EXPERIMENTAL STUDY

The effect of induction of endogenous CO by heme-oxygenase inducer, hemin versus heme-oxygenase blocker, zinc mesoporphyrin on gastric secretion and ulceration under different conditions in adult male albino rats

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Abstract: Although its role and importance is less well studied, carbon monoxide (CO) has been identified as the second gasotransmitter in the GI tract. This study was performed to investigate the effect of modifying the endogenous CO production by altering heme oxygenase (HO) activity either by induction through hemin administration or inhibition by zinc mesoporphyrin administration on gastric secretion and ulceration induced by either cold restraint stress (CRS) or indomethacin (IND) treatment in adult male albino rats. Our results revealed that hemin significantly increased HO-1 levels with an increase in carboxyhemoglobin (COHb) level while zinc mesoporphyrin significantly decreased HO-1 levels with a decrease in COHb level in all groups. Hemin pretreatment significantly attenuated the gastric mucosal lesions induced by CRS and IND administration, which was accompanied by significant reduction in free and total acidity of gastric secretion, decreased proteolytic activity and marked attenuation of lipid peroxidation inspite of decreased NO and PGE_2 levels. On the other hand, Inhibition of HO-1 activity by zinc mesoporphyrin prevented most of the effects caused by hemin administration except for its similar reduction in gastric mucosal NO and PGE_2 levels. On conclusion, Hemin exerts a protective effect against CRS and IND-induced gastric ulcers possibly via inducing HO-1 and increasing endogenous production of CO *(Tab. 2, Fig. 4, Ref. 75). Text in PDF www.elis.sk.*
Keywords: carbon monoxide, heme oxygenase, cold-restraint stress, indomethacin.

Carbon monoxide (CO) has been found to be produced in every living cell in a biochemical reaction catalyzed by heme oxygenase enzyme (HO) which degrades heme into biliverdin, CO and iron. Endogenous CO is not a waste product, but acts as a chemical messenger mediating and modulating many intracellular biochemical reactions which in turn regulate physiological functions (1). Nevertheless, knowing that inhalation of uncontrolled amounts of this gas can ultimately lead to serious systemic complications and neuronal derangements, the inherent toxic nature of CO cannot be ignored. From the clinical perspective, the key question is whether a safe and therapeutically effective threshold of CO can be reached locally in organs and tissues without delivering the potentially toxic amounts through the lungs (2).

The majority of endogenous CO is exhaled through the lungs (3) and the remaining part of endogenous CO is bound to hemoglobin and other heme proteins in tissues or oxidized to carbon dioxide in mitochondria (1). The physiological effects of CO are mediated either by cyclic guanosine monophosphate (cGMP)-dependent mechanism or via cGMP-independent (4) stimulation of different ion channels and via the Mitogen-Activated Protein Kinase (MAPK) pathways (1). In addition to inhibiting platelet aggregation, CO activates sGC in many different cell types either directly or via activation of nitric oxide synthase (NOS) which is responsible for CO-induced activation of neurotransmission, vasodilation (5).

CO affects free radical generation, lipid peroxidation, channel gating, and competes with NO for cGMP system. In addition, it binds to heme and alters the functions of heme-containing enzymes as cyclo-oxygenases, catalases, superoxide dismutases, nitric oxide synthases, and oxygenases (6). Apart from its generalized smooth muscle relaxant effect produced in nearly all systems including the gastrointestinal system, the effect of CO on gastric secretion and mucosal protection has not been yet studied under different conditions (7).

Peptic ulcer is an inflammatory debilitating disease characterized by a high rate of recurrence which creates a burden on the patient himself and on the economy of the society (8). There is not a single cause for peptic ulcer but its exact etiology lies in the presence of imbalance between aggressive factors (e.g., acidity, pepsin, inflammation and oxidative stress) and defensive factors (e.g., mucus, PGs, bicarbonate and GMB flow) (9). So the present work is a trial to evaluate the role of CO in pathophysiologic mechanisms of experimentally induced gastric ulceration caused by CRS and IND administration.

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Materials and methods

Animals

Fifty-four adult male albino (Sprague-Dawley strain) rats, of average weight 150–200 g, about 4 months old were used in the present study. Rats were purchased from the National Research Center, Cairo, Egypt. All rats were housed in stainless steel cages that contain barriers for each rat for individual housing, while the cage contained 5 rats and each rat had a tag number. They were fed commercial rat chow and left freely wandering in their cage for two weeks with 12 hours dark/light cycles for acclimatization before starting the experiment. All the experimental procedures were in accordance with our institutional guidelines. The ethics of the protocol was approved by The Laboratory Animals' Maintenance and Usage Committee of Faculty of Medicine, Minia University.

Drug protocol

Hemin (from Sigma, UT, USA) and zinc mesoporphyrin (from Aldrich, UT, USA) were freshly dissolved in 0.1 mol/L NaOH adjusted to pH 7.4 with 0.1 mol/L HCl and diluted with saline to the required volume (0.5 ml of this vehicle was given to non-treated rats). Hemin and zinc mesoporphyrin were prepared in darkness and protected from light (10).

Experimental procedures

All rats were fasted for 24 h prior to the study and housed in raised mesh-bottomed cages to minimize coprophagia with free access to water (11). All experiments were performed at the same time of the day to avoid variations due to diurnal rhythms of putative regulators of gastric functions.

Pyloric ligation

All rats were pylorically ligated under light ether anesthesia. The anterior abdominal wall was incised and the pyloric portion of the stomach was gently mobilized and ligated with a silk ligature around the pyloric sphincter taking great care not to interfere with the blood supply of the stomach, and the abdominal wall incision was closed (12).

Induction of cold-restraint stressed (CRS) ulcer

After pyloric ligation, the animals were immediately restrained by fixing the four limbs to a wooden board and placed in a refrigerator at 4 °C for 3 hours. The door of the refrigerator was opened every 0.5 hour for inspection and follow up (13). Rats were randomly divided into the following groups (6 rats each):

I. Non-stressed Groups:

1) Control group (C); in which each rat received 0.5 ml of the vehicle i.p. four times/week for four weeks before being subjected to pyloric ligation.

2) Hemin-treated group (H); each rat received hemin at a dose level of 25 mg/kg body weight, i.p. four times/week for four weeks (14).

3) Zinc mesoporphyrin treated group (ZM); each rat received zinc mesoporphyrin at a dose level of 2.5 μmol /Kg body weight/day, i.p. for five days (15).

II. CRS Groups:

1) CRS group; in which each rat received 0.5 ml of the vehicle i.p. four times/week for four weeks before exposure to CRS.

2) Stressed hemin-treated group (H+CRS); each rat received the same dose of hemin as non stressed group before exposure to CRS.

3) Stressed Zinc mesoporphyrin-treated group (ZM+CRS); each rat received the same dose of zinc mesoporphyrin as non stressed group before exposure to CRS.

III. Indomethacin-treated group:

1) IND-treated group; in which rats received no further treatment other than IND (40 mg/kg, s.c.). After two hours, pyloric ligation was performed (16).

2) Hemin and IND-treated group (H + IND); each rat received the same dose of hemin as non stressed group before the administration of IND.

3) Zinc mesoporphyrin and IND-treated group (ZM+IND); each rat received the same dose of zinc mesoporphyrin as non stressed group before the administration of IND.

Three hours after pyloric ligation, the rats were anesthetized by light ether anesthesia. Then their stomachs were removed, opened along the greater curvature and the gastric content of each stomach was collected. The stomachs were washed with ice-cold saline and examined for gross gastric mucosal lesions using a magnified lens by an observer not aware of the experiment.

Assessment of gastric mucosal lesions

The severity of the lesions was expressed in terms of the ulcer index (U.I.), and the severity factor was determined according to the method of Robert et al (17) The severity score of each stomach (M.S.S.) is the score of the severest ulcer of that stomach as measured from 0 to 5 as follows: 0 if no petechiae or erosions are present, 1 when lesions are only petechiae or erosion less than 1 mm, 2 when lesion size is 1–5 mm, or 3 if lesion exceeds 5 mm. The mean ulcer score (M.U.S) is the total number of ulcers divided by the number of rats/group.

The incidence rate of ulceration is the percentage of stomachs with ulcers in each group. The U.I. for each group was calculated from the following equation:

$$U.I. = \frac{\text{Incidence rate}}{10} + M.S.S + M.U.S.$$

Where U.I. is the ulcer index, M.S.S. is the mean severity score, and M.U.S. is the mean ulcer score.

The preventive index of a given drug was calculated from the equation according to Hano et al (18).

$$\text{Preventive index (P.I.)} = \frac{\text{U.I. of stressed group} - \text{U.I. of treated group}}{\text{U.I. of stressed group}} \times 100$$

Analysis of the gastric juice

The gastric juice collected after opening the stomachs was centrifuged at 1000 g for 10 minutes to remove any solid debris, and the volume of the supernatant was measured. The supernatant was then analyzed for the determination of free and total acid concentration and outputs, pepsin and mucin concentrations.

● Determination of free and total acidity of the gastric juice

The free acidity was determined by titration of a given volume of the gastric juice against 0.1 N sodium hydroxide up to 5.5 as guided by a pH meter. The total acidity which is composed of both mineral and combined organic acids in the gastric juice was determined by completing the titration in the above procedure for determining free acidity to pH 7 as guided by the pH meter (19). Free acid output and total acid output were calculated by multiplying the respective acid concentration by the volume collected at the end of the experiment and expressed as mEq/3 h (20) .

● Determination of proteolytic activity

This was determined by a modified spectrophotometric method (21). The pepsin activity is the major factor involved in the proteolytic activity of gastric secretion. This activity can be determined in terms of the amount of proteases produced after incubation of the hemoglobin substrate for 1/2 hour with standard pepsin or juice.

● Colorimetric assay for mucins and glycoproteins in gastric juice

It is a sensitive and specific method for saccharides, which are linked via N-acetylgalactosamine through O-glycosidic linkage to serine/threonine in mucins. The method is not affected by the carbohydrates present in other types of glycoproteins (22).

Biochemical analysis of gastric mucosa

The stomach of each rat was divided into two parts. One part was immersed in IND (10μg/ml) for 20 minutes to inhibit further formation and release of PGs (23), and 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 g for 10 minutes at room temperature. The supernatant was analyzed for determination of prostaglandin content. The other part of gastric mucosa was also scraped, homogenized in cold potassium phosphate buffer (0.05 M, pH 7.4) and centrifuged at 2000 g for 10 minutes at 4 °C. The supernatant was kept at −80 °C for subsequent measurement of lipid peroxides, NO and Heme-oxygenase (HO)-1.

● Determination of gastric mucosal NO

Gastric mucosal NO was determined using commercially available kits for colorimetric determination of NO (Biodiagnostic, Egypt) and 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 of the Griess reaction (24).

● Determination of gastric mucosal lipid peroxides

Malondialdehyde (MDA) levels in the gastric mucosa were determined as an indicator of lipid peroxidation by thiobarbituric acid method as previously described by Okhawa et al (25).

● Determination of gastric mucosal PGE_2

PGE_2 in the gastric mucosa was determined by enzyme-linked immunosorbent assay (ELISA) using PGE_2 assay kit (R&D Systems, USA), and based on the competitive binding technique in which PGE_2 present in a sample competes with a fixed amount of horseradish peroxidase (HRP)-labeled PGE_2 for sites on a monoclonal antibody (26).

● Determination of gastric mucosal HO-1

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 (27).

Measurement of endogenous carbon monoxide; carboxyhemoglobin (COHb) level

Carboxyhemoglobin (COHb) was measured by using spectrophotometer (BAUSCH & LOMB spectronic 2000). A 10 µl sample of aspirated blood from the retro-orbital sinus of anesthetized rats was added to 20 ml of diluent (2.5 mg/ml sodium dithionite was dissolved in 0.01 mol/L TRIS (hydroxymethyl) amino methane just before use), and its absolute derivative absorption at 420 nm was compared with the absolute derivative value at 420 nm for saturated blood samples (5 mL diluted blood was saturated by bubbling CO gas for 30 minutes) to give the percentage saturation of COHb (28).

Statistical analysis

All data were represented as mean ± standard error of the mean (M±SEM). Data were analyzed by repeated measure analysis of variance (ANOVA) followed by Bonferroni multiple comparison test. p value ≤ 0.05 was considered statistically significant.

Results

The results clearly demonstrated that hemin pretreatment proved to be the inducer of HO enzyme as evidenced by a significantly higher gastric mucosal HO-1 level, while administration of zinc mesoporphyrin proved to be the inhibitor to HO enzyme as

Tab. 1. Effect of hemin and zinc mesoporphyrin pretreatment on gastric juice parameters in CRS and IND treated rats as compared to non-treated rats.

Parameters	Groups								
	Control	Hemin treated	Zinc mesoporphyrin treated	CRS	Hemin + CRS	Zinc mesoporphyrin + CRS	IND	Hemin + IND	Zinc mesoporphyrin + IND
Volume (ml /3 h)	2.2 ± 0.1	2.4 ± 0.2	1.7 ± 0.1 [a]	1.6 ±0.1 [a]	1.25 ±0.09 [b]	0.36 ±0.07 [b]	1.7 ± 0.09 [a]	1.4 ± 0.08 [c]	0.41 ± 0.08 [c]
F.A.C. (mEq/L)	46.7± 3.6	50 ± 4.8	--	85 ± 2.6 [a]	62.5 ± 4.4 [b]	--	82.5 ± 7.7 [a]	56.7 ± 2.5 [c]	--
T.A.C. (mEq/L)	67.5 ± 2.8	81.7 ± 7	--	130 ± 2.6 [a]	100 ± 2.7 [b]	--	124.2 ± 6.6 [a]	100 ± 6.8 [c]	--
F.A.O. (mEq/3 h)	100.8±3.3	112.7 ± 6	--	136.5±11.2 [a]	79.6 ± 10.9 [b]	--	137.7 ± 9.2 [a]	82.7 ± 8.1 [c]	--
T.A.O. (mEq/3 h)	147.3±4.3	188 ± 18	--	209.3 ±17.4 [a]	123.3 ± 10 [b]	--	208.4 ± 4.1 [a]	143.6 ±10.9 [c]	--
Pepsin (mg/mL)	7.2 ± 0.7	8.7 ± 0.7	12 ± 0.8 [a]	12.7 ±0.3 [a]	8.3 ± 0.3 [b]	20.2 ± 1.2 [b]	13.5 ± 0.4 [a]	8.7 ± 0.4 [c]	18.3 ± 1.4 [c]
Mucin (mg/mL)	9.7 ± 0.8	9.6 ± 0.6	5.3 ± 0.3 [a]	7.7 ± 0.4 [a]	7.8 ± 0.3	2.7 ± 0.19 [b]	7.67 ± 0.6 [a]	9.1± 0.4	2.3± 0.1 [c]

Data represent the mean ± SEM of observations from 6 rats. [a], Significantly different from control group; [b], Significantly different from CRS; [c], Significantly different from IND treated group. P≤0.05. F.A.C.: Free Acid Concentration; T.A.C.: Total Acid Concentration.; FAO; Free acid output; TAO; Total acid output.
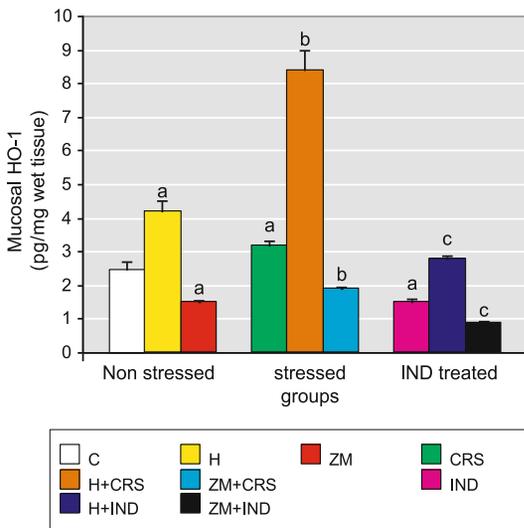
Fig.1. Effect of hemin and zinc mesoporphyrin pretreatment on the gastric mucosal HO-1 level in pylorically ligated rats under different treatment conditions.

Results are expressed as mean±SEM of 6 rats. C=Control, H=Hemin treated, ZM=Zinc mesoporphyrin treated, CRS=Cold restraint-stressed, IND= Indomethacin, H + CRS=Hemin treated + Cold restraint-stressed group, H+ IND=Hemin treated + Indomethacin treated group, ZM + CRS=Zinc mesoporphyrin treated + Cold restraint-stressed group, ZM + IND = Zinc mesoporphyrin + Indomethacin treated group. [a] – significantly different from control group; [b] –significantly different from CRS; [c] – significantly different from IND treated group. $p \leq 0.05$.
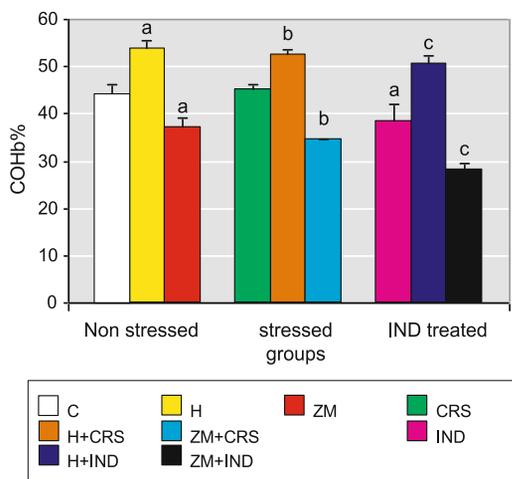
Fig. 2. Effect of hemin and zinc mesoporphyrin pretreatment on COHb level under different treatment conditions.

Results are expressed as mean±SEM of 6 rats. C=Control, H=Hemin treated, ZM=Zinc mesoporphyrin treated, CRS=Cold restraint-stressed, IND= Indomethacin, H + CRS=Hemin treated + Cold restraint-stressed group, H+ IND=Hemin treated + Indomethacin treated group, ZM + CRS=Zinc mesoporphyrin treated + Cold restraint-stressed group, ZM + IND = Zinc mesoporphyrin + Indomethacin treated group. [a] – significantly different from control group; [b] –significantly different from CRS; [c] – significantly different from IND treated group. $p \leq 0.05$.

evidenced by a significantly lower gastric mucosal HO-1 level in all treated groups. Exposure of the rats to CRS produced a significantly higher HO-1 level while IND administration significantly lowered HO-1 level when both were compared with corresponding non treated rats (Fig. 1). As regard to COHb levels which reflect endogenous CO production, hemin pretreatment showed a significantly higher COHb level while zinc mesoporphyrin significantly lowered COHb level in all treated groups. Exposure of the rats to CRS failed to alter significantly the COHb level while IND administration significantly lowered the COHb level when both were compared with control rats (Fig. 2).

CRS and IND administration significantly lowered the volume of gastric juice and mucin concentration, which was accompanied by a significantly higher free and total acidity of gastric juice and pepsin activity compared to the control rats. Hemin pretreatment failed to produce any significant change on the gastric juice parameters as compared to control rats. On the other hand, stressed and IND-treated hemin groups showed significantly lower levels of volume, proteolytic activity, and all acid parameters of the gastric juice without any significant change in mucin concentration when compared to both CRS- and IND-treated groups. Zinc mesoporphyrin pretreatment significantly lowered the volume of mucin with a significantly higher proteolytic activity level of the gastric juice when compared to control, CRS, and
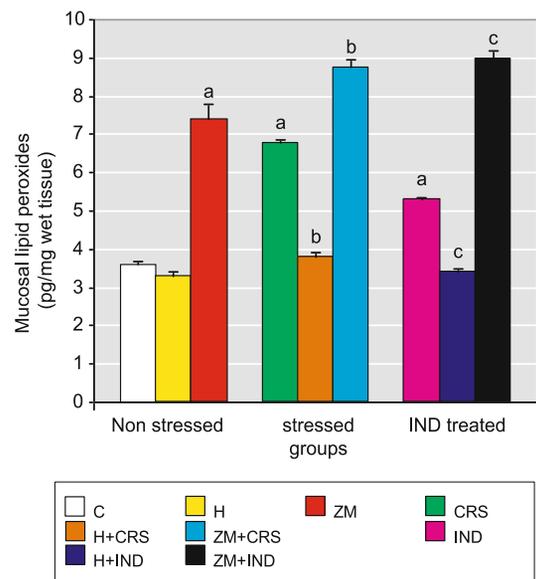
Fig. 3. Effect of hemin and zinc mesoporphyrin pretreatment on the gastric mucosal lipid peroxides level in pylorically ligated rats under different treatment conditions.

Results are expressed as mean±SEM of 6 rats. C=Control, H=Hemin treated, ZM=Zinc mesoporphyrin treated, CRS=Cold restraint-stressed, IND= Indomethacin, H + CRS=Hemin treated + Cold restraint-stressed group, H+ IND=Hemin treated + Indomethacin treated group, ZM + CRS=Zinc mesoporphyrin treated + Cold restraint-stressed group, ZM + IND = Zinc mesoporphyrin + Indomethacin treated group. [a] – significantly different from control group; [b] –significantly different from CRS; [c] – significantly different from IND treated group. $p \leq 0.05$.

IND-treated groups, but we could not determine acid parameters due to bloody juice (Tab. 1).

Subjecting the rats to CRS or administration of IND without any pretreatment was associated with high ulcer index reaching 19.25 and 32.5, respectively. Hemin pretreatment proved to be protective against the development of ulcerative lesions as evidenced by the decreased ulcer index in both CRS- and IND-treated groups. The preventive index was 40 and 39.1 %, respectively. On the other hand, Zinc mesoporphyrin proved to be injurious as evidenced by the increased ulcer index in non-stressed, CRS- and IND-treated groups reaching 6.75, 23.75 and 42.5, respectively (Tab. 2).

CRS and IND administration produced a significantly higher gastric mucosal lipid peroxides levels when compared to control rats. Hemin pretreatment failed to alter significantly the level when compared with control rats but it caused a significant reduction in gastric mucosal lipid peroxides in both CRS- and IND- treated rats. Administration of zinc mesoporphyrin, on the other hand, showed a significant higher gastric mucosal lipid peroxides level in all treated groups (Fig. 3).

Administration of IND to rats significantly lowered the gastric mucosal NO and PGE_2 levels while the exposure to CRS significantly lowered the gastric mucosal NO level but failed to produce any significant change in gastric mucosal PGE_2 level as compared to control rats. Hemin pretreatment significantly lowered the gastric mucosal NO in non-stressed and CRS-treated groups but failed to alter it significantly in IND-treated group. Hemin pretreatment significantly lowered PGE_2 levels in CRS-treated group but it failed to produce any significant change in both non-stressed and IND-treated groups. On the other hand, zinc mesoporphyrin pretreatment significantly lowered the gastric mucosal NO and PGE_2 levels in all groups (Fig. 4).

Discussion

The physiological function of CO has become subject to intensive research in recent years, while the studies on the gastro-intestinal tract have been at the forefront of these investigations (6). CO has long been considered a toxic air pollutant and known as a "silent killer" because of its strong affinity for hemoglobin thus resulting in death after inhalation in high concentrations (1).

The data of the present study clearly demonstrated that hemin pretreatment proved to be the inducer of HO enzyme as evidenced by increased HO-1 and COHb levels. These data are consistent with the findings of Duridanova et al (29). On the other hand, the pretreatment with zinc mesoporphyrin, the HO inhibitor in the present study, significantly reduced the gastric mucosal HO-1 level with significantly decreased COHb level. These findings are in accordance with Ueda et al (30) and Wang et al (31) even though they used a different HO inhibitor.

The precise mechanism of HO-1 induction is not known. Many inducible genes are expressed in response to activation of various transcriptional factors by a variety of inducing agents. The binding sites of many transcriptional factors have been identified in the promoter region of the HO-1 gene, and it appears that HO-1 expression is regulated by the activation and binding of such transcriptional factors to these regions. An increase in the binding of a number of transcriptional factors in response to hemin treatment has been demonstrated by Vessely et al (32), most significantly activator protein-2 and nuclear transcription factor-κB.

In the present study, CRS significantly increased the gastric mucosal HO-1 level as compared to control group which is in accordance with the data of Yang et al (33) who reported that HO-1 is one of the most critical cytoprotective mechanisms ac-
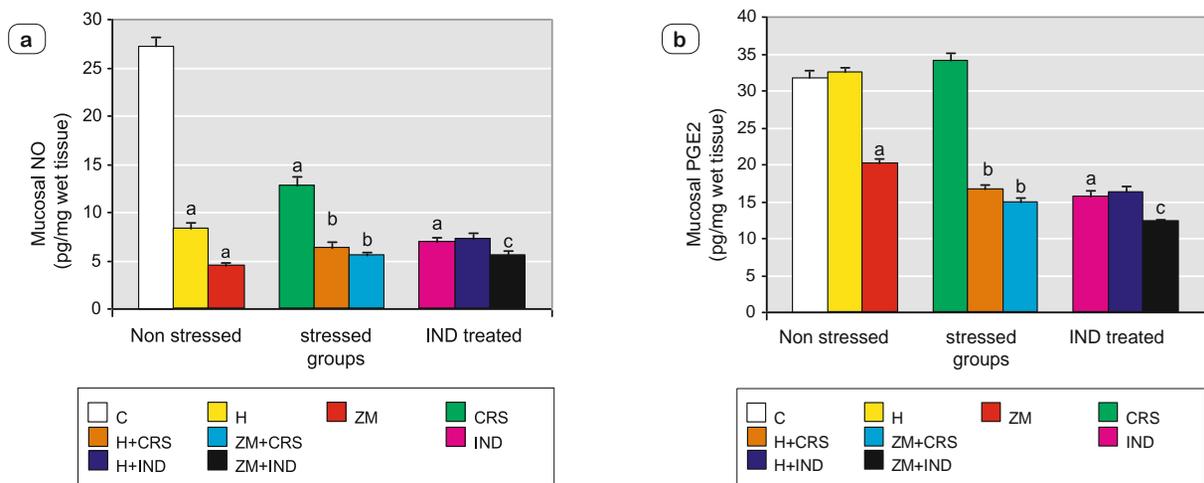
Fig. 4. Effect of hemin and zinc mesoporphyrin pretreatment on the gastric mucosal (a) NO and (b) PGE2 levels in pylorically ligated rats under different treatment conditions.
Results are expressed as mean±SEM of 6 rats. C=Control, H=Hemin treated, ZM=Zinc mesoporphyrin treated, CRS=Cold restraint-stressed, IND= Indomethacin, H + CRS=Hemin treated + Cold restraint-stressed group, H + IND=Hemin treated + Indomethacin treated group, ZM + CRS=Zinc mesoporphyrin treated + Cold restraint-stressed group, ZM + IND = Zinc mesoporphyrin + Indomethacin treated group. [a] – significantly different from control group; [b] –significantly different from CRS; [c] – significantly different from IND treated group. $p \leq 0.05$.

Tab. 2. Effect of hemin and zinc mesoporphyrin pretreatment on ulcer profile in cold restraint-stressed (CRS) or indomethacin (IND) treated rats as compared to non-treated rats.

Groups	% incidence	M.S.S.	M.U.S.	U.I.	P.I. (%)
Control	0	0	0	0	0
Hemin treated	0	0	0	0	-
Zinc mesoporphyrin treated	50	1	0.75	6.75	-
CRS	100	2.5±0.3	6.75±2.3	19.25	-
Stressed hemin treated	83.3	0.8±0.3[b]	2.5±1 b	11.6	40
Stressed Zinc mesoporphyrin treated	100	2.5±0.3[b]	11.25±0.7[b]	23.75	-19.5
IND treated	100	2.75±0.3	19.75±2.1	32.5	-
Hemin and IND treated	100	2.25±0.5	7.5±1[c]	19.8	39.1
Zinc mesoporphyrin and IND treated	100	2.5±0.3	30±1.5[c]	42.5	-30.8

M.S.S. – Mean Severity Score; M.U.S. – Mean Ulcer Score; U.I. – Ulcer Index; P.I. – Preventive Index; [b] – Significant difference from Cold restraint (C.R.S) group, $P \leq 0.05$; [c] – Significant difference from Indomethacin treated (IND) group, $P \leq 0.05$.

tivated during cellular stress, exerting anti-oxidative and anti-inflammatory functions, modulating the cell cycle and maintaining the microcirculation. On the other hand, IND significantly reduced the gastric mucosal HO-1 inspite of considering IND as one of cellular stress inducers as CRS. These results are supported by the studies of Song et al (43). Aburaya et al (7) reported that short-term treatment with a high concentration of IND as that used in this study did not upregulate HO-1 expression but lead to gastric mucosal damage through both necrosis and apoptosis mediated by increased membrane permeability and intracellular Ca^{2+}.

The obtained results of the present work revealed that CRS was not able to alter significantly the COHb level despite increased HO-1 level when compared with control non-treated rats. The explanation for this lies in the fact that CRS can induce tissue hypoxia (35) which leads to a rapid shift in CO from blood to extravascular compartments because hypoxia disrupts the balance between oxymyoglobin (O_2Mb) and carboxymyoglobin (COMb) by increasing the flow of CO from capillaries to extravascular tissue. In addition, hypoxia recruits muscle capillaries by effectively increasing the surface area for gas exchange, and increases cardiac output (36).

From the experimental point of view, the use of restraining in small animals such as rats has become one of the most popular methods for studying acute stress gastric ulcers. This ulcer model represents a tool to explain the importance of emotion and anxiety in the evolution of human peptic ulcers. The choice of CRS ulcer model in the present work was based on several factors as follows. Firstly stress is a very common contributor to ulcer formation in animals and humans. Secondly, pathophysiological mechanisms of stress-induced ulcers differ from those of other ulcer models, e.g., indomethacin-induced ulcers. Thirdly, this model is simple, rapid and effective in producing gastric ulcers. The choice of utilizing indomethacin in our second ulcer model was not only because non steroidal anti-inflammatory drugs (NSAIDs)-induced gastropathies are very common, but also because they have a different pathophysiology from CRS model. Moreover, similarly to CRS, indomethacin-induced ulceration model is simple, rapid and effective in producing ulcers.

The pathogenic mechanisms responsible for stress-induced gastric mucosal lesions include disturbance of gastric mucosal mi-

crocirculation, alteration of gastric secretion and abnormal gastric motility (37). Levenstein et al (38) reported that stress increases gastric acid secretion leading to peptic ulcer in the presence of other risk factors. In addition, decreased prostaglandin synthesis (39) and enhancement of lipid peroxidation (LPO) (40) are also involved in genesis of stress-induced ulcers.

The molecular basis for the gastrointestinal toxicity of NSAIDs is widely believed to be attributed to their inhibitory activity against cyclooxygenase (COX), 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. While the presence of acid in the lumen of the stomach may not be the primary factor in the pathogenesis of NSAID-induced gastropathy, it can make an important contribution to the severity of these lesions by impairing the restitution process, interfering with hemostasis and inactivating several growth factors that are important in mucosal defense and repair (41). In addition, neutrophil activation/infiltration leads to the release of reactive oxygen species (ROS) which damage the endothelium. In addition to being direct contributors to tissue necrosis, these free radicals can also influence the vascular tone by accelerating the inactivation of endothelium-derived relaxing factor (EDRF), i.e. nitric oxide (42).

The data of the present study clearly demonstrated that both CRS and IND evidently induced ulcers. The latter is in accordance with the observations of several researchers (43, 44). This occurs by enhancing the aggressive factors as evidenced by increased all acid parameters, proteolytic activity, and lipid peroxides level, as well as by counteracting the defensive factors as evidenced by decreased mucin, NO level and PGE_2 level (only in IND model).

The increased gastric acid secretion observed with CRS, in the present study, was also previously reported (45) and suggested to be due to increased vagal stimulation and increased histamine release (46). Similarly, IND significantly increased all acid parameters which are in accordance with the previously reported data (47). Increased gastric acid secretion parameters observed with IND owed to COX inhibition with reduction in PGs which are known to inhibit gastric acid secretion (48).

In the present study as well as in others (45), CRS was associated with a marked increase in gastric juice pepsin content. Stress can induce vagal overactivity (49) and disrupt the intact gastric mucosal barrier facilitating the acid back diffusion which stimulates pepsinogen release (50). Similarly, IND significantly increased gastric pepsin concentration which is in accordance with the findings of Khayyal et al (51). This effect was explained to be due to either diversion of arachidonic acid metabolism towards the lipooxygenase pathway, resulting in increased leukotriene synthesis and/or reduction in synthesis of PGs (51) which are potent inhibitors of pepsin secretion (48).

In agreement with other investigators, the present study observed CRS and IND to increase the gastric mucosal lipid peroxides level as compared to control group (43). 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 proinflammatory cytokines such as interleukin-1β (IL-1β) 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 (52).

In the present study, mucin concentration was significantly reduced in rats subjected to CRS which is in accordance with the findings of Bandyopadhyay et al (53). There is indirect evidence suggesting that the decreased NO observed with CRS in the present study reduced mucin synthesis as reported by Petersson et al (54) who suggested that NO induces mucus secretion by gastric mucosal cells without evidence of cellular damage and that cGMP is an intracellular mediator of mucus release in these cells. Similarly, IND significantly reduced gastric mucin which is in agreement with the studies of Khayyal et al (51). The effect of IND on mucin is in line with the known mechanism of IND as a non-selective inhibitor of COX enzyme which is involved in PGs synthesis, the potent stimulants of mucin synthesis (55).

In the present study, CRS significantly reduced gastric mucosal NO as compared to control group. These findings are in accordance with Shen et al (56) who reported a decrease in NO biosynthesis as a result of decreased NO synthase activity that correlated with an increase in the extent of damage. The CRS' reduced NOS activity contributing to decreased NO in the present work may be due to degradation of the heme located in the active site of NOS by the induced HO-1 (57). IND, also, significantly decreased gastric mucosal NO. Similar results were reported by Mollace et al (58) who reported that inhibition of COX markedly attenuates NOS activity. These effects were not associated with a change in NOS protein expression but mediated via increase in Ca^{2+} mobilization which leads to a decrease in intracellular Ca^{2+}. NOS activity can be activated via an enhanced level of intracellular (Ca^{2+}) and/or protein kinase stimulation. When intracellular Ca^{2+} increases, Ca^{2+} binds to calmodulin. Ca^{2+}/calmodulin complex dissociates the NOS-caveolin complex and activates NOS. Since NO is endothelium-derived relaxation factor (EDRF), during stress and secondary to PGs depletion by IND, the reduced NO synthesis can contribute to reduced mucosal blood flow by means of vasoconstriction response which becomes dominant.

The findings of the present work revealed that CRS was not able to alter significantly the gastric mucosal PGE_2 content which is in accordance with the data reported by Harada et al (59) Contrary to the present data, Bregonzio and his associates (11) reported that CRS was associated with a marked decrease in gastric mucosal PGE_2 level. The explanation for this lies in the fact that while COX-1 activity was found to decrease during CRS, COX-2 activity was observed to increase (47). Therefore, the observed non-alteration in the PGE_2 level by CRS in the present study may be due to the sum of COX-1 downregulation and COX-2 upregulation. On the other hand, IND significantly reduced mucosal PGE_2 which agrees with the previous reports (51) and with the known mechanism of action of IND as a non-selective COX inhibitor.

In the present study, Hemin significantly protected the gastric mucosa from ulceration induced by either CRS or IND and achieved preventive indices of 40 % and 39.1 %, respectively. This is in accordance with the previously reported data of Ueda et al (30) concerning CRS model, and Song et al (34) concerning IND model.

Hemin in non-stressed rats failed to alter significantly the gastric juice parameters and gastric mucosal lipid peroxides level but it significantly decreased the gastric mucosal NO level which is in accordance with the previous studies of Fouad et al (60) who reported that the reduced NO level may be due to inhibition of inducible NO synthase (iNOS) protein expression by increased HO level. On the other hand, Hemin did not produce any significant change in gastric mucosal PGE_2 level which may be due to the fact that hemin may act as a cofactor of COX. It can increase its substrate. arachidonic acid, as well as increase the expression and/or activity of COX, which would counteract the inhibitory influence of HO-1 activity on this enzyme (61).

The protective effect of hemin against ulcer development; in both ulcer models 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 calcium and increasing cAMP and cGMP levels (62). Blandizzi et al (63) reported that the reduction in acid production reduces pepsin activity and vice versa.

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-1 induction which is in agreement with other investigators (30). Nakao et al (64) reported that the possible explanation for the protective role of HO-1 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 H_2O_2 to more reactive hydroxyl radicals and promoting more severe tissue damage by propagating lipid peroxidation. Furthermore, because HO-1 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. Thus, in addition to removal of the pro-oxidant heme, in turn, the breakdown of heme to three byproducts has its own significance in essential cellular metabolism in contributing to the suppression of oxidative stress.

In the present study, hemin pretreatment in CRS ulcer model significantly decreased the gastric mucosal PGE_2 level. This is in accordance with the previously reported data of Li Volti et al (65). This could be attributed to HO-1 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 (61). Hemin pretreatment in CRS ulcer model also significantly decreased the NO level either by COX inhibition that decreased intracellular Ca^{2+} (since Ca^{2+} is a key regulator of NOS activity) (58), and also by HO-1 induction that degraded the heme located in the active site of NOS leading to a greater decrease in NO level when compared with CRS group (56). On the other hand, hemin pretreatment in IND ulcer model produced an insignificant change in PGE_2 level as COX enzyme has been already inhibited by IND administration (51). It also did not affect the NO level since NOS activity was supposed to have been inhibited by COX enzyme. That is why the PGE_2 and NO levels were not significantly different from IND-treated group.

NO donors were found to be protective against different types of gastric ulcer models while NO synthase inhibitors were ulcerogenic (66, 67). 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-1 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 endpoints 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 (1).

On the other hand, zinc mesoporphyrin pretreatment in the present study significantly increased the ulcerative lesions induced by CRS and achieved preventive index of -19.5%. This is in accordance with the previously reported data of Gomes et al (68) and Ueda et al (30) who reported that pretreatment with HO inhibitors aggravated the gastric ulcer.

The data of the present study clearly demonstrated that zinc mesoporphyrin pretreatment significantly decreased the volume, proteolytic activity, and mucin of gastric juice. Marked reduction in gastric mucosal NO and PGE_2 levels were exhibited in all groups. Similarly, Qin et al (69) and Chow et al (70) reported that

HO inhibitors downregulated the activity of iNOS and decreased the production of NO in a HO-1-independent manner, while Mancuso et al (71) reported that HO inhibitors may exert a direct inhibitory activity on prostaglandin endoperoxide synthase (PGHS), particularly the constitutive isoform, and therefore it decreased the PGE_2 production.

In two ulcer models, zinc mesoporphyrin pretreatment was observed to induce aggressive factors and reduce the defensive factors as evidenced by aggravation of gastric mucosal lesions, reduced volume of gastric secretion, increased proteolytic activity, reduced mucin production, and increased mucosal lipid peroxides with marked decrease in the gastric mucosal NO and PGE_2 levels associated with decreased COHb level. These findings are in agreement with Song et al (34) who reported that HO inhibitors aggravated ulcer index in a concentration-dependent manner. This ulcerogenic effect was probably due to inhibition of HO-1 resulting in marked decrease in CO production together with decreasing NO and PGE_2 levels.

Data of the present study clearly demonstrated that the HO inducer significantly decreased the rate of gastric secretion during stress. It is probable that CO is a double-faced actor as on the one hand, it increases the gastric secretion via its vasodilator action (6) and also by its direct inhibitory action on other mediators as NO and PGE_2 which inhibit the gastric secretion, whereas on the other hand, it acts via its direct action on gastric cells by means of which it can inhibit their gastric secretion. The final effect depends on the prominent action. In the present study, CO was observed to decrease the rate of gastric secretion due to its prominent inhibitory direct action. Furthermore, NO was also found to have a dual action on gastric secretion; a direct inhibitory effect on the parietal cells mediated by cGMP (72), as well as a stimulant action mediated by histamine release from histamine-containing cells or via its vasodilator action (73).

Current results demonstrated that the pathogenesis of gastric ulcer appears to be multifactorial, depending on the balance between the protective and aggressive factors. Endogenous CO is a double-faced gasotransmitter as it counteracts the aggressive factors, but also inhibits some of the protective factors. The net effect in the present study was a gastroprotective effect on both CRS and IND gastric ulcers. Nevertheless, endogenous CO does not seem to play a regulatory role under basal conditions as evidenced by the insignificant effect of HO inducers on gastric secretion in non stressed rats.

In conclusion, hemin pretreatment exerts a protective effect against CRS- and IND-induced gastric ulcers, possibly via the induction of HO-1 and increased endogenous production of CO, as well as via its antioxidant mechanisms. This effect was reversed by using zinc mesoporphyrin which decreased the production of CO and aggravated the ulcer index in both models. Therefore, HO inducers could open the door for an alternative and/or adjuvant regimen in the treatment of peptic ulcer disease by focusing on the strengthening of the gastric defensive mechanisms against endogenous and exogenous aggressors.

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