Research Article
The Potential Role of Hemopexin and Heme Oxygenase-1 Inducer in a Model of Sepsis

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1. Introduction

Sepsis is a devastating condition characterized by the activation of inflammatory and coagulation pathways in response to microbial systemic infection [1]. The septic response is a complex chain of adaptive and maladaptive alterations in homeostatic mechanisms [2]. Systemic inflammatory response to infection with the release of the proinflammatory mediators and the presence of toxins activate the coagulation cascade and evoke disseminated intravascular coagulation (DIC). This results in multiple organ failure, which is the leading cause of mortality in sepsis [3].

One cell type that is injured during sepsis is the erythrocyte. As red blood cells lyse, hemoglobin is released and oxidized, releasing free heme in the circulation. Free heme can cause tissue damage and programmed cell death through inducing the release of proinflammatory mediators and activation of coagulation system, and thus contributing to multiorgan dysfunction and death [4]. Therefore, it is critical to explore pathophysiologic alterations and interplay between coagulant responses, inflammatory responses, and heme scavenging mechanisms during sepsis in an effort to seek new therapeutic options [5].

There are a number of cytoprotective mechanisms against the deleterious effect of sepsis; among them are heme oxygenase-1 (HO-1, formerly heat shock protein 32) and hemopexin (HPx) mediated mechanisms. HO-1 is an inducible enzyme that converts heme into carbon monoxide (CO), biliverdin, and free iron [6]; it is associated with anti-inflammatory, anti-oxidative, and antiapoptotic activities. During sepsis, HO-1 was able to suppress neutrophil infiltration and protect organs during sepsis [7]. Therefore, upregulation of HO-1 has been associated with decreased injury during endotoxemia [8].

HPx is an acute-phase plasma protein expressed mainly in the liver, and its production is enhanced under inflammatory conditions. It has the highest binding affinity to heme, sub-sequent to heme binding; the heme-HPx complex is internalized in liver cells through LDL receptor-related...
protein1-(LRP1-) mediated endocytosis, resulting in cellular heme uptake [9]. Once inside the cell, heme is released into the cytoplasm and used to build new hemoproteins or is catabolized by HO-1 [10]. Some studies demonstrated the proinflammatory effect of HPx [11]. But, on the other hand, conflicting results showed that HPx preparations diminish the upregulation of the proinflammatory cytokines, TNF-α and IL-6, induced by lipopolysaccharide (LPS) stimulation of macrophages [12].

In this context, by using an animal model of sepsis, our study aimed to determine a new role for HO-1 and HPx in the coagulopathy induced by septic inflammation and whether they can affect the host defense mechanism by enhancing the production of the anti-inflammatory cytokine, IL-10, at early stages of sepsis.

The development of experimental sepsis models to elucidate the progression and pathophysiology of clinical sepsis extent over the past decades. The most frequently used sepsis model is cecal ligation puncture (CLP) which was employed in the current study [13].

2. Material and Methods

The study was conducted on 48 healthy male albino Wistar rats weighing 200–220 g; the animals were housed in an animal facility at the Faculty of Medicine, Alexandria University. A minimum temperature of 10°C in winter and a maximum one of 35°C in the summer were maintained. A period of 12–14 hours of daylight was provided. Food and water were available ad libitum. All experimental procedures were carried out based on the ethical guidelines for care and use of laboratory animals of Alexandria University. The study was approved by the Faculty of Medicine, Alexandria University Ethics Committee.

2.1. Experimental Design. The animals were divided into 4 experimental groups (12 rats/group): control group (CG): sham-operated rats, subjected to laparotomy and bowel manipulation, received an intraperitoneal injection of phosphate buffered saline (PBS) without CLP; CLP induced sepsis group (CLP): severe sepsis was induced by CLP as described below; CLP induced sepsis and hemin group (CLP + Hm): animals received single intraperitoneal injection of hemin 12 hrs before induction of sepsis at a dose of 50 μmol/kg (Sigma-Aldrich; Egypt) after being dissolved in dimethyl sulfoxide (DMSO) and diluted with PBS to make a solution with a concentration of 10 mmol/ml. [5]; CLP induced sepsis and hemopexin group (CLP + HPx): animals were injected with commercial HPx at a dose of 150 μg/rat i.v. (1.5 mg protein/ mL PBS) 30 min before induction of sepsis [14].

2.2. Cecal Ligation and Perforation. The rats were fasting for 12 hrs before the procedure but allowed free access to water. Anesthesia was induced by an intraperitoneal injection of pentobarbital (70 mg/kg). A midline laparotomy was performed, and the cecum was identified. Stool contents were milked to the tip of the cecum, which was subsequently ligated 1 cm from the tip with a 2-0 silk tie. The cecum was then perforated four punctures with a 22 G needle to induce severe sepsis and returned into the abdomen. The abdominal wall was closed with a continuous 3-0 silk suture. No antibiotics were used, and the animals had free access to food and water postoperatively. All animals in all experiments received saline to prevent dehydration (60 mL/kg/day subcutaneously) [5].

2.3. Blood and Specimen Collection. 48 hrs after CLP, mortality rate was calculated; then animals were deeply anesthetized with 100 mg/kg pentobarbital. The blood was collected via cardiac puncture with EDTA-treated syringe needles immediately mixed with 3.2% sodium citrate at a ratio of 9 : 1 (blood vol/citrate vol). Blood was then centrifuged at 1,500 g for 15 min to separate the plasma that was frozen in aliquots at −70°C until assay. The lungs and livers were excised for histological assessment.

2.4. Coagulation Parameters

2.4.1. Estimation of Prothrombin Time (PT). Thawed plasma was transferred into a polypropylene aliquot tube and resuspended at 1,500 g for 15 minutes to obtain platelet poor plasma (PPP) where platelet count was <10,000/μl. The top 0.1 mL of plasma was removed and placed in a water bath kept at a 37°C, 0.1 mL of thromboplastin (tissue factor) and 0.1 mL of calcium chloride (CaCl₂) were added, and the contents were mixed thoroughly to initiate coagulation [15].

2.4.2. Estimation of the Activated Partial Thromboplastin Time (APTT). PPP was obtained as previously mentioned; then 0.1 mL was incubated at 37°C. Phospholipid (cephalin) and contact activator (Kaolin) were added followed by the calcium (all prewarmed at 37°C) and the contents were mixed thoroughly to initiate coagulation [16].

Both assays were performed in an Amelung KC4 coagulation semiautomated analyzer using electromechanical method of clot detection (Sigma Chemical Co., St. Louis, MO).

2.4.3. Activated Protein C (APC) Chromogenic Assay. PPP was incubated at 37°C with the protein C activator (Protac) for 5 minutes. Then a chromogenic substrate for APC was added. The change in optical density was measured and by comparison against a standard reference curve the protein C level was determined [17].

2.5. Enzyme-Linked Immunosorbent Assay (ELISA). Liver homogenate HO-1, plasma, and liver homogenate IL-10 levels were measured by using ELISA kits (Shanghai BlueGene Biotech Co., Ltd., Shanghai, China) according to the manufacturer’s instructions [18, 19].

Liver tissues were homogenized in homogenization buffer (20 mmol/L potassium phosphate buffer (pH 7.4), 250 mmol/L sucrose, 2 mmol/L ethylene diamine tetra-acetic acid, 2 mmol/L phenylmethylsulfonyl fluoride, and 10 g/mL leupeptin). The homogenates were centrifuged at 10,000 × g for 30 min, and the supernatant was further centrifuged at 100,000 xg for 1h at 4°C. The pellet was suspended with
potassium phosphate buffer followed by sonication for 2 sec at 4°C (microsome fraction) [5].

2.6. Histologic Analysis. Liver and lung specimens were fixed with 10% buffered formalin, embedded in paraffin, sectioned, and examined under light microscopy. Transverse sections were made at five different levels to cover the entire organ, and ten fields were selected randomly from each section and examined for counting the number of thrombi. The mean number of thrombi was calculated for statistical analysis.

2.7. Statistical Analysis. Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. Quantitative data were described using mean and standard error. The distributions of quantitative variables were tested for normality using Kolmogorov-Smirnov test, Shapiro-Wilk test, and D'Agostino test. Also, histogram and QQ plot were used for vision test. Data were normally distributed, and, thus, comparison between different studied groups was analyzed using F-test (ANOVA) and post hoc test (LSD) for pairwise comparisons. Significance of the obtained results was judged at the 5% level.

3. Results

3.1. Survival Rates. Survival rates were calculated during 48-hour period after the induction of sepsis. Control rats survived the entire period, while rats subjected to CLP showed survival rates of 83.3%, 75%, and 58.3% after 6, 12, and 18 hrs of induction, respectively. Hemin improved the survival rates to 91.6 and 83.3% 6 and 12 hrs after induction of sepsis; also HPx treated rats had the same rates as hemin group after 6 and 12 hrs of sepsis, but survival then decreased to be 75% after 18 hrs and for the rest of the study period (Figure 1).

3.2. Coagulation Parameters. The PT and APTT values were significantly lower in CLP group in comparison to the control one (P < 0.001). The administration of hemin or HPx significantly prolonged the PT and APTT in CLP + Hm and CLP + HPx groups, in comparison to the CLP group. However, prolongation of both tests in CLP + Hm group was significantly more than that in CLP + HPx group. Regarding the APC, the results showed a significant reduction in its level in CLP compared to the control group. However, preadministration of hemin was almost able to increase APC level, as its level was significantly increased in CLP + Hm group in comparison to control and CLP groups. Similarly, HPx administration was associated with significant enhancement of APC levels compared to control and CLP groups, but its level remains significantly lower than the hemin administrated group (CLP + Hm) (Table 1).

Liver HO-1 Concentration (ng/mg Tissue). Liver HO-1 levels were measured using ELISA technique. CLP induced a significant increase in HO-1 level in the liver (P < 0.001) compared to its level in the control group; its mean value was 321.32 ± 42.42 and 201.33 ± 66.87, respectively. Pretreatment of hemin or HPx exerted a significant increase in HO-1 in the liver tissue 48 hrs after CLP; its mean value in the former was 501.91 ± 76.89 and 408.21 ± 97.83 in the latter. However, its level in CLP + Hm group was significantly higher than its level in CLP + HPx group (Figure 2). On average, hemin pretreatment increased HO-1 levels by 56% in the liver tissue while HPx increased it only by 27%.

IL-10 Levels. In control group, rats had low levels of serum IL-10 (pg/mL) with mean value equal to 19.06 ± 4.38. Following CLP, IL-10 was significantly increased by approximately two-and-half-fold than control rats; its mean value was 45.92 ± 8.99. IL-10 was, fortunately, increased significantly after either

### Table 1: Comparison between experimental groups regarding the coagulation parameters.

<table>
<thead>
<tr>
<th></th>
<th>Control group (CG)</th>
<th>Sepsis group (CLP)</th>
<th>CLP + Hm</th>
<th>CLP + HPx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prothrombin time (seconds)</td>
<td>17.08 ± 0.2</td>
<td>13.6 ± 0.2*</td>
<td>18.6 ± 0.4**</td>
<td>15.9 ± 0.5***</td>
</tr>
<tr>
<td>Partial thromboplastin time (seconds)</td>
<td>15.8 ± 0.04</td>
<td>9.2 ± 0.3*</td>
<td>15.6 ± 0.2**</td>
<td>12.1 ± 0.3***</td>
</tr>
<tr>
<td>APC (IU/dL)</td>
<td>96.8 ± 2.2</td>
<td>59 ± 1.6*</td>
<td>83 ± 0.6**</td>
<td>75.5 ± 1.05***</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM (7–12 per group); one-way ANOVA was conducted and the results of post hoc least significance difference comparison were shown. Results are significant at P < 0.001. *Significant versus control group, **significant versus CLP, and ***significant versus CLP + Hm.

APC: activated protein C.
hemin or HPx treatment compared with CLP rats where the mean values were 60.78 ± 8.65 and 76.68 ± 25.48, respectively. However, HPx pretreatment was associated with a significant increase in serum IL-10 compared to hemin pretreated group. IL-10 level in liver (pg/mg tissue) was only significantly increased in HPx pretreated group (580.72 ± 158.69) in comparison to control (420.32 ± 65.43) and CLP (434.04 ± 199.64) groups (Figures 3(a) and 3(b)).

Histopathological Examination. The number of thrombi was counted in 10 different fields per organ. Results showed that the number of thrombi was significantly higher in all groups in comparison to the control group. There was a significant difference between the number of thrombi among groups, in a way that they were significantly higher in CLP group in comparison to CLP + Hm and CLP + HPx groups. Although the number of thrombi was decreased in the latter groups, still they were significantly lower in CLP + Hm group than in CLP + HPx group. The results run in the same pattern for liver and lung specimens (Figure 4).

Histological examination of liver and lung sections showed the appearance of some inflammatory changes that were most prominent in CLP group. These inflammatory changes were moderate in the group CLP + Hm and mild in CLP + HPx group as shown in Figure 5.

4. Discussion

Despite the development of advances in diagnostic and prognostic biomarkers, the presence of promising preclinical animal studies examining the fundamental cellular and biological mechanisms underlying septic physiology, and a number of clinical trials targeting treatment of thromboinflammatory mediators and pathways, there are only few therapeutic agents passing to phase III clinical trials and currently none have witnessed sustained clinical use [20]. For example, two of the most promising therapeutics recently met unfortunate endings: activated protein C (APC) was pulled from the market and an anti-toll-like-receptor 4 compound failed in a phase III clinical trial [21]. Moreover, there is growing evidence suggesting that single therapeutic agents may not be an effective solution for a dynamic, complicated disease like sepsis [22].

So, currently, more researches continue in this scope to identify and study new therapies that hold promise. So, agents targeting heme oxygenase-1 acting as a cytoprotective enzyme catalyzing the oxidative degradation of toxic heme released during the pathophysiology of sepsis [6], in addition to heme sequestering protein “hemopexin” [9], may fill part of the gap in this arena and hold an important goal to develop novel and effective adjuvant therapies in the treatment of sepsis aiming to prevent sepsis related morbidity and mortality.

Sepsis is associated with impaired hemostasis, with tilt of the balance towards the procoagulant state. In sepsis, the clotting cascade is enhanced through induction of tissue factor as a result of endotoxemia [23].

The current study, in line with previous research [24], had demonstrated that CLP induced sepsis was associated with shortening in the PT and APTT, together with significant reduction in the APC level compared to the control rats. The reduction in protein C level in sepsis was reported [25] to be due to consumption and reduction of thrombomodulin (TM) level in the cell surface, thus impairing its activation [26]. APC had direct activity in blocking thrombin formation and enhancing fibrinolysis [27].

Low plasma PC levels can be considered as predictive parameter of early death after CLP as reported by Berg et al. (2006), who showed an enhancement of the survival rate in animals that did not have significant reduction in PC level [28]. Previous studies had reported anti-inflammatory effects of APC through blocking TNF production. APC also can decrease endothelial cells apoptosis in response to inflammation and ischemia [29].

Multiorgan failure is a leading cause of death in sepsis. No specific therapeutic agent, till now, is available to prevent multiorgan failure in sepsis. However, among the promising approaches is using agents that upregulate endogenous intracellular defenses against cellular toxins. One of such agents is hemin, which upregulates cellular levels of HO-1 [30].

However, there are contradictory studies concerning the role of HO-1 in sepsis physiopathology. One study indicates that the HO-1 pathway leads to increased susceptibility to severe sepsis as it found that treatment of animals with an HO-1 inhibitor increases their survival rate [31]. On the other hand, Chung et al. [6] demonstrated that HO-1 deficient mice are highly susceptible to developing severe sepsis and high mortality compared to mice with normal HO-1. Our study was designed to elucidate this contradiction, and it showed the protective effect of HO-1 during microbial sepsis. Enhancing the HO-1 level with hemin pretreatment was significantly evident. The increment of HO-1 48 hrs after sepsis in response to hemin pretreatment was associated with reduction of sepsis-induced thrombosis in the liver and lung, increased
Figure 3: Concentrations of IL-10 (a) in the serum (pg/mL) and (b) in the liver homogenate (pg/mg tissue), measured 48 hours after CLP, using ELISA technique. Data are expressed as mean ± SEM (n = 7–12 per group). ∗ significant versus CG, ∗# significant versus CLP group, and # significant versus CLP + Hm group (one-way ANOVA test, P < 0.001 for serum IL-10, P < 0.05 for liver IL-10). (c) The correlation between serum and liver IL-10 in septic rats is a positive correlation, where r (Pearson correlation coefficient) = 0.926 and P < 0.05.

level of APC, and prolonged prothrombin and partial thromboplastin times after CLP.

The protective role of HO-1 during microbial sepsis has been partly attributed to its ability to suppress the deleterious effect of free heme produced during the course of infection. Therefore, it protects against irreversible tissue damage [4]. Suzuki et al. (2000) had also reported an increase in hepatic, renal HO-1 mRNA and HO-1 activity 6–24 hrs following LPS administration that finally returned to the control level by 72 hrs [32].

Other reports showed similar results, even though they used a different regimen of hemin administration, being given to rats 12 hrs after the induction of sepsis [33, 34]. A contradicting finding to our study was held by Freitas et al. (2011) who reported an increase in survival rate after the treatment with HO-1 inhibitor, denoting that HO-1 with its metabolites CO and biliverdin downregulates neutrophil migration to the infectious focus, leading to bacterial dissemination. HO-1 inhibitor pretreatment was associated with reduced systemic inflammatory events with less cytokines release and consequently protection against organ damage and hypotension compared to nontreated animals [35]. Thus conclusively, it can be said that HO-1 may result in prevention of organ failure through reducing the coagulability and inhibiting the damaging effects of heme. On the contrary, it may enhance organ failure through the activation of systemic inflammatory response and increased proinflammatory cytokines. Our study results support the first statement and oppose the second. As results showed that HO-1 significantly increased the level of the anti-inflammatory cytokine IL-10, IL-10 was shown to be produced in a greater amount during sepsis; its amount may be related to the severity of sepsis and to the survival. The cytokines release timing and the balance between pro- and anti-inflammatory cytokines determine the severity of infection and the rate of survival [36].

Inflammation and hypercoagulable state are the main features of sepsis [37]. However, coagulation and inflammation relationship is complex and, as yet, not completely
understood. It was postulated that inflammation induced activation of coagulation is mediated by overproduction of inflammatory cytokines as IL-6 and TNF-α. Both are able to activate coagulation by increasing the expression of tissue factor and inhibiting normal anticoagulant pathway [38]. Therefore, several mechanisms were found to explain the anticoagulant effect of HO-1; among them is its ability to inhibit the production of IL-6 and TNF-α. Another mechanism is through induction of PC, APC, and TM in tissues [5].

A recent therapeutic approach against tissue damage of sepsis is the heme scavenger protein, HPx. In the current study, HPx was administered by intraperitoneal route to rats 30 min before the induction of sepsis. The results showed a significant prolongation in the PT and APTT with an increased level of APC. Consequently, control of coagulation was improved; their liver HO-1 and IL-10 (serum and liver) were significantly increased compared to septic rats.

HPx is able to neutralize the rising amounts of free heme. Binding of heme to HPx neutralizes its prooxidant effect. Such binding also inhibits the release of free radicals in hepatocytes due to the presence of heme and thus prevents their apoptosis [4]. Previous study reported that binding of heme to HPx changes the conformation of the protein and permits binding of the complex to a specific receptor found mostly on hepatocytes but also on some other cell types. After binding to this receptor (LDL receptor-related protein, LRP1), HPx–heme complexes are internalized, thereby initiating an intracellular signaling cascade, which leads to the upregulation of HO-1 expression [39].

Larsen et al., 2010, found that HPx serum concentration within 48 hrs of presentation with septic shock was positively associated with patient survival time. That is, patients with lower HPx serum concentrations died at earlier time points compared to patients with higher HPx serum concentrations.
concentrations [4]. Similarly, Jung et al., 2015, reported that low serum HPx levels were related to sepsis severity and could indicate poor prognosis for septic shock patients [40].

Therefore, we consider that administration of exogenous HPx might be used therapeutically to increase tolerance to infection and hence prevent the development of severe sepsis in rats and therefore decrease the mortality rate.

The reported beneficial effect of HPx most probably requires the expression of HO-1 to catabolize HPx-bound heme and to decrease IL-6 and TNF-α secretion from LPS stimulated macrophages from one side and increase the expression of IL-10 from the other side [39]. However, this finding was contradicted by several observations. First, the anti-inflammatory effects of HPx do not require the presence of heme, but HO-1 induction is known to be triggered by the binding and internalization of HPx-heme complexes. Second, HPx can directly attenuate the inflammatory cytokines secretion by LPS stimulated macrophages [12]. Third, HO-1 induction was not observed in murine macrophages incubated with HPx under the same conditions shown to inhibit LPS-induced cytokine secretion [39].

Therefore, the anti-inflammatory activity of HPx is attributed to its direct effect on the anti-inflammatory key counter-regulatory protein, IL-10. IL-10 is an anti-inflammatory cytokine that was significantly increased after CLP as evidenced in our study. There was a positive correlation between its level in serum and liver in CLP group. This denotes that sepsis induces the production of IL-10 mainly through the stimulation of Kupffer cells, which are considered to be a primary source of IL-10 during a model of abdominal sepsis as demonstrated by Traegera et al. (2010) [41]. HPx administration to septic rats was associated with a significant increase in serum and liver IL-10 levels, when compared to both nontreated septic rats and rats treated with hemin. The latter
group showed a significant increase in serum IL-10 only but not in liver IL-10 level. This finding could be interpreted by the ability of HO-1 to stimulate sources for IL-10 expression other than the Kupffer cells.

Kasten et al., 2010 [42], reported significant IL-10 production by peritoneal neutrophils present at the site of infection during the first 24 hrs after CLP, suggesting that neutrophils are an additional source through which serum IL-10 is elevated during sepsis.

Role of IL-10 in sepsis is complex; it could be shown that IL-10 acts posttranscriptionally to downregulate TNF expression as it increases TNF mRNA instability [43]. Thus, IL-10 shortens the half-life of TNF and is necessary to turn off TNF production, which explains the overwhelming secretion of TNF after Kupffer cells depletion.

However, Song et al., 1999, reported that inhibition of IL-10 12 hrs after CLP can improve survival [44]. Therefore, timing of the increased rate of IL-10 is an important factor to determine its effect. In the latter study, IL-10 was given, 5 hrs after CLP, and reported 50% improvement, while treatment with IL-10 at time of CLP does not increase survival. Therefore, additional investigations are required into IL-10 and its interaction with HO-1 and HPx.

5. Conclusion

The present study extends these previous reports by demonstrating that hemin and HPx administration upregulate liver HO-1 and reduce CLP induced thrombosis. Hemin or HPx administration was able to prolong the PT and APTT and enhance APC. Also, their administration was able to reduce the inflammatory infiltrate in liver and lung parenchyma and to reduce the number of apoptotic cells. All effects may be partially due to the increased production of the anti-inflammatory cytokine IL-10.

Despite showing the same results, still hemin was superior to HPx in controlling the coagulation and in enhancing the HO-1 production, while HPx was a more potent stimulant for the expression of IL-10.

Abbreviations

APTT: Activated partial thromboplastin time
APC: Activated protein C
CLP: Cecal ligation puncture
HO-1: Heme oxygenase-1
HPx: Hemopexin
LPS: Lipopolysaccharide
PBS: Phosphate buffered saline
PPP: Platelet poor plasma
PT: Prothrombin time
TM: Thrombomodulin.

Conflict of Interests

The authors declare that they have no competing interests.

Authors’ Contribution

All the authors contributed to the study design, collection of the data, and writing of the paper. All authors read and approved the final paper. All authors contributed equally to funding this research.

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