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ORIGINAL RESEARCH

Alpha-Chymotrypsin Protects Against Acute Lung, Kidney, and Liver Injuries and Increases Survival in CLP-Induced Sepsis in Rats Through Inhibition of TLR4/NF-κB Pathway

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Abstract: Inflammation and oxidative stress play a major role in the development of sepsis and its associated complications, leading to multiple organ failure and death. The lungs, liver, and kidneys are among the early affected organs correlated with mortality in sepsis. Alpha-chymotrypsin (α-ch) is a serine protease that exerts anti-inflammatory, anti-edematous, and anti-oxidant properties.

Purpose: This study was undertaken to elucidate if the anti-inflammatory and anti-oxidant effects of α-ch observed in previous studies can alleviate lung, liver, and kidney injuries in a cecal ligation and puncture (CLP)-induced sepsis model, and thus decrease mortality.

Materials and Methods: Septic animals were given α-ch 2 h post CLP procedure. Sepsis outcomes were assessed in the lungs, liver, and kidneys. Separate animal groups were investigated for a survival study.

Results: CLP resulted in 0% survival, while α-chymotrypsin post-treatment led to 50% survival at the end of the study. Administration of α-chymotrypsin resulted in a significant attenuation of sepsis-induced elevated malonaldehyde (MDA) and total nitrite/nitrate (NOx) levels. In addition, there was a significant increase in reduced glutathione (GSH) content and superoxide dismutase (SOD) activity in the lungs, liver, and kidneys. Administration of α-ch reduced elevated tissue expression of toll-like receptor-4 (TLR4), nuclear factor kappa-B (NF-κB), myeloperoxidase (MPO), and inducible nitric oxide synthase (iNOS). Alpha-chymotrypsin resulted in a significant reduction in serum levels of tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6). Alpha-chymotrypsin attenuated the rise in serum creatinine, cystatin C, blood urea nitrogen (BUN), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) levels that was observed in the septic group. In addition, α-ch significantly reduced the lung wet/dry weight ratio, total protein content, and leukocytic counts in bronchoalveolar lavage fluid (BALF). Histopathological examination of the lungs, liver, and kidneys confirmed the protective effects of α-ch on those organs.

Conclusion: α-ch has protective potential against sepsis through lowering tissue expression of TLR4, NF-κB, MPO, and iNOS leading to decreased oxidative stress and inflammatory signals induced by sepsis. This effect appeared to alleviate the damage to the lungs, liver, and kidneys and increase survival in rats subjected to sepsis.

Keywords: Sepsis, CLP, TNF-α, Cytokines, ROS, TLR-4, iNOS

Introduction

Sepsis, a critical complication seen commonly in post-operative patients, is caused by an infection that results in a systemic inflammatory response syndrome and ends with multiple organ dysfunction.[1,2] It is a major cause of morbidity and mortality in intensive care unit patients. The mortality rate associated with septic shock is high (between 25% and 52%), causing a global clinical problem.[3] The lung, liver, and kidney injuries associated with sepsis are a substantial cause of death.[4]

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Although the acute respiratory distress syndrome (ARDS) associated with sepsis is not yet fully understood, some studies elucidated that proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) or interleukin-1β (IL-1β), prompt alveolar barrier injury, neutrophils extracellular traps formation, and platelet activation with microthrombi formation. This results in impaired gas exchange, severe hypoxemia, and hypercapnia.[5] The kidneys, through the regulation of fluid and blood pressure, have a pivotal role in organ perfusion and in the general circulation.[6] The exact underlying mechanism of the acute kidney injury (AKI) associated with sepsis is not yet completely understood. However, inflammatory signals, including oxidative stress, ischemia-reperfusion injury, tubular apoptosis, and local microcirculation, were expected to be the causative mechanism of acute tubular necrosis.[7] In sepsis, hepatocytes are hypothesized to play a crucial role through the release of acute-phase proteins into the systemic circulation, thus, playing a regulatory role in excessive inflammation or immunosuppression.[8] Liver injury associated with sepsis is due to two main complications: hypoxic hepatitis and cholestasis. Hypoxic hepatitis results from hemodynamic alterations, microthrombi formation, endothelium dysfunction, and sinusoidal obstruction. Sepsis-induced cholestasis results from the diminished formation of bile and its impaired flow.[9]

In spite of the guidelines outlined for sepsis management and the advancements in treatments, the mortality rate for septic patients with acute lung injury (ALI), acute hepatic injury (AHI), and acute kidney injury (AKI) is still as high as 70%. Therefore, establishing novel, safe, and effective therapeutics and preventive approaches is critical for successfully managing sepsis and reducing its complications.

Two hemodynamic phases, which may overlap together, are manifested during sepsis: a hyperdynamic and a hypodynamic phase. Pro-inflammatory cytokines, such as TNF-α, IL-1, and IL-6 and reactive oxygen species (ROS), such as superoxide anions and nitric oxide are released in the first phase. This phase is the main cause of the high mortality associated with sepsis, which is correlated with multiple organ injuries.[10–12] To terminate the inflammatory response after the infection has been controlled, anti-inflammatory cytokines such as IL-10 are released in the second phase.[13] Such a phase, also if gone uncontrolled, can result in severe suppression of the immune system, which makes the patient more susceptible to secondary infections.[14] A secondary infection leads to repeated episodes of hyper- and hypo-inflammatory phases that worsen the sepsis complications.[15] Binding of lipopolysaccharide (LPS) to toll-like receptor-4 (TLR4) and the subsequent translocation of nuclear factor kappa-B (NF-κB) into the nucleus and the increased transcription of inducible nitric oxide synthase (iNOS) results in the excessive production of nitric oxide (NO). The interaction of NO with superoxide radicals forms peroxynitrite that massively damages the functions of normal cells.[1] Targeting this signaling pathway and decreasing the oxidative stress induced during sepsis can in turn decrease multiple organ dysfunction, and so decrease mortality. In addition, myeloperoxidase (MPO) of activated neutrophils produces hypochlorous acid that interacts with superoxide anion, causing direct damage to the host cells.[16,17]

Serine proteases are members of the protease family and are released from inflammatory cells, such as neutrophils.[18] Alpha-chymotrypsin (α-ch) is a serine protease that is released after its cleavage from chymotrypsinogen in the intestine. α-ch exerts potent anti-inflammatory effects that accelerate the resorption of inflammatory oedema, haematoma, or oedema, following an operation or trauma. During subacute or chronic inflammatory processes, chymotrypsin exerts proteolytic properties that dissolve the fibrinous formations.[19] In vitro, the inflammatory state of neutrophils was suppressed by chymotrypsin.[20] Another study revealed that pleural edema was substantially minimized in chymotrypsin-treated rats.[21] Low-doses of chymotrypsin were effective in inhibiting neutrophil migration into inflammatory foci in vivo.[22] In addition, in both acute and subacute models of hind paw inflammation in rats, chymotrypsin demonstrated anti-inflammatory activity.[23] Moreover, in a study to elucidate the anti-oxidant properties of chymotrypsin, it was found to act indirectly through the reduction of lipid peroxidation products, as well as through the maintenance of superoxide dismutase (SOD), catalase, glutathione peroxidase, and glutathione-S-transferase.[24]

Due to its diverse biological activities, including its anti-edematous, anti-inflammatory, anti-oxidative, anti-infective, and fibrinolytic effects, chymotrypsin has been widely used to hasten the repair of traumatic injuries and burns, as well as to relieve sciatica.[25] In addition, it has been used to manage upper respiratory conditions and chronic pulmonary disease.[26]

In light of the previous studies, we explored the prospective beneficial effects of α-ch in a well-established experimental sepsis model in rats induced by cecal ligation and puncture (CLP). This model is regarded as a standard

model for the induction of sepsis as it imitates the pathways involved in sepsis in humans.[27,28] We investigated if α-ch can alleviate CLP-induced ALI, AHI, and AKI, given its anti-inflammatory and antioxidant effects that were previously described, and whether such an enhancement could increase survival in septic rats.

Materials and Methods

Experimental Animals and Design

Female albino Wistar rats (200 ± 20 g) were purchased from Nahda University at Beni Suef (NUB) Animal House (Beni Suef, Egypt). Rats were kept at room temperature (25 ± 2 ˚C) in plastic cages and under a 12 h dark–light cycle. Before the experiment was conducted, all rats were accommodated in laboratory conditions for at least one week. All rats were maintained under the same conditions throughout the experiment. Diet and water were allowed *ad libitum*. All the procedures in the experiment were conducted according to the international ethical guidelines, and the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals and were approved by the Commission on Ethics of Scientific Research, Faculty of Pharmacy, Minia University (Code number of the project: ES12/2020).

Alpha-chymotrypsin was obtained from αChymotrypsin (Amoun Pharmaceutical Company S.A.E., Egypt). Twenty-four rats were randomly assigned to three groups, as follows: Group 1: (n = 6) sham-operated (sham group); Group 2: (n = 12) septic animals (CLP group); Group 3: (n = 6) CLP rats receiving α-ch (8.1 unit/rat, i.m, single dose, 2 h following CLP surgery).[29]

Induction of Sepsis by CLP

Polymicrobial sepsis was induced surgically by CLP operation, as described previously.[30,31] A severe model was performed through the ligation of more than 75% of the cecum (nearly below the ileo-cecal valve) and puncturing twice with an 18-gauge needle. Aside from the ligation and puncture of the cecum, all sham-operated animals went through the same steps.

Survival Study

In another experiment, thirty rats (same weight and sex as the previous experiment) were randomly assigned to three groups, ten rats each, as follows: sham-operated group, septic-untreated group (CLP group), and α-ch-treated septic group. All rats were allowed free access to water and food. The mortality of rats was monitored daily for 7 days.[32]

Blood Collection and Tissue Isolation

Twenty-four hours after CLP induction, blood samples were collected by cardiac puncture after inducing anesthesia by using sodium thiopental (50 mg/kg). Serum samples were collected after centrifugation for 10 min at 2500 rpm. Bronchoalveolar lavage fluid (BALF) was obtained by intubating the lungs and lavaging them with cold phosphate buffer saline (PBS).[33] The lungs were carefully dissected, washed, and divided as follows: the upper right lobe was used for measurement of lung wet/dry weight, the lower lobe of the right lung was kept for histopathological examination,[34] and the left lung was homogenized for further measurements.

A segment of the liver's medial lobe and left kidney of each animal was rapidly dissected, blotted dry on filter paper, weighed, and processed for histopathological examination. The other kidney and liver samples were flash-frozen in liquid nitrogen and stored at −80°C.[35]

Prior to analysis, lung, liver, and kidney tissues were placed in PBS to prepare a 5% W/V homogenate. Homogenates were centrifuged for 15 minutes at 4000 rpm and the supernatant was separated for various analyses.

Histopathological Examination

Samples were fixed in neutral buffered formalin solution (10%). Standard hematoxylin and eosin (H&E) staining was carried out after processing the lung, kidney, and liver tissues. Slides were examined using a light microscope.

The microscopical assessment was carried out by a pathologist blind to the experimental groups. Infiltration of inflammatory cells, hemorrhage, congestion, and edema were evaluated in lung tissues and scored 1 to 4 as follows: 0,

absent; 1, light; 2, moderate; 3, strong; and 4, intense. The lung injury score was calculated as the mean of the scores of individual parameters. The percentage of tubules that exhibit cellular necrosis was used for the evaluation of the kidney injury score as follows: 0 = none, 1 = <20%, 2 = >20% and <50%, 3 = >50% and <70%, 4 = >70%. At least ten fields were examined for each animal.[31]

The following four criteria were used to assess liver injury: congestion, edema, infiltration of polymorphonuclear leukocytes and monocytes, and necrosis on a scale of 0–4 as follows: 1 = congestion, 2 = edema, 3 = infiltration of polymorphonuclear leukocytes and monocytes, 4 = necrosis. The total score is calculated based on the sum of the scores given for the individual parameters.[36]

Measurement of Lung W/D Weight Ratio, Total Leukocytic Counts, and Protein Content in BALF

Lung edema was assessed using lung W/D weight ratio.[34] The upper right pulmonary lobe was placed in an oven at 80°C for 24 h. It was weighed using a sensitive electric scale (analytical balance 220/C/2, RADWAG, Poland) before (wet weight) and after (dry weight) its placement in the oven.

Bronchoalveolar lavage fluid (BALF) was centrifuged at 1000 rpm for 10 min at 4°C. The total cell count was obtained after resuspension of the cell pellet in 0.5 mL PBS using Mindray Bc-20s Auto Hematology Analyzer.[37]

Total protein concentration in the supernatant of the BALF was determined colorimetrically, following the procedure of George and Kingsley,[38] as per the manufacturer's recommendation (BioMed kit, Egypt).

Measurement of Serum Creatinine, Urea, ALT, and AST

Serum creatinine (CR), blood urea nitrogen (BUN), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) levels were assessed as previously described,[39,40,42] using commercially available kits (Biodiagnostic, Egypt).

Measurement of Pulmonary, Renal, and Hepatic Antioxidant Defense

Reduced glutathione (GSH) in lung, liver, and kidney homogenates was measured colorimetrically at 412 nm following previously described methodology.[41] Superoxide dismutase (SOD) activity was determined in all tissue homogenates spectrophotometrically by the method described by Marklund and Marklund.[42]

Measurement of Oxidative Stress Markers in Lung, Liver, and Kidney Homogenates

After the reduction of nitrates to nitrites by cadmium, the Griess reaction was used to determine the total nitrates content colorimetrically.[43] Malondialdehyde (MDA) concentration was assessed colorimetrically in all tissue homogenates following a previously described method.[44]

Measurement of Serum TNF-α, IL-6, IL-1β and Cystatin C

Serum inflammatory cytokines and cystatin C were measured using commercially available ELISA kits (Elabscience Biotechnology, Houston, Texas, USA). Standard curves were established using reference standard proteins. Serum concentrations were determined at 450 nm spectrophotometrically.

Immunohistochemical Detection of TLR4, NF-κB, MPO, and iNOS in Pulmonary, Hepatic, and Renal Tissues

The tissue sections (5 um) were immuno-stained for Toll-like receptor-4 (TLR4) (Rabbit polyclonal antibody, Catalog No. GB11186, Servicebio, Wuhan, China), Nuclear factor-kappaB (NF-κB) (Rabbit monoclonal antibody, Catalog No. A19653, ABclonal technology, Woburn, Massachusetts, USA), Myeloperoxidase (MPO) (Rabbit polyclonal antibody, Catalog No. RB-373-A, Thermo Fisher Scientific, Waltham, Massachusetts, USA), and inducible nitric oxide synthase (iNOS) (Rabbit polyclonal antibody, Catalog No. GB11119, Servicebio, Wuhan, China), according to the manufacturer's guidelines. The enzymatic method described by Nakane and Pierce Jr[45] was used to detect the expression. Hematoxylin was used as a counterstain.

Statistical Analysis

Statistical analysis of the data was performed using GraphPad Prism (version 7.0; San Diego, CA, USA). Shapiro–Wilk test was used to determine the normality of the data. Results were normalized to the mean of the sham group. Analysis of variance (ANOVA) test was used to test the significance of the results. Tukey's post-hoc test was used for multiple comparisons. Log-rank (Mantel-Cox) test was used for analysis of the survival data. If the *p*-values were <0.05, then the differences were considered statistically significant.

Results

Alpha-Chymotrypsin Improves Survival and Ameliorates CLP-Induced Inflammatory Signals and Oxidative Stress in Septic Rats

Survival analysis revealed a significant difference ($p < 0.05$) in survival between the sham group and the CLP group, as well as between the α-ch-treated group and the untreated septic group (Figure 1A). No rats survived beyond the end of the first 48 h in the CLP group. Interestingly, the administration of α-ch 2 h post-CLP significantly reduced mortality and resulted in 100%, 90%, and 50% survival after the first, second, and seventh day, respectively, in the treated group.

Induction of sepsis caused a significant increase ($p < 0.05$) in serum TNF-α, IL-1β, and IL-6 levels when compared with sham-operated rats. The administration of a single dose of α-ch 2 h post-CLP surgery significantly reduced ($p < 0.05$) serum TNF-α, IL-1β, and IL-6 levels compared with the CLP group (Figure 1B).

Induction of sepsis resulted in a significant decrease ($p < 0.05$) in SOD activity and GSH content in lung, hepatic, and renal tissues when compared with the sham group. Treatment with α-ch 2 h after CLP resulted in a significant increase ($p < 0.05$) in GSH and SOD activity in all the examined tissues (Figure 1C–E).

Sepsis induction led to a significant elevation ($p < 0.05$) in tissue MDA, as an indicator of thiobarbituric acid reactive substances (TBARS), when compared with sham-operated rats. Administration of α-ch 2 h after CLP significantly reduced ($p < 0.05$) the elevated TBARS in the septic lung, liver, and kidney tissues. Similar changes were observed in the levels of nitrates, as shown in Figure 2A–C.

Alpha-Chymotrypsin Protects Against Sepsis-induced ALI

The wet/Dry weight ratio, leukocytic count, total protein content in BALF, and lung injury scores significantly increased ($p < 0.05$) in CLP rats compared with sham rats. Administration of α-ch ameliorated such observations (Figure 2A–D).

Figure 2 shows H&E-stained lung sections and histopathological scores of lung tissues. The sham-operated group showed normal alveoli with intact alveolar membranes. The CLP group showed thickened alveolar membranes due to edema and inflammatory infiltrate. Sections from the sham group showed normal alveolar spaces with intervening bronchioles. Meanwhile, congested and dilated alveolar spaces were evident in the CLP group. In addition, interstitial tissue congestion, areas of inflammatory infiltrates (alveolar macrophages and lymphocytes), and focal areas of alveolar membrane damage were also seen in the CLP group. After the administration of α-ch, intact alveolar membrane and non-congested alveolar spaces were seen. The absence of signs of rupture, hemorrhage, edema, and inflammatory infiltrates was also noted (Figure 2E). These findings are suggestive of a potentially remarkable protective effect.

Alpha-Chymotrypsin Protects Against Sepsis-induced AHI

Alanine aminotransferase (ALT), aspartate aminotransferase (AST) levels, and liver injury scores increased dramatically following the induction of sepsis. Injection of α-ch reduced such levels and scores (Figure 3A–C).

The H&E-stained liver sections in Figure 3D show that the sham group displayed non-congested central vein and normally arranged hepatocyte cords. In the CLP group, the central vein was dilated and congested. It was surrounded by peripherally arranged hepatocyte cords with a faint eosinophilic cytoplasm and a central basophilic nucleus (mild-to-moderate vacuolar degeneration). Hepatic sinusoids were dilated and congested, as opposed to the normal sinusoids seen in the sham group. Clusters of lymphocytes and macrophages were also observed. The prominence of Kupffer cells with oval to triangular-shaped nuclei was noted. The protective effect of α-ch was

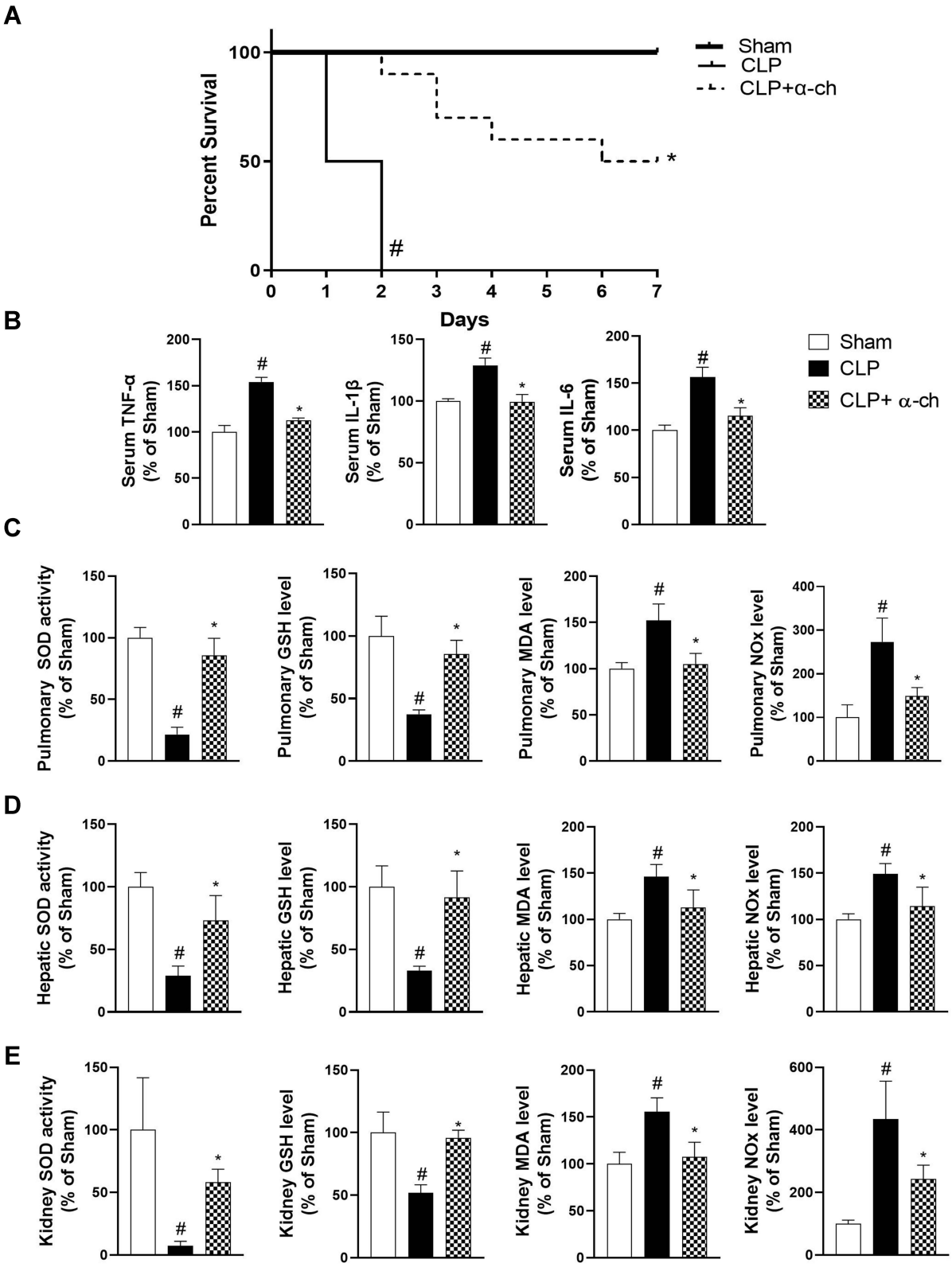

Figure 1 Effect of alpha-chymotrypsin treatment on survival, serum inflammatory signals, and tissue oxidative stress. (**A**) Administration of α-ch treatment (8.1 unit/rat, i.m, single dose) 2 h after CLP markedly improved the 7-day survival of septic rats by 50% compared with the untreated septic group (n = 10 rats per group). (**B**) Bar charts showing the effect of CLP and α-ch on serum TNF-α, IL-1β, and IL-6. (**C**) Pulmonary SOD activity, GSH, MDA, and NOx levels are shown. (**D**) Hepatic SOD activity, GSH, MDA and NOx levels. (**E**) Renal SOD activity, GSH, MDA and NOx levels. Data were normalized to the mean of the Sham group. Data were analyzed with one-way ANOVA followed by Tukey's test for multiple comparisons, n = 6 for all groups. #Denotes significant difference compared with sham ($p < 0.05$). *Significantly different from CLP ($p < 0.05$).

Abbreviations: CLP, Cecal Ligation and Puncture; α-ch, alpha-chymotrypsin.

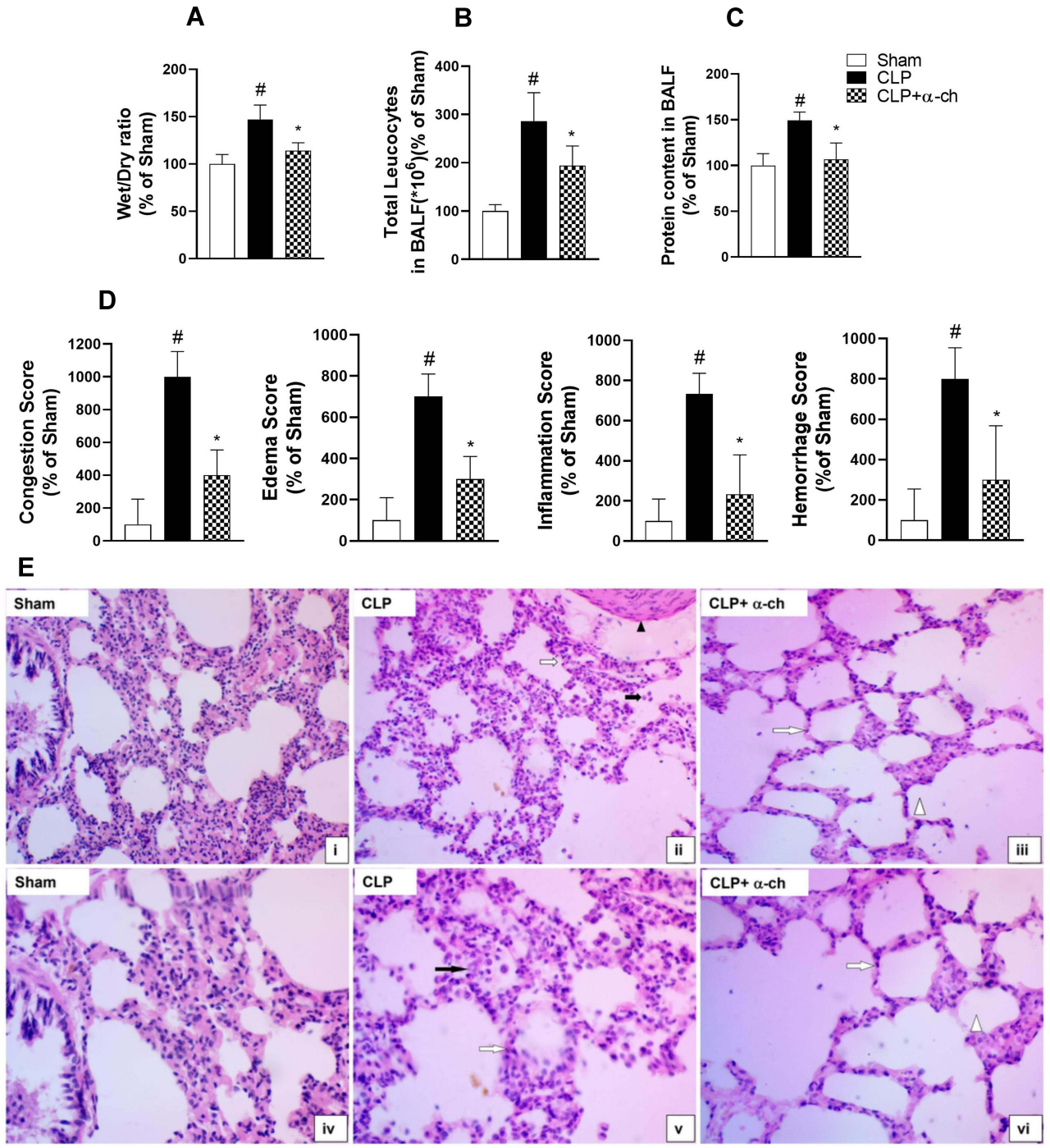

Figure 2 Protective effect of alpha-chymotrypsin against sepsis-induced ALI. Bar charts showing the effect of CLP and α-ch on (**A**) W/D weight ratio of lung tissue, (**B**) Total number of leukocytes in BALF, and (**C**) Total protein content in BALF. (**D**) Lung injury score analysis. Data were normalized to the mean of the Sham group. Data were analyzed with one-way ANOVA followed by Tukey's test for multiple comparisons, n = 6 for all groups. #Significantly different from sham ($p < 0.05$). *Significantly different compared with CLP ($p< 0.05$). (**E**) α-ch treatment reduces sepsis-induced histopathological lesions in rat lungs (H&E stain, original magnification ×200 for i, ii, and iii and ×400 for iv, v, and vi). The sham group showed no signs of injury. In the CLP group, inflammatory infiltrate and edema caused the alveolar membrane to be thickened (white arrow). Focal areas of alveolar membrane damage resulted in congested dilated alveolar spaces (arrow head), and areas of inflammatory infiltrates (alveolar macrophages and lymphocytes) and interstitial tissue congestion (black arrow). The protective effect of α-ch was apparent in the form of intact alveolar membrane of the multiple alveoli (arrow), empty alveolar space, and absence of signs of edema or hemorrhage (arrow head).
Abbreviations: CLP, Cecal Ligation and Puncture; α-ch, alpha-chymotrypsin.

evident by the presence of non-congested central vein and sinusoidal spaces. Peripherally arranged hepatocytes with eosinophilic cytoplasm and a central nucleus with no fatty vacuoles or swelling were observed. Neither necrotic focal regions nor inflammatory infiltrates were observed.

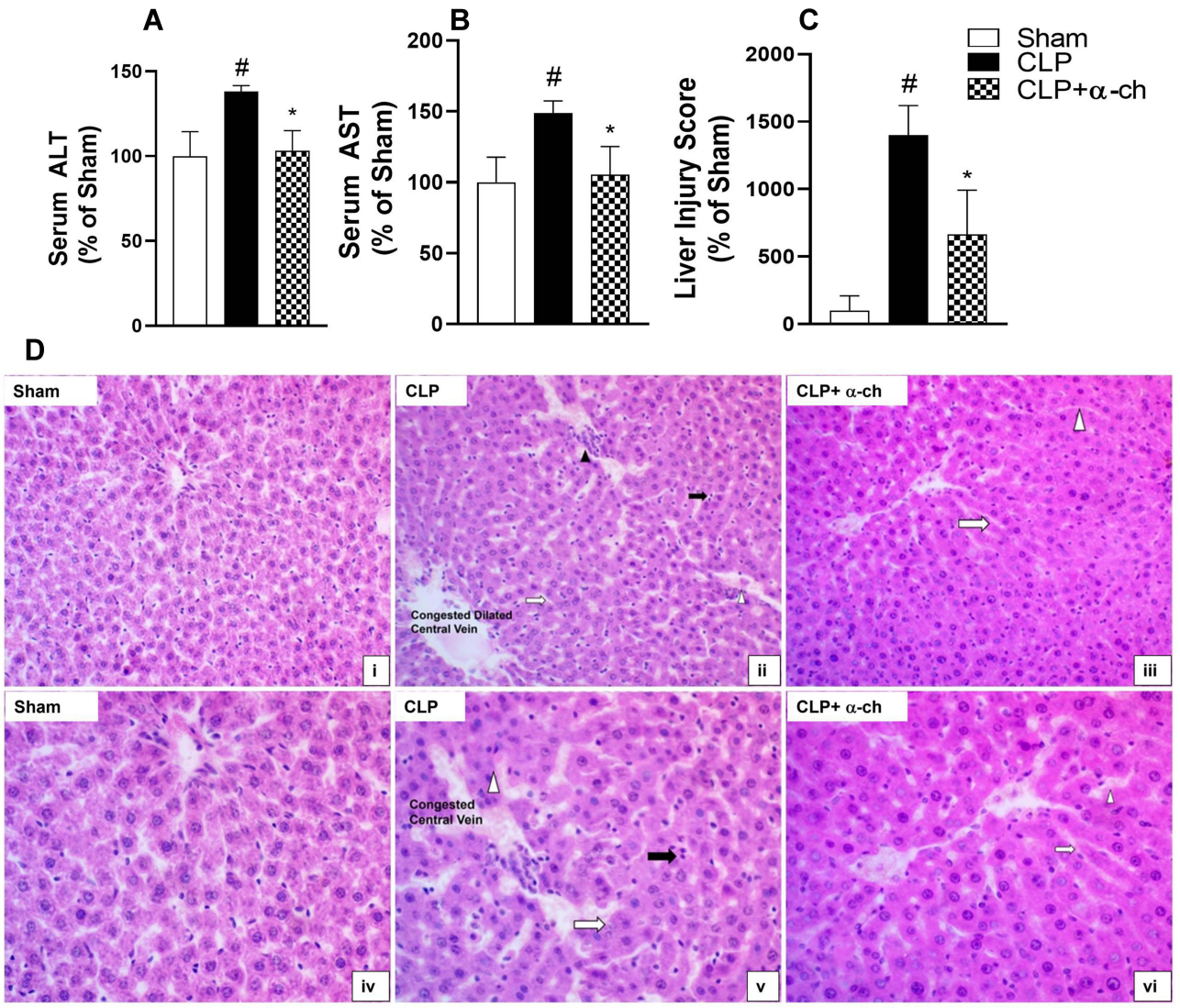

Figure 3 Protective effect of alpha-chymotrypsin against sepsis-induced AHI. Bar charts showing the effect of CLP and α-ch on (**A**) Serum ALT, (**B**) Serum AST, and (**C**) Liver injury score analysis. Data were normalized to the mean of the Sham group. Data were analyzed with one-way ANOVA followed by Tukey's test for multiple comparisons, n = 6 for all groups. #Significantly different compared with sham ($p < 0.05$). *Significantly different compared with CLP ($p < 0.05$). (**D**) α-ch treatment reduced sepsis-induced histopathological lesions in rat liver (H&E stain, original magnification ×200 for i, ii, and iii and ×400 for iv, v, and vi). The sham group showed no signs of injury. CLP group showed mild to moderated vacuolar degeneration (white arrow), and congested and dilated hepatic sinusoids (white arrow head). Triangular to oval-shaped nucleus of prominent Kupffer cell (black arrow) and clusters of lymphocytes and macrophages (infiltrates) (black arrow head) are shown. The protective effect of α-ch was apparent in the form of non-congested central vein with peripherally arranged hepatocyte cords (arrow). Non-congested sinusoidal spaces with non-necrotic focal regions or inflammatory infiltrates (arrow head) also were observed.

Abbreviations: CLP, Cecal Ligation and Puncture; α-ch, alpha-chymotrypsin.

Alpha-Chymotrypsin Protects Against Sepsis-induced AKI

Serum CR, BUN, serum cystatin C, and kidney injury scores markedly increased in the CLP group compared with the sham-operated animals. Treatment with α-ch significantly reduced such levels and scores (Figure 4A–D).

As shown in the H&E-stained kidney sections (Figure 4E), normal renal glomeruli were evident in the sham group. In the CLP group, signs of glomerular injury such as congested and fibrosed glomerular capillaries and narrow or obliterated Bowman's spaces with mesangial cell proliferation were observed. In addition, focal areas of tubular damage, tubular edema, and intratubular casts were signs of tubular injury seen in the CLP group. Interstitial tissue edema and inflammatory cellular infiltrates (aggregates of lymphocytes) were also observed. The protective effect of α-ch was reflected in the normal structure of the kidney and the normal tubular structures with no edema or tubular casts. The interstitial tissue was thin, uncongested, free from inflammation, and had no signs of destruction.

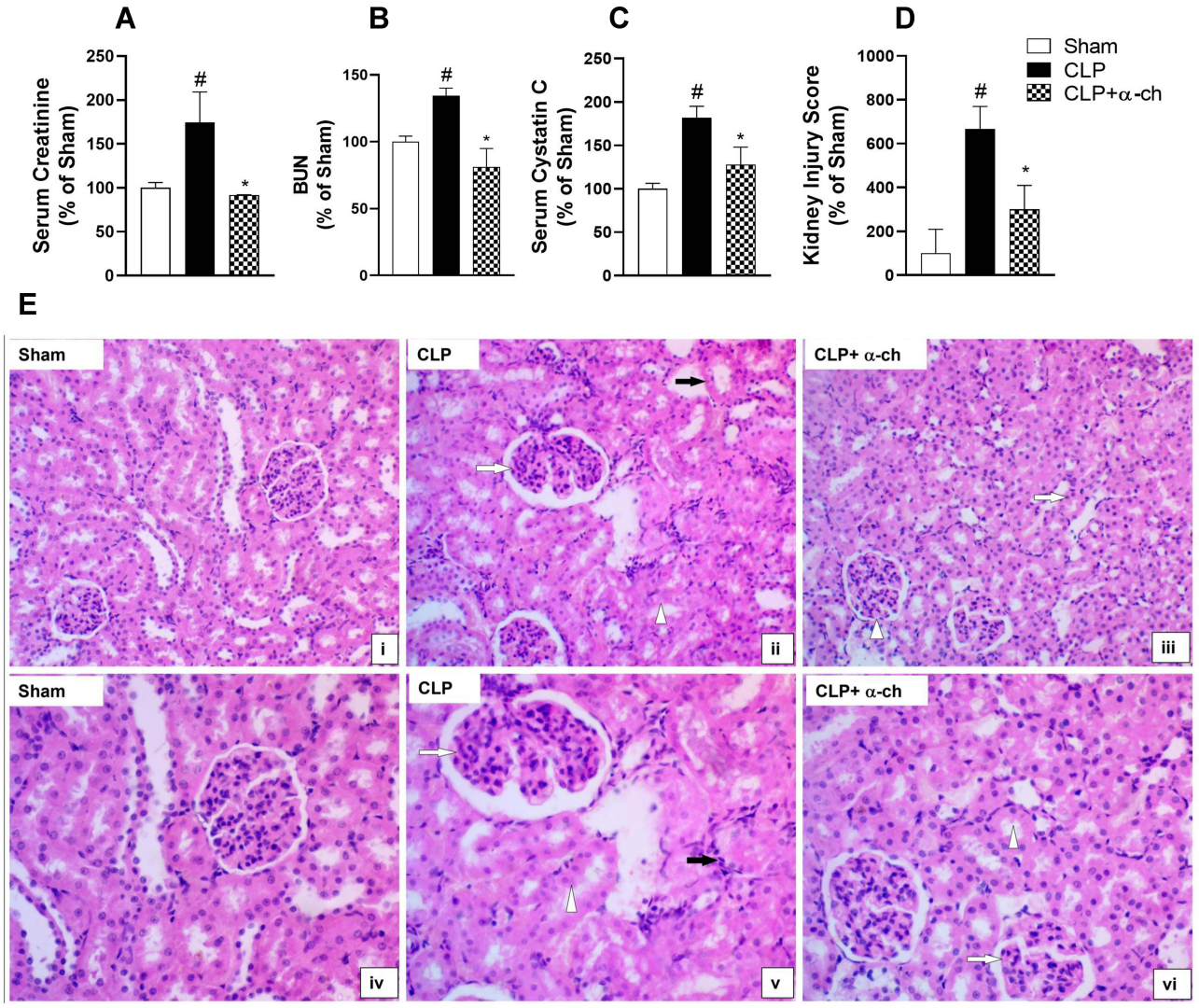

Figure 4 Protective effect of alpha-chymotrypsin against sepsis-induced AKI. Bar charts showing the effect of CLP and α-ch on (**A**), Serum CR, (**B**) BUN, (**C**) Serum cystatin C, and (D) Kidney injury score. Data were normalized to the mean of the Sham group. Data were analyzed with one-way ANOVA followed by Tukey's test for multiple comparisons, n = 6 for all groups. #Significantly different compared with sham ($p < 0.05$). *Significantly different compared withCLP ($p < 0.05$). (**E**) α-ch administration reduced sepsis-induced histopathological lesions in the kidneys of treated rats (H&E stain, original magnification ×200 for i, ii, and iii and ×400 for iv, v, and vi). The sham group showed no signs of injury. In the CLP group, narrow to obliterated Bowman's spaces with mesangial cell proliferation and congested and fibrosed glomerulus capillary (white arrow) are shown. Tubular edema, focal areas of tubular damage, intratubular casts (arrow head), and interstitial tissue (black arrow) were observed. Tissue edema in the form of lymphocytic aggregates (black arrow) is illustrated. Protective effect of α-ch was in the form of normal structure of the glomerulus (arrow) and normal tubular structures (arrow head).
Abbreviations: CLP, Cecal Ligation and Puncture; α-ch, alpha-chymotrypsin.

Alpha-Chymotrypsin Ameliorates Sepsis-Induced Increased TLR4 Immunoreactivity

To explore the underlying protective mechanism of α-ch, we measured the tissue expression of TLR4. As shown in Figure 5, the sham group showed no detectable expression of TLR4 in the alveolar cells (lung section), hepatocytes (liver section), or the cells lining the renal tubules (kidney section). On the other hand, strong immunolabeling for TLR4 was observed in the CLP group in different organ sections. A weak immunoexpression was detected after the administration of α-ch.

Alpha-Chymotrypsin Ameliorates Sepsis-Induced Elevated NF-κB Immunoreactivity

As shown in Figure 6, the cells lining the alveoli (lung section) showed no immunoreactivity for NF-κB in the sham group, but a positive immunostaining was detected in the CLP group. Alpha-chymotrypsin showed very weak expression

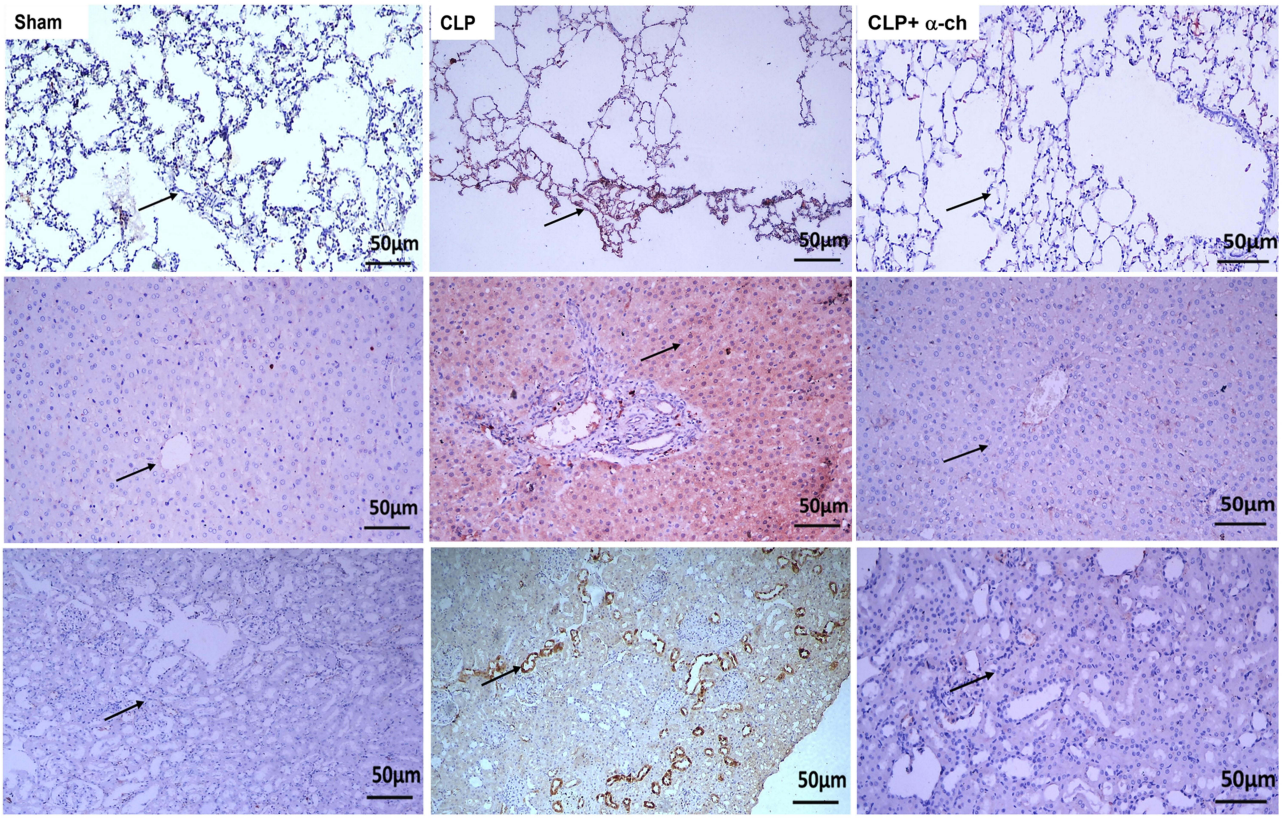

Figure 5 Protective effect of alpha-chymotrypsin against sepsis-induced high TLR4 immunoreactivity. Upper panel: Representative photomicrographs of rat lung tissues immuno-stained for TLR4; From the sham group (top-far left) showing no expression in the cells lining the alveoli (arrow), From the (CLP) group with strong cytoplasmic immunostaining (arrow) and from the (CLP+ α-ch) treated group showing very weak immunostaining for TLR4 (arrow). Middle panel: Representative photomicrographs of rat liver tissues immuno-stained for TLR4; From the sham group (left) showing no expression in the hepatocytes (arrow), From the (CLP) group with strong cytoplasmic expression in the hepatocytes and the endothelial cells lining the sinusoids (arrow) and from the (CLP+ α-ch) treated group showing weak immunoexpression for TLR4 (arrow). Middle panel: Representative photomicrographs of rat liver tissues immuno-stained for TLR4; From the sham group (left) showing no expression in the hepatocytes (arrow), From the (CLP) group with strong cytoplasmic expression in the hepatocytes and the endothelial cells lining the sinusoids (arrow) and from the (CLP+ α-ch) treated group showing weak immunoexpression for TLR4 (arrow). Lower panel: Representative photomicrographs of rat renal tissues immuno-stained for TLR4; From the sham group (bottom-far left) showing no expression in the cells lining the renal tubules (arrow), from the (CLP) group with strong cytoplasmic immunostaining in the cells lining the renal tubules and the mesangial cells of the glomeruli (arrow) and from the (CLP+ α-ch) treated group showing weak immunostaining for TLR4 (arrow).
Abbreviations: CLP, Cecal Ligation and Puncture; α-ch, alpha-chymotrypsin.

for NF-κB. In addition, hepatocytes showed no cytoplasmic immunoreactivity in the sham group for NF-κB, compared with the strong immunoreactivity observed in the CLP group. Administration of α-ch attenuated the rise of expression after CLP induction. The cells lining the renal tubules showed a strong increase in the expression of NF-κB in the CLP group, which was inhibited following the administration of α-ch. No detectable expression for NF-κB was observed in the sham group in all examined tissue sections.

Alpha-Chymotrypsin Ameliorates Sepsis-Induced Increase in iNOS Immunoreactivity

Figure 7 shows that following the induction of CLP, a strong immunoreactivity of iNOS was observed in alveolar cells (lung section), hepatocytes (liver section), and the cells lining the renal tubules (kidney section). The α-ch-treated group showed weak immunolabeling for iNOS in all examined tissue sections. This was not detectable in the sham group.

Alpha-Chymotrypsin Ameliorates Sepsis-Induced Elevated MPO Immunoreactivity

The CLP procedure resulted in strong MPO immunoreactivity in alveolar cells (lung section), hepatocytes (liver section), and the cells lining the renal tubules (kidney section). This decreased after the administration of α-ch. The sham group showed no noticeable immunolabeling for MPO in all examined tissue sections (Figure 8).

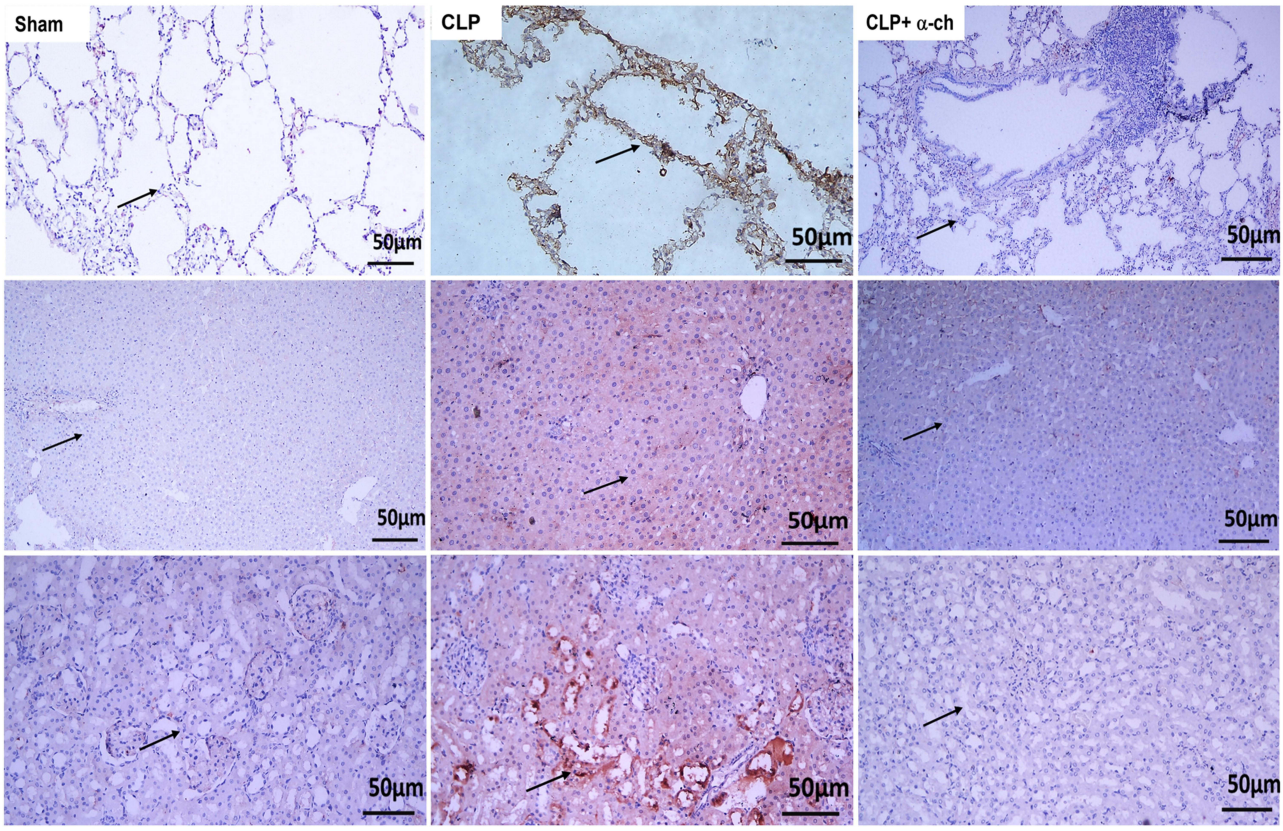

Figure 6 Protective effect of alpha-chymotrypsin against sepsis-induced high NF-κB immunoreactivity. Upper panel: Representative photomicrographs of rat lung tissues immuno-stained for NF-κB; from the sham group (top-far left) showing no expression in the cells lining the alveoli (arrow), from the (CLP) group with strong cytoplasmic immunoexpression (arrow) and from the (CLP+ α-ch) treated group showing weak immunoexpression for NF-κB (arrow). Middle panel: Representative photomicrographs of rat liver tissues immuno-stained for NF-κB; from the sham group (left) showing no expression in the hepatocytes (arrow), from the (CLP) group with strong cytoplasmic immunoexpression in the hepatocytes and the endothelial cells lining the sinusoids (arrow) and from the (CLP+ α-ch) treated group showing weak immunoexpression for NF-κB (arrow). Lower panel: Representative photomicrographs of rat renal tissues immuno-stained for NF-κB; from the sham group (bottom-far left) showing no expression in the cells lining the renal tubules (arrow), from the (CLP) group with strong cytoplasmic immunoexpression in the cells lining the renal tubules (arrow) and from the (CLP+ α-ch) treated group showing very weak immunoexpression for NF-κB (arrow).
Abbreviations: CLP, Cecal Ligation and Puncture; α-ch, alpha-chymotrypsin.

Discussion

In this study, we investigated for the first time the effect of α-ch against sepsis-induced mortality and its pulmonary and hepatorenal protective effects. Based on the previously documented antioxidant and anti-inflammatory effects of chymotrypsin, we initially hypothesized that α-ch would be protective against sepsis-induced organ damage and mortality. The results of the current study revealed that chymotrypsin at a dose of 8.1 U/rat significantly improved the survival of septic rats. This protective effect is correlated with scavenging free radicals and increasing intracellular concentrations of GSH and SOD by α-ch and its previously discussed anti-inflammatory effects.[20,24]

A novel finding in this study is the role of α-ch in attenuating tissue expression of TLR4, NF-κB, MPO, and iNOS. These effects could explain the underlying protective mechanism of α-ch in sepsis. The induction of polymicrobial sepsis and the resultant release of endotoxins make the CLP model the preferable model for induction of sepsis.[28,46] Induction of sepsis by CLP in the current study resulted in a severe reduction in survival; no animals survived beyond the first 48 h, which is consistent with previous studies.[47] This increased mortality in untreated septic rats can be attributed to the evoked cytokine storm that characterizes sepsis and septic shock.[5,48] High levels of serum TNF-α, IL-1β, and IL-6 were observed following induction of sepsis in rats. In addition, the induction of CLP increased MDA and total nitrite levels and decreased GSH levels and SOD activity, indicative of enhanced ROS production and oxidative stress. The role of oxidative stress in the pathogenesis of sepsis-induced multiple organ dysfunction and the associated high mortality rate in

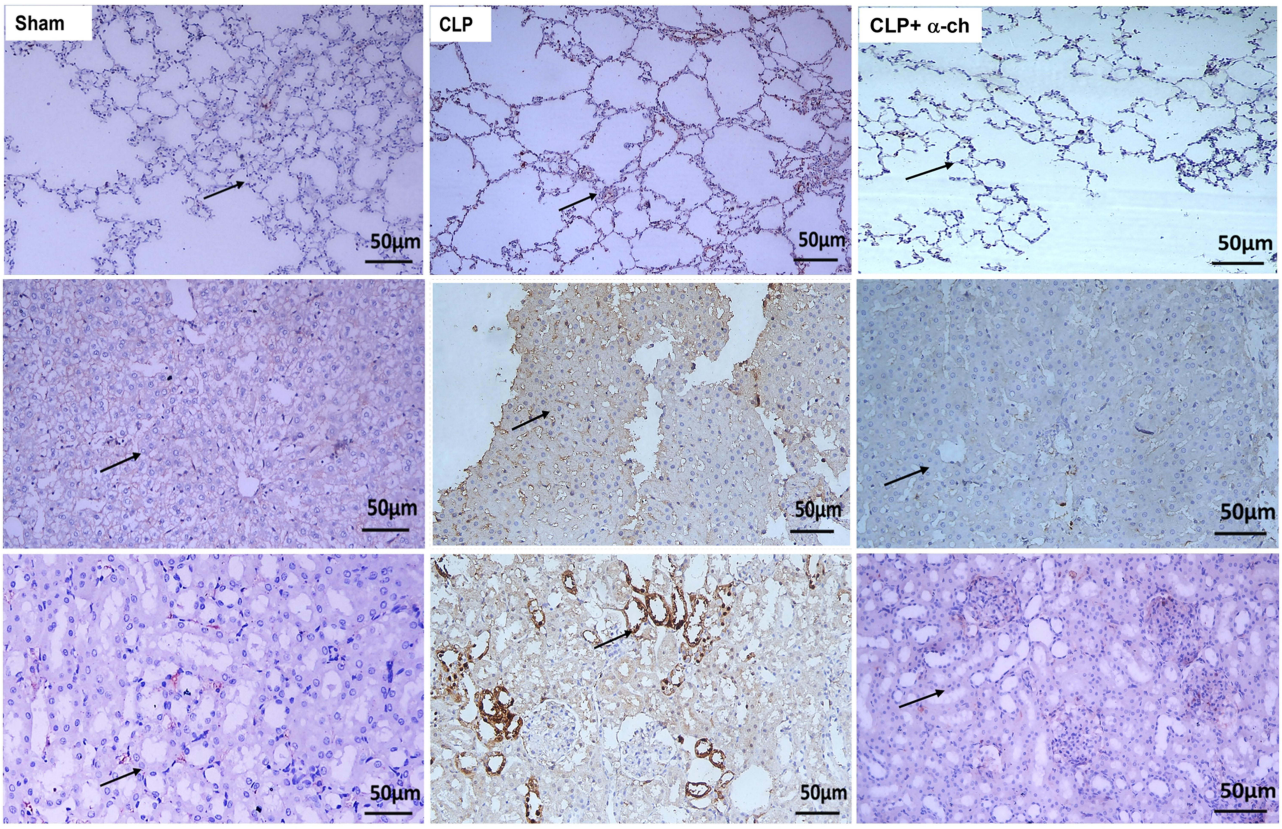

Figure 7 Protective effect of alpha-chymotrypsin against sepsis-induced high iNOS immunoreactivity. Upper panel: Representative photomicrographs of rat lung tissues immuno-stained for iNOS; from the sham group (top-far left) showing no expression in the cells lining the alveoli (arrow), from the (CLP) group with strong cytoplasmic immunoexpression (arrow) and from the (CLP+ α-ch) treated group showing weak immunoexpression for iNOS (arrow). Middle panel: Representative photomicrographs of rat liver tissues immunostained for MPO; from the sham group (left) showing no expression in the hepatocytes (arrow), from the (CLP) group with moderate cytoplasmic immunoexpression in the hepatocytes and the endothelial cells lining the sinusoids (arrow) and from the (CLP+ α-ch) treated group showing weak immunoexpression for iNOS (arrow). Lower panel: Representative photomicrographs of rat renal tissues immuno-stained for iNOS; from the sham group (bottom-far left) showing no expression in the cells lining the renal tubules (arrow), from the (CLP) group with strong cytoplasmic immunoexpression in the cells lining the renal tubules and the endothelial cells lining the glomeruli (arrow) and from the (CLP+ α-ch) treated group showing weak immunoexpression for iNOS (arrow).
Abbreviations: CLP, Cecal Ligation and Puncture; α-ch, alpha-chymotrypsin.

sepsis was stated in many studies.[2,49–52] Interestingly, the increased serum MDA levels, indicative of oxidative stress, correlate with poor prognosis in septic patients.[53]

In the present study, increased kidney and liver dysfunction parameters and signs of lung injury were evident in the untreated septic rats. Damage to these organs was confirmed with the histopathological changes seen in the examined tissue sections. Signs of lung injury were consistent with the results of a previous study[31]. In the study performed by Aboyoussef and colleagues,[54] acute hepatic injury resulted from the induction of sepsis, which was confirmed by the rise in the serum levels of ALT and AST. Following the induction of the CLP model,[55] the serum levels of cystatin C, creatinine, and urea were assessed as indicators of renal injury, and the levels of AST and ALT were assessed as indicators of liver injury.

In the current study, treatment of septic animals with α-ch 2 h after induction of sepsis improved animal survival and decreased ALI, AHI, and AKI associated with sepsis. During or after the experimental induction of sepsis, TNF-α, IL-1β, and IL-6 are released from monocytes and endothelial cells; this results in free radical formation and oxidative tissue injury.[56,57] Inhibition of cytokine synthesis was suggested as a strategy to increase survival during sepsis.[58,59] Following burn injury, signaling by IL-1β and IL-6 were proposed to play a critical role in the development of sepsis.[60] The study conducted by RaviKumar and colleagues[60] showed that a combination of chymotrypsin with trypsin (Chymoral Forte D. S. preparation) decreased the levels of serum IL-1β and IL-6. This treatment hastened wound epithelialization and burn wound healing, consequently obtaining better scar outcomes compared with the untreated group. Another study

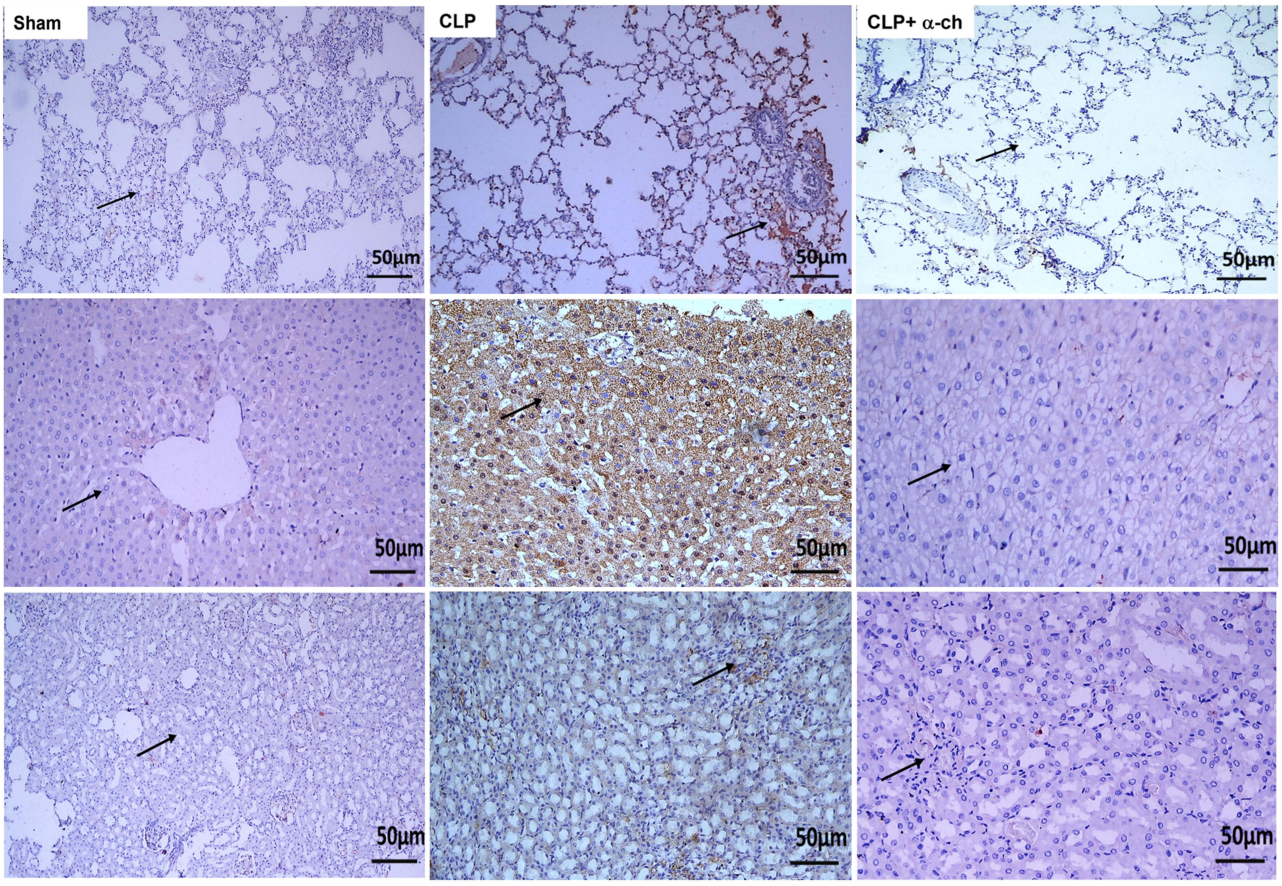

Figure 8 Protective effect of alpha-chymotrypsin against sepsis-induced high MPO immunoreactivity. Upper panel: Representative photomicrographs of rat lung tissues immuno-stained for MPO; from the sham group (top-far left) showing no expression in the cells lining the alveoli (arrow), from the (CLP) group with strong cytoplasmic immunostaining (arrow) and from the (CLP+ α-ch) treated group showing very weak immunostaining for MPO (arrow). Middle panel: Representative photomicrographs of rat liver tissues immuno-stained for MPO; from the sham group (left) showing no expression in the hepatocytes (arrow), from the (CLP) group with strong cytoplasmic immunoexpression in the hepatocytes and the endothelial cells lining the sinusoids (arrow) and from the (CLP+ α-ch) treated group showing weak immunoexpression for MPO (arrow). Lower panel: Representative photomicrographs of rat renal tissues immuno-stained for MPO; from the sham group (bottom-far left) showing no expression in the cells lining the renal tubules (arrow), from the (CLP) group with moderate cytoplasmic immunoexpression in the epithelial cells of tubules (arrow) and from the (CLP+ α-ch) treated group showing very weak immunoexpression for MPO (arrow).
Abbreviations: CLP, Cecal Ligation and Puncture; α-ch, alpha-chymotrypsin.

investigating the effect of chymotrypsin on orally administered IL-6 revealed that longer incubation periods of IL-6 with chymotrypsin were essential to shut the biological activity of this cytokine in vivo.[61] Another study elucidated that incubating TNF-α with pancreatic proteases resulted in marked degradation by chymotrypsin, which indicates that TNF-α is vulnerable to chymotrypsin activity.[62]

In a study conducted by Swamy and Patil,[23] the anti-inflammatory effects of chymotrypsin were observed in a model of acute and sub-acute inflammation induced by hind paw edema and cotton pellet-induced granuloma, respectively. Such an anti-inflammatory effect was superior to that of aspirin in the sub-acute model of inflammation and exhibited a synergistic effect with aspirin in both models. This effect may be attributed to the inhibition of migration of neutrophils into the inflamed tissues[22] and the stimulation of neutrophil apoptosis.[63] In a study of bovine mastitis, chymotrypsin was considered an effective treatment when combined with beta-lactams as it reduced the production of fibrin and hence allowed more effective distribution of the antibiotic drugs, in addition to its proteolytic activity.[64]

In line with its protective effects, rats given α-ch had significantly lowered MDA and NO levels and higher GSH levels and SOD activity compared with the septic rats. Several studies supported the antioxidant effect of α-ch. A study that comprised 30 patients with 20–30% deep second-degree burns revealed that the protective effect of α-ch may be due to an increase in the activity of the antioxidant enzymes: glutathione peroxidase, glutathione-S-transferase, SOD,

catalase, and ceruloplasmin, which allowed for effective scavenging of the oxygen free radicals and the reduction of lipid peroxidation products.[24]

Activated neutrophils are an important source of reactive oxygen species (ROS) during sepsis.[65] Since chymotrypsin was found to induce neutrophil apoptosis dramatically,[63] the effect of α-ch on tissue expression of TLR4, NF-κB, MPO, and iNOS can be explained in light of its effect on neutrophils. During bacterial infection, activated neutrophils release MPO that forms hypochlorous acid (HOCl), and NADPH oxidase, which produces ROS (O_2^- and H_2O_2) to repel the bacteria but at the same time cause damage to the host cells.[16,17] In a previous clinical trial, early excessive stimulation of neutrophils and high levels of MPO on day 1 predicted the 90-day mortality.[66] NO is produced excessively in case of inflammation by iNOS. This is considered dangerous to the host cells, especially when NO reacts with superoxide radicals, as it causes direct damage to the functions of the normal cells.[67]

LPS, a component of the cell wall of gram-negative bacteria, starts the inflammatory signal by binding to TLR4 in the form of CD14-LPS complex. The inflammatory signal initiated through adaptor proteins results in the activation of a pleiotropic transcription factor involved in inflammation: NF-κB.[68] After the translocation of the active NF-κB complex to the nucleus, the p65-p50 complex stimulates iNOS transcription. Other inflammatory cytokines released from infected cells, such as TNF-α and IL-1β also trigger NO production.[69] Inhibiting the binding of LPS to TLR4 or inhibiting the translocation of NF-κB into the nucleus will eventually lead to inhibition of NO production. The ability of α-ch to reduce TLR/NF-κB was shown in a recent study investigating its effect in a model of adjuvant-arthritis.[70] This effect was also accompanied by a reduction in serum inflammatory cytokines.

A study to investigate the mechanism of chymotrypsin (combined with trypsin) in tissue healing and repair found that it prevents a sharp rise in the protease inhibitor α1-antitrypsin, which, if unregulated, can cause uncontrolled inflammation and impaired healing. This rise results in fibrinolytic shutdown due to inhibition of plasmin. Alpha 1-antitrypsin, if maintained for a long duration, could cause a reduction in the inflammatory cascade, ROS production, and oxidative stress, leading to a faster healing process.[71] The combination of chymotrypsin with trypsin resulted in resolving the inflammatory edema and restoring the microcirculation, in addition to the shortening of the fibrinolytic shutdown to facilitate tissue repair.[72,73] Another study investigated the effect of removing MEL-4, a mouse cell surface antigen involved in the neutrophil-endothelial cell interactions, by using low doses of chymotrypsin for a short time to inhibit the migration of neutrophils into the inflamed tissues, in order to separate the antigen from the cell surface.[22]

Conclusion

Treatment of septic rats with a single dose of α-ch showed a great potential in the management of sepsis, which was observed as a significant increase in survival due to the significant attenuation of ALI, AHI, and AKI. This protection by α-chymotrypsin was confirmed using histopathological studies and decreased injury scores. Such protective effects can be attributed to the anti-inflammatory and antioxidant actions of α-chymotrypsin by lowering the tissue expression of TLR4, NF-κB, MPO, and iNOS.

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Disclosure

The authors report no conflicts of interest in this work.

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