

## Gastroprotective effect of ghrelin against indomethacin-induced gastric injury in rats: possible role of heme oxygenase-1 pathway

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**Abstract.** Ghrelin has been shown to ameliorate gastric injury by several mechanisms in experimental animal models. The present study aimed to investigate the effect of pretreatment with ghrelin on indomethacin-induced gastric injury in rats and the role of heme oxygenase-1 (HO-1) pathway as a novel mechanism underlying the gastroprotective effect of ghrelin. In all groups studied, ulcer score (U.S), ulcer index (U.I) and preventive index (P.I) were evaluated and the gastric inflammatory biomarkers including levels of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ) and myeloperoxidase (MPO) activity as well as prostaglandin E2 (PGE2), malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD), HO-1 and bilirubin as an indicator of heme oxygenase activity were measured. Indomethacin induced significant elevation in U.S and U.I as well as the inflammatory and the oxidative markers and reduced the PGE2 in addition to HO-1 level and activity. Pretreatment with ghrelin reversed these results. In order to elucidate the possible role of HO-1 in mediating the protective effects of ghrelin, tin protoporphyrin (SnPP) HO-1 blocker was administered; it significantly attenuated the gastroprotective effect of ghrelin. In conclusion HO-1 activity significantly contributes toward ghrelin-mediated gastroprotection.

**Key words:** Ghrelin — NSAIDs — Indomethacin — Gastric injury — Heme oxygenase-1

**Abbreviations:** CO, carbon monoxide; COX, cyclooxygenase; GHS-R, growth hormone secretagogue receptor; GSH, glutathione peroxidase; HO, heme oxygenase; IL-1 $\beta$ , interleukin 1 $\beta$ ; IND, indomethacin; MDA, malondialdehyde; MPO, myeloperoxidase; NO, nitric oxide; NSAIDs, nonsteroidal anti-inflammatory drugs; PG, prostaglandin; PGE2, prostaglandin E2; P.I, preventive index; PMN, polymorphonuclear; ROS, reactive oxygen species; SnPP, Tin-protoporphyrin; SOD, superoxide dismutase; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; U.I, ulcer index; U.S, ulcer score.

### Introduction

Gastric ulcers associated with the utilization of nonsteroidal anti-inflammatory drugs (NSAIDs) remain one of the major clinical problems all over the world (Dhiyaaldeen et al. 2014). The frequency of usage is attributed to the fact that NSAIDs-induced gastric ulcers are the second most common etiology of gastric ulcers (Adinortey et al. 2013). The pathophysiology of gastric ulcer has generally focused on an imbalance between aggressive and defensive factors of the gastric mucosa (Tulassay and Herszényi 2010). Indometha-

cin (IND), a representative of NSAIDs family, is known to produce serious side effects in the mucosa of the stomach such as erosions, and ulcerative lesions (Prasad et al. 2012): It was reported that oral administration of IND in rats is well known to cause ulcerative lesions in the gastric mucosa (Kim et al. 2011). Several mechanisms underlie the effect of IND including inhibition of prostaglandin synthesis and generation of reactive oxygen species (ROS) (Takeuchi et al. 2011).

Ghrelin is a 28-amino acid peptide that exerts several biological activities including regulation of food intake and energy balance (Harrison et al. 2008). Ghrelin has been discovered in the gastrointestinal tract, particularly in gastric mucosa, as an endogenous ligand for growth hormone secretagogue receptor (GHS-R) (Kojima and Kangawa 2005). Specifically, the majority of plasma ghrelin originates in the

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oxyntic gland where A-like cells exist (Gaytan et al. 2003), it was proposed that nitric oxide (NO) pathway, antioxidant and anti-inflammatory effects may underlie the gastroprotective effect of ghrelin (Sibilia et al. 2003; Gamal El-Din et al. 2012). However, other underlying protective mechanisms of ghrelin in gastric injury remain to be investigated.

Heme oxygenase (HO) is the rate-limiting enzyme in the oxidation of heme to carbon monoxide (CO) and biliverdin, which is changed over into bilirubin (Wagener et al. 1999). HO-1, an inducible isoform is produced under stressful states such as hypoxia (Ryter et al. 2002), free radicals and chemical stressor (Shibahara 2003). Induction of the important cytoprotective molecule HO-1 has been shown to have vasodilatory, and anti-inflammatory effects (Wagener et al. 2001).

HO-1 is expressed in gastric epithelium and previous works have shown increased HO-1 expression in an inflamed stomach (Barton et al. 2003) and during the healing of gastric ulcers (Guo et al. 2003). HO-1 and its metabolites have the potential to counteract the NSAIDs-induced gastric injury by different mechanisms, including antioxidant properties and their ability to restore mucosal blood flow (Uc et al. 2012). Previous studies had demonstrated an inducible effect of ghrelin on HO-1 that provides efficient cytoprotection in ischemia-reperfusion liver injury secondary to transplantation or hemorrhage resuscitation in rats (Bauer and Bauer 2002) and in case of paracetamol-induced acute liver injury rat model (Ahmed et al. 2014), also it was reported that the neuroprotective effects of ghrelin may be mediated by the up-regulation of gene expression of HO-1 (Jazwa and Cuadrado 2010). The present study was designed to investigate whether HO-1 dependent pathway would be one of the mechanisms underlying the gastroprotective effect of ghrelin against NSAIDs-induced gastric injury in rats.

## Material and Methods

### Drugs and chemicals

Ghrelin was obtained from Sigma (St. Louis, MO, USA) as powder, it was dissolved in saline. IND was obtained from Kahira Pharm & Chem. Ind. Co. (Egypt) as powder, it was suspended in carboxymethylcellulose 1% (El-Nasr pharmaceutical & Chemical Co, Egypt). Tin protoporphyrin (SnPP) was obtained from (Tocris Bioscience, UK) and was prepared in 0.1 mol/l NaOH and phosphate-buffered saline, pH was adjusted to pH 7.4. All drugs solutions and suspensions were freshly prepared. All other chemicals were of analytical grade and obtained from standard commercial suppliers.

### Animals

This study was conducted on 40 adult Wistar albino male rats, 6–8 weeks old, animals were purchased from Abou-Rawash

Animal House (Giza, Egypt), weighing  $200 \pm 20$  g. Rats were housed at room temperature in metal cages with a 12:12-h light/dark cycle, four rats in each cage. The present study was carried out in accordance with the guidelines set by the Research Ethics Committee of Benha Faculty of Medicine.

### Experimental design

The rats were fasted for 24 h prior to the experiment in mesh bottomed cages to minimize corporopohagia. The animals had free access to water except for the last hour before the experiment. All experiments were performed during the same time of the day to avoid variations due to diurnal rhythms of putative regulators of gastric functions (Bregonzio et al. 2003). The rats were randomly assigned into five groups (eight rats in each group):

Group I: control group; rats were injected with saline subcutaneously (s.c.) as a vehicle.

Group II: rats were injected with ghrelin (40  $\mu$ g/kg; s.c.) (Khalefa et al. 2010).

Group III: IND was administered orally *via* gavage at a single dose (30 mg/kg) (Bhargava et al. 1973) to induce the gastric ulcer in rats.

Group IV: rats were pretreated with ghrelin (40  $\mu$ g/kg; s.c), 30 min before induction of gastric ulceration by IND.

Group V: rats were pretreated with a potent inhibitor of HO-1 activity, tin-protoporphyrin (SnPP) 30 mg/kg; intravenously under light ether anesthesia, 10 min before administration of ghrelin, which was followed by IND (Yoda et al. 2010).

### Assessment of gastric mucosal lesions

Twenty four hours later after administration of IND during which they were fasting (Yoda et al. 2010), rats of all groups were anaesthetized with urethane (1.5 g/kg; i.p.) before cervical dislocation, stomachs were rapidly removed, opened by an incision along the greater curvature and washed by cold saline solution to remove the gastric content remnants and blood clots. The stomachs were stretched and pinned out flat on a cork board and photographed, the number and the severity of discrete areas of damage in the mucosa were inspected using a magnifier and scored by trained independent observer who was unaware of the drug treatment, the ulcer score (U.S) was determined according to the method described by Dekanski et al. (1975) as follows: 0, no damage; 1, blood at the lumen; 2, pinpoint erosions; 3, one to five small erosions < 2 mm; 4, more than five small erosions < 2 mm; 5, one to three large erosions >2 mm; 6, more than three large erosions >2 mm. The ulcer index (U.I) was calculated by the following equation: U.I. = mean ulcer score of similarly treated group  $\times$  percentage of ulcerated animals of the same group and the preventive index (P.I) was calculated by the equation (Hano et al. 1976):

$$P.I = \frac{(U.I \text{ of IND group} - U.I \text{ of pretreated group})}{U.I \text{ of IND group}} \times 100$$

#### Biochemical analysis of gastric mucosa

Sample of the stomach of each rat was immersed in IND (10 µg/ml) for 20 min to inhibit further formation and release of PGs, then stored at -80°C. Subsequently, the gastric mucosa was scraped, homogenized in 2 ml normal saline containing 0.1 M dithiothreitol and centrifuged at 2000 rpm for 10 min at room temperature. The supernatant was used for determination of prostaglandin E2 (PGE2). PGE2 in the gastric mucosa was determined by enzyme-linked immunosorbent assay (ELISA) using PGE2 assay kit (R&D Systems, USA), and based on the competitive binding technique in which PGE2 present in a sample competes with a fixed amount of horseradish peroxidase (HRP)-labeled PGE2 for sites on a monoclonal antibody (Brzozowski et al. 2007).

The mucosa of another part of the stomach was also scrapped, homogenized in cold phosphate buffer (0.05 M, pH 7.4) and centrifuged at 2000 rpm for 10 min; the supernatant was then kept at -80°C for measurement of (i) malondialdehyde (MDA) content in the gastric mucosa as an indicator of lipid peroxidation, (ii) the levels of pro-inflammatory cytokines; tumor necrosis factor (TNF-α) and interleukin 1β (IL-1β), and (iii) hemeoxygenase-1 (HO-1) level.

MDA levels in the gastric mucosa were determined as an indicator of lipid peroxidation by thiobarbituric acid method (Cayman Chemical Co., Ann Arbor, MI, USA) (Okhawa et al. 1979), the glutathione (GSH) activity (Sedlak et al. 1968) and superoxide dismutase (SOD) activity using Cayman assays (Cayman Chemical Co., Ann Arbor, MI, USA) activity as antioxidants (Das et al. 2000). The levels of pro-inflammatory cytokines TNF-α and IL-1β were determined by using ELISA assay using standard kits (Ray Biotech, Inc., USA) (Cunha et al. 1993), myeloperoxidase (MPO) activity; an index of polymorphonuclear (PMN) cell accumulation into gastric tissue was determined according to Bradley et al. (1982). The glandular stomach was weighed and subsequently homogenized in phosphate buffer. The samples were freeze-thawed 3 times and centrifuged at 15,000 × g for 20 minutes at 4°C. Finally the change in absorbance at 460 nm over 3 min was measured with a spectrophotometer.

Gastric mucosal HO-1 was determined by ELISA using Rat HO-1 immunoassay kit (Biovendor, USA), and based on the competitive binding technique in which HO-1 present in a sample is captured by the immobilized antibody and is detected with an IgG antibody conjugated to horseradish peroxidase (Chen et al. 2003) and bilirubin level as a measure of HO activity was measured spectrophotometrically using commercial kit from BioMed Diagnostics (White City, OR, USA) (Motterlini et al. 1996).

#### Histological evaluation of gastric damage

For histological evaluation, stomach samples were fixed in 10% formalin solution where they remained for 24 h. After fixation, the samples were transferred to a solution of 70% alcohol. The material was then embedded in paraffin and sectioned. Sections (4 µm thick) were deparaffinized, stained with hematoxylin and eosin (H&E), and then examined under a light microscope by an experienced pathologist who was blinded to the treatment. Gastric microscopic damage was scored on a 0–14 scale according to the criteria described by Laine and Weinstein (1988). Briefly, a 1 cm segment of each histological section was examined for epithelial cell loss (score: 0–3), edema in the upper mucosa (score: 0–4), hemorrhagic damage (score: 0–4), and the presence of inflammatory cells (score: 0–3).

#### Statistical Analysis

All the data are presented as mean ± standard deviation (SD). Evaluation of differences between groups was performed using one-way ANOVA with post hoc test (LSD) between groups with SPSS 19.0 software. A value  $p < 0.05$  was considered statistically significant.

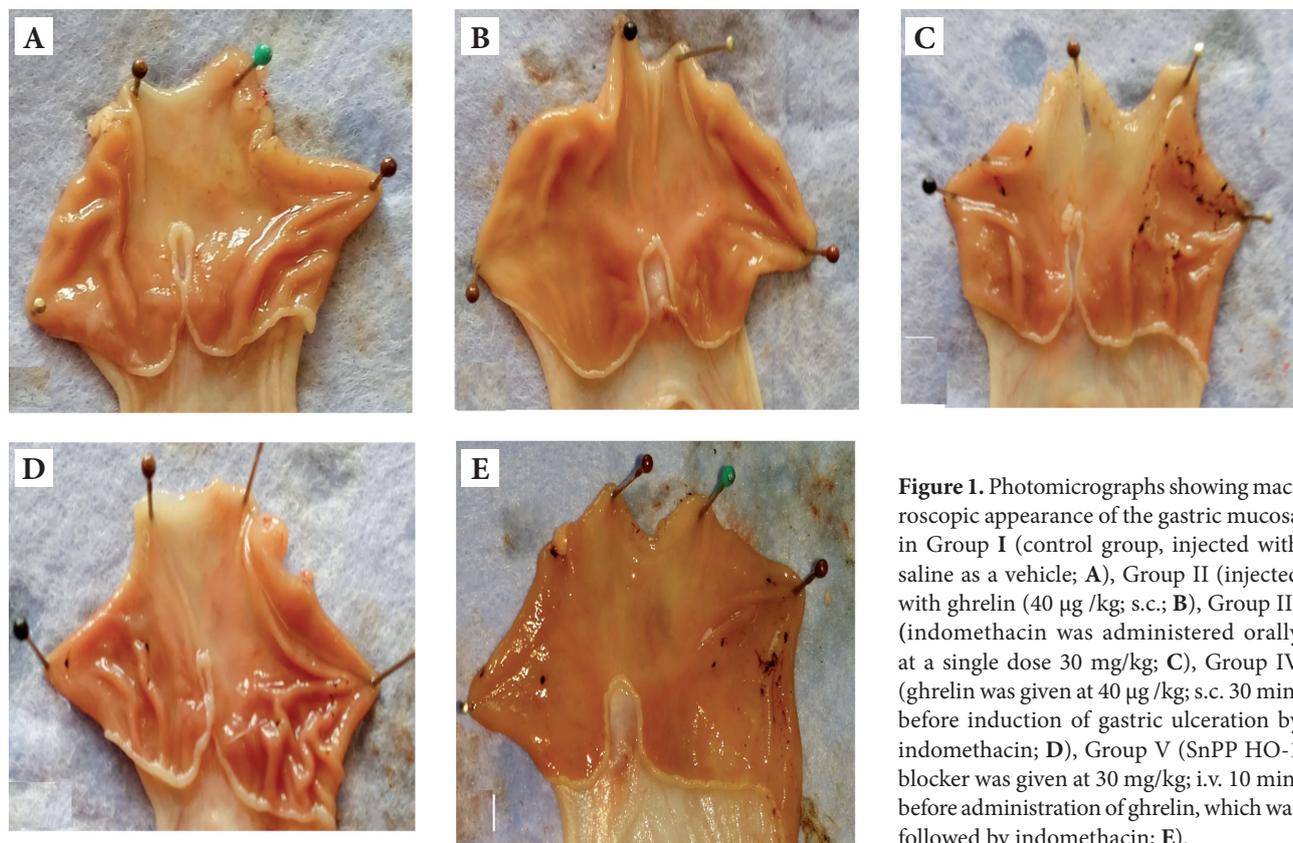
## Results

#### *Effect of ghrelin administration on IND-induced gastric lesions in rats and the role of HO-1*

The present work revealed that IND administration in Group III resulted in a marked ulcerative lesion, it was accompanied by a significant ( $p < 0.05$ ) elevation in the levels of U.S and U.I as compared with that of control rats. Pretreatment with ghrelin in Group IV significantly ( $p < 0.05$ ) reduced the U.S and U.I showing P.I of 77.1% as compared to Group III. On the other hand, the HO-1 blocker (SnPP) pretreatment (Group V) abolished the protective effect of ghrelin in rats subjected to gastric lesions as it significantly ( $p < 0.05$ ) increased the U.S and U.I with P.I of 32.98% as compared to ghrelin-pretreated group (Group IV) (Table 1 and Fig. 1).

#### *Effect of ghrelin administration on inflammatory changes that associate with the IND-induced gastric lesions in rats and the role of HO-1*

Quantification data for the levels of the pro-inflammatory cytokines (TNF-α and IL-1β) in the IND-treated rats (Group III) showed a significant ( $p < 0.05$ ) elevation compared to control rats. However, these cytokines showed a significant ( $p < 0.05$ ) reduction in rats pretreated with ghrelin (Group IV) compared to the IND-treated rats group (Group III). Furthermore, the pretreatment with



**Figure 1.** Photomicrographs showing macroscopic appearance of the gastric mucosa in Group I (control group, injected with saline as a vehicle; **A**), Group II (injected with ghrelin (40  $\mu\text{g}/\text{kg}$ ; s.c.; **B**), Group III (indomethacin was administered orally at a single dose 30 mg/kg; **C**), Group IV (ghrelin was given at 40  $\mu\text{g}/\text{kg}$ ; s.c. 30 min. before induction of gastric ulceration by indomethacin; **D**), Group V (SnPP HO-1 blocker was given at 30 mg/kg; i.v. 10 min. before administration of ghrelin, which was followed by indomethacin; **E**).

the HO-1 blocker (SnPP) (Group V) abolished the effect of ghrelin pretreatment as it resulted in a significant ( $p < 0.05$ ) increase of TNF- $\alpha$  and IL-1 $\beta$  as compared to Group IV (Table 2).

Similarly the MPO activity in IND-treated group (Group III) showed a significant ( $p < 0.05$ ) elevation compared to the control group (Group I), however, pretreatment with ghrelin as in Group IV resulted in its decrease significantly ( $p < 0.05$ ) as compared to Group III. On the other hand, the pretreatment with the HO-1 blocker (SnPP)

(Group V) abolished the effect of ghrelin pretreatment as it resulted in a significant ( $p < 0.05$ ) increase of MPO activity as compared to Group IV (Table 2).

The PGE2 levels were significantly ( $p < 0.05$ ) reduced in the IND-treated group (Group III) compared to the control group, however, pretreated rats with ghrelin (Group IV) showed a significant ( $p < 0.05$ ) elevation as compared to Group III. HO-1 blocker (SnPP) pretreatment (Group V) resulted in its significant ( $p < 0.05$ ) decrease as compared to Group IV (Table 2).

**Table 1.** Changes in ulcer score (severity), percentage of ulceration, ulcer index, preventive index and microscopic damage scores in control and differently treated groups

	Group				
	I	II	III	IV	V
Ulcer score	0	0	4.82 $\pm$ 0.11 <sup>a</sup>	1.47 $\pm$ 0.23 <sup>a,b</sup>	3.23 $\pm$ 0.30 <sup>a,b,c</sup>
% of ulceration	0	0	100%	75%	100%
Ulcer index	0	0	482 <sup>a</sup>	110.25 <sup>a,b</sup>	323 <sup>a,b,c</sup>
Preventive index	–	–	–	77.1%	32.98%
Microscopic score	0.6 $\pm$ 0.29	0.3 $\pm$ 0.12	11.9 $\pm$ 0.67 <sup>a</sup>	6.2 $\pm$ 0.70 <sup>a,b</sup>	8.3 $\pm$ 3.49 <sup>a,b,c</sup>

Data are represented as mean  $\pm$  SD,  $n = 8$ . <sup>a</sup> $p < 0.05$  significant difference compared with control group; <sup>b</sup> $p < 0.05$  significant difference compared with Group III; <sup>c</sup> $p < 0.05$  significant difference compared with Group IV. (For more information, see section Materials and Methods).

**Table 2.** Changes in pro-inflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$ ), myeloperoxidase (MPO) activity and prostaglandin E2 (PGE2) in gastric mucosa of control and differently treated groups

	Group				
	I	II	III	IV	V
TNF- $\alpha$ (pg/g)	1239.1 $\pm$ 35.6	1215.7 $\pm$ 59.1	2518.7 $\pm$ 48.6 <sup>a</sup>	1427.6 $\pm$ 73.1 <sup>a,b</sup>	2096.2 $\pm$ 73.4 <sup>a,b,c</sup>
IL-1 $\beta$ (pg/g)	538.7 $\pm$ 15.5	536.4 $\pm$ 17.7	928.9 $\pm$ 24.6 <sup>a</sup>	628.5 $\pm$ 16.6 <sup>a,b</sup>	827.8 $\pm$ 20.8 <sup>a,b,c</sup>
MPO (U/g)	9.6 $\pm$ 0.48	9.3 $\pm$ 0.42	25.4 $\pm$ 2.9 <sup>a</sup>	14.9 $\pm$ 1.4 <sup>a,b</sup>	20.1 $\pm$ 0.71 <sup>a,b,c</sup>
PGE2 (ng/g)	538.0 $\pm$ 24.9	552.3 $\pm$ 13.0	256.6 $\pm$ 30.5 <sup>a</sup>	454.4 $\pm$ 24.1 <sup>a,b</sup>	364.7 $\pm$ 8.5 <sup>a,b,c</sup>

Data are represented as mean  $\pm$  SD,  $n = 8$ . <sup>a</sup>  $p < 0.05$  significant difference compared with Group I; <sup>b</sup>  $p < 0.05$  significant difference compared with Group III; <sup>c</sup>  $p < 0.05$  significant difference compared with Group IV. TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ; IL-1 $\beta$ , interleukin 1 $\beta$ . (For more information, see section Materials and Methods).

#### *Effect of ghrelin administration on oxidative stress changes that associate with the IND-induced gastric lesions in rats and the role of HO-1*

The gastric injury induced by the IND (Group III) increased gastric MDA significantly ( $p < 0.05$ ) with a parallel significant ( $p < 0.05$ ) decrease in SOD and GSH content as compared to control rats. Pretreatment with ghrelin (Group IV) resulted in a significant ( $p < 0.05$ ) decrease in MDA and a significant ( $p < 0.05$ ) increase in GSH and SOD content as compared to Group III. Similar to other parameters, the HO-1 blocker (SnPP) pretreatment (Group V) abolished the protective effect of ghrelin as it resulted in a significant ( $p < 0.05$ ) increase regard MDA and a significant ( $p < 0.05$ ) decrease regard GSH and SOD as compared to Group IV (Table 3).

#### *Effect of ghrelin administration on HO-1 and bilirubin gastric levels*

From our data, we observed that IND administration (Group III) reduced gastric HO-1 level significantly ( $p < 0.05$ ) as compared to control group. Pretreatment with ghrelin (Group IV) significantly ( $p < 0.05$ ) increased the HO-1 level as compared to Group III, however, the pretreatment with the HO-1 blocker (SnPP) (Group V) resulted in

non-significant change in the level of the gastric HO-1 as compared to Group IV (Table 4).

The gastric injury induced by the IND (Group III) reduced the gastric bilirubin significantly ( $p < 0.05$ ) as compared to the control group, however, ghrelin pretreatment (Group IV) significantly ( $p < 0.05$ ) increased the bilirubin level as compared to Group III. Pretreatment with the HO-1 blocker (SnPP) (Group V) significantly ( $p < 0.05$ ) reduced the bilirubin level compared with ghrelin-treated group (Group IV) (Table 4).

#### *Histopathological results*

Histopathological results show that stomach of rats from control and ghrelin groups (Fig. 2A and B, respectively), by microscopic examination, revealed features of normal intact mucosa with normal mucosal thickness and perpendicular alignment of the fundic glands with microscopic scores  $0.6 \pm 0.29$  and  $0.3 \pm 0.12$ , respectively (Table 1). On the other hand, the stomach of rats treated with IND (Group III) showed sever epithelial cell loss, sever edema, marked congestion of blood vessels, necrosis, moderate hemorrhage and marked inflammatory cells infiltration (Fig. 2C and D) with a microscopic score  $11.9 \pm 0.67$  (Table 1). The microscopic examination of stomach from rats pretreated with ghrelin (Group IV) (Fig. 2E) revealed mild epithelial cell loss with maintenance of basal epithelium,

**Table 3.** Changes in oxidative stress markers, lipid peroxidation (MDA) and antioxidants (GSH, SOD) in gastric mucosa of control and differently treated groups

	Group				
	I	II	III	IV	V
MDA (nmol/g)	4.6 $\pm$ 0.2	4.5 $\pm$ 0.2	15.4 $\pm$ 0.4 <sup>a</sup>	8.3 $\pm$ 0.3 <sup>a,b</sup>	13.5 $\pm$ 0.5 <sup>a,b,c</sup>
GSH ( $\mu$ mol/g)	1.4 $\pm$ 0.1	1.4 $\pm$ 0.2	0.66 $\pm$ 0.1 <sup>a</sup>	1.1 $\pm$ 0.1 <sup>a,b</sup>	0.8 $\pm$ 0.05 <sup>a,c</sup>
SOD ( $\mu$ g)	340.5 $\pm$ 21.0	351.9 $\pm$ 16.5	229.3 $\pm$ 17.3 <sup>a</sup>	277.2 $\pm$ 8.5 <sup>a,b</sup>	246.7 $\pm$ 10.5 <sup>a,b,c</sup>

Data are represented as mean  $\pm$  SD,  $n = 8$ . <sup>a</sup>  $p < 0.05$  significant difference compared with Group I; <sup>b</sup>  $p < 0.05$  significant difference compared with Group III; <sup>c</sup>  $p < 0.05$  significant difference compared with Group IV. MDA, malondialdehyde; GSH, glutathione peroxidase; SOD, superoxide dismutase. (For more information, see section Materials and Methods).

mild congestion and edema with moderate inflammatory cells infiltration which all confirms the gross findings of protection of the mucosa with ghrelin (Fig. 1D) with a microscopic score  $6.2 \pm 0.70$  (Table 1), sever epithelial loss, moderate congestion, edema and moderate inflammatory cells infiltration with a microscopic score  $8.3 \pm 3.49$  which also confirms the reduction of the protective effect of ghrelin was observed with the use of HO-1 blocker (SnPP) in Group V (Fig. 2F).

## Discussion

The diversity of etiological factors underlying gastric ulcers and the complex nature of pathways participating in its treatment always make peptic ulcers prevention and treatment a complicated challenge. Keeping the balance between the aggressive and protective factors of the gastric mucosa is a critical objective in peptic ulcer management (El-Moselhy et al. 2009). The present study was designed to investigate the role of the HO-1 pathway in the gastroprotective effect of ghrelin against NSAIDs-induced gastric injury in rats, exploring these underlying mechanisms might provide some useful information for treatment of the gastric injury.

The results of the present study showed that administration of IND at a single dose (30 mg/kg; p.o.) caused multiple mucosal injuries by gross examination along with significant increase in gastric U.I as compared with control rats. This result was in agreement with previous studies (Heeba et al. 2009; Kim et al. 2011) that reported an increase in U.S and U.I in IND-treated rats. Moreover, these results were confirmed by histopathological examination of gastric mucosa in IND-administered rats, as it showed epithelial loss, congested blood vessels with inflammatory cells infiltration. IND-induced mucosal injury is attributed to various processes, including infiltration of leukocytes, inhibition of PGE<sub>2</sub>, initiation of lipid peroxidation, decreasing the levels of antioxidants, and induction of apoptosis (Sabina et al. 2007).

In the present study, IND significantly elevated gastric TNF- $\alpha$  and IL-1 $\beta$  levels along with increased MPO activity that is considered an index of PMN cells accumulation into gastric tissue as compared to control group. These results are in accordance with the work of other investigators (Sabina

et al. 2007; Aliye et al. 2012). PMN migration is an early and critical event in the pathogenesis of gastric mucosal injury caused by IND. TNF- $\alpha$  causes PMN migration through upregulating the expression of adhesion molecules in both neutrophil and endothelial cells (de Souza et al. 2011).

PG, a key molecule that has a gastroprotective effect and stimulates the ulcer healing mechanism, is synthesized in the mucosal cells by cyclooxygenase (COX) enzymes. It stimulates the secretion of bicarbonates and mucus, maintains mucosal blood flow, regulates mucosal turnover, and induces angiogenesis (Adhikary et al. 2011). Our experimental results were in line with previous data (Brzozowski et al. 2005), that showed that exposure to IND significantly reduced gastric mucosal PGE<sub>2</sub> level. This result might be explained by the inhibition of COX activity and by the conversion of PGs into products of oxidation such as 8-iso-PGF<sub>2</sub> alpha in the presence of oxidative damage (Natale et al. 2004). In addition, oxidative stress could inhibit COX activity, thus reducing PG levels (Fujimoto et al. 2004).

Experimental studies have shown that ROS play certain roles in IND-induced gastric mucosal damage (Suleyman et al. 2009). Agents such as IND initiate lipid peroxidation by functioning as oxidants and cause damage by producing ROS. A number of enzymatic and non-enzymatic defense mechanisms such as SOD and GSH respectively reduce or prevent the oxidative tissue damage of gastric mucosa after administration of NSAIDs (Polat et al. 2011). These enzymes play an important role in the elimination of oxygen free radicals and lipid hydroperoxides in the gastric mucosal cell (Kim et al. 2011). Previous studies demonstrated an increase in MDA with a decrease in antioxidants such as GSH and SOD in gastric mucosa of IND-treated rats (Abdallah et al. 2011; Kim et al. 2011). Consistent with these findings, this work revealed that administration of IND significantly increased the lipid peroxidation (MDA) and decreased the level of non-enzymatic (GSH) and the activity of enzymatic (SOD) antioxidants in the gastric mucosa of an IND-administered rat.

In the present work IND significantly reduced the gastric mucosal HO-1 in spite of considering IND as one of cellular stress inducers. These results are supported by the studies of Song et al. (2008) and Aburaya et al. (2006) who reported that IND did not upregulate HO-1 expression but lead to gastric

**Table 4.** Changes in heme oxygenase-1 (HO-1) and bilirubin levels in gastric mucosa of control and differently treated groups

	Group				
	I	II	III	IV	V
HO-1 (pg/mg)	1.7 $\pm$ 0.1	2.4 $\pm$ 0.2 <sup>a</sup>	1.3 $\pm$ 0.1 <sup>a</sup>	2.4 $\pm$ 0.1 <sup>a,b</sup>	2.3 $\pm$ 0.05 <sup>a,b</sup>
Bilirubin (mg/dl)	0.07 $\pm$ 0.006	0.3 $\pm$ 0.03 <sup>a</sup>	0.02 $\pm$ 0.007 <sup>a</sup>	0.36 $\pm$ 0.03 <sup>a,b</sup>	0.04 $\pm$ 0.01 <sup>a,b,c</sup>

Data are represented as mean  $\pm$  SD,  $n = 8$ . <sup>a</sup>  $p < 0.05$  significant difference compared with Group I; <sup>b</sup>  $p < 0.05$  significant difference compared with Group III; <sup>c</sup>  $p < 0.05$  significant difference compared with Group IV. (For more information, see section Materials and Methods).

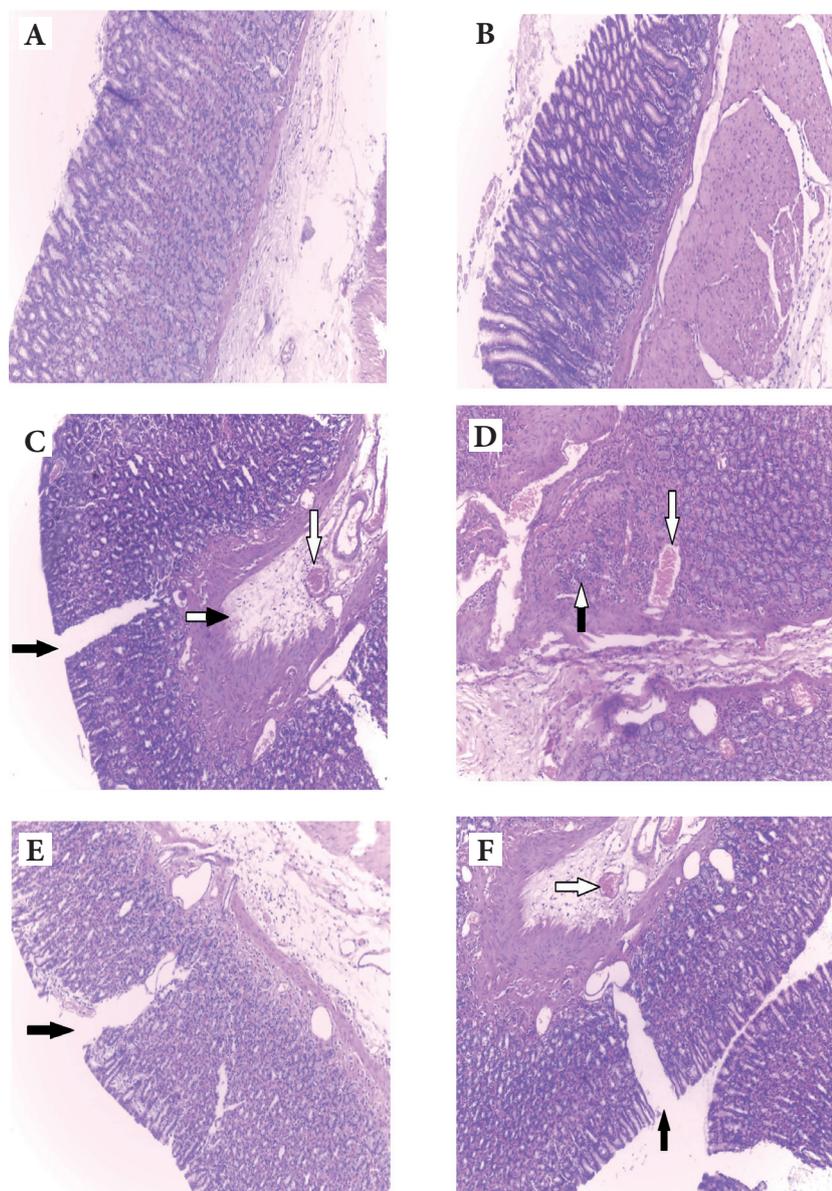
mucosal damage through both necrosis and apoptosis. This reduction was associated with a subsequent decrease in the gastric bilirubin level as an indicator of the HO-1 activity (Huang et al. 2005).

Pretreatment of rats with ghrelin (40  $\mu\text{g}/\text{kg}$ ; s.c.), before induction of gastric injury, resulted in amelioration of gastric injury. Specifically, it significantly decreased the gastric U.I showing P.I 77.1% as compared with IND group. These observations were confirmed by histopathological examination that showed a reduction of the depth and severity of IND induced gastric mucosal lesions. These results are in agreement with previously published results (Sibilia et al. 2003; Konturek et al. 2004; Khalefa et al. 2010); it was reported that exogenous ghrelin administration attenuates the experi-

mentally induced gastric mucosal lesions and exert certain gastroprotective effect with several possible mechanisms underlying the protective effect of ghrelin.

In contrast to our findings, Suzuki et al. (2006) reported that plasma ghrelin levels were significantly higher in patients with peptic ulcer than in those with gastritis without ulcer, which suggests a possible relationship between mucosal injury susceptibility and the elevated plasma ghrelin.

In order to explain the protective effect of ghrelin on gastric injury induced by IND plasma  $\text{TNF-}\alpha$  and  $\text{IL-1}\beta$ , as markers for inflammation, together with gastric  $\text{PGE}_2$  were measured. We also measured MDA, GSH, and SOD content, as markers for the oxidative stress and the HO-1 gastric level together with bilirubin.



**Figure 2.** Histological evaluation (H&E) of the gastric mucosa in Group I (A); Group II (B) showing normal intact mucosa with normal mucosal thickness and perpendicular alignment of the fundic glands; Group III (C, D) showing epithelial cell loss, submucosal edema, congestion of blood vessels and inflammatory cells infiltration (white arrowhead); Group IV (E) showing mild epithelial cell loss with reduction in the severity of gastric mucosal lesions; Group V (F) showing congested blood vessels with epithelial loss. Black arrow; epithelial cell loss; black arrowhead, submucosal edema; white arrow, congestion of blood vessels; white arrowhead, inflammatory cells infiltration. (Magnification  $\times 200$ ). (For more information, see section Materials and Methods).

The reduction of the inflammatory markers including, gastric levels of TNF- $\alpha$  and IL-1 $\beta$  and the MPO activity, suggesting that the gastroprotective effect of ghrelin is in part dependent on its inhibitory effect on neutrophil infiltration and the neutrophil-associated TNF- $\alpha$  and IL-1 $\beta$  response. This is in accordance with Konturek et al. (2004), who demonstrated that the treatment with ghrelin caused a dose-dependent decrease in TNF- $\alpha$  mRNA expression indicating an important anti-inflammatory effect of this peptide and Dixit et al. (2004) who reported that ghrelin also is known to exert certain effects on the immune system including inhibition of expression of the pro-inflammatory cytokines by human monocytes and T lymphocytes, this effect of ghrelin is mediated directly through the ghrelin receptors present on immune cells.

The gastric PGE<sub>2</sub> level increased in pretreated rats with ghrelin indicating that PGs contribute to the gastroprotective effect of ghrelin. Our results are in agreement with those of Brzozowski et al. (2006) and Sibilia et al. (2008), who proved that COX-1-derived PGs are mainly involved in ghrelin gastroprotection activity. In addition pretreatment with ghrelin in rats subjected to gastric injury was associated with reduction in the lipid peroxidation indicated by the reduction of MDA gastric level together with increased antioxidant activity indicated by GSH and SOD gastric levels these results are going with those of İşeri et al. (2005), who demonstrated that ghrelin administration ameliorates the oxidative injury of the gastric tissue, which appears to involve the inhibition of toxic oxygen metabolite generation, and restoration of tissue GSH which is considered one of the major mechanisms of reducing oxidative stress, either by increasing the synthesis or by inhibiting the depletion of this crucial antioxidant. Also Yada et al. (2006) provided *in vitro* studies verified that ghrelin increases the mRNA levels of SOD, implicating an antioxidant mechanism of ghrelin. All these data suggest that ghrelin directs the redox reactions toward the reduction state thus reducing the reactivity of oxygen free radicals with other molecules.

Our results have shown that the gastric HO-1 level and its activity increased with pretreatment of ghrelin in IND-treated rats. HO-1 is considered to be a cytoprotective enzyme because each of the products of heme breakdown including CO, iron and biliverdin which is changed into bilirubin plays its own protective role (Ryter et al. 2002). Recent studies have shown that induction of HO-1 has a gastroprotective effect due to the formation of biliverdin and bilirubin with their antioxidant properties, and release of CO, which has anti-inflammatory properties (Uc et al. 2012), another possible explanation was reported by Nakao et al. (2008), who reported that a possible explanation for the protective role of HO-1 may lie in the removal of free heme. Free heme has been implicated in the conversion of H<sub>2</sub>O<sub>2</sub> to more reactive hydroxyl radicals and promoting more severe tissue damage by propagating lipid peroxidation; however,

the pretreatment with SnPP, HO-1 blocker, (30 mg/kg; i.v) resulted in reduction of HO-1 activity without affection of its level as compared with ghrelin-pretreated group, moreover SnPP ameliorated the gastroprotective effect of ghrelin pretreatment as it resulted in significantly increased U.I with P.I 32.98% as compared with pretreated group with ghrelin, which was confirmed histopathologically. These findings suggest that the gastroprotective effect of ghrelin against IND-induced gastric injury involves the HO-1 pathway. For our knowledge, this is the first study to show that the decrease in severity of gastric mucosal injury in rats pretreated with ghrelin depends on the HO-1 enzyme activity.

## Conclusion

In conclusion, the present study revealed that ghrelin pretreatment could improve the outcome of IND-induced gastric injury in rats. This protective effect could be explained on the basis of PG formation, anti-inflammatory and antioxidant effects. We also demonstrate for the first time that HO-1 activation significantly contributes to the gastroprotective effect of ghrelin.

**Conflict of interest.** The authors declare that they have no conflict of interest.

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