Research Article

Protective effect of hemin against indomethacin-induced gastric ulcer; role of hemoxygenase enzyme

Rasha K. Khalifa, Ashraf Taye, Mohamed A. El-Moselhy
Department of pharmacology & Toxicology, Faculty of Pharmacy, Minia University, Egypt.

Abstract
Non steroidal anti-inflammatory drugs are widely used and effective against inflammatory diseases. However, its clinical use is limited by its ulcerogenic effect. This study aimed to investigate the gastro-protective effect of hemin on IND- induced gastric ulcer in rats and if by using HO-١ inducer hemin and HO-١ blocker ZnPP. Rats were assigned into four groups, group١ served as control, group ٢ treated i.p with IND (٥٠mg/kg b.w), group ٣ pretreated with hemin (١٠mg/kg b.w), group ٤ pretreated with ZnPP+ hemin all drugs taken for ٧ successive days and the last day IND was injected to all rats. Our results revealed that IND induced gastric damage as evidenced by observed ulcers, histological changes and increased oxidative and inflammatory parameters and we showed that hemin has a gastro-protective effect and increased the PH of the gastric juice, increased catalase enzyme, HO-١ activity and this gastro-protective effect was deccreased in combination with HO-١inhibitor ZnPP .so our study revealed that HO-١has a role in the gastro-protective effect of hemin.
Key words: hemin, ulcer, HO-١

Introduction

Peptic ulcers are common and represent a health problem. Its incidence is increasing due to rapid development and civilizational constraints. The estimates of peptic ulcer incidence vary ranging from ٣٪ to ١٠٪ worldwide**.**

Although the introduction of histamine $H_٢$ receptor blockers and proton pump inhibitors has allowed great progress in the treatment of peptic ulcer search for new drugs continues.

Gastric ulcers are circumscribed erosions of the gastric mucosa that may penetrate the muscle layer and perforate the stomach wall. Gastric ulceration is characterized by episodes of burning, epigastric pain, belching and nausea especially when the stomach is empty or after ingesting certain foods. Gastric ulceration is a multifaceted disease with a complex pluricausal etiology that is not fully understood**.** It is associated with an imbalance between defensive mecha-nisms and aggressive factors allowing the latter to predominates and cause mucosal damage.

Non steroidal anti-inflammatory drugs like indomethacin are widely used in the treatment of pain, fever and inflammation. However, these drugs have some side effects, especially on the gastrointestinal tract. Indomethacin and other non steroidal anti inflammatory drugs (NSAIDs), cause gastric erosion and ulceration (Fries, Miller et al., ١٩٨٩).

These injurious effect have been attributed to depletion of prostaglandin via the inhibition of the enzyme cycloxygenase (Simmons, Botting et al., ٢٠٠٤) however, this may not be the sole mechanism of injury. In rats low doses of asprin that significantly inhibit gastric mucosal prosta-glandin synthesis may not induce gastric mucosal injury (Ligumsky, Golanska et al., ١٩٨٣). other possible mechanisms of NSAIDs include increased mucosal production of pro-inflammatory cytokines (Koizumi, Odashima et al., ٢٠٠٩) increased production of reactive oxygen species and increased lipid peroxidation (Naito and Yoshikawa ٢٠٠٦). In addition it was reported that the increased ulcerogenic response to indomethacin is mediated through over-expression of inducible nitric oxide synthase (i-NOS) (Maity, Banerjee et al., ٢٠٠٩). Therefore it was obvious that gastric damage induced by indomethacin is multi-factorial involving leukocyte infilt-ration, free radicals formation and disturbance in nitric oxide production in gastric tissue. Heme oxygenase-١(HO-١) is a stress inducible protein ,which stimulate oxidative degradation of heme, so eliminating the potentially toxic free-heme, but releasing bilverdin ,carbon monoxide (CO) and ferrous iron.Biliverdin is then converted to biliruben by biliverdin reductase (Pae and Chung ٢٠٠٩).

The biological importance of hemeoxy-genase (HO) originates from its function as the rate-limiting enzyme in heme cata-bolism. Heme is oxidatively cleaved by the HO system into equimolar quantities of carbon monoxide (CO), biliverdin, and $Fe^{٢+}$; and in a coupled reaction biliverdin is rapidly converted into bilirubin via biliver-din reductase (Kapitulnik and Maines ٢٠٠٩). Three distinctive HO isoforms have

been identified and although they catalyze the same biochemical reaction they are the products of different genes with different expression patterns in cells and tissues (Maines ١٩٩٧). HO-٣ is a poor heme catalyst that has been found only in rat brain with no activity reported in humans (Elbirt and Bonkovsky ١٩٩٩); HO-٢ which contributes to cell homeostasis is constitutively expressed in many tissues including neuronal and testicular tissue; whereas HO-١, also known as heat shock protein-٣٢ (Hsp٣٢), is stress-inducible and expressed at a relatively low level in most tissues. In addition to its substrate heme, HO-١ is upregulated by heavy metals (Maines ١٩٨٤) and stimuli that cause oxidative stress such as heat shock, ischemia, hemorrhagic shock , reactive oxygen species (ROS) (Cooper, Liu et al., ٢٠٠٩), radiation, and hypoxia. Many reports have also shown that inflammatory mediators such IL-١, TNF-α, LPS, ROS and reactive nitrogen species (RNS) are able to upregulate HO-١ in vitro.

HO-١ induction is usually associated with a protective response (Niess, Passek et al., ١٩٩٩); classically the beneficial nature of HO is attributed to its ability of removing free heme, which has cytotoxic effects. However, new evidence indicates that although HO-١ as such does not directly catalyze an antioxidant reaction, its upregulation, and the production of CO and biliverdin, influences many biological events linked to a cytoprotective and anti-inflammatory response against oxidative stress (Abraham, Rezzani et al., ٢٠٠٤). For instance CO is believed to act as a signaling molecule in a similar manner as nitric oxide (NO), with anti-inflammatory and anti-apoptotic properties (Otterbein, Bach et al., ٢٠٠٠). Biliverdin and bilirubin are reducing agents with antioxidant properties and have the ability to efficiently scavenge proxyl radicals and inhibit lipid peroxidation (Stocker, Yamamoto et al., ١٩٨٧). Although biliverdin is rapidly converted to bilirubin and has a short half-life, in an recycling process bilirubin as a potent antioxidant oxidizes itself back to biliverdin. Treatment with biliverdin decrease mRNA expression of inducible nitric oxide synthase (Ueda, Ueyama et al.,), cyclooxygenase ٢, and the inflammatory cytokines IL-٦ and IL-١β, as well as decrease neutrophil infiltration into the jejunal muscularis in rat model of small intestinal transplants (Nakao, Otterbein et al., ٢٠٠٤). Moreover, biliverdin is an endogenous ligand of the aryl hydrocarbon receptor (El-Ashmawy, Khedr et al.,), which upon activation protects against experimental acute pancreatitis by induction of IL-٢٢ (Balla, Jacob et al., ١٩٩٢). Similarly; $Fe^{٢+}$ is involved in gene regulation including that of NO synthase (NOS). Although potentially toxic, $Fe^{٢+}$ leads to the opening of channels that export $Fe^{٢+}$ from the cells inducing the upregulation of ferritin, an iron storing protein which protects cells against oxidant damage by oxidation of low-density lipoproteins (Balla, Jacob et al., ١٩٩٢).

ZnPP is a metalloprotein has also been used chronically to inhibit HO-١ activity so the aim of this work is to study the gastro-protctive effect of hemin alone or in combination with ZnPP as HO-١ inhibitor.

Materials and methods

Chemicals:

In the present study, Indomethacin, hemin, ZnPP were obtained from Sigma, alpha lipoic acid was a kind gift from Mepaco Pharmaceutical Egypt, Commercial kits for detection of Malonaldialdhyde cat# STA-٨٣٢, catalase cat# STA-٣٤١ and nitrate/nitrites were purchased from Biodiagnostic company, Egypt, PGE٢ kits cat# EHPGE٢ was purchased from Thermo-fisher

Animals:

A total of ٣٢ male Sprague-Dawley rats, weighing ١٥٠ to ٢٠٠ g purchased from (Othman animal house, Giza). Animals were housed at room temperature with ١٢:١٢ h light/dark cycles and were given food and water libitum. Experiments were conducted in accordance with the international ethical guidelines for animal care of the United States Naval Medical Research Center, Unit No. ٣, Abbaseya, Cairo, Egypt, Accredited by the Association for Assessment and Accreditation of Laboratory Animal Care international (AAALAC international). The adopted guidelines are in accordance with "Principles of Laboratory Animal Care "(NIH publication no. ٨٥-٢٣, revised ١٩٨٥). The study protocol was approved by members of" the research ethics committee" and by the pharmacology and toxicology department, Faculty of Pharmacy , Minia University, Egypt.

Drug protocol:

Hemin and ZnPP were freshly dissolved in ٠.١ mol/L NaOH adjusted to PH ٧.٤ with ٠.١ mol/L HCl and diluted with saline to the required volume. Hemin and ZnPP were prepared in darkness and protected from light (Ibrahim, El-Sayed et al., ٢٠١٢)

Experimental procedures (induction of gastric ulceration):

All rats were fasted for ٢٤ h prior to the study and housed in raise mesh –bottomed cages to minimize coprophagia, with free access to water . All experiments were performed at the same time of the day to avoid variations due to diurnal rhythms of putative regulators of gastric function. Rats were administered either IND (٥٠ mg/kg i.p, groups) or vehicle (control group). Each drug was administered i.p for ٧ days before induction of gastric ulceration by IND.

١- Control group: injected with saline i.p in a volume equal to the total volume of liquid administered to the experimental rats

٢- Non- pretreated indomethacin group received no further medication other than

١ml indomethacin (٥٠ mg/kg i.p) (Hangaishi, Ishizaka et al., ٢٠٠٠)
٣- Hemin (١٠ mg/ kg i.p) (Costa, Silva et al., ٢٠١٣)+ IND in which hemin administered for ٧ days prior to induction with IND.
٤- ZnPP+ hemin+ IND gp in which ZnPP ٥٠ µg/kg was administered one hour before hemin ١٠mg/kg for ٧ days prior to IND administration in the seventh day.

The animals were sacrified using sodium thiopental ٦ hours after IND. Stomachs were removed and opened along the greater curvature, washed with normal saline solution and scored for macroscopic mucosal lesions. Another sets of stomachs from each subgroup was fixed in isotonic ١٠٪ formalin solution for histo-pathological examination.

Assessments of gastric mucosal lesions:
Gastric mucosal lesions were expressed in terms of ulcer index (U.I) as previously described which depend on the calculation of lesions index by using a ٠-٣ scoring system with score ٣ denoting the highest severity. The U.I for each group was calculated as the total number of lesions multiplied by their severity score.

Determination of the gastric juice pH:
The pH of the gastric juice was determined as previously described (Moore ١٩٦٨) using a pH meter. Briefly the pH meter was first caliberated using commercial pH buffers, pH of the unknown juice was then determined. In between a given run of readings, measurements were intermittently taken with a known pH buffers to check for the possible drift during analysis.

Analysis of the gastric mucosa:
Immediately before analysis, stomachs of rats in the various groups, were left to reach room temperatures and by a scalpel blade were scrapped gently on a piece of parafilm to separate the gastric mucosa. The gastric mucosa was then used afterwards for the determination of the levels of lipid peroxides,, NO, PGE٢, the acyivity of HO-١and catalase .

Determination of lipid peroxidation in gastric mucosa:
Lipid peroxidation was determined as thiobarbituric acid reactive species (TBARS), as previously described (Mihara and Uchiyama ١٩٧٨) and the results were extrapolated from a standard curve of (MDA), which is the break-down product of lipid peroxides.

Determination of catalase activity:
Catalase activity was determined according to (Aebi ١٩٨٤). Briefly, decomposition of $H_٢O_٢$ was followed at ٢٤٠ nm. Catalase activity was defined as the amount of enzyme required to decompose ١ millimole of $H_٢O_٢$ per minute at ٢٥^{o} C and pH ٧.٨. Results are expressed as millimole per minute per milligram tissue (mmol/min/mg tissue).

Determination of $PGE_٢$ level in gastric mucosa:
Gastric mucosal $PGE_٢$ was determined by an enzyme- linked immunosorbent assay (ELISA) using immuno assay kit (Thermofisher according to manufacturer's instruction). And based on the competitive binding techniques in which $PGE_٢$ present in a sample competes with a fixed amount of horseradish peroxidase (HRP)- labeled $PGE_٢$ for sites on the mono-clonal antibody (Fries, Miller et al., ١٩٨٩).

Determination of gastric mucosal NO:
Gastric mucosal NO was determined using colorimetric determination of NO (Biodiagnostic, Egypt). The assay is based on the enzymatic conversion of nitrate to nitrite by nitrate reductase. The reaction is followed by colorimetric detection of nitrite as an azo-dye product and the concentration of nitrite is determined by the Griess reaction .

Determination of HO-١ activity:
Activity of HO-١ in mucosal tissue homogenates was carried out as previously described**,** Briefly, ١٠٠ mg of frozen gastric tissue were homogenized in ١ml of saline, aliquots (٥٠٠ µl) of homogenates were added to ٢٥٠ mg of $BaCl_٢$ and vortex-mixed thoroughly then, ٠.٧٥ ml of benzene was added to the mixture, and tubes were vigorously vortex- mixed again. The benzene phase containing extracted bilirubin was separated from the aqueous phase by centrifugation at ١٣,٠٠٠ rpm for ٣٠ minutes. A standard bilirubin curve was measured spectrophotometrically, as absor-bance difference between ٤٥٠ and ٦٠٠ nm and expressed as mg/dl.

Statistical analysis:
Data is expressed as Mean±S.E.M,. tatistical evaluation was performed by ANOVA followed by the Tukey's multiple comparison test. All analysis were made with the statistical software Graphpad prism (version ٥.٠٠ for windows, Graph pad software, San Diego California.

Results

١- Effect of hemin (١٠mg/kg i.p) on gastric lesion development and its alteration by ZnPP combinations:
Figure (١A) showed that IND (٥٠mg/kg,ip) significantly induced the ulcer index compared to control group with p value <٠.٠٥ IND (٥٠mg/kg,ip) significantly induced the ulcer index compared to control group with p value<٠.٠٥ while pretreatment of rats with hemin(١٠mg/kg) significantly reduced the U.I from ٩.٠٦±٠.٢٤ for IND treated gp to ٣.٥+٠.٢٨ for hemin+IND gp. On the other hand using combination of ZnPP+

hemin before IND non significantly reduced the U.I compared to IND and significantly increased the U.I compared to hemin +IND gp.

٢- Effect of hemin (١٠mg/kg) on pH of gastric juice and its alteration by ZnPP combination:

Figure (١B) show that IND (٥٠mg/kg ip) significantly reduced the PH of the gastric juice compared to control group (p<٠.٠٥), while pretreatment of IND treated rats with hemin significantly elevated the PH from ٢.٨±٠.١ for IND treated gp to ٤.١٢±٠.٠٤ for hemin+IND gp. On the other hand pretreatment of rats with combination of ZnPP+ hemin non significantly produced any change compared to IND treated gp and significantly reduced the PH compared to hemin pretreated gp.

٣- Effect of hemin on lipid peroxidation and its alteration by ZnPP combination:

Figure (٢A) show that IND significantly increased lipid peroxidation compared to control group while pretreatment of IND treated rats with hemin significantly reduced the MDA level from ٥.٩٦+٠.١٦ for IND gp to ٢.٤+٠.٠٤ to hemin +IND gp. Also ZnPP+ hemin pretreatment significantly decreased the MDA level compared to IND gp. On the other hand ZnPP+hemin+IND gp significantly increased the MDA level compared to hemin pretreatment.

٤- Effect of ALA on gastric mucosal catalase activity and its alteration by other combination

Figure (٢B) show that IND significantly decreased the catalase activity compared to control group while pretreatment of IND treated rats with hemin significantly increased the catalase activity from ٠.٥٢±.٠٣٣ for IND treated rats to ١.٨±٠.٠١ for hemin +IND gp. Also pretreatment of induced rats with ZnPP+hemin significantly increased the catalase activity compared with IND gp but with lower significance. On the other hand ZnPP+ hemin +IND gp significantly reduced catalase activity compared to hemin +IND gp.

٥- Effect of hemin on HO-١ activity and its alteration by ZnPP combination:

Figure (٣) show that HO-١ activity was significantly decreased by IND which was modulated by pretreatment with hemin which significantly increased HO-١ activity from ٢٩٧.٣±٥.٢٣ for IND treated gp to ٦٨١.٣±٢٢.٦٧ for hemin pretreated gp. Morever pretreatment with ZnPP+hemin produced no change compared to IND treated gp while reduced the HO-١ activity compared to hemin+IND gp.

٦- Effect of hemin on total gastric mucosal nitrites content and its alteration by ZnPP combination:

Figure (٤A) show that IND significantly increased gastric mucosal nitrites compared to control gp and pretreatment of IND induced rats with hemin decreased the total gastric mucosal nitrites compared to IND treated gp Also pretreatment of induced rats with ZnPP+ hemin significantly reduced the total gastric mucosal nitrites compared to IND treated gp. On the other hand Znpp+hemin+Ind gp significantly increased the NO compared to hemin+IND gp.

٧- Effect of hemin on PGE٢ and its alteration by ZnPP combination:

Figure (٤B) show that IND significantly decreased PGE٢ compared to control group and pretreatment of IND induced rats with hemin drugs significantly decreased PGE٢ compared to IND. Also using combination ZnPP with hemin significantly decreased PGE٢ compared to IND but increased PGE٢ compared to hemin+IND gp.

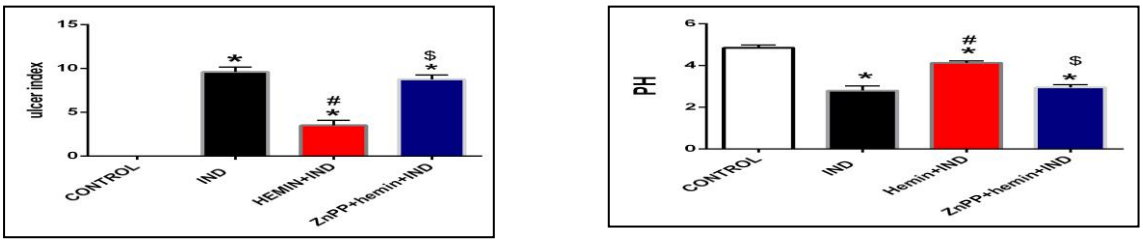

Figure.١. Effect of hemin alone or in combination with ZnPP as HO-١blocker on (A) Ulcer index of the gastric juice, (B) PH, Data are presented as mean±SEM n=٨ rats per group *p<٠.٠٥ vs control, #p<٠.٠٥ vs IND, $ P<٠.٠٥ vs hemin+IND.

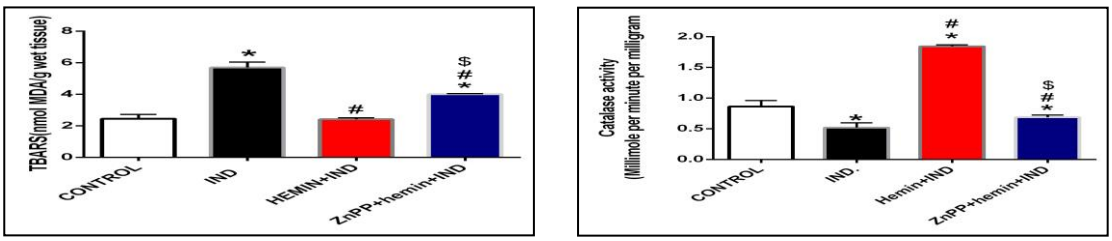

Figure.٢. Effect of hemin alone or in combination with ZnPP as HO-١ blocker on (A)MDA and(B) catalase activity. Data are presented as mean±SEM n=٨ rats per group * p<٠.٠٥ vs control,# p<٠.٠٥ vs IND, $ P<٠.٠٥ vs hemin +IND .

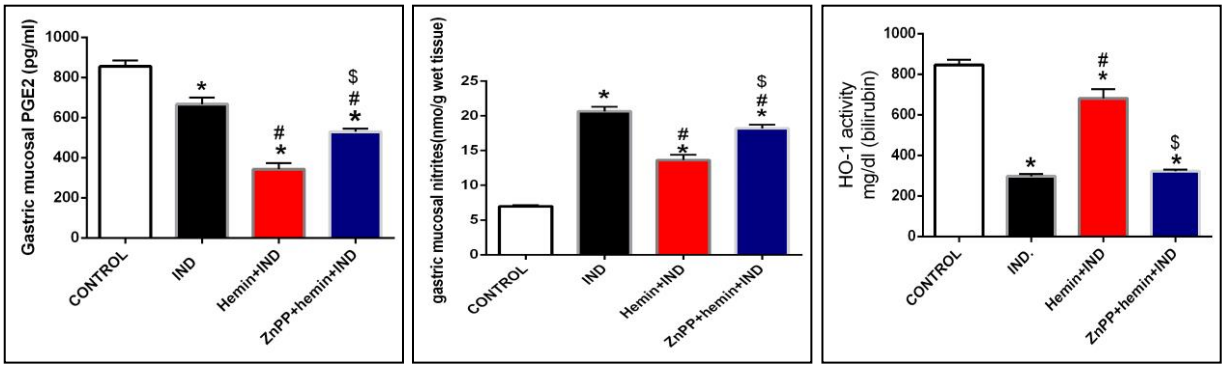

Figure.٣. Effect of hemin alone or in combination with ZnPP as HO-١blocker on (A) PGE٢ (B) gastric mucosal nitrites, (C) HO-١ activity. Data are presented as mean±SEM n=٨ rats per group * p<٠.٠٥ vs control,# p<٠.٠٥ vs IND, $ P<٠.٠٥ vs hemin +IND

Discussion:

Many reports have demonstrated that hemeoxygenase and in particular HO-١ may play a role in the resolution of an acute inflammatory reactions. The present study aimed to investigate the possible role of HO-١ inducer hemin in IND-induced gastric ulcer formation in rats.In the present study, we demonstrated that hemin significantly increased HO-١ activity in IND-induced ulcerated rats and significantly reduced the ulcer index, lipid peroxidation PGE٢ and increased catalase activity and the PH of the gastric juice .Furthermore, we showed that these effects were reversed by using a combination of hemin and HO-١ inhibitor, ZnPP.

HO is the rate limiting enzyme in heme catabolism leading to the generation of biliverdin, free iron, and carbon monoxide(CO)(Pae and Chung ٢٠٠٩) the physiological function of CO has become subject to intensive research in recent years, while the studies on the gastrointestinal tract have been at the forefront of these investigations

The data of the present study clearly demonstrated that hemin pretreatment proved to be the inducer of HO enzyme as evidenced by increased HO-١. These data are consistent with the findings of. On the other hand, the pretreatment with zinc protoporphyrin, the HO inhibitor in the present study, significantly reduced the gastric mucosal HO-١ level. These findings are in accordance with (Ueda, Ueyama et al., ٢٠٠٨). The precise mechanism of HO-١ induction is not known. Many inducible genes are expressed in response to activation of various transcriptional factors by a variety of inducing agents. IND significantly reduced the gastric mucosal HO-١ inspite of considering IND as one of cellular stress inducers. These results are supported by the studies of (Song, Shin et al., ٢٠٠٨) . (Aburaya, Tanaka et al., ٢٠٠٦) reported that short-term treatment with a high concentration of IND as that used in this study did not upregulate HO-١ expression but lead to gastric mucosal damage through both necrosis and apoptosis mediated by increased membrane permeability and intracellular Ca^{++}.

The choice of utilizing indomethacin in our ulcer model was because non steroidal anti-infl ammatory drugs (NSAIDs)-induced gastropathies are very common. The molecular basis for the gastrointestinal toxicity of NSAIDs is widely believed to be attributed to their inhibitory activity against cyclooxygenase, which causes them to block the production of prostaglandins. Suppression of prostaglandin synthesis is associated with reduction in gastric mucosal blood flow (GMB), disturbance of microcirculation and decrease in mucus secretion, which are involved in the pathogenesis of gastrointestinal mucosal disorders (Naito, Iinuma et al., ٢٠٠٨).

The data of the present study clearly demonstrated that IND evidently induced ulcers in accordance with the observations of several researchers (Bhattacharya, Ghosal et al., ٢٠٠٦). This occurs by enhancing the aggressive factors as evidenced by increased all acid parameters, proteolytic activity, and lipid peroxide level, as well as by counteracting the defensive factors as evidenced by decreased PGE٢ level. IND significantly increased gastric acid secretion and decreased the PH of the gastric juice (Konturek, Konturek et al., ٢٠٠٥).
Increased gastric acid parameters observed with IND owed to COX inhibition with reduction in PG which are known to inhibit gastric acid secretion (Simmons, Botting et al., ٢٠٠٤).

In agreement with other investigators, the present study observed that IND increase the gastric mucosal lipid peroxides level as compared to control group (Bhattacharya, Ghosal et al., ٢٠٠٦). This increase in lipid peroxidation is a result of the state of oxidative stress and ROS induced by stress.

Infiltration and activation of phagocytes (especially neutrophils) brought about by proinfl amatory cytokines such as interleukin-١β (IL-١β) and tumor necrosis factor-α (TNF-α), and the activation of phagocyte xanthine oxidase and NADPH oxidase enzymes in the gastric mucosa are among the most important sources of ROS under stress conditions (Utsumi, Yasukawa et al., ٢٠٠٦). In the present work IND significantly reduced mucosal PGE٢ which agrees with the previous reports (Khayyal, Seif-El-Nasr et al., ٢٠٠٦).

In our study IND increased NO level this is in accordance with (Takeuchi, Yokota et al., ٢٠٠٦) that showed that i-NOS activity was increased in animals with gastric inflammation and in patients with crohn's disease or ulcerative colitis. (Kimura, Miura et al., ١٩٩٧) showed that after IND induction an initial rise in TNF-α and IL-١β increases i-NOS expression and therby increase NO level resulting in gastric injury. The mechanism by which TNF-α modulates i-NOS is un clear but NF-κB has been implicated in invitro studies (Baeuerle and Baltimore ١٩٩٦).

The protective effect of hemin against ulcer development; in IND ulcer model may be due to its inhibitory effect on gastric acid secretion and proteolytic activity found in this study. The inhibitory effects of hemin on gastric acidity could be due to the effect of the produced CO in decreasing histamine release by downregulating mast cell function through decreasing the free cytosolic calciumand increasing cAMP and cGMP levels (Di Bello, Berni et al., ١٩٩٨)

Another explanation for the protective effect of hemin pretreatment may be due to the anti-oxidative effect of this drug as evidenced by decreasing the gastric mucosal lipid peroxides level. This could be attributed to HO-١ induction which is in agreement with other investigators (Vesely, Exon et al., ١٩٩٨). (Naito, Iinuma et al., ٢٠٠٨) reported that the possible explanation for the protective role of HO-١ may lie in the removal of free heme. Free heme has been implicated as the source of catalytic iron that would participate in the Fenton reaction, converting H٢O٢ to more reactive hydroxyl radicals and promoting more severe tissue damage by propagating lipid peroxidation.Furthermore, because HO-١ functions by catabolizing the heme to biliverdin, iron and CO, these byproducts of heme degradation are believed to be effector molecules underlying the potent cytoprotection observed with the HO system.

In the present study, hemin pretreatment in ulcer model significantly decreased the gastric mucosal PGE٢ level. This is in accordance with the previously reported data of (Li Volti, Ientile et al., ٢٠٠٤) .This could be attributed to HO-١ induction reducing the cellular heme. This influences the rate of arachidonic acid acylation or reacylation, the balance of which determines the amount of arachidonic acid available for prostaglandin synthesis (Haider, Olszanecki et al., ٢٠٠٢). Hemin pretreatment in IND ulcer model also significantly decreased the NO level either by COX inhibition that decreased intracellular Ca٢+ (since Ca٢+ is a key regulator of NOS activity) (Mollace, Muscoli et al., ٢٠٠٥), and also by HO-١ induction that degraded the heme located in the active site of NOS leading to a greater decrease in NO level when compared with IND group (Shen, Zhou et al., ٢٠٠٦).

NO donors were found to be protective against different types of gastric ulcer models while NO synthase inhibitors were ulcerogenic. These results seem contra-dictory to the results of the present work since hemin pretreatment significantly decreased the gastric mucosal NO level. These findings support a protective effect of endogenous CO independent of NO production. NO donors were found to be protective against different types of gastric ulcer models while NO synthase inhibitors were ulcerogenic. These results seem contradictory to the results of the present work since hemin pretreatment significantly decreased the gastric mucosal NO level. These findings support a protective effect of endogenous CO independent of NO production.

NO has a beneficial hemodynamic effect as well as a cytotoxic effect, depending on the site and rate of NO production and chemical fate of the NO produced. The cytotoxicity of NO is mediated by generation of peroxynitrite and nitrosylation of thiols, as well as by impairment of iron-sulfur clusters of proteins. The detrimental effects of nitric oxide reactive species including NO and peroxinitrite can be partially compensated by the induced expression of HO-١ as it offers a strong antioxidant protection. Furthermore, increased CO production has the potential to inactivate NOS, and thus to reduce the production of nitric oxide reactive species. The ndpoints of this feedback loop would be that the reduced NO transformation reduces oxidative stress and that increased CO production has NO-equivalent signaling functions such as stimulation of sGC and activation of K channels (Wu and Wang ٢٠٠٥).

On the other hand in our result using combination of hemin and zinc protoporphyrin as HO-١ inhibitor didn't produce any change in IND ulcer model

and antagonized the protective effect of hemin. The data of the present study clearly demonstrated that hemin+ZnPP combination pretreatment significantly decreased the gastric mucosal NO and PGE٢ level of the ulcered model, similarly (Chow, Lin et al., ٢٠٠٩) reported that HO inhibitors downregulated the activity of iNOS and decreased the production of NO in a HO-١-independent manner, while (Mancuso, Pistritto et al., ١٩٩٧) reported that HO inhibitors may exert a direct inhibitory activity on prostaglandin endoperoxide synthase (PGHS), particularly the constitutive isoform, and therefore it decreased the PGE٢ production.

So in our results, combination of hemin and ZnPP aggrevate gastric mucosal lesions, increased mucosal lipid peroxides with marked increase in NO and PGE٢ level compared to pretreatment with hemin only. These findings are in agreement of (Song, Shin et al., ٢٠٠٨) who reported that HO-١ inhibitors aggrevated ulcer index in a concentration dependent manner. This ulcerogenic effect was probably due to ZnPP the inhibitor of HO-١ resulted in marked decrease in CO production.

In conclusion current results demonstrated that hemin pretreatment exerts a protective effect against IND-induced gastric ulcer, possibly via the induction of HO-١ and increased endogenous production of CO as well as via its antioxidant mechanism. This effect was reversed by using ZnPP a HO-١b inhibitor with hemin which decreased the production of CO and aggrevated the ulcer index compared to hemin only.

Therefore HO-١ inducer could open the door for an adjuvant regimen in the treatment of peptic ulcer disease by focusing on the strengthening of the gastric defensive mechanisms against endogenous an exogenous aggressors.

References

١. Abraham, N. G., R. Rezzani, L. Rodella, A. Kruger, D. Taller, G. Li Volti, A. I. Goodman and A. Kappas (٢٠٠٤). "Overexpression of human heme oxygenase-١ attenuates endothelial cell sloughing in experimental diabetes." American Journal of Physiology - Heart and Circulatory Physiology ٢٨٧(٦): H٢٤٦٨.

٢. Aburaya, M., K. Tanaka, T. Hoshino, S. Tsutsumi, K. Suzuki, M. Makise, R. Akagi and T. Mizushima (٢٠٠٦). "Heme oxygenase-١ protects gastric mucosal cells against non-steroidal anti-inflammatory drugs." J Biol Chem ٢٨١(٤٤): ٣٣٤٢٢-٣٣٤٣٢.

٣. Aebi, H. (١٩٨٤). "Catalase in vitro." Methods Enzymol ١٠٥: ١٢١-١٢٦.

٤. Baeuerle, P. A. and D. Baltimore (١٩٩٦). "NF-kappa B: ten years after." Cell ٨٧(١): ١٣-٢٠.

٥. Balla, G., H. S. Jacob, J. Balla, M. Rosenberg, K. Nath, F. Apple, J. W . Eaton and G. M. Vercellotti (١٩٩٢). "Ferritin: a cytoprotective antioxidant strategem of endothelium." J Biol Chem ٢٦٧(٢٥): ١٨١٤٨-١٨١٥٣.

٦. Bhattacharya, A., S. Ghosal and S. K. Bhattacharya (٢٠٠٦). "Effect of fish oil on offensive and defensive factors in gastric ulceration in rats." Prostaglandins Leukot Essent Fatty Acids ٧٤(٢): ١٠٩-١١٦.

٧. Chow, J. M., H. Y. Lin, S. C. Shen, M. S. Wu, C. W. Lin, W. T. Chiu, C. H. Lin and Y. C. Chen (٢٠٠٩). "Zinc protoporphyrin inhibition of lipopolysaccharide-, lipoteichoic acid-, and peptidoglycan-induced nitric oxide production through stimulating iNOS protein ubiquitination." Toxicol Appl Pharmacol ٢٣٧(٣): ٣٥٧-٣٦٥.

٨. Cooper, K. L., K. J. Liu and L. G. Hudson (٢٠٠٩). "Enhanced ROS production and redox signaling with combined arsenite and UVA exposure: contribution of NADPH oxidase." Free Radic Biol Med ٤٧(٤): ٣٨١-٣٨٨.

٩. Costa, N. R., R. O. Silva, L. A. Nicolau, L. T. Lucetti, A. P. Santana, K. S. Aragao, P. M. Soares, R. A. Ribeiro, M. H. Souza, A. L. Barbosa and J. V. Medeiros (٢٠١٣). "Role of soluble guanylate cyclase activation in the gastroprotective effect of the HO-١/CO pathway against alendronate-induced gastric damage in rats." Eur J Pharmacol ٧٠٠(١-٣): ٥١-٥٩.

١٠. Di Bello, M. G., L. Berni, P. Gai, C. Mirabella, J. F. Ndisang, E. Masini, T. Bani Sacchi and P. F. Mannaioni (١٩٩٨). "A regulatory role for carbon monoxide in mast cell function." Inflamm Res ٤٧ **Suppl** ١: S٧-٨.

١١. El-Ashmawy, N. E., E. G. Khedr, H. A. El-Bahrawy and H. M. Selim (٢٠١٦). "Nebivolol prevents indomethacin-induced gastric ulcer in rats." J Immunotoxicol ١٣(٤): ٥٨٠-٥٨٩.

١٢. Elbirt, K. K. and H. L. Bonkovsky (١٩٩٩). "Heme oxygenase: recent advances in understanding its regulation and role." Proc Assoc Am Physicians ١١١(٥): ٤٣٨-٤٤٧.

١٣. Fries, J. F., S. R. Miller, P. W. Spitz, C. A. Williams, H. B. Hubert and D. A. Bloch (١٩٨٩). "Toward an epidemiology of gastropathy associated with nonsteroidal antiinflammatory drug use." Gastroenterology ٩٦(٢ Pt ٢ Suppl): ٦٤٧-٦٥٥.

١٤. Haider, A., R. Olszanecki, R. Gryglewski, M. L .Schwartzman, E. Lianos, A. Kappas, A. Nasjletti and N.

G. Abraham (٢٠٠٢). "Regulation of cyclooxygenase by the heme-heme oxygenase system in microvessel endothelial cells." J Pharmacol Exp Ther ٣٠٠(١): ١٨٨-١٩٤.

١٥. Hangaishi, M., N. Ishizaka, T. Aizawa, Y .Kurihara, J. Taguchi, R. Nagai, S. Kimura and M. Ohno (٢٠٠٠). "Induction of heme oxygenase-١ can act protectively against cardiac ischemia/reperfusion in vivo." Biochem Biophys Res Commun ٢٧٩(٢): ٥٨٢-٥٨٨.

١٦. Ibrahim, I., S. El-Sayed, S. Abdel-Hakim, M. Hassan and N. Aziz (٢٠١٢). "Inhibition of endogenous CO by ZnPP protects against stress-induced gastric lesion in adult male albino rats." Journal of Physiology and Biochemistry ٦٨(٣): ٣١٩-٣٢٨.

١٧. Kapitulnik, J. and M. D. Maines (٢٠٠٩). "Pleiotropic functions of biliverdin reductase: cellular signaling and generation of cytoprotective and cytotoxic bilirubin." Trends Pharmacol Sci ٣٠(٣): ١٢٩-١٣٧.

١٨. Khayyal, M. T., M. Seif-El-Nasr, M. A. El-Ghazaly, S. N. Okpanyi, O. Kelber and D. Weiser (٢٠٠٦). "Mechanisms involved in the gastro-protective effect of STW ٥ (Iberogast) and its components against ulcers and rebound acidity." Phytomedicine ١٣ **Suppl** ٥: ٥٦-٦٦.

١٩. Kimura, H., S. Miura, T. Shigematsu, N. Ohkubo, Y. Tsuzuki, I. Kurose, H. Higuchi, Y. Akiba, R. Hokari, M. Hirokawa, H. Serizawa and H. Ishii (١٩٩٧). "Increased nitric oxide production and inducible nitric oxide synthase activity in colonic mucosa of patients with active ulcerative colitis and Crohn's disease." Dig Dis Sci ٤٢(٥): ١٠٤٧-١٠٥٤.

٢٠. Koizumi, S., M. Odashima ,M. Otaka, M. Jin, J. Linden, S. Watanabe and H. Ohnishi (٢٠٠٩). "Attenuation of gastric mucosal inflammation induced by indomethacin through activation of the A٢A adenosine receptor in rats." J Gastroenterol ٤٤(٥): ٤١٩-٤٢٥.

٢١. Konturek, S. J., P. C. Konturek and T. Brzozowski (٢٠٠٥). "Prostaglandins and ulcer healing." J Physiol Pharmacol ٥٦ **Suppl** ٥: ٥-٣١.

٢٢. Li Volti, G., R. Ientile, N. G. Abraham, A. Vanella, G. Cannavò, F. Mazza, M. Currò, G. Raciti, R. Avola and A. Campisi (٢٠٠٤). "Immunocytochemical localization and expression of heme oxygenase-١ in primary astroglial cell cultures during differentiation: effect of glutamate." Biochemical and Biophysical Research Communications ٣١٥(٢): ٥١٧-٥٢٤.

٢٣. Ligumsky, M., E. M. Golanska, D. G. Hansen and G. L. Kauffman, Jr. (١٩٨٣). "Aspirin can inhibit gastric mucosal cyclo-oxygenase without causing lesions in rat." Gastroenterology ٨٤(٤): ٧٥٦-٧٦١.

٢٤. Maines, M. D. (١٩٨٤). "New developments in the regulation of heme metabolism and their implications." Crit Rev Toxicol ١٢ :(٣) ٢٤١-٣١٤.

٢٥. Maines, M. D. (١٩٩٧). "The heme oxygenase system: a regulator of second messenger gases." Annu Rev Pharmacol Toxicol ٣٧: ٥١٧-٥٥٤.

٢٦. Maity, B., D. Banerjee, S. K. Bandyopadhyay and S. Chattopadhyay (٢٠٠٩). "Regulation of arginase/nitric oxide synthesis axis via cytokine balance contributes to the healing action of malabaricone B against indomethacin-induced gastric ulceration in mice." Int Immunopharmacol ٩(٤): ٤٩١-٤٩٨.

٢٧. Mancuso, C., G. Pistritto, G. Tringali, A. B. Grossman, P. Preziosi and P . Navarra (١٩٩٧). "Evidence that carbon monoxide stimulates prostaglandin endoperoxide synthase activity in rat hypothalamic explants and in primary cultures of rat hypothalamic astrocytes." Molecular Brain Research ٤٥(٢): ٢٩٤-٣٠٠.

٢٨. Mihara, M. and M. Uchiyama (١٩٧٨). "Determination of malonaldehyde precursor in tissues by thiobarbituric acid test." Anal Biochem ٨٦(١): ٢٧١-٢٧٨.

٢٩. Mollace, V., C. Muscoli, E. Masini, S. Cuzzocrea and D. Salvemini (٢٠٠٥). "Modulation of prostaglandin biosynthesis by nitric oxide and nitric oxide donors." Pharmacol Rev ٥٧(٢): ٢١٧-٢٥٢.

٣٠. Moore, E. W. (١٩٦٨). "Cation measurements in biological materials." Ann N Y Acad Sci ١٤٨(١): ٩٣-١٠٩.

٣١. Naito, Y., S. Iinuma, N. Yagi, Y. Boku, E. Imamoto, T. Takagi, O. Handa, S. Kokura and T. Yoshikawa " .(٢٠٠٨)Prevention of Indomethacin-Induced Gastric Mucosal Injury in Helicobacter pylori-Negative Healthy Volunteers: A Comparison Study Rebamipide vs Famotidine." J Clin Biochem Nutr ٤٣(١): ٣٤-٤٠.

٣٢. Naito, Y. and T. Yoshikawa (٢٠٠٦). "Oxidative stress involvement and gene expression in indomethacin-induced gastropathy." Redox Rep ١١(٦): ٢٤٣-٢٥٣.

٣٣. Nakao, A., L. E. Otterbein, M. Overhaus, J. K. Sarady, A. Tsung, K. Kimizuka, M. A. Nalesnik, T. Kaizu, T. Uchiyama, F. Liu, N. Murase, A. J. Bauer and F. H. Bach (٢٠٠٤). "Biliverdin protects the functional integrity of a transplanted syngeneic small bowel." Gastroenterology ١٢٧(٢): ٥٩٥-٦٠٦.

٣٤. Niess, A. M., F. Passek, I. Lorenz, E. M. Schneider, H. H. Dickhuth, H. Northoff and E. Fehrenbach (١٩٩٩). "Expression of the antioxidant stress protein heme oxygenase-١ (HO-١) in human leukocytes." Free Radic Biol Med ٢٦(١-٢): ١٨٤-١٩٢.

٣٥. Otterbein, L. E., F. H. Bach, J. Alam, M. Soares, H. Tao Lu, M. Wysk, R. J. Davis, R. A. Flavell and A. M. Choi (٢٠٠٠). "Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway." Nat Med ٦(٤): ٤٢٢-٤٢٨.

٣٦. Pae, H. O. and H. T. Chung (٢٠٠٩). "Heme oxygenase-١: its therapeutic roles in inflammatory diseases." Immune Netw ٩(١): ١٢-١٩.

٣٧. Shen, G. M., M. Q. Zhou, G. S. Xu, Y. Xu and G. Yin (٢٠٠٦). "Role of vasoactive intestinal peptide and nitric oxide in the modulation of electroacupuncture on gastric motility in stressed rats." World J Gastroenterol ١٢(٣٨): ٦١٥٦-٦١٦٠.

٣٨. Simmons, D. L., R. M. Botting and T. Hla (٢٠٠٤)" .(Cyclooxygenase isozymes: the biology of prostaglandin synthesis and inhibition." Pharmacol Rev ٥٦(٣): ٣٨٧-٤٣٧.

٣٩. Song, H. J., C. Y. Shin, T. Y. Oh and U. D. Sohn (٢٠٠٨). "The protective effect of eupatilin on indomethacin-induced cell damage in cultured feline ileal smooth muscle cells: involvement of HO-١ and ERK." J Ethnopharmacol ١١٨(١): ٩٤-١٠١.

٤٠. Stocker, R., Y. Yamamoto, A. F. McDonagh, A. N. Glazer and B. N. Ames (١٩٨٧). "Bilirubin is an antioxidant of possible physiological importance." Science ٢٣٥(٤٧٩٢): ١٠٤٣-١٠٤٦.

٤١. Takeuchi, K., A. Yokota, A. Tanaka and Y. Takahira (٢٠٠٦). "Factors Involved in Upregulation of Inducible Nitric Oxide Synthase in Rat Small Intestine Following Administration of Nonsteroidal Anti-inflammatory Drugs." Digestive Diseases and Sciences ٥١(٧): ١٢٥٠-١٢٥٩.

٤٢. Ueda, K., T. Ueyama, K. Yoshida, H. Kimura, T. Ito, Y. Shimizu, M. Oka, Y. Tsuruo and M. Ichinose (٢٠٠٨). "Adaptive HNE-Nrf٢-HO-١ pathway against oxidative stress is associated with acute gastric mucosal lesions." Am J Physiol Gastrointest Liver Physiol ٢٩٥(٣): G٤٦٠-٤٦٩.

٤٣. Utsumi, H., K. Yasukawa, T. Soeda, K.-i. Yamada, R. Shigemi, T. Yao and M. Tsuneyoshi (٢٠٠٦). "Noninvasive Mapping of Reactive Oxygen Species by in Vivo Electron Spin Resonance Spectroscopy in Indomethacin-Induced Gastric Ulcers in Rats." Journal of Pharmacology and Experimental Therapeutics ٣١٧(١): ٢٢٨.

٤٤. Vesely, M. J., D. J. Exon, J. E. Clark, R. Foresti, C. J. Green and R. Motterlini (١٩٩٨). "Heme oxygenase-١ induction in skeletal muscle cells: hemin and sodium nitroprusside are regulators in vitro." Am J Physiol ٢٧٥(٤ Pt ١): C١٠٨٧-١٠٩٤.

٤٥. Wu, L. and R. Wang (٢٠٠٥). "Carbon monoxide: endogenous production, physiological functions, and pharmacological applications." Pharmacol Rev ٥٧(٤): ٥٨٥-٦٣٠.