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Therapeutic potential of ramipril, losartan, and spironolactone against sepsis-associated liver tissue injury induced by cecal ligation and puncture in rats

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Abstract. – OBJECTIVE: Sepsis-associated liver injury is responsible for the high morbidity and mortality rates seen with septic shock. Activation of the renin-angiotensin-aldosterone system (RAAS) is an essential counteractive mechanism during the hypotensive phase of sepsis; however, excessive activation is associated with exaggerated pro-oxidant and inflammatory response, which aggravates organ damage. This study aimed to evaluate the effect of RAAS inhibition on sepsis-induced liver damage.

MATERIALS AND METHODS: The cecal ligation and puncture (CLP) model was employed as a model of sepsis. Rats were divided into five groups: sham-operated, vehicle-treated septic rats, septic rats treated with ramipril in a dose of 10 mg/kg, septic rats treated with losartan in a dose of 20 mg/kg, and finally septic rats treated with spironolactone in a dose of 25 mg/kg. Rats received the treatment one hour after induction. Twenty-four hours later, rats were euthanized, and serum samples and liver tissue were collected to evaluate liver function and hepatic oxidative, anti-oxidative, inflammatory, and apoptotic markers. The microscopic integrity of the hepatic tissue was also assessed.

RESULTS: The results of our study showed that all the treatments used ameliorated sepsis-induced liver injury. This was reflected by improved liver function parameters and histopathological appearance of liver tissue. Treatment with ramipril, losartan, or spironolactone reduced tissue malondialdehyde (MDA), nitric oxide, activated caspase-3, and TNF-α. Moreover, these drugs increased hepatic reduced-glutathione (GSH) levels, superoxide dismutase (SOD) activity, and proliferating cell nuclear antigen (PCNA) expression.

CONCLUSIONS: Administration of ramipril, losartan, or spironolactone after CLP produced a hepatoprotective effect in rats, possibly by reducing oxidative stress, inflammation, and apoptosis.

Key Words:
Liver injury, CLP, TNF, PCNA, Caspase-3.

Introduction

Due to its role in phagocytosis and clearance of bacterial cells, the liver is one of the earliest organs affected by sepsis, with a recorded incidence rate ranging from 34% to 46% and liver dysfunction-associated mortality rates ranging from 54% and 68%[1-4]. Several mechanisms are involved in the pathogenesis of sepsis-induced acute liver injury. They include the induction of an exaggerated systemic inflammatory reaction, hepatic ischemia, coagulopathy, and unregulated apoptosis. The mechanisms of sepsis-associated liver dysfunction are divided into primary and secondary mechanisms. Hypovolemic shock followed by resuscitation results in ischemia-reperfusion injury, causing primary dysfunction[5]. Secondary injury results from Kupffer cells activation by endotoxins, leading to hepatocyte damage. He-

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patocyte damage is also induced by reactive oxygen radicals released from neutrophils[6]. When liver injury takes place, its pivotal protective role is adversely affected[7]. Bacterial clearance, inactivation of toxins, and production of inflammatory mediators are among the protective functions of the liver[8]. In experimental models of sepsis, such as cecal ligation and puncture (CLP), liver injury is observed within 1.5 hours after CLP[9]. Since liver impairment is a major contributing factor to sepsis progression and death, sepsis-mediated mortality can be effectively reduced by combating hepatocellular damage and improving liver function[3].

Among the most important functions of the liver is the production of angiotensinogen, the precursor of angiotensin II (Ang-II). Ang-II is produced by the activity of the angiotensin-converting enzyme (ACE) and is considered the starting point of the renin-angiotensin-aldosterone system (RAAS). RAAS activation during septicemia is a well-known phenomenon observed in experimental[10] and clinical studies[11]. It is essential in regulating blood pressure, hypovolemia, and electrolyte balance during sepsis. In addition to its systemic effects, RAAS is also expressed locally in different tissues and plays a role in cellular growth, inflammation, and apoptosis[12]. Ang-II is also an inflammatory agent that plays a role in sepsis-induced mortality and organ failure. Ang-II causes oxidative stress through the excessive production of oxygen free radicals, activating multiple intracellular signaling pathways, such as NF-κB and MAPK[13]. Ang-II stimulates the production of aldosterone, which acts on mineralocorticoid receptors and exerts fibroproliferative effects, leading to the formation of fibroblasts and inflammatory mediators[14]. Ang-II contributes to apoptosis by activating of NF-κB, which mediates the phosphorylation of JNK and the cleavage of caspase-3[15].

RAAS inhibition at several points along its pathway showed protective effects against a number of disease models[10]. Thus, we hypothesized that interference with RAAS through the use of ramipril, which inhibits angiotensin-converting enzyme, or losartan, which blocks angiotensin receptors, or spironolactone, which antagonizes aldosterone receptors, would exert a protective effect against sepsis-induced liver damage. To the best of our knowledge, our study is the first to compare the effects of three agents affecting the RAAS system in sepsis-induced hepatic injury.

Materials and Methods

Animals

Wistar rats (3-month-old) weighing 190±10 g were obtained from the animal care facility at Nahda University in Beni-Suef, Egypt. Animals were acclimatized for two weeks and housed under controlled temperature and humidity with a 12 h light-dark cycle. All rats had access to food and water *ad libitum*. The study was approved by the Commission on Ethics of Scientific Research for the Ethical Principles and Guidelines of the Care and Use of Laboratory Animals, Faculty of Pharmacy, Minia University (Code number of the project: 37/2019).

Experimental Design

Based on survival data, 39 rats were randomly assigned to five experimental groups [Group 1: sham-operated (n=6), Group 2: CLP non-treated group (vehicle-treated, n=15), Group 3: CLP treated with 10 mg/kg of oral ramipril (n=6), Group 4: CLP treated with 20 mg/kg of intraperitoneal losartan (n=6), and Group 5: CLP treated with 25 mg/kg of oral spironolactone (n=6)][16]. The drugs under investigation were administered one hour after the CLP procedure. Twenty-four hours after CLP surgery, all rats were euthanized using an overdose of sodium pentobarbital (50 mg/kg).

The CLP Procedure

The CLP-induced sepsis was performed as previously described[17]. Rats were anesthetized by ketamine-xylazine combination (50 and 10 mg/kg, respectively). The abdomen was shaved and disinfected, and then a ventral incision was made in the lower left quadrant. The cecum was identified, exposed, and then ligated with a 3/0 silk sterile surgical suture. Two through-and-through punctures were done in the ligated cecum using an 18-gauge syringe needle. To limit the variability in the severity of sepsis, a fixed proportion (75%) of the cecum length was subjected to ligation throughout the study. The ligated part was then gently squeezed to extrude some fecal matter and carefully returned to the abdominal cavity. At the end of the procedure, the incision was sutured, and normal saline in a dose of 30 ml/kg was subcutaneously injected for resuscitation. The same steps were followed with the exception of cecum ligation and puncture for the sham-operated rats.

The survival study was performed first to determine the number of animals needed in each group. For the survival studies, 10 rats/group were monitored for three days, and death events were recorded. All surviving animals were then euthanized. The CLP procedure was performed by trained doctors, and the procedure was carried out under general anesthesia. Efforts were made to minimize animal suffering. The rats were all put on heating pads to ensure they did not develop hypothermia. They were then transferred to their cages on a sterile surgical absorbing pad to minimize infections of the suture site. Food pellets were made available inside the cage to reduce the effort made by the animal to reach for food 24 hours after surgery. The health status of each animal was assessed twice daily, according to Morton and Griffiths[18], and the rats were euthanized immediately if their score indicated severe illness. The use of analgesics was not compatible with this study as they affect inflammatory cytokines. No rats were euthanized before the three-day survival observation period. For the 24-hour study, only rats in the sepsis non-treated group were found dead after 24 hours, and the cause of death was suspected to be disseminated intravascular coagulopathy, a consequence of severe septic shock.

Tissue Isolation and Preparation

After the injection of sodium pentobarbital, blood was collected directly from the heart, and then animals were euthanized by decapitation. Blood was left at room temperature to coagulate and was then centrifuged (10 minutes at 15,000 rpm; Centurion Scientific Limited, UK). The liver was dissected and divided into different parts; one part was immediately placed in liquid nitrogen for biochemical analyses, and another part was fixed in buffered 10% formalin for histopathological and immunohistochemical assessments. The liver homogenate was prepared as 10% w/v in phosphate buffer solution (PBS) using a tissue homogenizer (Cole-Parmer, Vernon Hills, IL, USA) The supernatant was collected after centrifugation using a cooling centrifuge (Scilogex, Rocky Hill, CT, USA).

Measurement of Serum Parameters

Liver enzymes, specifically ALT and AST, were identified in the serum samples utilizing colorimetric assay kits obtained from Diamond Diagnostics, Egypt, and following the manufacturer's instructions.

Serum lactate was measured colorimetrically using a commercial kit obtained from Biomed, Egypt.

Histopathological and Morphometric Studies

Fixed liver tissues were processed for staining with Hematoxylin and Eosin (H&E) using previously reported techniques[19]. Photomicrographs were obtained using a digital camera mounted on an Olympus microscope (Tokyo, Japan). Hematoxylin and eosin findings were scored as follows for histopathological alterations (-): normal. (+): between normal and mild levels. (++): mild level (less than 25% of the total fields examined revealed histopathological alterations). (+++): moderate level (25-50% of the total fields examined revealed histopathological alterations). (++++): severe level (50- 75% of the total fields examined revealed histopathological alterations).

Assessment of Liver Oxidative Stress Markers

Tissue malondialdehyde (MDA) was measured in liver homogenates as a marker of lipid peroxidation. The assay kit was obtained from Biodiagnostic, Egypt (Cat. No. MD 25 29), and the procedures were carried out in accordance with the supplier's instructions. Total nitrate/nitrite (NOx) in the liver homogenate was assayed after reduction of nitrate into nitrite using cadmium as previously described[20].

Determination of Hepatic Antioxidant Capacity

Hepatic content of reduced glutathione (GSH) and superoxide dismutase (SOD) enzyme activity levels were measured using commercially available kits obtained from Biodiagnostic, Egypt (Cat. No. GR 25 11 and Cat. No. SD 25 21; respectively).

Assessment of Apoptosis

Liver homogenates from the different experimental groups were assessed for apoptosis by using a rat Caspase 3 ELISA Kit (MyBioSource, Cat. No: MBS018987) to measure caspase-3 in accordance with the manufacturer's instructions.

Immunohistochemical Assessment of Tumor Necrosis Factor-α (TNF-α) and Proliferating Cell Nuclear Antigen (PCNA)

Paraffin sections were mounted on positively charged slides using the avidin-biotin-peroxidase complex (ABC) method, mouse TNF-α mono-

clonal antibody (Elabscience, Cat# E-AB-22159, Dil.: 1:100), and mouse PCNA monoclonal antibody (Novusbio, Cat# NBP2-44730, Dil.: 0.5 ug/ml). Sections from each group were incubated with these antibodies, and then the reagents required for the ABC method were added (Vectastain ABC-HRP kit, Vector laboratories, Newark, NJ, USA). Marker expression was labeled with peroxidase and colored with diaminobenzidine (DAB, produced by Sigma, USA) to detect antigen-antibody complex. Negative controls were included using non-immune serum in place of the primary or secondary antibodies. IHC-stained sections were examined using an Olympus microscope (BX-53, Tokyo, Japan). Quantitation of the extent of staining was performed by measuring areas of immune-positive staining using ImageJ program, LOCI, University of Wisconsin in 10 non-overlapping liver sections/group[21].

Statistical Analysis

Animal survival among the different groups was compared using the Log-rank Mantel-Cox test. The correlation between parameters of liver injury was determined by calculation of the Pearson correlation coefficient r. The correlation was considered weak if $r<|0.3|$, moderate if $|0.3| \leq r \leq |0.7|$ and strong if $r>|0.7|$. Data is presented as mean ± S.E.M. To find the statistical significance among groups, an analysis of variance (ANOVA) test followed by the Tukey post-hoc test was performed where p-values lower than 0.05 were considered significant. All statistical analyses were carried out using GraphPad Prism® 8 software (San Diego, CA, USA).

Results

Effect of CLP and Different Treatments on Mortality

Rats in different groups were monitored for 3 days after surgery. Figure 1 shows the survival rate in different groups. Only 10% of septic non-treated rats survived while rats treated with, losartan, ramipril, or spironolactone had an overall survival of 80%, 70%, and 80%, respectively. In the vehicle-treated CLP group, only 40% of the rats survived the first 24 hours after surgery. In the spironolactone treated group, all rats survived the first 24 hours after surgery. In the ramipril treated group, 90% of rats survived the first 24 hours after surgery. In the losartan treated group, 80% of rats survived the first 24 hours after surgery. In the sham-operated group, no mortality occurred.

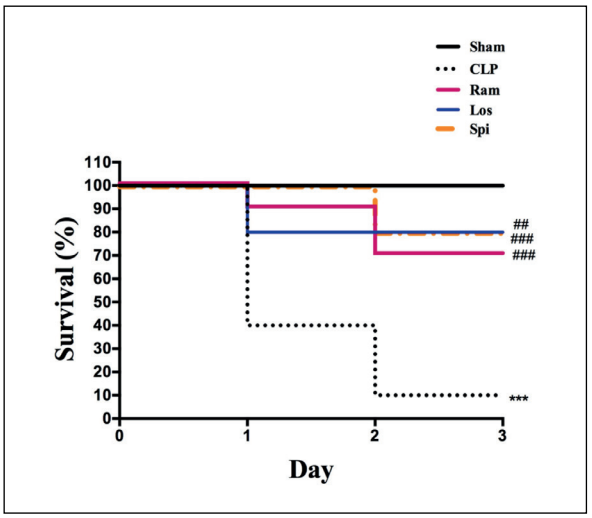

Figure 1. Effect of CLP and different treatments on mortality. Surgical induction of sepsis by the cecal ligation and puncture (CLP) procedure resulted in 100% mortality within 3 days. RAAS inhibition, significantly reduced the survival of CLP rats (n=10). Sham rats (n=10) showed no mortality. Survival analysis was carried out using the Log-rank Mantel-Cox test. ***: Significantly different from the sham-operated group at $p<0.001$, respectively. # and ##: Significantly different from the vehicle-treated CLP group at $p<0.01$ and $p<0.001$, respectively.

Liver Function Impairment and Liver Tissue Injury Induced by CLP and its Alteration by Different Treatments

As shown in Figure 2 (upper panel), the serum liver enzymes, ALT and AST, were elevated in CLP rats compared to sham animals ($p<0.05$). On the other hand, administration of each of the drugs under investigation individually inhibited sepsis-induced increases in ALT and AST. It is worth noting that although serum albumin and bilirubin levels were measured, no significant changes were observed among the different experimental groups (data not shown).

Histopathological examination of liver tissues revealed normal lobular architecture in sham-operated rats. Hepatocytes were arranged in cords characterized by radial arrangement around the central vein (CV). In contrast, samples from the untreated CLP group showed numerous degenerative changes in hepatocytes. Some showed vacuolated cytoplasms with deformed nuclei. Others showed apoptotic cells with acidophilic cyto-

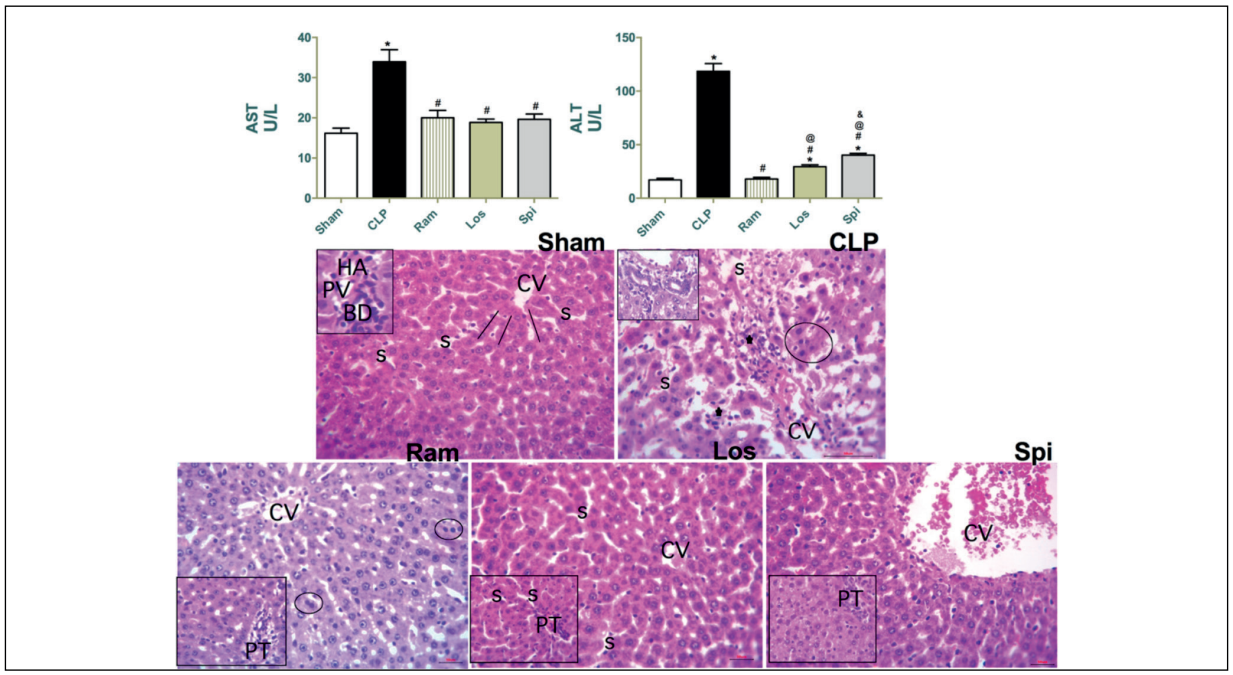

Figure 2. Effect of different treatments on the serum liver enzymes and histological appearance of the liver of septic rats.Upper panel: Bar charts showing the effect of CLP and different treatments on serum ALT and AST. Data were analyzed with ANOVA followed by the Tukey test for multiple comparisons. *Significantly different from sham-operated rats (p<0.05); #Significantly different from untreated CLP group (p<0.05), @Significantly different from ramipril-treated CLP group (p<0.05), CLP = cecal ligation and puncture**;** Spirono=spironolactone. Lower panels: Representative photomicrographs of rat liver tissues (H&E ×400) from the sham control group showing normal lobular appearance. Polygonal hepatocytes with an acidophilic granular cytoplasm and rounded vesicular nuclei (arrows) radiating from the central veins (CV) in plates (lines) and separated by blood sinusoidal (S). Inset: branches of the portal vein PV, hepatic artery (HA), and bile duct (D) in the portal tract. The untreated CLP-group shows disturbed lobular architecture surrounding congested CV showing necrotic foci (stars), sinusoidal dilatation and congestion (S), disorganized and degenerated apoptotic hepatocyte with deeply acidophilic cytoplasm and dark small nuclei either in clusters (circle) or scattered (arrows). Numerous hepatocytes with foamy vacuolated cytoplasm (dashed arrows, right inset). Notice proliferation of bile ducts (BD in inset). (Ram) Ramipril-group, (Los) Losartan-group, and (Spi) Spironolactone-group showing restored normal lobular architecture with apparently normal hepatocytes (arrows) radiating from a slightly congested central vein (CV) and separated by slightly dilated blood sinusoids (S). Notice a few scattered vacuolated cells around PT (arrows in inset) or a few scattered apoptotic cells around CV (circles). H&E ×400.

plasm and small dark nuclei. Moreover, sinusoidal dilatation and congestion, areas of hepatocyte atrophy, and proliferation of bile ducts were clearly observed in the untreated CLP group. On the contrary, treated CLP animals showed preserved lobular architecture and no evidence of major morphological injury. However, minimal congestion of CV and few vacuolated cells around the portal tract were observed in the spironolactone-treated CLP group (Figure 2, lower panels).

Morphometric Analysis of Liver Tissue from the Different Groups

Examination and scoring of hepatic tissue revealed significant changes in the liver of septic animals in the following parameters:

1. Hepatocytic apoptosis around the central vein and portal vein.
2. Vascular congestion of small vessels surrounding the central vein and portal vein.
3. Space of Disse (the space between sinusoidal endothelium and hepatocytes).
4. Congestion of the central vein and portal vein.

As shown in Table I, septic rats receiving different treatments showed improvements in the parameters mentioned above.

Effect of CLP on Serum Lactate and its Alteration by Different Treatments

Serum lactate is a biomarker of sepsis[23]. We measured lactate concentration in the serum of rats in the different experimental groups. As shown in Figure 3, CLP caused a significant increase in serum lactate compared with sham

Table I. Scoring of morphological changes in control and experimental groups by light microscope (H & E-stained sections).

Finding	Sham	CLP	Ramipril	Losartan	Spironolactone
Hepatocyte Apoptosis					
Around CV	-	++++	+	+	++
Around PT	-	+	-	-	+
Vascular congestion	-				
Around CV	-	++	-	-	+
Around PT	-	++++	-	-	++
Space of Disse					
Widening	-	++++	+	+	+
Congestion	-	++	-	-	-
CV congestion	-	+++	+	+	+
PV congestion	-	+++	-	-	-

Central veins (CV), sinusoidal spaces (S). portal tract (PT), portal vein (PV), hepatic artery (HA), and bile duct (D). Scoring system: (-) normal. (+) between normal and mild level. (++) mild level (less than 25% of the total fields examined revealed histopathological alterations). (+++) moderate level (less than 50% of the total fields examined revealed histopathological alterations). (++++) severe level (less than 75% of the total fields examined revealed histopathological alterations).

rats. Rats treated with losartan, ramipril, or spironolactone had significantly lower lactate levels ($p<0.05$) compared with CLP untreated rats.

Effect of CLP on Liver Oxidative Stress and Antioxidants Markers and their Alteration by Different Treatments

In order to evaluate the oxidative stress status of liver tissue, hepatic MDA content (as a marker of lipid peroxidation), total nitrates (NOx), SOD activity, and GSH concentration (as markers of antioxidant capacity) were determined. Septic rats showed significantly higher levels of hepatic MDA and total nitrates compared with the sham-operated group (Figure 4 lower panel). CLP rates showed decreased SOD activity and GSH concentrations compared with the sham-operated rats ($p<0.05$). Treatment of septic rats with any of the studied drugs led to a significant reduction ($p<0.05$) in the level of MDA and NOx and the preservation of SOD and GSH levels, when compared with rats subjected to CLP (Figure 4; upper panel).

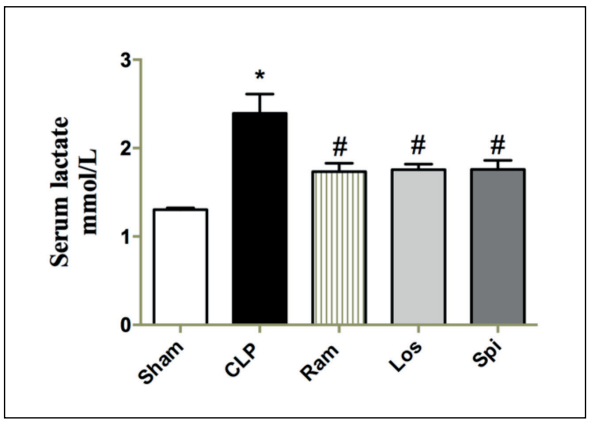

Figure 3. Effect of different treatments on serum lactate. Bar charts showing the effect of CLP and different treatments on serum lactate. Data were analyzed with one-way ANOVA followed by Tukey test for multiple comparisons post-test, n=6. *Denotes a significant difference compared to sham. #Denotes a significant difference compared to untreated CLP rats. CLP = cecal ligation and puncture, MDA = malondialdehyde, NO = total nitrated, SOD = superoxide dismutase, Ram = Ramipril, Los = Losartan, Spi = Spironolactone, CLP = cecal ligation and puncture.

Caspase-3 Level in Sham-Operated Rats and Different CLP Groups

The CLP group showed high levels of activated caspase-3 in comparison to the sham group, which showed no detectable activated caspase-3. The liver samples from rats treated with ramipril, losartan, or spironolactone had significantly lower caspase-3 levels (Figure 5).

Effect of CLP and Different Treatments on TNF-α and PCNA Immunoreactivity

Figures 6 and 7 show sections from the livers of the sham group showing mild cytoplasmic staining for TNF-α and evident nuclear staining of PCNA. The hepatocytes of the CLP group showed extensive immunopositive staining for TNF-α and reduced immunopositive staining for PCNA. Rats treated with ramipril, losartan, or spironolactone had a significantly lower expression of TNF-α and a higher expression of PCNA compared to non-treated septic rats ($p<0.05$).

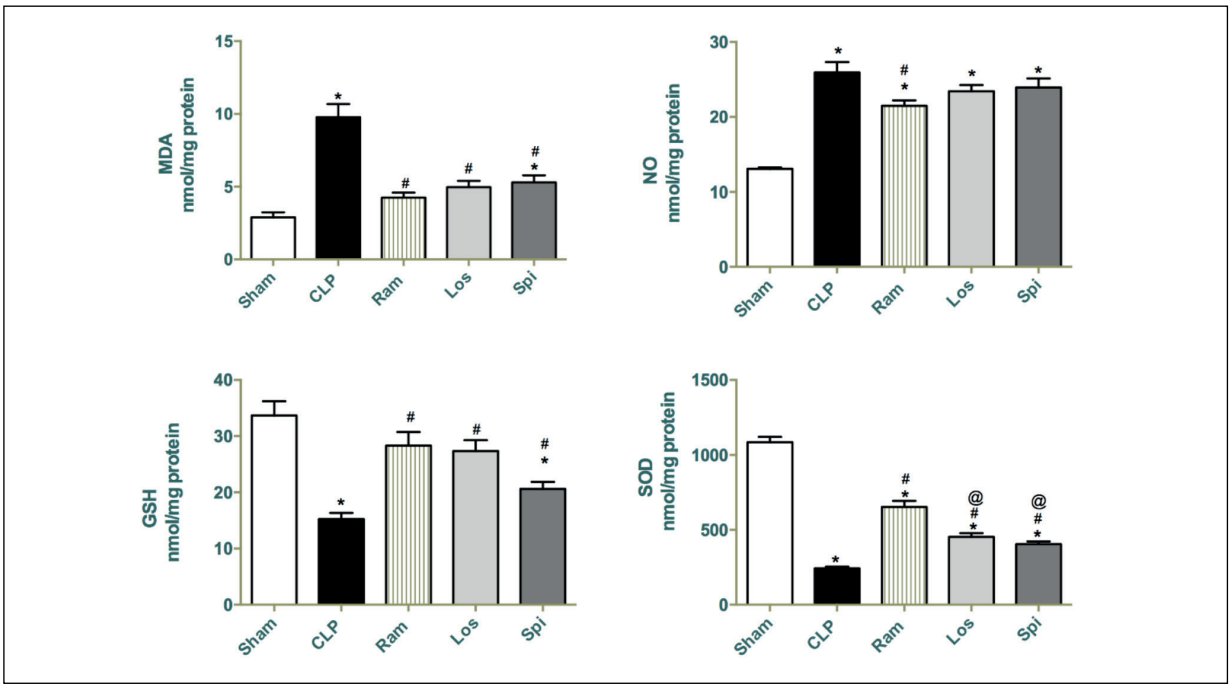

Figure 4. Effect of different treatments on hepatic oxidative and antioxidant profile of septic rats. Bar charts showing the effect of CLP and different treatments on hepatic MDA, NO, GSH and SOD. Data were analyzed with one-way ANOVA followed by the Tukey test for multiple comparisons post-test, n = 6. *Denotes a significant difference compared to sham. #Denotes a significant difference compared to untreated CLP rats. @Denotes a significant difference compared to ramipril-treated rats. CLP = cecal ligation and puncture, MDA = malondialdehyde, NO = total nitrated, SOD = superoxide dismutase, Ram = Ramipril, Los = Losartan, Spi = Spironolactone, CLP = cecal ligation and puncture.

Correlation Analysis Between the Measured Parameters of Liver Injury

Correlation analysis shows that liver injury (represented as serum liver enzymes ALT and AST) possesses a strong positive correlation with the levels of serum lactate, MDA, NO, caspase-3, and TNF-α. As shown in Figure 8, a strong negative correlation is also seen with the level of SOD, GSH, and PCNA. Data are presented as a correlation matrix of Pearson's correlation coefficient between the measured parameters.

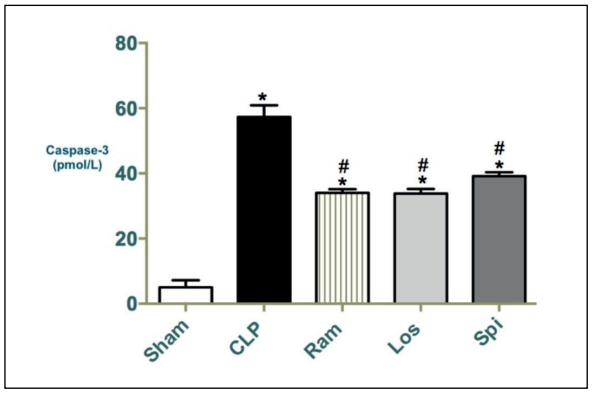

Figure 5. Effect of CLP and different treatments on hepatic caspase-3 concentration. Bar charts showing the concentration of caspase-3 in liver tissue from different experimental groups. Data were analyzed with the ANOVA test followed by the Tukey-Kramer test for multiple comparisons. *Significantly different from sham-operated rats (p<0.05); #Significantly different from untreated CLP group (p<0.05).

Discussion

A decline in liver function and disrupted structural integrity are observed early on in sepsis[7]. This diminishes the liver's protective role during sepsis and leads to a deterioration in other organ functions, ultimately resulting in high morbidity and mortality rates[7]. In this study, we compared the effects of different drugs interfering with the RAAS system on CLP, a well-established experimental sepsis model. We especially focused on their hepatoprotective role. The liver injury induced by CLP was demonstrated as elevated serum ALT and AST levels, the most frequently used indicators and early response markers of liver injury[23].

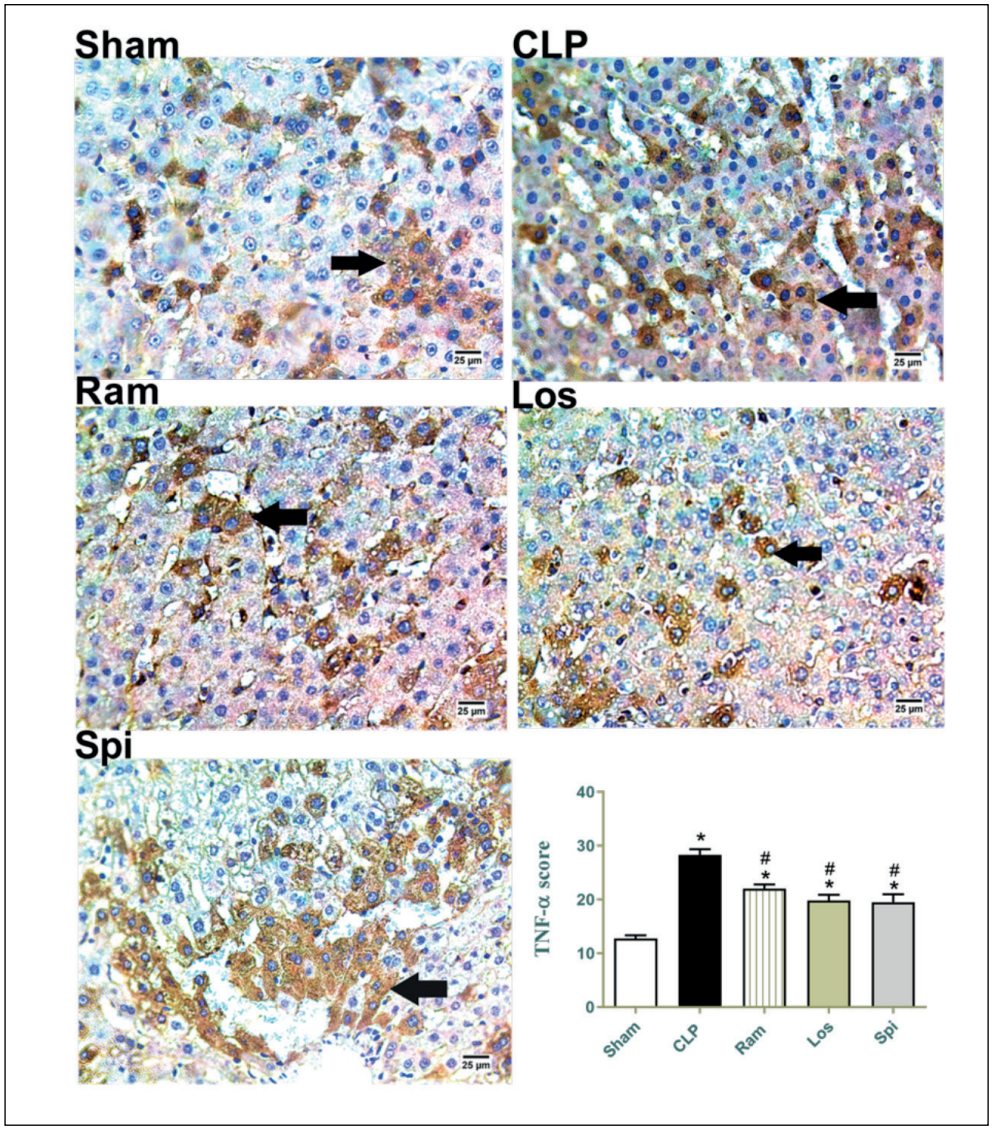

Figure 6. Effect of CLP and different treatments on TNF-α immunoreactivity**.** Representative photomicrographs showing cytoplasmic TNF-α immunoreactivity in liver sections from sham and different CLP groups. A positive reaction for TNF-α was observed in the cytoplasm of hepatocytes (Black arrow) (IHC-Peroxidase-DAB). Magnification bar 25 mm (100×). Bar charts showing semi-quantitative determination of TNF-α immunoreactivity in liver tissue from different experimental groups. Data were analyzed using the ANOVA test followed by the Tukey test for multiple comparisons. *Significantly different from sham-operated rats (p<0.05); #Significantly different from untreated CLP group (p<0.05).

A prior investigation on doxorubicin-induced liver injury revealed notable hepatic structural damage, as evidenced by elevated serum markers of ALT and AST[24]. The hepatic tissue structure was also distorted, showing signs of inflammation and apoptosis. Septic rats treated with ramipril, losartan, or spironolactone showed a reduction in serum ALT and AST. The histopathological examination of the liver tissue integrity revealed an attenuation of the injury and restoration of the general lobular architecture of liver tissue. Interestingly, our data shows that treatment with ramipril was associated with enhanced protection when compared to other RAAS inhibitors.

Lactate is produced by most tissues under anaerobic conditions, with most production observed in muscles[25]. Under normal conditions, the majority of lactate is cleared by the liver, and a smaller portion is cleared by the kidneys[22]. Gen-

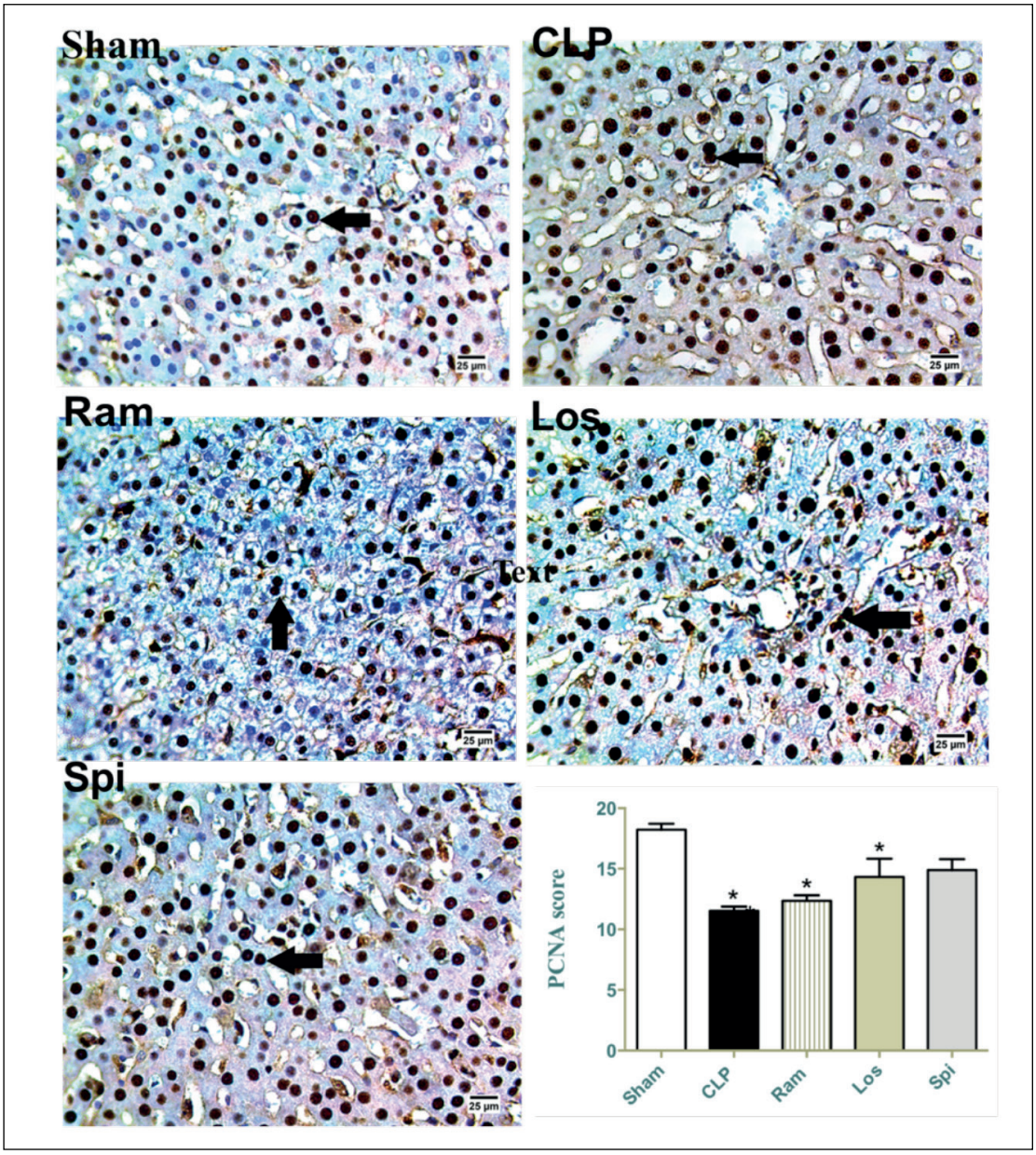

Figure 7. Effect of CLP and different treatments on PCNA immunoreactivity. Representative photomicrographs showing cytoplasmic PCNA immunoreactivity in liver sections from sham and different CLP groups. Positive reaction for in nuclei of hepatocytes (Black arrow) (IHC-Peroxidase-DAB). Magnification bar 25 mm (100×) Bar charts showing semi-quantitative determination of PCNA immunoreactivity in liver tissue from different experimental groups. Data were analyzed using the ANOVA test followed by the Tukey test for multiple comparisons. *Significantly different from sham-operated rats (p<0.05); #Significantly different from untreated CLP group (p<0.05).

erally, lactate overproduction, reduced clearance, or a combination of the two results in elevated lactate levels, which is evident during septic shock. Circulatory dysfunction and liver dysfunction lead to hypoperfusion, which contributes to both the increased production and decreased clearance of lactate[26]. Serum lactate was reported as one of the biomarkers for determining the severity and mortality in experimental sepsis induced by cecal ligation and puncture[22]. In our study, elevated serum lactate was evident in the sera of the rats subjected to CLP. All treated groups showed lower lactate levels, compared with CLP. These results are in alignment with the survival data and the other parameters measured, as shown in our correlation analysis.

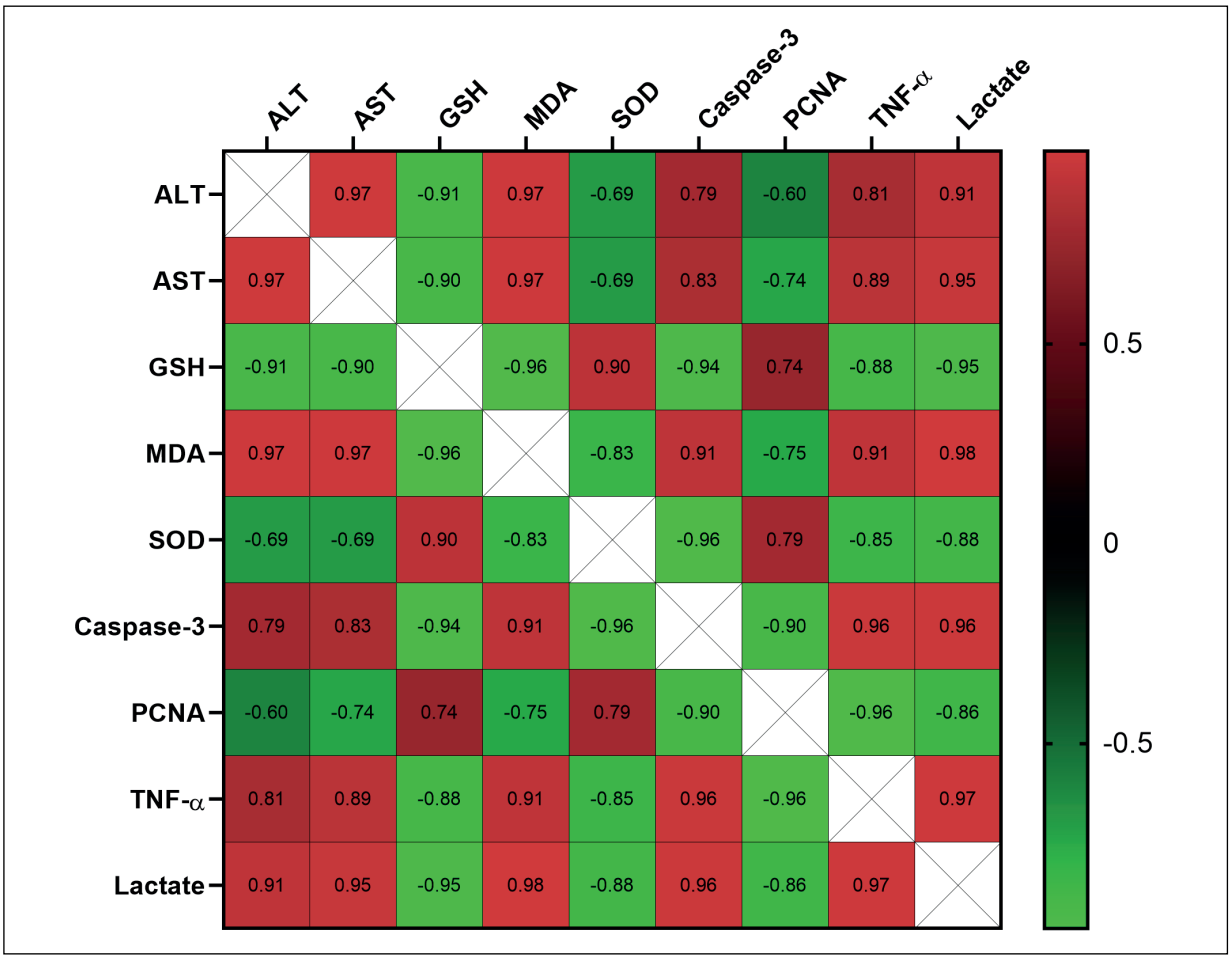

Figure 8. Correlation analysis of Liver enzymes and measured parameters. Correlation matrix between liver enzymes and related parameters. Data from different measurements was analyzed for correlation using the Pearson correlation coefficient (r). Positive values indicate a positive correlation while negative values indicate a negative one. When r is in the range of |0.3|–|0.7|, this indicates a moderate correlation, and when r>|0.7|, this means a strong correlation. MDA: malondialdehyde; SOD: superoxide dismutase; GSH: reduced glutathione.

Oxidative stress plays a vital role in sepsis-induced liver damage[27]. The generation of oxygen free radicals initiates liver damage, which stimulates the production of profibrogenic mediators and induces hepatic fibrogenesis[28]. Lipid peroxidation is one of the most important markers of oxidative stress[29]. Similar to our results, Ahmed et al[17] showed an increase in the levels of MDA and total nitrites in animals with CLP-induced liver damage compared with those in the sham group. Also, in a model of ischemia, reperfusion-induced hepatic injury is associated with elevated MDA levels as well as a reduction in GSH activity[30]. Blocking the RAAS system at different points prevented sepsis-induced oxidative stress within the liver tissues. Previous studies[32,33] on angiotensin-converting enzyme inhibitors (ACEIs) showed their ability to reduce oxidative stress. For example, ramipril treatment reduced hepatic oxidative stress in a model of liver injury induced by carbon tetrachloride[31]. The effect of another member of the ACEIs, captopril, was also shown to attenuate LPS-induced oxidative stress[32]. ACEIs reduce oxidative stress by inhibiting angiotensin II (AngII) synthesis as they are powerful pro-oxidant agents. AngII was found to stimulate reactive oxygen species (ROS) production from NADPH oxidase *in vivo* and *in vitro*[33]. In addition, AT1 knockout mice were observed to have reduced oxidative stress in a model of chronic liver injury[34].

Blockade of the AngII receptor was also associated with low oxidative stress. Losartan showed protective effects on aging mice by reducing inflammation and oxidative stress[35]. Moreover, losartan showed antioxidant and hepatoprotective

effects in a model of fatty liver[36], and lowered MDA and NOx in LPS-induced septic shock[37]. In addition, a clinical study[38] showed that valsartan, another ARB, inhibits ROS generation from human leukocytes. In another study[39], valsartan was used in the treatment of methotrexate-induced cardiotoxicity. By measuring MDA level and catalase activity levels, it was found to decrease oxidant molecules and increase antioxidant molecules.

In 2011, a study showed that losartan reduces oxidative stress by blocking a functional mitochondrial AngII type 2 receptor. This receptor is coupled to mitochondrial nitric oxide production, thus contributing to nitrosative and oxidative stress[40]. Barut et al[41] suggested that spironolactone markedly reduced MDA and MPO levels in the lungs after ischemia-reperfusion injury. They attributed this effect to a reduction in the tissue iNOS. Spironolactone also showed an ability to reduce cardiac oxidative stress in a model of diabetes[42]. In addition, spironolactone showed an antioxidant effect in a model of kidney injury[43]. These reported effects can be explained by the ability of spironolactone to block aldosterone-induced activation of NADPH oxidase, thus decreasing ROS production[16].

To maintain the integrity of the cell membrane and cellular proteins, SOD and GSH play a defensive role in neutralizing oxygen free radicals[44]. As shown in previous studies[35], we observed a reduction in SOD and GSH levels in the liver of the septic group in our study. Our results showed restored antioxidant defense in response to ramipril, losartan, or spironolactone. This further emphasizes their ability to limit the generation of oxygen-free radicals. Inhibition of the RAAS system at earlier points along its pathway by ramipril or losartan showed a more pronounced hepatoprotective effect compared to aldosterone blockade. This is plausible as inhibition of AngII signaling will inhibit all downstream effects, including aldosterone release.

A positive correlation between oxidative stress and inflammation is evident in previous studies[45,46]. Similar to our results, a previous study[47] demonstrated that CLP-induced sepsis-induced elevated levels of renal TNF-TNF-α and MDA. Our data, in line with previous reports[17,48-50], showed elevated hepatic TNF-TNF-α in rats subjected to sepsis, which positively correlated with elevated oxidative stress. TNF-TNF-α was shown to enhance the expression of inducible nitric oxide synthase (iNOS), which leads to increased production of nitric oxide[51], elevated oxidative stress, increased production of proinflammatory cytokines, activation of neutrophil infiltration, and ultimately hepatic damage[49,52]. Our results showed reduced hepatic TNF-α and decreased oxidative stress in all treated septic rats, supporting the hepatoprotective effects of drugs interfering with the RAAS system. The effect of RAAS system blockade on TNF-α production was previously reported in different disease models[53-56].

One of the outcomes of elevated oxidative stress is the activation of intrinsic-mitochondrial apoptotic pathways, as well as the activation of extrinsic pathways *via* death receptors. The caspase enzymes are key players in apoptosis. They exist in an inactive form composed of predomain and catalytic subunits. Caspases are activated by a cleavage reaction that releases the catalytic unit. Caspase 3 is found in the cytoplasm. Detection of cleaved caspase-3 has become a popular tool for the evaluation of cell death as it is a signal in both mitochondrial and extrinsic apoptotic pathways[57]. In CLP-nontreated rats, the liver tissue showed enhanced caspase-3 immunoreactivity and histological evidence of apoptotic cells. As a result, attenuation of apoptosis is expected to counteract hepatocellular damage induced by sepsis[58]. Neutrophils and macrophages infiltrate the hepatic tissue during sepsis, contributing to the activation of proapoptotic signaling through the production of cytokines (e.g., TNF-α) and NO[59]. On the other hand, liver tissue from the different treated septic groups showed a marked reduction in activated caspase-3. The ability of RAAS inhibitors to suppress apoptosis was previously demonstrated in other studies using perindopril[60], losartan[15] and spironolactone[61].

Proliferating-cell nuclear antigen or PCNA, is a nuclear factor involved in cell proliferation and DNA replication, which was found to correlate with hepatocellular regeneration[62,63]. Elevated hepatic PCNA levels indicate recovery and regeneration after hepatic damage[64]. PCNA reduces apoptosis by binding and inactivating procaspases[65]. In sepsis, a reduced gene expression of hepatic PCNA was associated with increased hepatic inflammation and apoptosis, as shown in this study and other studies[21,50,66]. The decreased hepatic PCNA levels in septic rats correlated with liver dysfunction parameters, serum lactate, and apoptosis. The liver tissue from rats in different treatment groups showed increased expression of PCNA. Abcejo et al[67] showed a negative correlation between PCNA expression and mortality in

sepsis. Animals experiencing severe sepsis and high mortality had lower PCNA levels compared with those having less severe disease[67]. Our survival data is similar to the survival data reported in the study by Abcejo et al[67].They also reported low expression of PCNA in septic rats similar to our findings. Thus, the ability of ramipril, losartan, and spironolactone to preserve hepatic PCNA, although partial, might provide an explanation for the improved survival of treated CLP rats. It is noteworthy to point out that sham group showed the expression of PCNA, which does not indicate a liver injury in sham animals. It is unclear whether this effect is a consequence of their ability to reduce sepsis severity *via* reducing oxidative stress and inflammation or is a direct effect of these drugs. Further studies are needed to address this. Our finding of elevated PCNA in response to inhibition of RAAS was previously reported in different animal models[68-70].

It is important to note that there are some case reports[71-73] describing liver injury induced in patients using losartan or ramipril. However, this effect was reported as an "uncommon" and idiosyncratic reaction and its mechanism has not yet been explained. Nonetheless, the literature contains a lot of experimental evidence supporting the hepatoprotective effect of these drugs in different models of liver injury[31,74-77]. One factor that may be responsible for the observed benefit in our study and in the literature is the short-term use of these drugs in experimental settings, contrary to the reported cases from patients who are chronic users.

Although combining the studied agents in future studies may seem appealing, it is important to consider that in clinical settings, neither additive nor potentiating effects were reported in response to the ACEI + ARB combination[78,79]. Moreover, it is important to avoid combining anti-hypertensives in sepsis therapy, as hypotension and hypoperfusion are associated with poor prognoses and negative outcomes.

Conclusions

In conclusion, ramipril, losartan, and spironolactone can act as protective agents against sepsis-induced liver injury through their antioxidant, anti-inflammatory, and antiapoptotic effects (Figure 9). Further research is warranted to characterize their beneficial effect on clinical sepsis and associated organ damage.

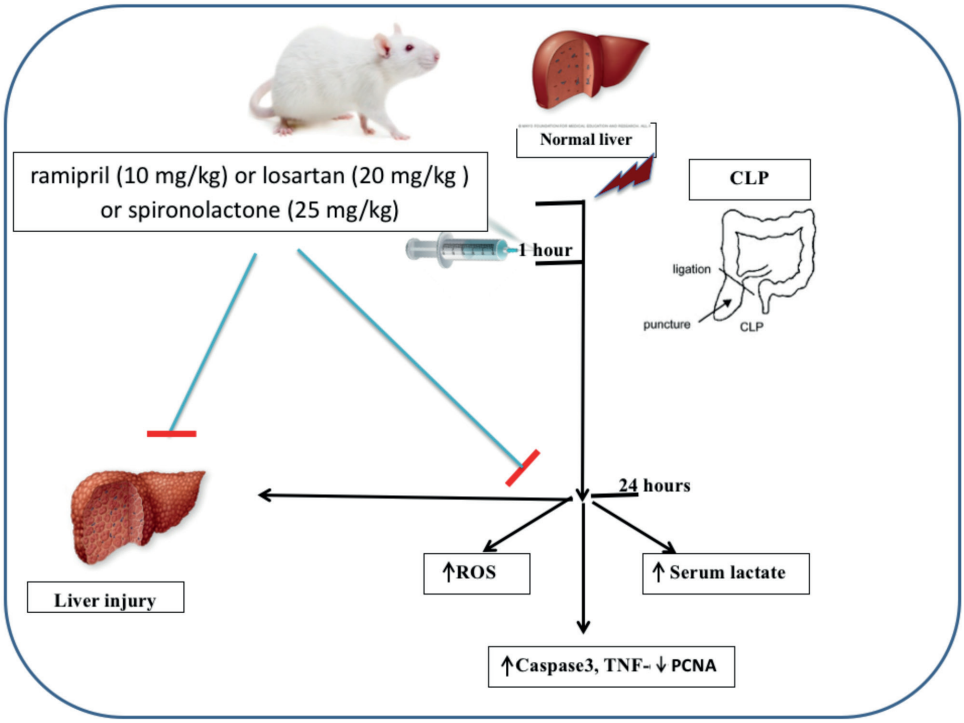

Figure 9. Summary of the study.

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Informed Consent
Not applicable.

Ethics Approval
The study was approved by the Commission on Ethics of Scientific Research for the Ethical Principles and Guidelines of the Care and Use of Laboratory Animals, Faculty of Pharmacy, Minia University (Code number of the project: 37/2019).

Conflict of Interest
The authors declare that they have no conflict of interest.

Authors' Contributions
A.A.: execution of experiments, sample collection, data handling, manuscript writing, and approval of the final form; M.E.-D., A.-S.F.A., and M.M.A.K.: design, supervision, manuscript writing, revision, and approval of the final form; O.F. and A.B.A.: revision, approval of the final form, and funding source. All authors have read and agreed to the published version of the manuscript.

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Availability of Data and Materials
All data are contained within the article.

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