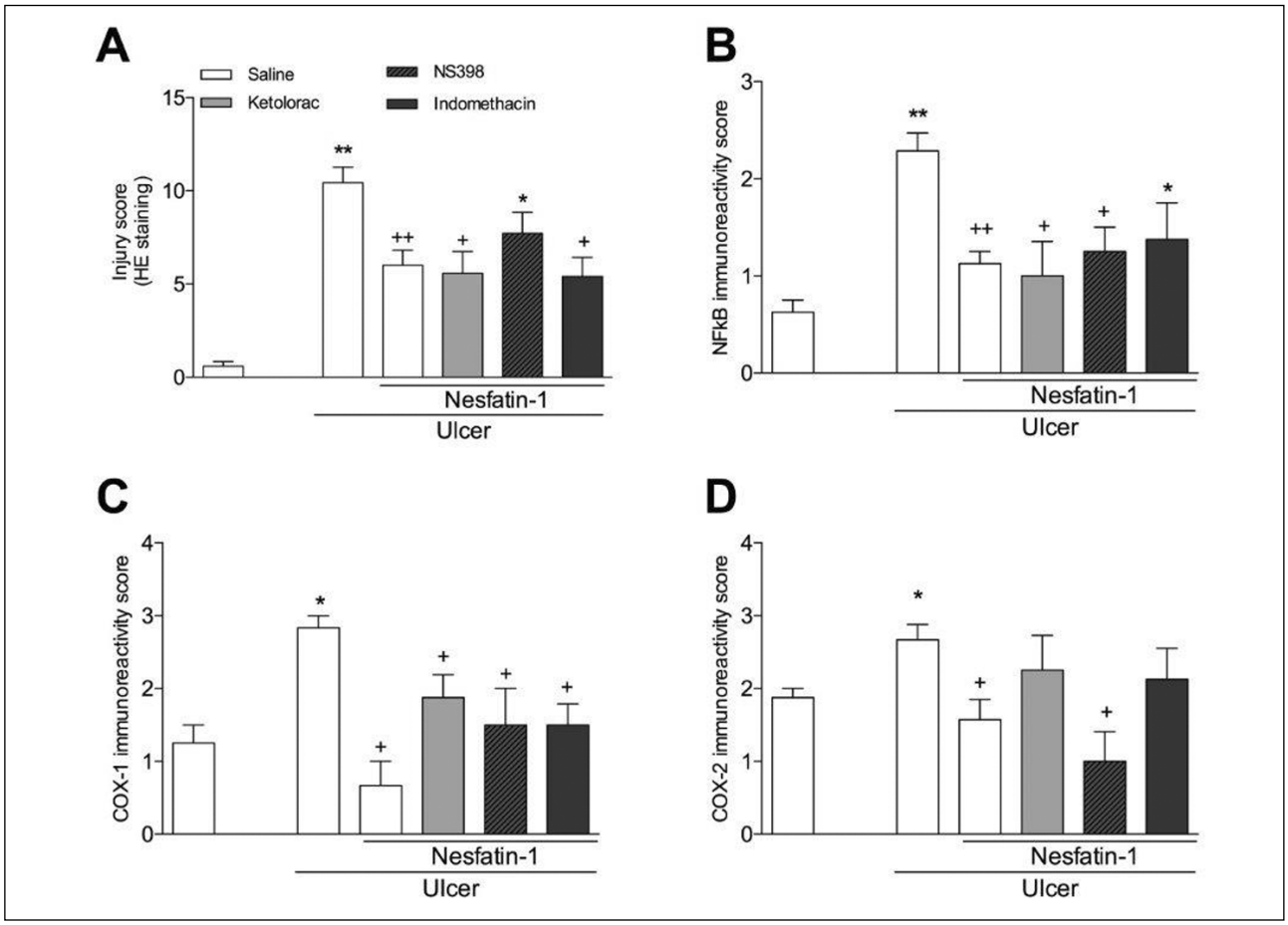

Fig. 6. (*A*) Injury scores in gastric tissues stained with hematoxylene and eosin (HE), and immunoreacitivty scores of (*B*) NF-κB (*C*) COX-1, (*D*) COX-2. *P < 0.05, **P < 0.01 versus saline-treated control group, +P < 0.05, ++P < 0.01 versus saline-treated ulcer group. The results are expressed as the mean ± S.E.M. One-way analysis of variance (ANOVA) and Tukey-Kramer multiple comparison tests were used.

control group, showing a moderate COX-2 immunoreactivity in the gastric glands (*Fig. 7G*). A lower and moderate staining was observed in the gastric glands of the nesfatin-1 (P < 0.05; *Figs. 6D* and *7I*), ketorolac plus nesfatin-1 (*Fig. 7J*) or indomethacin plus nesfatin-1- treated groups (*Fig. 7L*). COX-2 immunoreactivity was mild in the ulcer group treated with nesfatin-1 plus NS-398 (P < 0.05; *Figs. 6D* and *7K*).

Immunohistochemistry of prostaglandin E_2 and prostacyclin

Except for the saline-treated ulcer group (*Fig. 8B*) and the NS-398 plus nesfatin-1-treated group (*Fig. 8E*), which demonstrated a moderate PGE_2 immunoreactivity in the gastric glands, PGE_2 immunoreactivity was mild in the control (*Fig. 8A*), the nesfatin-1 (*Fig. 8C*), the nesfatin-1 plus ketorolac (*Fig. 8D*) and the nesfatin-1 plus indomethacin treated ulcer groups (*Fig. 8F*). Mild PGI_2 synthase immunoreactivity was observed in the gastric mucosa of the control group (*Fig. 8G*) and the nesfatin-1 plus indomethacin-treated ulcer group (*Fig. 8L*). In all the other ulcer groups, moderate PGI_2 synthase staining was evident in the gastric gland cells (*Fig. 8I-8K*). However, no statistical difference was present among the PGI_2 synthase and PGE_2 immunoreactivity scores of the groups (data not shown).

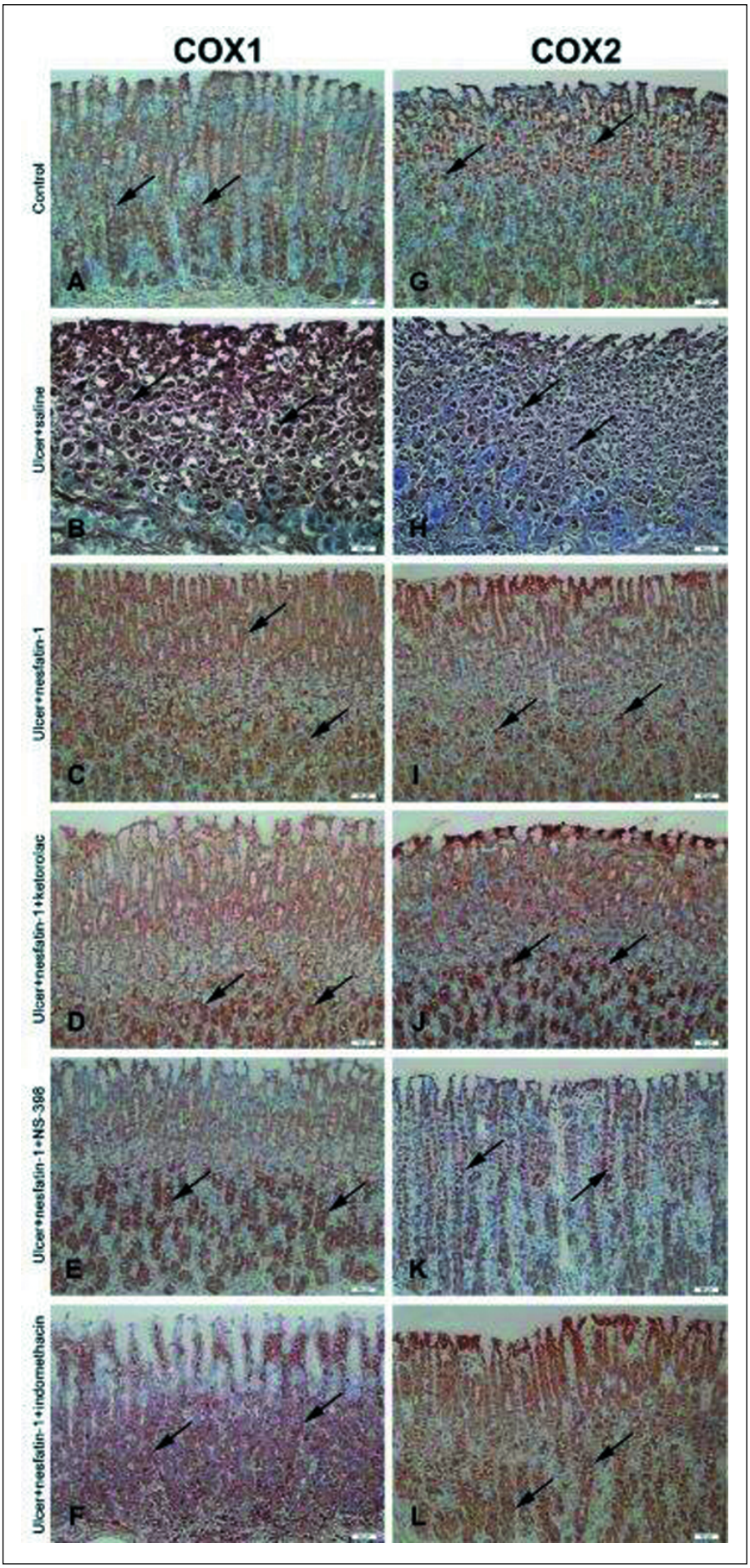

Fig. 7. COX-1 immunohistochemistry (*A-F*). Mild COX-1 immunoreactivity (arrow) in the gastric glands of the control group (*A*). Intense COX-1 immunoreactivity (arrow) in the severely degenerated gastric glands in the saline-treated ulcer group (*B*). Mild COX-1 immunoreactivity (arrow) in the gastric glands of the saline plus nesfatin-1-treated ulcer group (*C*). Moderate COX-1 immunoreactivity (arrow) in the gastric glands of the ulcer groups treated with nesfatin-1 plus ketorolac (*D*), nesfatin-1 plus NS398 (*E*), and nesfatin-1 plus indomethacin (*F*). COX-2 immunohistochemistry (*G-L*). Moderately stained COX-2 immunoreactive cells (arrow) in the gastric glands of the control group (*G*). Intensely stained COX-2 immunoreactive cells (arrow) in the gastric glands in the saline-treated ulcer group (*H*). Moderate staining in the gastric gland cells (arrow) in the saline plus nesfatin-1 treated (*I*) and nesfatin-1 plus ketorolac treated (*J*) ulcer groups. Mild staining in the gastric gland cells (arrow) of the nesfatin-1 plus NS398 treated ulcer group (*K*). Moderate staining in the gastric gland cells (arrow) of ulcer group treated with nesfatin-1 plus indomethacin (*L*). *A-L*: × 200; bar: 50 µm.

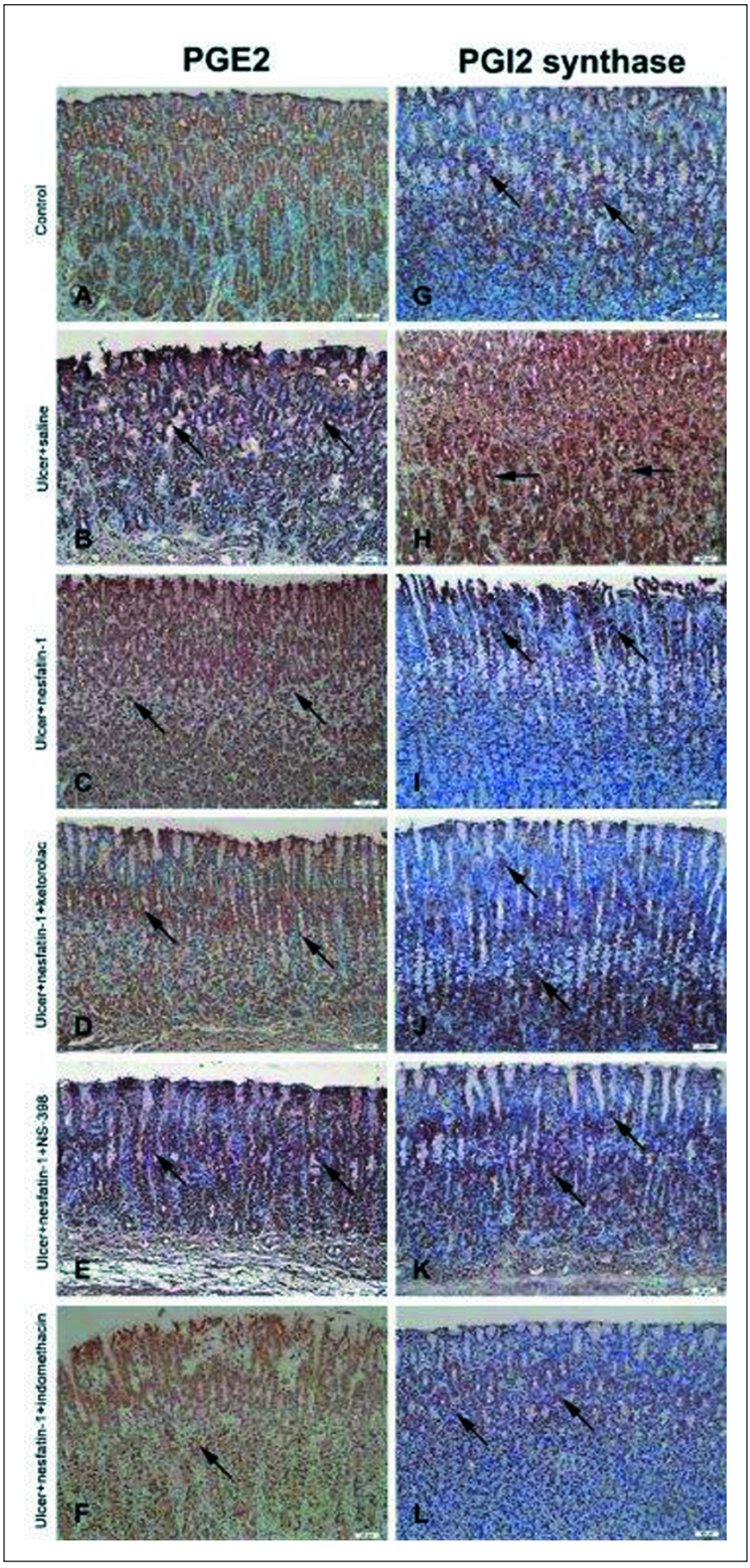

Fig. 8. PGE_2 immunohistochemistry (*A-F*). Mild PGE_2 immunoreactivity in the gastric glands of the control group (*A*). Moderate staining (arrow) in the gastric gland cells of the ulcer group (*B*). Mild staining (arrow) in the gastric glands of the nesfatin-1-treated (*C*) and nesfatin-1 plus ketorolac treated (*D*) ulcer groups. (*E*) Moderately stained gastric glands (arrow) in the ulcer group treated with nesfatin-1 plus NS398. (*F*) Mild PGE_2 immunoreactivity in the gastric glands of the ulcer group treated with nesfatin-1 plus indomethacin. PGI_2 synthase immunohistochemistry (*G-L*). PGI_2 synthase immunoreactive cells (arrow) in the control group (*G*). Moderately stained gastric glands (arrow) in the saline-treated (*H*), saline plus nesfatin-1-treated (*I*), nesfatin-1 plus ketorolac-treated (*J*), and nesfatin-1 plus NS398-treated (*K*) ulcer groups. (*L*) Mild PGI_2 synthase immunoreactivity (arrow) in the gastric glands of the ulcer group treated with nesfatin-1 and indomethacin. *A-L*: × 200, bar: 50 µm.

DISCUSSION

The findings of the current study demonstrate that nesfatin-1 treatment reversed acetic acid-induced oxidative damage of the stomach, by inhibiting neutrophil infiltration and pro-inflammatory cytokine release, as well as by augmenting its antioxidant capacity. In addition, ulcer-induced upregulation of COX-1, COX-2 and NF-κB immunoreactivity were abolished by nesfatin-1 treatment. Blockade of selective COX-1 or COX-2 enzymes or non-selective indomethacin, reversed most of the alleviations induced by nesfatin-1, but COX-2-blockade was consistently more effective in reversing nesfatin-1-induced changes. This suggests that the antioxidant effects of nesfatin-1 are mediated mainly *via* the activation of COX-2 enzyme.

Mucosal reconstruction during the healing process of chronic gastric ulcer is performed by the proliferation and migration of the epithelial cells and connective tissue, which involves the upregulation of COX-2 expression and the synthesis of prostaglandins (5, 35, 36). The present study in which acetic acid induced an ulcer of the gastric tissue of the rats which is well known to become chronic within 3 days (24), demonstrated an oxidative injury accompanied by elevated COX-1 and COX-2 immunoreactivity scores. This suggests the involvement of both COX enzymes in the early healing process of a chronic ulcer. Similarly, the elevation in gastric levels of COX-1, COX-2, PGE_1 and PGI_2 supports that augmented prostaglandin synthesis has accompanied the elevated activity of COX enzymes during the early phase of healing, when oxidative injury was dominating the ulcerated gastric tissue. Increased recruitment of neutrophils and generation of pro-inflammatory cytokines, along with enhanced release of ROM and depleted antioxidants, were the indicators of oxidative injury in the gastric tissue. On the other hand, treatment with nesfatin-1, following ulcer induction, depressed the extent of oxidative injury, and replenished the antioxidant capacity without altering the gastric levels of COX enzymes or prostaglandins. However, immunoreactivity scores of both COX-1 and COX-2 enzymes, as well as NF-κB in the ulcerated gastric tissue, were elevated as a compensatory mechanism, suggesting that both COX-1 and COX-2 participate in the proliferation of epithelium, angiogenesis and generation of prostaglandins during the healing process of gastric ulcers (25). On the other hand, COX-1 and COX-2 immunoreactivity scores were reduced back to control levels by nesfatin-1 treatment, suggesting that this compensatory upregulation of COX enzymes and NF-κB has become unnecessary because gastric damage was alleviated by nesfatin-1 treatment. In support of that, Szlachcic *et al*. (23) have proposed that endogenous prostaglandins are not the only mediators, and several other agents, such as nitric oxide, could be participating in the protective effect of nesfatin-1 on gastric damage.

As it was first shown in the central nervous system, peripheral inflammatory stimuli act on nesfatin-1 expressing hypothalamic and brainstem neurons, which were proposed to be responsible of the anorexigenic feature of inflammation (13, 37). Plasma level of nesfatin-1 and its mRNA expression in the hypothalamus were elevated in rats exposed to acute stress, but not chronic stress (38). This implicates the compensatory release of nesfatin-1 upon the exposure to a stressful event. On the other hand, peripheral administration of nesfatin-1 treatment was observed to ameliorate oxidative damage of renal (39), cardiac (40), hepatic (41) and cerebral (14) injury, by suppressing oxidative stress and cell apoptosis. Its gastroprotective and healing-promoting effects were shown in acute (23) and chronic (21) gastric ulcers. We have previously shown that nesfatin-1 facilitated surgical wound healing occurred through the suppression of neutrophil recruitment, apoptosis, vascular endothelial growth factor activation (15), and alleviated indomethacin-induced gastric injury (20). In our previous studies, we have shown that nesfatin-1 ameliorates oxidative injury, as observed by reduced lipid peroxidation and neutrophil infiltration to the inflamed tissues, while the antioxidant status of the tissues was maintained and the generation of ROM and proinflammatory cytokines was depressed (14, 15, 20). However, the involvement of COX enzymes in the antioxidant and healing-promoting effects of nesfatin-1 was not identified before. Present findings demonstrated that healing of the acetic acid-induced ulcer was facilitated through the suppression of ROM production and pro-inflammatory cytokine release. This is concomitant with the replenishment of antioxidants, which appears to involve the activation of both COX enzymes, with a more enhanced effect of COX-2 enzyme.

Brzozowski *et al*. (7) have reported that either of the selective COX-1 or COX-2 inhibitors were equally potent in mimicking the injurious effects of NSAIDs, because gastric mucosa is protected against injuries by prostaglandins, which depend on prostaglandin endoperoxide synthase/COX for its synthesis from arachidonic acid (42). It has been described that constitutively produced COX-1 enzyme maintains mucosal integrity, microcirculation, motor and secretory functions in intact stomach, while expression of the inducible COX-2 is elevated in damaged stomachs (43, 44), as well as in macrophages and fibroblasts (45, 46). It has been postulated that expression of IL-1β in macrophages/monocytes and fibroblasts at the ulcer area upregulate the mRNA expression of COX-2 and participate in ulcer healing (47, 48). Regarding these studies, it appears that ulcer healing involves increased activity of COX-2 enzyme, and agents that can induce COX-2 activity may have anti-ulcer effects. Current data demonstrates that nesfatin-1 reduced IL-1β and ROM levels, elevated antioxidant capacity (GSH, SOD and CAT), and the facilitatory effect of nesfatin-1 on the healing process of chronic gastric ulcer, could be operating by the upregulation of the COX-2 system. This is because of the suppressive effect of nesfatin-1 on neutrophil infiltration and lipid peroxidation in the gastric tissue was reversed when COX-2 activity was blocked. Thus, this data suggests that antioxidant and radical scavenging effects of nesfatin-1 during the early healing phase of gastric ulcer depends on COX activity, and specifically COX-2 activity. On the other hand, once the ulcer was improved by nesfatin-1 gastric COX-2 immunoreactivity was reduced, suggesting that COX-2 operated protection carried out by nesfatin-1, was completed by consuming COX-2 enzyme. Thus, our data suggests that COX-2 inhibitors, which delay gastric healing, similarly are responsible in reducing the gastroprotective actions of nesfatin-1. Furthermore, this verifies the role of elevated COX-2 activity in ulcer healing promoted by nesfatin-1. In support of our findings, nesfatin-1 was shown to increase COX-2 mRNA expression in human and murine chondrocytes (49), implicating the involvement of COX enzymes in its regulatory functions.

In conclusion, our results suggest that nesfatin-1 ameliorates ulcer-induced inflammatory response through the modulation of the balance between oxidants and antioxidants. These gastroprotective effects of nesfatin-1 are dependent on COX-1 and COX-2 activity, with a more COX-2 dominant mechanism being active in inhibiting inflammatory mediators and neutrophil migration during the ulcer healing process. Likewise, nesfatin-1 was previously shown to ameliorate acetic acid-induced colitis by inhibiting neutrophil infiltration and ROM formation (50). Although the effects of nesfatin-1 were mediated by a G protein-coupled receptor (51), a specific receptor is yet to be identified. Identification of the nesfatin-1 receptor(s) and development of selective nesfatin-1 antagonists would provide more information about the endogenous role of nesfatin-1 and its interaction with the COX enzymes during the healing process of gastric ulcers.

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