Research Article

A Potential Protective Role of Hemin, a Heme Oxygenase-١ Inducer in L-arginine-Induced Acute Pancreatitis in Rats

Yassmen M. M. A. Khalifa*, Ashraf Taye and Ihab T. Abdel-Raheem*****
* Department of Pharmacology & Toxicology, Faculty of Pharmacy, Deraya University, Minia, Egypt.
** Department of Pharmacology & Toxicology, Faculty of Pharmacy, Minia University, Minia, Egypt.
***Department of Pharmacology & Toxicology, Faculty of Pharmacy, Damanhour University, Damanhour, Egypt.

Abstract
Oxidative stress is a known mechanism mediating pancreatic β-cell injury. As a response, the cell triggers the synthesis of antioxidant and stress response elements like heme oxygenase. Heme oxygenase-١ (HO-١), which elicits antioxidant and anti-inflammatory activity, is up-regulated by various stimulants, such as oxidative stress, inflammation and thus is implicated in various inflammatory diseases. HO-١ inducers such as hemin may be useful for preventing L-arginine-induced pancreatic inflammation. The purpose of this study is to investigate whether HO-١ upregulation when using hemin, a heme oxygenase-١ inducer, could protect pancreatic β-cells from L-arginine-induced acute pancreatitis. Acute pancreatitis was induced by injection of L-arginine (٥٠٠ mg/١٠٠g, i.p) as a single dose. Effects of hemin (٥mg/kg, i.p.) against acute pancreatitis in the presence or absence of zinc protoporphyrin (ZnPP) were evaluated. **Results:** Our results show that the pancreatic weight/body weight ratios, pathological scores, amylase and lipase activities as well as the expression levels of TNF-α, COX-II and iNOS were significantly reduced in the pancreatic tissues of the rats in the hemin- pretreated group compared with those of the rats in the vehicle group. Administration of hemin before induction of AP significantly increased HO-١ activity in the pancreas. The suppression of HO-١ activity in the ZnPP-treated group significantly abolished the protective effects of hemin. Collectively, our findings demonstrate that hemin protects rats from acute pancreatitis by inducing HO-١ mediated anti-inflammatory and antioxidant effects.
Key words: Acute pancreatitis, L-arginine, Hemin, oxidative stress, heme oxygenase-١.

Introduction
Acute pancreatitis (AP) is a potentially life threatening inflammatory disease develop-ping within the pancreatic gland with high mortality rates[١]. Despite intensive research and improved medical treatment, overall lethality is still high reaching up to ٢٠-٣٠ % especially in presence of infected pancreatic necrosis[٢]. Recent studies have indicated that the release of prion-flammatory cytokines and oxidative stress play an important role in the pathogenesis of acute pancreatitis[٣,٤]. AP starts as local inflammation of the pancreas that induces the development of multiple organ dysfunction syndromes. In the early phase of inflammatory response, oxygen free radicals and basic proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-١β (IL-١β) and interleukin-٦ (IL-٦) have been suggested to be responsible for the local tissue damage and systemic inflammatory response syndrome (SIRS)[٥,٦]. Thus, inhibiting the synthesis of these cytokines and altering the balance between pro- and anti-inflammatory cytokines in the early phase might significantly affect the severity of pancreatitis and the survival rate [٧,٨]. Heme oxygenase-١ (HO-١) is a heat-shock protein[٩] and also a stress protein induced by several agents that cause oxidative damage[١٠]. Heme oxygenase-١ (HO-١) is highly inducible by a vast array of stimuli, including oxidative stress, heat shock, ultraviolet radiation, heavy metals,

cytokines, nitric oxide (NO) and inflammatory stimuli. It catalyzes the oxidative degradation of heme to equimolar amounts of biliverdin, free iron and carbon monoxide (CO). Of these metabolites, bilirubin acts as a direct antioxidant whereas CO may exert tissue-protective actions primarily through its vasodilator and antiplatelet effects. The pharmacological applications of CO and biliverdin/bilirubin can mimic the HO-١ dependent cytoprotection and anti-inflammation in many injury models. HO-١, once expressed under various pathological conditions, has an ability to metabolize high amounts of potentially toxic prooxidant molecule heme[١١] to produce high concentrations of its enzymatic by-products that can influence various biological events and has recently been the focus of considerable medical interest. The degradation of heme is now considered critical in cellular defense for two contrasting reasons. First, the pro-oxidant heme is removed. Second, the increased production of bilirubin and CO is now regarded as beneficial and critical to cellular defense mechanisms. Several studies have demonstrated the cyto-protective effects of HO-١ in pancreatic β-cells. For example, HO-١ upregulation in islets of Langerhans leads to protection of β-cells from the detrimental effect that is mediated by various apoptotic stimuli, including cytokines and Fas[١٢-١٥]. HO-١ induction by hemin reduced the production of proinflammatory cytokines from macrophages by inhibiting the activation of inflammatory signaling molecules (JNK and NF-κB) in these cell types. In view of the aforementioned studies, the present work was conducted to assess the outcome of hemin, HO-١ inducer on the experimental model of AP in rats.

Materials and methods

Chemicals

L-arginine, powder (Euromedex, France) was prepared as a solution by dissolving in ٠.٩٪ saline to an ultimate concentration of ٥٠٠ mg/ml and the pH was adjusted to ٧ with ٥ N HCl. Hemin and Zinc protoporphyrin were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). All other chemicals were of analytical grade and were obtained from commercial sources.

Animals

Adult male wistar rats weighing ٢٠٠-٢٥٠ g obtained from the animal care unit (Faculty of Agriculture, Minia University, Egypt) were used after ١ week for proper acclimatization to the animal house conditions (١٢:١٢ h light/dark cycles and ٢٥ ± ٢°C temperature) and had free access to standard rodent chow and water ad libitum. Experiments were conducted in accordance with the international ethical guidelines for animal care of the United States Naval Medical Research Centre, Unit no. ٣, Abbaseya, Cairo, Egypt, accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International). 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.

Animal groups

The rats were randomly divided into ٤ groups of six rats each, as following:

١. Control normal group: rats were normal non-pancreatic rats, received ١ml of physiologic saline by i.p.injection.

٢. Acute pancreatic group (AP): rats were treated with L-arginine; acute pancreatitis was induced by single i.p. injection of ٢٠٪ l-arginine hydrochloride in saline (٥٠٠ mg/١٠٠g).

٣. Acute pancreatic group pre-treated with hemin (AP+H.): pancreatic rats were intraperitoneally treated with hemin in a dose of ٥ mg/kg ١ h before AP modeling for ٧٢ hr.

٤. Acute pancreatic group pre-treated with a combination of zinc protoporphyrin and hemin (AP+Znpp+H.): pancreatic rats were intraperitoneally injected with ZnPP and hemin at ٥٠µg/Kg and ٥ mg/kg ١ h before AP modeling. for ٧٢ hr.

Sample collection

At the end of the experiment, rats were fasted overnight and each rat was weighed then killed. Rats were dissected to obtain the pancreases, which were weighted. Pancreatic weight index ([Pancreas weight/

body weight] x ١٠٠٠) that reveals the degree of pancreatic edema, expressed as (mg/g) was calculated. Blood samples were withdrawn from jugular vein in clean tubes immediately, left for ٣٠ minutes, and sera were separated by centrifugation at ٣٠٠٠ rpm for ١٥ min. at ٤°C and stored at -٧٠ °C until being used. Then, the abdomen and the thorax were opened and the pancreas was dissected, washed three times in ice cold saline and blotted individually on ash-free filter paper. One piece was kept into ١٠٪ formalin solution and fixed for histopathological examination, and the remaining pieces were frozen in liquid nitrogen and stored at -٨٠°C till being used for preparation of tissue homogenates. Tissue homogenates were centrifuged at ٧,٧٠٠ x g for ٣٠ minutes at ٤°C, and the resultant supernatant was assayed for the different estimations.

Biochemical analysis

Assessment of serum amylase and lipase activities Serum amylase and lipase activities expressed as (U/L) were determined according to the method previously described of[١٦,١٧] using commercially available kits, serum amylase (BIOLABO SAS.) and lipase (BioVision, USA).

Assessment of antioxidant enzyme activities, oxidative stress biomarkers, and nitric oxide bioavailability. The catalase pancreatic content expressed as (units/mg protein) was determined spectrophotometrically as previously described[١٨] using commercially available kits, (Biodiagnostic, Egypt). Pancreatic superoxide dismutase (SOD) activity expressed as (units/mg protein) was evaluated as previously described[١٩] using commercially available kits, (Biodiagnostic, Egypt). Pancreatic MDA level expressed as (nmol/mg protein) was obtained spectrophotometrically as previously described[٢٠] using commercially available kits, (Bio-diagnostic, Egypt). Finally, the pancreatic nitrite/nitrate ratio (as an indicator of nitric oxide bioavailability) expressed as (nmol/mg protein) was assessed spectrophotometrically as previously described[٢١] using commercially available kits, (Bio-diagnostic, Egypt).

Assessment of inflammatory biomarkers (TNF-α, COX-II and iNOS) by western blot analysis

Pancreatic tissues were homogenized in ice-cold RIBA lysis buffer PL٠٠٥ provided by Bio BASIC INC. (Marhham Ontario L٣R ٨T٤ Canada) for ٣٠ min. on ice. Then, the extracts were transferred to a new tube for further protein concentration determination analysis. Bradford Protein Assay Kit (SK٣٠٤١) for quantitative protein analysis was provided by BIO BASIC INC. (Markham Ontario L٣R ٨T٤ Canada). A Bradford assay was performed according to manufacture instructions. Equal amounts of total protein (٤٠ μg) were fractionated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories, TNC, USA) at ١٧٠ mA for ١-٢hr. The membranes were blocked at room temperature for ١hr. in blocking buffer (TBS, ٠.١٪ Tween ٢٠ and ٣٪ bovine serum albumin (BSA)) and then immunoblotted overnight at ٤°C with primary antibodies targeted against the following: TNF-α rabbit polyclonal antibody (Thermo Fisher Scientific, USA), COX-II rabbit polyclonal antibody (Thermo Fisher Scientific, USA), iNOS rabbit polyclonal antibody (Thermo Fisher Scientific, USA) and β-actin (١:١٠٠٠). After being rinsed ٣-٥ times with TBST for ٥ min each, the membranes were incubated for ١hr. at room temperature with horse-radish peroxidase-conjugated secondary antibodies (Goat antirabbit IgG-HRP-lmg Goat mab -Novus Biologicals) and then rinsed ٣-٥ times for ٥ min with TBST. Finally, the protein blots on the membranes were visualized using a (Clarity™ Western ECL substrate - BIO-RAD, USA). The density values of the protein bands were calculated as a ratio to β-actin and quantified using Image analysis software with an imaging densitometer (Chemi Doc MP imager).

Assessment of HO-١ activity in pancreatic tissue

The activity of HO in the supernatant was determined according to the method previously described of[٢٢,٢٣]. The supernatants were incubated at ٣٧ °C for ١

hour with heme (٥٠ mmol/L), rat liver cytosol (٥ mg/mL), $MgCl_٢$ (٢ mmol/L), glucose-٦-phosphate dehydrogenase (١ unit), glucose-٦-phosphate (٢mmol/L) and NADPH (٠.٨ mmol/L) in ٠.٥ mL of ٠.١ mol/L phosphate buffer saline (pH ٧.٤). Reaction was stopped by putting the tubes on crushed ice, then the bilirubin generated was extracted by chloroform and its amount was determined with a scanning spectro-photometer and was defined as the difference between the absorbance at ٤٦٣ and ٥٢٠ nm, while using a standard bilirubin curve. The results of the measurements were expressed as the formation of bilirubin (in picomoles) per milligram of protein within ١ hr.

Histopathological examination

Pancreatic samples isolated from rats after scarification were fixed in ١٠٪ neutral buffered formalin and embedded in paraffin by standard methods. Paraffin sections of ٥ µm thickness were cut and stained with hematoxylin and eosin, assessed under dark field microscope and examined blind by histologist for grading histopathological changes. Pancreatic damage was assessed and scored by grading acinar cell degeneration, interstitial inflammation, edema and hemorrhage as described by[٢٤].

Statistical analysis

Data are expressed as mean ± S.E.M. (standard error of the mean) and were analyzed using the one-way ANOVA followed by the Tukey–Kramer post analysis test for multiple comparisons. A probability value (P) below ٠.٠٥ was considered to indicate a statistically significant difference. Statistical analysis was performed using Graph Pad Prism ٥ (GraphPad Software, San Diego, California, USA).

Results

General parameters

The results obtained in the present investigation revealed that L-arginine induced acute pancreatitis exhibited a significant increase in serum levels of amylase, lipase and C-reactive protein. Moreover, L-arginine significantly increased the pancreatic tissue content of inflammatory biomarkers, oxidative stress parameters and NO bioavailability. In addition, L-arginine significantly increased pancreatic tissue activity of HO-١ while depleted antioxidant enzyme capacities of SOD and catalase compared to control group. Treatment with Hemin significantly attenuated L-arginine-induced biochemical changes. The changes in the measured parameters in both serum and pancreatic tissues in the studied groups were shown in Table ١.

Table (١): Effect of pre-hemin treatment (٥mg/kg, i.p.) in presence and absence of zinc protoporphyrin (Znpp) (٥٠ µg/Kg, i.p.) on the serum and pancreatic tissue levels of the pancreatic markers and the oxidative status in L-arginine induced acute pancreatitis

Parameter	Control	AP	AP+H.	AP+Znpp+H.
Pancreatic weight index (mg/g)	٢.٢±٠.١٧	*٣.٣±٠.٢٦	#١.٧±٠.١٣	#١.٨٣±٠.١٥
Serum amylase (U/L)	٣٠٨.٥±٧	*٢٤٣٨±١٦٨	#١٣٢٠±١٣١	#١٧٧٩±١٠١.٩
Serum lipase (U/L)	٣٠٩±٣.٧	*٢٥٦٦±٢٥٦	#١٤٠٣±١٨٦	+٢١٦٧±٦٩.٢٧
Serum C-reactive protein (mg/L)	١.٢±٠.١٧	*١٠.٩±١.٧٤	#٤.٣٥٣±٠.٤	+٨.٢٣٣±٠.٧٣٠
Pancreatic SOD(U/mg)	٢.٨٨±٠.١	*٠.٨٠±٠.١٢	#١.٥٦±٠.١٢	+٠.٩٥±٠.٠٣٢
Pancreatic Catalase(U/mg)	٢٧٠±٢٣.٨	*١٠٦±٥.٩٦	#١٩١.٨±٧.٥	+١٤٣.١±٧.٨٦٨

Data are expressed as mean ± S.E.M. of ٦ rats in each group. *P‹٠.٠٥ vs. control group; #P‹٠.٠٥ vs. acute pancreatic group (AP); +P‹٠.٠٥ vs. hemin pre-treated AP group (AP+H.). AP: Acute pancreatitis; H.: Hemin; Znpp: Zinc protoporphyrin

Effect of pre-hemin treatment in presence and absence of zinc protoporphyrin (Znpp) on the serum levels of amylase and lipase in L-arginine-induced acute pancreatitis

Pancreatic injury markers (serum amylase and lipase) were significantly increased in AP group compared to control group (P<٠.٠٠١). However, pretreatment with hemin significantly decreased the serum amylase and lipase levels as compared to AP rats (P<٠.٠٠١). In addition, the serum amylase and lipase levels were almost totally reversed in the rats in AP+ZnPP+H. group compared with those in the AP+H. group (P<٠.٠٥) as shown in Table ١.

Effect of pre-hemin treatment in presence and absence of zinc protoporphyrin (Znpp) on the serum level of C-reactive protein in L-arginine-induced acute pancreatitis

Serum level of C-reactive protein (CRP) was significantly higher in the AP group than that in the control group (P<٠.٠٠١). In addition, compared with the AP group, pre-treatment with hemin significantly decreased serum C-reactive protein level (P<٠.٠٠١). Furthermore, the serum level of C-reactive protein was also significantly higher in the rats in the AP+Znpp+H. group than those in the AP+H. group (P<٠.٠٥) as shown in Table ١.

Effect of pre-hemin treatment in presence and absence of zinc protoporphyrin (Znpp) on the oxidative status in the pancreatic tissue in L-arginine-induced acute pancreatitis

Oxidative stress markers (SOD and CAT activities) were significantly lower in AP group than in the control group (P<٠.٠٠١). However, pretreatment with hemin significantly increased the pancreatic SOD and CAT activities as compared to AP rats (P<٠.٠٠١). On the other hand, pre-treatment with hemin in the presence of zinc protoporphyrin (AP+Znpp+H.) abolished the effect of hemin (AP+H.) in the pancreatic levels of SOD and CAT as compared to AP rats (P<٠.٠٥) as shown in Table ١.

Effect of pre-hemin treatment in presence and absence of zinc protoporphyrin (Znpp) on malondialdehyde and NO bioavailability in the pancreatic tissue in L-arginine-induced acute pancreatitis

The MDA and NO levels in the pancreatic tissue were significantly increased in AP group compared to control group (P<٠.٠٠١), and were significantly reduced by pre-treatment with hemin (AP+H.) (P<٠.٠٠١). On the other hand, the inhibitory effect of hemin on the MDA and NO levels was abolished by treatment with ZnPP (AP+Znpp+H.) group (P<٠.٠٥) as shown in Fig.١A. & ١B.

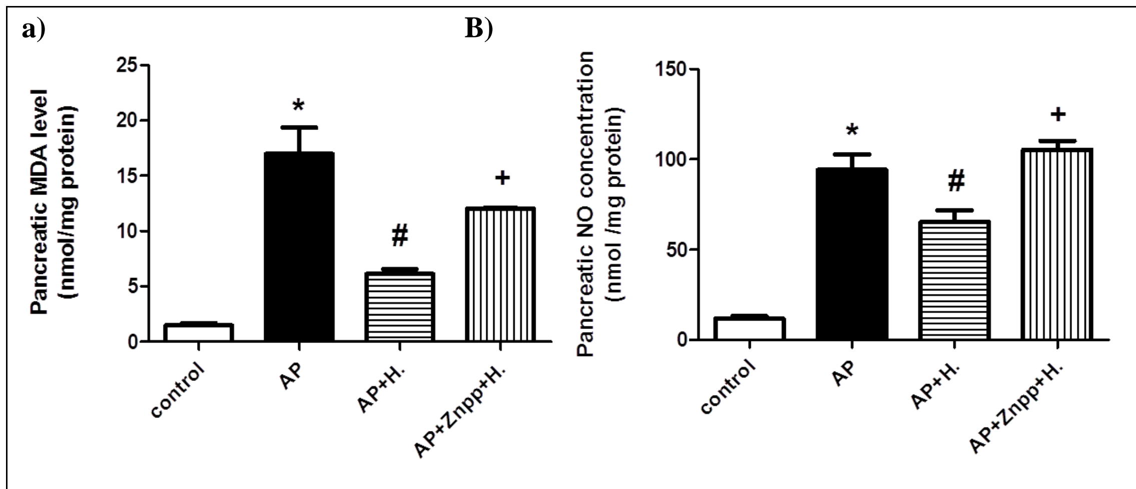

Fig. ١A. & ١B. Effect of pre-hemin treatment (٥mg/kg, i.p.) in presence and absence of zinc protoporphyrin (Znpp) (٥٠µg/Kg, i.p.) on pancreatic malondialdehyde (MDA) and nitric oxide (NO) levels of L-arginine-induced acute pancreatic group. Data represent the mean ± S.E.M., n=٦ rats; *P<٠.٠٥ vs. control group; $^{\#}$P<٠.٠٥ vs. acute pancreatic group (AP); $^{+}$P<٠.٠٥ vs. AP+H.-treated group. AP: Acute pancreatitis; H.: Hemin; Znpp: Zinc protoporphyrin

Effect of pre-hemin treatment in presence and absence of zinc protoporphyrin (Znpp) on pancreatic HO-١ activity in L-arginine-induced acute pancreatitis

Considering the HO-١ is a potential target for the treatment of AP, the effect of hemin pre-treatment on HO-١ activity was analyzed. The HO-١ activity was significantly increased in the hemin treated group compared with AP group (P<٠.٠٠١). On the other hand, the results revealed that HO-١ activity was significantly inhibited in the pancreatic tissues of rats in the AP+Znpp+H. group (P<٠.٠٥) as shown in Fig.٢.

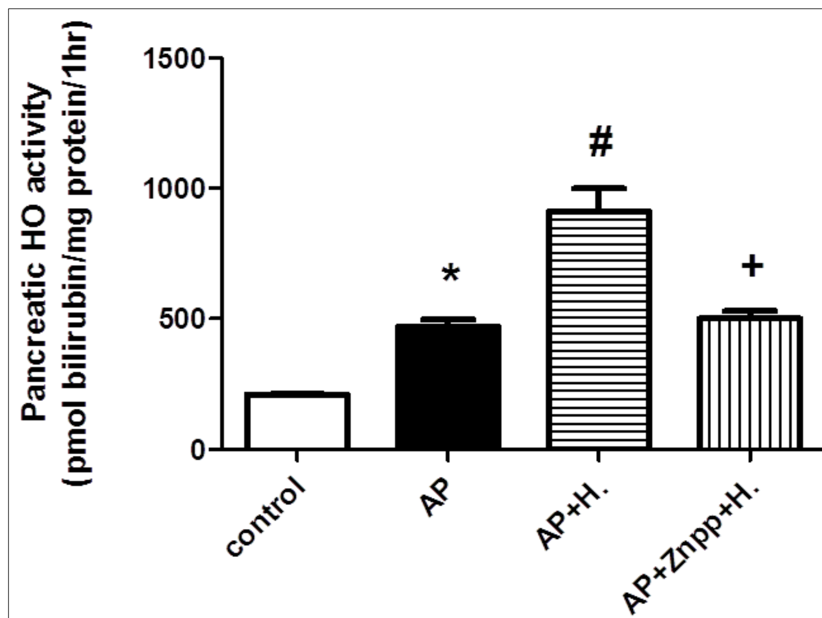

Fig. ٢. Effect of pre-hemin treatment (٥mg/kg, i.p.) in presence and absence of zinc protoporphyrin (Znpp) (٥٠µg/Kg, i.p.) on pancreatic HO-١ activity of L-arginine-induced acute pancreatic group. Data represent the mean ± S.E.M., n=٦ rats; *P<٠.٠٥ vs. control group; $^{\#}$P<٠.٠٥ vs. acute pancreatic group (AP); $^{+}$P<٠.٠٥ vs. AP+H.-treated group. AP: Acute pancreatitis; H.: Hemin; Znpp: Zinc protoporphyrin

Effect of pre-hemin treatment in presence and absence of zinc protoporphyrin (Znpp) on pancreatic inflammatory biomarkers (TNF-α, COX-II, iNOS) levels in L-arginine-induced acute pancreatitis

Western blot results showed that the levels of pro-inflammatory cytokines, including TNF-α, COX-II and iNOS in pancreatic tissue were significantly increased in AP group compared to control group (P<٠.٠٠١) and were significantly inhibited in in hemin pre-treated group (P<٠.٠٠١). Based on the effective inhibition of HO-١ activity by Znpp, tissue necrosis and inflammatory infiltration were more severe in the rats in the AP+Znpp+H. group than those in the AP+H. group (P<٠.٠٥) as shown in Fig.٣. A, B and C.

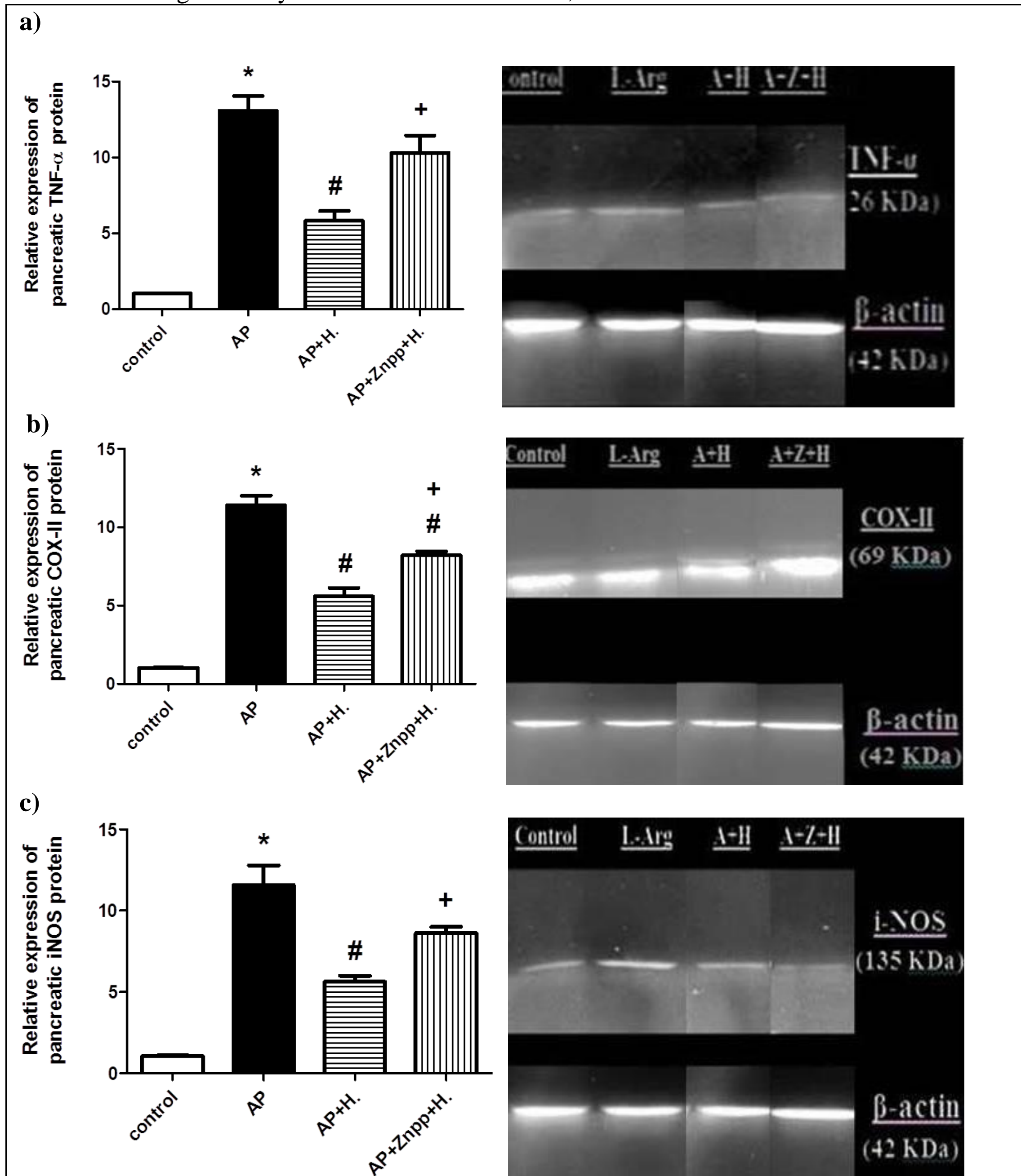

Fig.٣. A, B and C. Effect of pre-hemin treatment (٥mg/kg, i.p.) in presence and absence of zinc protoporphyrin (Znpp) (٥٠µg/Kg, i.p.) on pancreatic inflammatory biomarkers (TNF-α, COX-II and iNOS) levels of L-arginine-induced acute pancreatic group. Bar graphs indicate results of densitometric analysis of the bands as normalized to the quantity of β-actin protein. Data represent the mean ± S.E.M., n=٦ rats; *P<٠.٠٥ vs. control group; #P<٠.٠٥ vs. acute pancreatic group (AP); +P<٠.٠٥ vs. AP+H.-treated group. AP: Acute pancreatitis; H.: Hemin; Znpp: Zinc protoporphyrin

Effect of hemin on histological findings in the pancreatic tissue

Histopathological scoring included assessment of edema, acinar necrosis, inflammatory cell infiltration and hemorrhage. While regular pancreas morphology was observed in the control group, varying degrees of acinar swelling, pancreatic tissue necrosis, hemorrhage and inflammatory cell infiltration were observed in the AP group. The AP significantly increased the total histological scores of the pancreatic tissue as compared to the control group. However, pre-hemin treatment significantly lowered these scores but pre-hemin treatment in the presence of zinc protoporphyrin (AP+Znpp+H.) abolished this effect as compared to the (AP+H.) group (Table ٣). Evaluation of histological signs of pancreatic inflammation is summarized as follow (Table ٢) and illustrated in (Fig. ٤).

Table (٢): Effect of pre-hemin treatment (٥mg/kg, i.p.) in presence and absence of zinc protoporphyrin (Znpp) (٥٠µg/Kg, i.p.) on severity of histopathological lesions in pancreatic tissue in L-arginine-induced acute pancreatitis

Pancreatic histopathological alterations	Control	AP	AP+H.	AP+ ZnPP+H.
Diffuse inflammatory cells infiltration	-	+++	-	-
Congestion in blood vessels	-	++	-	+
Atrophy in islands of langerhans	-	+	-	-
Focal inflammatory cells infiltration	-	-	-	+
Atrophy in the acini	-	-	-	-

Score level – is considered normal; Scores +, ++ and +++ are mild, moderate and severe levels, revealing less than ٢٥٪, ٥٠٪ and ٧٥٪ histopathological alterations of total fields examined, respectively.
AP: Acute pancreatitis; H.: Hemin; Znpp: Zinc protoporphyrin

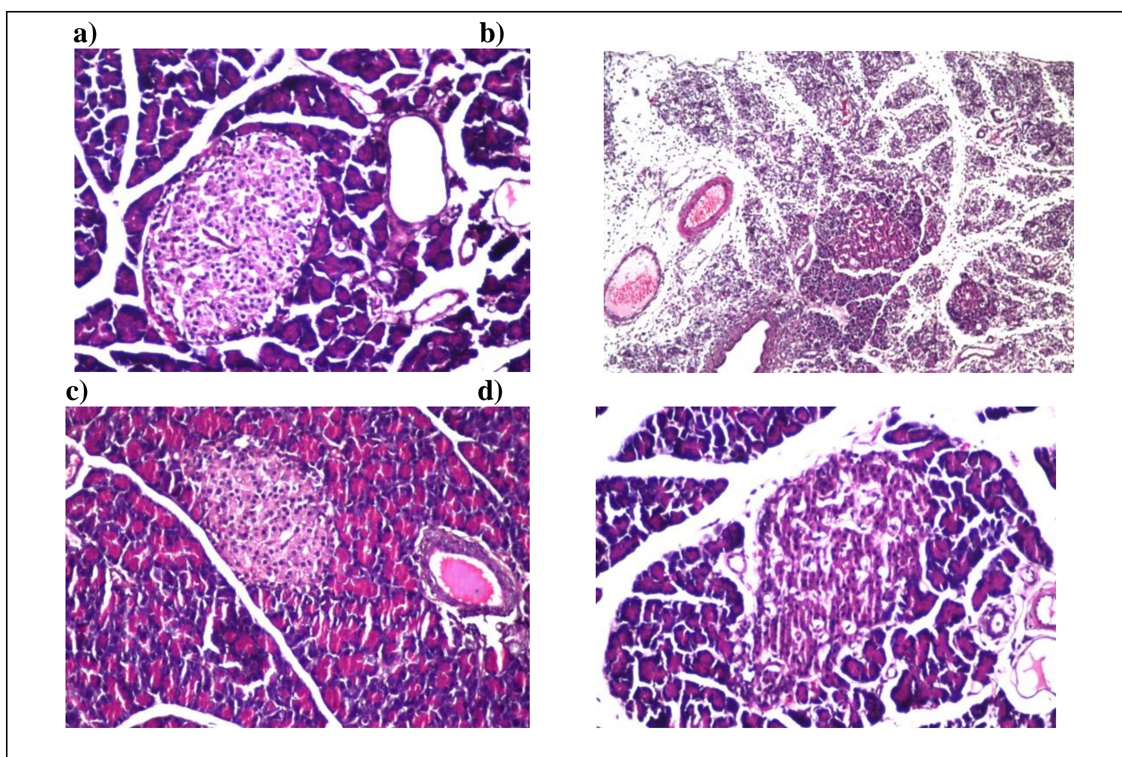

Fig. ٤. The hematoxylin and eosin-stained photomicrographs (H&E; x٤٠٠) of a section in the pancreas of (A) Control group showing normal histological structure of islands of langerhans cells as endocrine portion as well as acini and duct system as exocrine one. (B) Acute pancreatitis (AP) group showing diffuse inflammatory cells infiltration in between the acini as well as in the interlobular stromal tissue with congestion in blood vessels and atrophy of islands of langerhans cells. (C) Hemin pre-treated group showing normal histological structure. (D) Zinc protoporphyrin (ZnPP) plus hemin (H.) pre-treated group showing congestion in blood vessels with few inflammatory cells infiltration in the periphery of the lobules.

Discussion

Acute pancreatitis (AP) is an inflammatory disease with high mortality rates; particularly in presence of multiorgan failure. Inflammatory infiltration and oxidative stress are critically involved in the development of AP [٢٥]. In the present work, intraperitoneal injection of L-arginine was used to induce acute pancreatitis. One of the mechanisms by which L-arginine induces AP includes the metabolism of L-arginine to NO by nitric oxide synthase causing oxidative stress in the pancreas. Moreover, L-arginine may also stimulate induction of AP through increased oxygen free radicals generation and inflammatory cytokines production such as TNF-α and IL-٦ which have a key role in the development of the disease. The results obtained in the present investigation revealed that L-arginine induced-pancreatitis exhibited a significant increase in serum levels of amylase and lipase enzymes with severe pancreatic histo-architectural changes. The serum levels of both enzymes have high sensitivity and specificity for the diagnosis and assessment of severity of AP [٢٦].

Additional biomarkers for diagnosis of acute pancreatitis include C-reactive protein (CRP), oxidative stress biomarkers as well as pro-inflammatory cytokines (TNF-α, COX-II and iNOS). In agreement with the data of other reports[٢٧, ٢٨], the current study showed significant increase in pancreatic NO level in L-arginine received rats. The increase in NO level is due to the induction of iNOS activity in the pancreas [٢٩]. NO level may lead to acinar cell damage and pancreatic edema. In consistent with previous studies[٣٠], the current study showed significant decrease in parameters

related to the enzymatic antioxidant system, including SOD and CAT activities in L-arginine-treated rats. The decrease of these enzymes activities in turn increases free

radicals generation and enhances lipid peroxidation. MDA, as indicator of lipid peroxidation, was elevated in L-arginine-treated rats. Lipid peroxidation is a process mediated by free radicals causes damage to membranes of the endothelial cells of capillaries due to oxidative deterioration of polyunsaturated fatty acids of cell membrane. Lipid peroxidation could be attributed to the increased generation of free radicals produced by L-arginine[٢٧].

Regarding such concepts, it is logic to introduce drugs that possess anti-inflammatory and/or antioxidant actions to control underlying pathogenesis of acute pancreatitis and improve its course. We first found that the HO-١ induction by hemin treatment when given before L-arginine-induced AP significantly restored the serum amylase and lipase levels. Oxidative stress plays a vital role in the pathogenesis of AP and the beneficial effects of hemin might be associated with the suppression of neutrophil mediated lipoperoxidation, as demonstrated by reduced MDA level; event that reflects its antioxidant action[٣١]. Additionally, our results showed that hemin- mediated increase of HO-١ activity was proved enough to decrease the NO-dependent pathological and inflammatory conditions as reported by[٣٢]. All these findings indicate that hemin has protective effects from pancreatitis and may improve the functions of pancreas. These data are in agreement with the findings of Nakamichi et al.,[٣٣] who have demonstrated that pre-treatment, as a prophylactic, with hemin or hemin-activated cell protects against experimentally induced pancreatic injury.

Moreover, pre-hemin administration significantly reversed the elevation of TNF-α, COX-II and iNOS levels in the treated group. Our results reported that hemin attenuated the pancreatic tissue injury through inhibiting the release of inflammatory cytokines. This inhibition could be because hemin increased the expression of HO-١ as observed in our study, which can show several potential protective mechanisms. The inhibitory effect of hemin on the activation of the inflammatory signaling molecules may be, at least in part, associated with CO production by HO-١, leading to reduced inflammatory cytokines. CO, a by-product of heme catabolism by HO-١, exerts potent anti-inflammatory effects by inhibiting JNK/AP-١ binding[٣٤] and/or NF-κB binding[٣٥]. Also, the histopathological examination revealed that pre-hemin administration significantly decreased the histopathological scores of all pancreatic changes. The effect of hemin was completely blunted by the HO-١ inhibitor ZnPP. These observations indicate that induction of HO-١by hemin exerts anti-inflammatory activities by producing three anti-inflammatory metabolites (i.e. CO, ferrous iron as well as biliverdin) via its enzyme activity[٣٦]. CO decreases pro-inflammatory cytokine production[٣٧], reduces apoptosis[٣٨, ٣٩], improves organ function[٤٠] and increases the survival[٤١]. Biliverdin and bilirubin, the end bile pigments of heme degradation, protect cells against oxidative stress by their antioxidant properties[٤٢,٤٣].

Iron potentially can stimulate the expression of secondary antioxidant protein, ferritin[٤٤,٤٥]. The increase in HO-١ in pancreatic macrophages in response to hemin raised the hypothesis that hemin selectively stimulated macrophage polarization towards the anti-inflammatory M٢-phenotype which in turn is responsible for the observed protection afford by hemin [٤٦]. M٢ macrophage reduces inflammation and promote tissue repair. They have a distinctive ability to metabolize arginine to the "repair" molecule ornithine and by producing anti-inflammatory cytokines like IL-١٠ [٤٧]. These findings indicate that pre-hemin administration is a suitable candidate as an effective strategy against AP through its anti-inflammatory and antioxidant effects.

References

١. Malleo, G., et al., Role of tumor necrosis factor-alpha in acute pancreatitis: from biological basis to

clinical evidence. Shock, ٢٠٠٧. ٢٨(٢): p. ١٣٠-٤٠.
٢. Hegyi, P., et al., L-arginine-induced experimental pancreatitis. World Journal of Gastroenterology : WJG, ٢٠٠٤. ١٠(١٤): p. ٢٠٠٣-٢٠٠٩.
٣. Bhatia, M., et al., Inflammatory mediators in acute pancreatitis. J Pathol, ٢٠٠٠. ١٩٠(٢): p. ١١٧-٢٥.
٤. Yang, Z.W., X.X. Meng, and P. Xu, Central role of neutrophil in the pathogenesis of severe acute pancreatitis. J Cell Mol Med, ٢٠١٥. ١٩(١١): p. ٢٥١٣-٢٠.
٥. Carnovale, A., et al., Mortality in acute pancreatitis: is it an early or a late event? Jop, ٢٠٠٥. ٦(٥): p. ٤٣٨-٤٤.
٦. Sah, R.P., P. Garg, and A.K. Saluja, Pathogenic mechanisms of acute pancreatitis. Curr Opin Gastroenterol, ٢٠١٢. ٢٨(٥): p. ٥٠٧-١٥.
٧. Walley, K.R., et al., Balance of inflammatory cytokines related to severity and mortality of murine sepsis. Infect Immun, ١٩٩٦. ٦٤(١١): p. ٤٧٣٣-٨.
٨. Weiss, Y.G. and C.S. Deutschman, Modulation of gene expression in critical illness: a new millennium or a brave new world? Crit Care Med, ٢٠٠٠. ٢٨(٨): p. ٣٠٧٨-٩.
٩. Mitani, K., et al., Heat shock induction of heme oxygenase mRNA in human Hep ٣B hepatoma cells. Biochemical and Biophysical Research Communications, ١٩٨٩. ١٦٥(١): p. ٤٣٧-٤٤١.
١٠. Nascimento, A.L., P. Luscher, and R.M. Tyrrell, Ultraviolet A (٣٢٠-٣٨٠ nm) radiation causes an alteration in the binding of a specific protein/protein complex to a short region of the promoter of the human heme oxygenase ١ gene. Nucleic Acids Res, ١٩٩٣. ٢١(٥): p. ١١٠٣-٩.
١١. Stocker, R., et al., Bilirubin is an antioxidant of possible physiological importance. Science, ١٩٨٧. ٢٣٥: p. ١٠٤٣-١٠٤٧.
١٢. Pileggi, A., et al., Heme oxygenase-١ induction in islet cells results in protection from apoptosis and improved in vivo function after transplantation. Diabetes, ٢٠٠١. ٥٠(٩): p. ١٩٨٣-١٩٩١.
١٣. Ribeiro, M.M., et al., Heme oxygenase-١ fused to a TAT peptide transduces and protects pancreatic β-cells. Biochemical and Biophysical Research Communications, ٢٠٠٣. ٣٠٥(٤): p. ٨٧٦-٨٨١.
١٤. Tobiasch, E., L. Gunther, and F.H. Bach, Heme oxygenase-١ protects pancreatic beta cells from apoptosis caused by various stimuli. J Investig Med, ٢٠٠١. ٤٩(٦): p. ٥٦٦-٧١.
١٥. Lee, E.-M., et al., Protective Effect of Heme Oxygenase-١ on High Glucose-Induced Pancreatic β-Cell Injury. Diabetes & Metabolism Journal, ٢٠١١. ٣٥(٥): p. ٤٦٩-٤٧٩.
١٦. Mondragon, A., et al., Divergent effects of liraglutide, exendin-٤, and sitagliptin on beta-cell mass and indicators of pancreatitis in a mouse model of hyperglycaemia. PLoS One, ٢٠١٤. ٩(٨): p. e١٠٤٨٧٣.
١٧. Winn-Deen, E.S., et al., Development of a direct assay for alpha-amylase. Clin Chem, ١٩٨٨. ٣٤(١٠): p. ٢٠٠٥-٨.
١٨. Kakkar, R., et al., Increased oxidative stress in rat liver and pancreas during progression of streptozotocin-induced diabetes. Clin Sci (Lond), ١٩٩٨. ٩٤(٦): p. ٦٢٣-٣٢.
١٩. Szabolcs, A., et al., Effect of melatonin on the severity of L-arginine-induced experimental acute pancreatitis in rats. World Journal of Gastroenterology : WJG, ٢٠٠٦. ١٢(٢): p. ٢٥١-٢٥٨.
٢٠. Buege, J.A. and S.D. Aust, Microsomal lipid peroxidation. Methods Enzymol, ١٩٧٨. ٥٢: p. ٣٠٢-١٠.
٢١. Sun, J., et al., Measurement of Nitric Oxide Production in Biological Systems by Using Griess Reaction Assay. Sensors, ٢٠٠٣. ٣(٨): p. ٢٧٦.
٢٢. Abraham, N.G., J.D. Lutton, and R.D. Levere, Heme metabolism and erythropoiesis in abnormal iron states: role of delta-aminolevulinic acid synthase and heme oxygenase. Exp Hematol, ١٩٨٥. ١٣(٨): p. ٨٣٨-٤٣.
٢٣. Tenhunen, R., H.S. Marver, and R. Schmid, Microsomal heme oxygenase. Characterization of the enzyme. J Biol Chem, ١٩٦٩. ٢٤٤(٢٣): p. ٦٣٨٨-٩٤.
٢٤. Abdin, A.A., et al., Effect of pentoxifylline and/or alpha lipoic acid

on experimentally induced acute pancreatitis. European journal of pharmacology, ٢٠١٠. ٦٤٣(٢): p. ٢٨٩-٢٩٦.

٢٥. Gomez-Cambronero, L.G., et al., Role of cytokines and oxidative stress in the pathophysiology of acute pancreatitis: therapeutical implications. Curr Drug Targets Inflamm Allergy, ٢٠٠٢. ١(٤): p. ٣٩٣-٤٠٣.

٢٦. Akyazi, I., et al., Long-term aspirin pretreatment in the prevention of cerulein-induced acute pancreatitis in rats. World J Gastroenterol, ٢٠١٣. ١٩(١٩): p. ٢٨٩٤-٩٠٣.

٢٧. Biradar, S. and B. Veeresh, Protective effect of lawsone on L-Arginine induced acute pancreatitis in rats. Indian J Exp Biol, ٢٠١٣. ٥١(٣): p. ٢٥٦-٦١.

٢٨. Xu, X.W., et al., Treatment with ginkgo biloba extract protects rats against acute pancreatitis-associated lung injury by modulating alveolar macrophage. Prz Gastroenterol, ٢٠١٤. ٩(١): p. ٤٣-٨.

٢٩. Takacs, T., et al., The role of nitric oxide in edema formation in L-arginine-induced acute pancreatitis. Pancreas, ٢٠٠٢. ٢٥(٣): p. ٢٧٧-٨٢.

٣٠. Czako, L., et al., Oxidative stress in distant organs and the effects of allopurinol during experimental acute pancreatitis. Int J Pancreatol, ٢٠٠٠. ٢٧(٣): p. ٢٠٩-١٦.

٣١. Collino, M., et al., Beneficial effect of prolonged heme oxygenase ١ activation in a rat model of chronic heart failure. Dis Model Mech, ٢٠١٣. ٦(٤): p. ١٠١٢-٢٠.

٣٢. Al-Kahtani, M.A., et al., Hemin attenuates cisplatin-induced acute renal injury in male rats. Oxid Med Cell Longev, ٢٠١٤. ٢٠١٤: p. ٤٧٦٤٣٠.

٣٣. Nakamichi, I., et al., Hemin-activated macrophages home to the pancreas and protect from acute pancreatitis via heme oxygenase-١ induction. J Clin Invest, ٢٠٠٥. ١١٥(١١): p. ٣٠٠٧-١٤.

٣٤. Morse, D., et al., Suppression of inflammatory cytokine production by carbon monoxide involves the JNK pathway and AP-١. J Biol Chem, ٢٠٠٣. ٢٧٨(٣٩): p. ٣٦٩٩٣-٨.

٣٥. Sarady, J.K., et al., Carbon monoxide modulates endotoxin-induced production of granulocyte macrophage colony-stimulating factor in macrophages. Am J Respir Cell Mol Biol, ٢٠٠٢. ٢٧(٦): p. ٧٣٩-٤٥.

٣٦. Liu, X.H., et al., Induction of Heme Oxygenase-١ by Sodium ٩-Hydroxyltanshinone IIA Sulfonate Derivative Contributes to Inhibit LPS-Mediated Inflammatory Response in Macrophages. Cell Physiol Biochem, ٢٠١٥. ٣٦(٤): p. ١٣١٦-٣٠.

٣٧. Durante, W., Carbon monoxide and bile pigments: surprising mediators of vascular function. Vasc Med, ٢٠٠٢. ٧(٣): p. ١٩٥-٢٠٢.

٣٨. Brouard, S., et al., Carbon monoxide generated by heme oxygenase ١ suppresses endothelial cell apoptosis. J Exp Med, ٢٠٠٠. ١٩٢(٧): p. ١٠١٥-٢٦.

٣٩. Nikolic, I., et al., Pharmacological application of carbon monoxide ameliorates islet-directed auto-immunity in mice via anti-inflammatory and anti-apoptotic effects. Diabetologia, ٢٠١٤. ٥٧(٥): p. ٩٨٠-٩٩٠.

٤٠. Yao, L., et al., Carbon monoxide-releasing molecules attenuate postresuscitation myocardial injury and protect cardiac mitochondrial function by reducing the production of mitochondrial reactive oxygen species in a rat model of cardiac arrest. Journal of cardiovascular pharmacology and therapeutics, ٢٠١٥. ٢٠(٣): p. ٣٣٠-٣٤١.

٤١. Wang, X., et al., A novel role of exogenous carbon monoxide on protecting cardiac function and improving survival against sepsis via mitochondrial energetic metabolism pathway. Int J Biol Sci, ٢٠١٤. ١٠(٧): p. ٧٧٧-٧٨٨.

٤٢. Clark, J.E., et al., Dynamics of haem oxygenase-١ expression and bilirubin production in cellular protection against oxidative stress. Biochem J, ٢٠٠٠. ٣٤٨ Pt ٣: p. ٦١٥-٩.

٤٣. Ben-Amotz, R., et al., Intraperitoneal bilirubin administration decreases infarct area in a rat coronary ischemia/reperfusion model. Frontiers in physiology, ٢٠١٤. ٥: p. ٥٣.

٤٤. Juckett, M.B., et al., Ferritin protects endothelial cells from oxidized low density lipoprotein in vitro. Am J Pathol, ١٩٩٥. ١٤٧(٣): p. ٧٨٢-٩.
٤٥. Yegin, Z., et al., The interplay among iron metabolism, endothelium and inflammatory cascade in dysmetabolic disorders. Journal of endocrinological investigation, ٢٠١٥. ٣٨(٣): p. ٣٣٣-٣٣٨.
٤٦. Nakamichi, I., et al., Hemin-activated macrophages home to the pancreas and protect from acute pancreatitis via heme oxygenase-١ induction. The Journal of clinical investigation, ٢٠٠٥. ١١٥(١١): p. ٣٠٠٧-٣٠١٤.
٤٧. Mills, C.D., M١ and M٢ Macrophages: Oracles of Health and Disease. Crit Rev Immunol, ٢٠١٢. ٣٢(٦): p. ٤٦٣-٨٨.