ORIGINAL ARTICLE

CONTENTS

Renal protective effects of Eprosartan in sepsis: Targeting NF-κB and apoptotic pathways

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ABSTRACT

Aim: To reveal possible protective impact of Eprosartan on sepsis-induced acute kidney injury within the sepsis model.

Materials and Methods: Albino male Swiss mice (n=40) were allocated into four distinct groups: (I) Normal group, (II) Cecal ligation & puncture group, (III) Vehicle group, and (IV) CLP + Eprosartan group (60 mg/kg one hour before CLP intraperitoneally). Blood and tissue biochemical/routine indicators, renal function, SA-AKI-related pathophysiological processes, and nuclear factor kappa B p65 gene expression in septic mice were assessed by histological hematoxylin and eosin staining, quantitative real-time polymerase chain reaction, and Enzyme-Linked Immunosorbent Assay.

Results: Our findings highlight that Eprosartan reversed CLP-provoked increased serum blood urea nitrogen, creatinine (as well as kidney injury molecule levels. It also significantly inhibited the elevated concentrations of tumor necrosis factor alpha and caspase-3 within the tissue. Additionally, NF-κB gene expression level was notably lessened in the group of CLP+ Eprosartan than that of CLP ($p<0.05$). Eprosartan treatment attenuated considerable tubular injuries in the sepsis murine group $p<0.05$.

Conclusions: our findings unveil that Eprosartan could serve as a promising therapeutic agent in the context of sepsis-induced AKI.

KEY WORDS: cecal ligation & puncture, Eprosartan, sepsis, NF-κB p65 gene expression, sepsis-associated acute kidney injury

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INTRODUCTION

Sepsis is a life-threatening condition caused by and leading to infection-induced organ dysfunction syndrome [1]. Sepsis is a life-threatening medical condition that occurs when infection leads to systemic impaired function of the tissues and organs in response to this infection, leading to immunosuppression [2]. Particularly, sepsis is a leading reason for intensive care unit (ICU) admission nationwide; it was present in 291 of every 1000 ICU admissions [3]. This is often due to uncontrolled immune response, cytokine storm, and oxidative stress that cause multiple organ failure, eventually leading to death [4]. They frequently result from microorganisms such as bacteria, viruses, and fungi that can lead to organ dysfunction in most cases [5]. Sepsis is related to several morbidities, such as the heart, kidney, liver, and central nervous system [6]. A common outcome associated with the clinical scenario of sepsis is acute kidney injury (AKI) [7]. The pathogenesis of AKI in the context of sepsis is multifaceted. Among those contributing factors, oxidative stress and inflammation arise as pivotal etiological agents of septic AKI [8]. Sepsis-associated acute kidney injury (SA-AKI) originates from intricate and heterogeneous mechanisms that culminate in renal injury. These mechanisms may either arise directly from the infectious agent and corresponding host immune response or they may represent indirect ramifications of sepsis or its therapeutic intervention. Various pathophysiological mechanisms may interact and participate in AKI in patients suffering from sepsis, encompassing systemic and renal inflammation, macrocirculatory anomalies, microcirculatory dysfunction, metabolic reprogramming, macrocirculatory impairment, and dysregulation of the renin-angiotensin-aldosterone system (RAAS) [9]. Inflammation, recognized as a critical element of sepsis, appears to exert a significant influence on the pathogenesis of SA-AKI. Specifically, pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) have the potential to stimulate toll-like receptors (TLRs). TLR-2 and TLR-4 are expressed on the surfaces of tubular epithelial cells within the

renal system [10, 11]. The engagement of TLR-2 and TLR-4 initiates a cascade of inflammatory responses, which is marked by the secretion of pro-inflammatory cytokines, including interleukin (IL): IL-1α, IL-6, IL-8, and tumor necrosis factor alpha (TNF-α) [12, 13]. Eprosartan was specifically chosen because angiotensin II plays a crucial role in the pathogenesis of sepsis-induced renal injury through activation of the nuclear factor kappa B (NF-κB) pathway and promotion of inflammatory, oxidative, and apoptotic responses; therefore, blocking its action with Eprosartan was hypothesized to provide renoprotective effects.

AIM

The aim of this research is to reveal possible protective impact of Eprosartan on sepsis-induced acute kidney injury within the sepsis model.

MATERIALS AND METHODS

ANIMALS

The current investigation was conducted utilizing a cohort of forty albino Swiss mice weighing between 25 and 30 grams and aged 8 to 12 weeks, procured from the University of Kufa's science college. These mice were accommodated in the animal housing facility within designated enclosures, maintained under a controlled photoperiod of 12 hours of light and 12 hours of darkness, at a stable room temperature of 25°C, with humidity levels between 60% and 65%, and were provided with unrestricted access to food and water *ad libitum*.

CECAL LIGATION AND PUNCTURE

Within the current research, the cecal ligation and puncture **(**CLP) used in earlier studies, including those done by [14, 15], was employed to induce sepsis in the animals. In this case, CLP produces a persistently draining, multifocal infectious source located in the peritoneal cavity. It is introduced by making a midline incision measuring approximately 1.5 cm while the subject is under general anesthesia, wherein xylazine of (20mg/ml) and ketamine of (100mg/ml) are mixed (2:1) and administered [16]. The caecum is ligated, and the caecum is situated below the ileocecal junction and punctured twice with a cutting cannula to inflict kidney organ damage during the acute sepsis phase, first 24 hrs. After that process, the puncture hole is used to squeeze a tiny amount of fecal material from behind the site of perforation. Thereafter, to prevent leakage, the anterior abdominal wall is closed with a single button stitch and tissue adhesive after the cecum is secured back in the stomach. The sham mice were given the same procedures as the experimental groups, except that CLP was not done.

EXPERIMENTAL GROUPS

Mice were classified into the subsequent four distinct groups (n=10):

1. Sham group: Evidently, mice exhibited no apparent signs of disease.
2. CLP cohort: Mice belonging to this cohort experienced a CLP surgical procedure.
3. Vehicle group: Mice classified within this category were administered an equivalent volumetric measurement of the solvent dimethyl sulfoxide (DMSO) intraperitoneally; CLP was performed after 1 hour, and then the animals were sacrificed after 24 hours.
4. Eprosartan group: The mice in this group were given Eprosartan 60 mg/kg intraperitoneally [17]; CLP was performed after 1 hour, then the animals were sacrificed after 24 hours.

SAMPLE PREPARATION AND TISSUE ISOLATION

After 24 hours, mice were euthanized under anesthesia, and blood was collected using the direct heart puncture method. The blood was left in a gel tube rack for about twenty minutes to allow for clot formation, after which it was centrifuged at 10,000×g for about 10 minutes. Then the supernatant was retained at -20 °C for Enzyme-Linked Immunosorbent Assay (ELISA) and the analysis of renal function [18]. After blood sample collection, the abdominal cavity was opened along the midline of the abdomen. After removing the kidney capsule and perirenal fat, the kidneys were carefully dissected and washed, and the right parts of the kidney tissue were fixed in 10% formaldehyde for histological investigation: Hematoxylin and eosin (H&E) [19], while the left kidney of different groups was collected and divided into two sections. The initial segment was subjected to homogenization at 7.4 pH in a cold phosphate-buffered saline (PBS) solution, followed by centrifugation at 3000×g. The resultant supernatant was employed for measurement of TNF-α and caspase 3 by ELISA, the second section was stored at -80 °C for real-time PCR tests for gene expression measurement [20].

EVALUATION OF RENAL HISTOLOGY

After being fixed for twenty-four hours with 4% paraformaldehyde, the right kidney was embedded in

Table 1. Sequences of primers and the housekeeping gene

Target Gene	Primer Direction	Sequence (5′→3′)
NF-κB/p65	Forward	GGCCTCATCCACATGAACTT
	Reverse	CACTGTCACCTGGAAGCAGA
HKG	Forward	TCTTGGGCTACACTGAGGAC
	Reverse	TGTTGCTGTAGCCGTATTCA

paraffin. H&E and periodic acid-Schiff (PAS) reagents were used to stain the 4 μm thick sections cut from the wax blocks containing renal tissue. Using a standard light microscope, the pathological alterations in the kidney tissue of mice were seen. The percentage of injured tubules was used to score tissue damage, which was examined in a blinded manner: 0 is "no harm"; 1 is 0%–25%; 2 – 25%–50%; 3 – 50%–75%; 4 – >75% [21].

EVALUATION OF RENAL FUNCTION

Measurement of serum BUN and creatinine (Cr) concentrations was conducted utilizing Biolis colorimetric assay kits (Biolis, Japan).

ASSAY FOR ENZYME-LINKED IMMUNOSORBENT

Instructions of the ELISA kit (Sunlong, China; TNF-α, catalog no. SL0547Mo; caspase-3, catalog no. SL0679Mo; KIM-1, catalog no. SL0339Mo) were followed while processing tissue samples kept in a refrigerator at -80°C for evaluation of TNF-α and caspase-3, in addition to measurement of serum Kidney Injury Molecule (KIM-1) levels. A microplate reader detected the absorbance at 450 nm to create a standard curve and determine the concentration.

QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION ANALYSIS

By the manufacturer's instructions, total RNA was extracted using TRIzol, and the RNA recovery kit's instructions were closely followed to purify the recovered RNA. The objective of this process was to assess the target genes' mRNA expression levels. We used the ABI 7500 real-time PCR machine and the Power SYBR Green PCR master mix to run real-time PCR in triplicate on cDNA that was produced by a reverse transcription reaction. Every process was carried out in compliance with the manufacturer's instructions. Table 1 displays the primer sequences that were employed, with GAPDH acting as the internal control. The 2^-ΔΔCt technique determined the target genes' relative expression levels.

STATISTICAL ANALYSIS

The statistical analysis in this study was performed using GraphPad Prism version 9.3.1. To investigate differences between groups, a one-way ANOVA was used. Subsequently, the Bonferroni method for multiple comparisons was utilized to conduct post hoc tests. Additionally, the Kruskal-Wallis test was used to analyze non-parametric variables of histopathological outcomes. All tests were deemed statistically significant when p was less than 0.05. All data are presented as mean ± SEM.

RESULTS

EFFECT OF EPROSARTAN ON RENAL FUNCTION

The results indicated that the CLP cohorts had remarkably heightened BUN and Cr levels ($p<0.001$), contrasting with the sham cohort. Additionally, BUN (Fig.1) and Cr (Fig.2) levels of Eprosartan groups had significantly decreased ($p<0.001$), contrary to the CLP cohort.

EFFECT OF EPROSARTAN ON INFLAMMATORY CYTOKINES

According to the findings, the CLP cohort exhibited a notably heightened level of TNF-α ($p< 0.001$), which was in stark contrast to the sham cohort. Furthermore, the TNF-α (Fig.3) concentration of the Eprosartan cohort was observed to be significantly diminished ($p< 0.001$) in comparison to the CLP cohort.

EFFECT OF EPROSARTAN ON APOPTOTIC FACTOR (CASPASE-3)

The results indicated that caspase-3 tissue levels within the CLP cohort were remarkably raised ($p<0.001$), contrary to the sham cohort. Additionally, the Eprosartan group had significantly lower levels ($p<0.001$) of caspase-3, contrasting with the CLP group (Fig.4).

EFFECT OF EPROSARTAN SERUM KIM-1

The results indicated that the CLP cohort had remarkably heightened serum KIM-1 levels compared to the sham cohort. Additionally, KIM-1 levels of the Eprosartan group had significantly decreased, contrary to the CLP cohort (Fig.5).

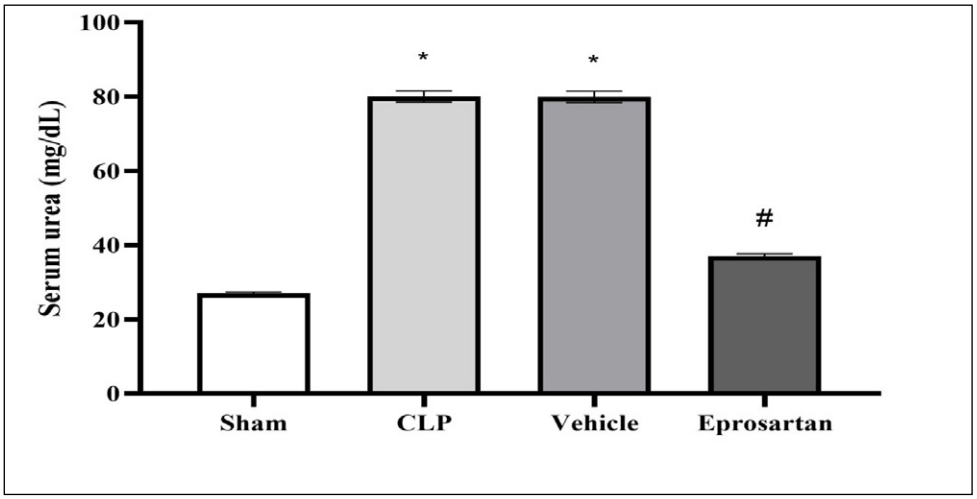

Fig. 1. Mean ± SEM concentrations of the urea in the different experimental cohorts; *: $p < 0.001$, *vs.* Sham group; #: $p < 0.001$, *vs.* CLP or vehicle group, CLP: cecal ligation & puncture
Source: Own materials

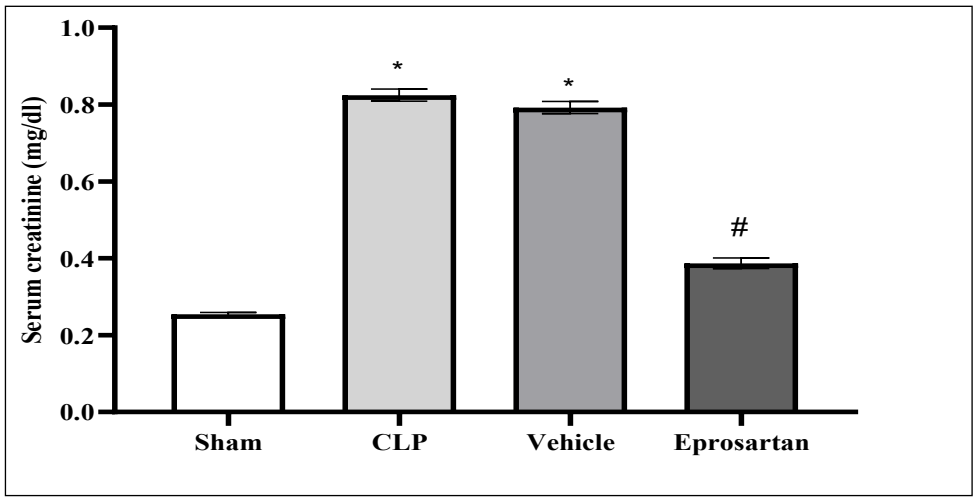

Fig. 2. Mean ± SEM concentrations of serum creatinine in the different experimental cohorts; *: $p < 0.001$, *vs.* Sham group; #: $p < 0.001$, *vs.* CLP or vehicle group, CLP: cecal ligation & puncture
Source: Own materials

IMPACT OF EPROSARTAN ON RENAL HISTOPATHOLOGICAL DAMAGE

As it illustrated by figures 6&7, significant pathological alterations were observed in both the CLP and vehicle cohorts, encompassing interstitial edema, loss of the brush border, vacuolar degeneration, cast formation, inflammation, vascular congestion/hemorrhage, and tubular necrosis. Nevertheless, the renal injury induced by CLP were markedly ameliorated through pretreatment with Eprosartan (Fig. 6-7).

EFFECT OF EPROSARTAN ON mRNA EXPRESSION OF NF-KB P65 GENE

As shown in figure 8, the CLP group had a lower ΔCT than the sham group, indicating a significant increase in NF-κB p65 gene mRNA expression ($p<0.001$). Additionally, there is a substantial ΔCT surge ($p<0.001$) in the Eprosartan group compared to the CLP group, representing a decrease in NF-κB p65 gene mRNA expression.

DISCUSSIONS

Polymicrobial sepsis is a life-threatening situation characterized by the dysfunction of multiple organs resulting from the aberrant response of the body towards microbial invasion [22]. Sepsis in the United States is regarded as the third most common cause of death and contributes significantly to mortality rates [23]. A plethora of experimental and clinical investigations show that the immunosuppressive state induced by sepsis is typified by decreased antimicrobial effector functionalities, thereby heightening vulnerability to infections [24]. The immunosuppression associated with sepsis is multifaceted and is believed to arise from compromised cytokine production and a reduction in the phagocytic capabilities of myeloid cells. The present investigation elucidated that concentration of the pro-inflammatory cytokine TNF-α was markedly elevated in the CLP cohort in comparison to the ostensibly healthy cohort. This investigation corroborates prior findings [25], which demonstrated that in models of sepsis, the concentrations of

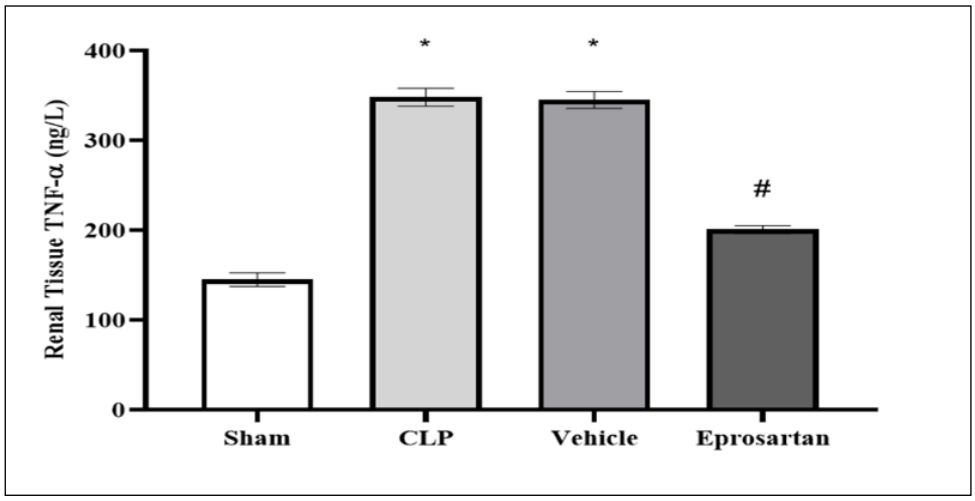

Fig. 3. Mean ± SEM concentrations of renal tissue TNF-α (ng/L) among the various experimental cohorts; *: $p < 0.001$, *vs.* Sham group; #: $p < 0.001$, *vs.* CLP or vehicle group, CLP: cecal ligation & puncture; TNF-α: tumor necrosis factor alpha
Source: Own materials

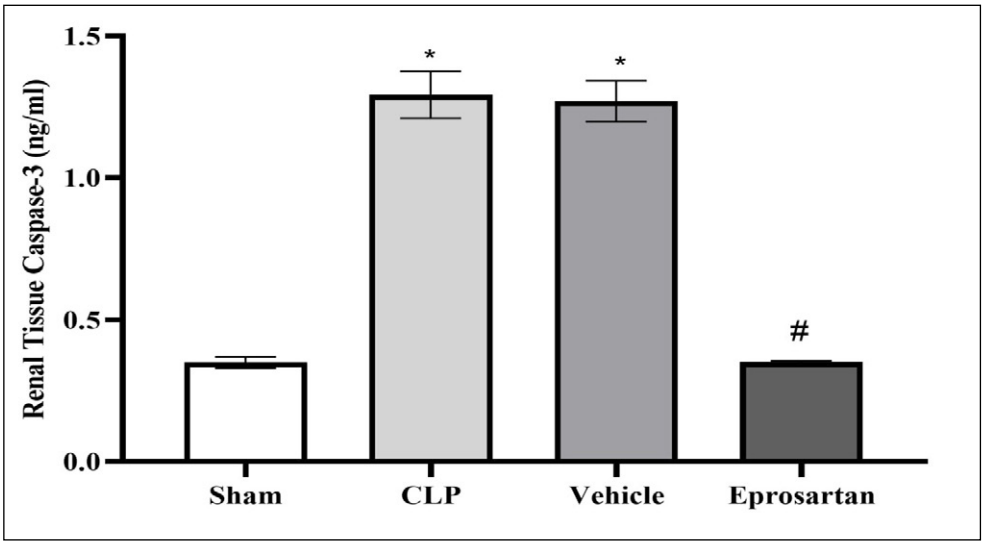

Fig. 4. Mean ± SEM caspase-3 (ng/mL) levels of the experimental groups; *: $p < 0.001$, vs. Sham group; #: $p < 0.001$, *vs.* CLP or vehicle group, CLP: cecal ligation & puncture

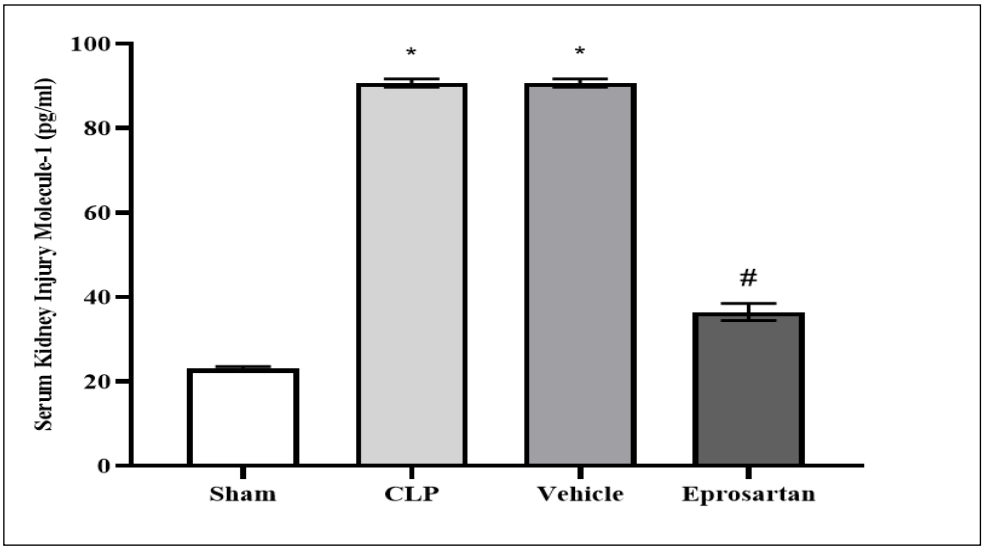

Fig. 5. Mean ± SEM concentrations of serum KIM-1 (pg/mL) in the different experimental cohorts; *: $p < 0.001$, *vs.* Sham group; #: $p < 0.001$, *vs.* CLP or vehicle group, CLP: cecal ligation & puncture
Source: Own materials

proinflammatory cytokines, particularly TNF-α, were elevated in CLP murine. Also, this result, allied with a study [26], showed that renal ischemia-reperfusion injury (IRI) is a major cause of AKI, characterized by significant inflammation that exacerbates tissue damage. Another study investigated the effect of resveratrol in IRI rats. They found that the level of TNF-α became altered (increased significantly) in ischemic rats [27]. Within the confines of the present study, concerning the impact of Eprosartan on TNF-α

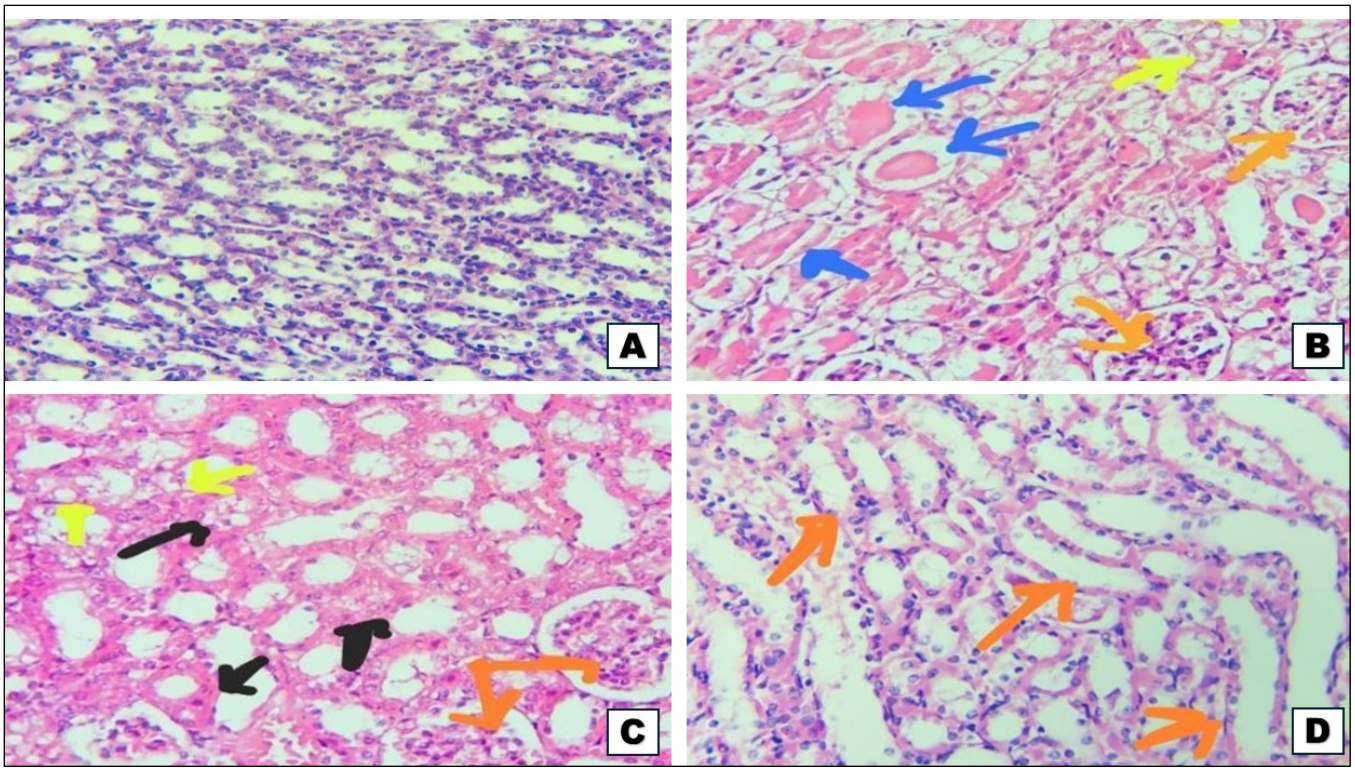

Fig. 6. Eprosartan mitigates the pathological impairment of renal tissues in septic rodent models. H&E staining (400x): **A)** Sham group, mouse kidney with normal renal tubules; **B)** CLP group, mouse kidney with 95% renal tubule damage. Cytoplasmic vacuoles (yellow arrows), eosinophilic casts (blue arrows), and a normal glomerulus (orange arrows); **C)** vehicle groups, mice kidneys with 90% renal tubule damage. Cytoplasmic swelling and increased cytoplasmic eosinophilia (black arrows), cytoplasmic vacuoles (yellow arrows), and a normal glomerulus (orange arrows); **D)** Eprosartan group, mice kidney with 40 % renal tubule damage. Normal tubules (orange arrows)
Source: Own materials

levels, it was observed to significantly diminish TNF-α levels in the renal tissue in comparison to the CLP cohort. To the utmost extent of our knowledge, this was the first study to show how this agent affected the renal TNF-α in mice with sepsis in the CLP model. This observation may be ascribed to NF-κB's noticeable downregulation alongside its related cytokines that promote inflammation [17]. The current work found that CLP and vehicle groups had significantly greater tissue levels of caspase-3 than the sham group. Similar results found that the sepsis group had higher caspase-3 levels than the physiological normal state [28]. Moreover, this work concurs with a prior study that examined renal damage induced by renal IRI and employed a new effective therapeutic approach. They found that renal IRI caused a significant surge in kidney markers of apoptosis, caspase-3, compared to the sham group [29, 30]. Also, in the present study, concerning the effect of Eprosartan on the level of caspase-3, the concentration of caspase-3 was remarkably lower within the Eprosartan cohort opposing to the CLP cohort. This study, as far as we know, was the first to validate the agent's effect on caspase-3 in the murine CLP pattern of sepsis. The pro-apoptotic mechanisms are efficiently thwarted by the role of Eprosartan in the downregulation of the NF-κB pathway, the upregulation of Bcl2, and the downregulation of BAX, in addition to stabilizing the permeability of the mitochondrial membrane and prohibiting cell death via the stimulation of the Sirtuin 1/PGC1 α /Sirtuin 3 pathway [17]. The present study proved that KIM-1 level was notably increased within the CLP cohort in comparison to the sham cohort. This work agrees with a previous study that confirmed levels of KIM-1 in *Lyn* mice who underwent sepsis were raised remarkably when compared with Lyn murine [31]. Additionally, the current study aligns with another research effort that demonstrated the upregulation of the inflammatory marker KIM-1 in both the renal IRI and vehicle groups, when compared to the sham groups [29, 30]. Moreover, concerning the influence of Eprosartan on the KIM-1 concentration, it is observed that this agent significantly attenuates the KIM-1 level when juxtaposed with the CLP cohort. To the extent of our knowledge, this investigation represents the inaugural work elucidating the impact of this pharmacological agent on renal KIM-1 levels within the CLP sepsis model in mice. The rationale underlying such an observation may be attributed

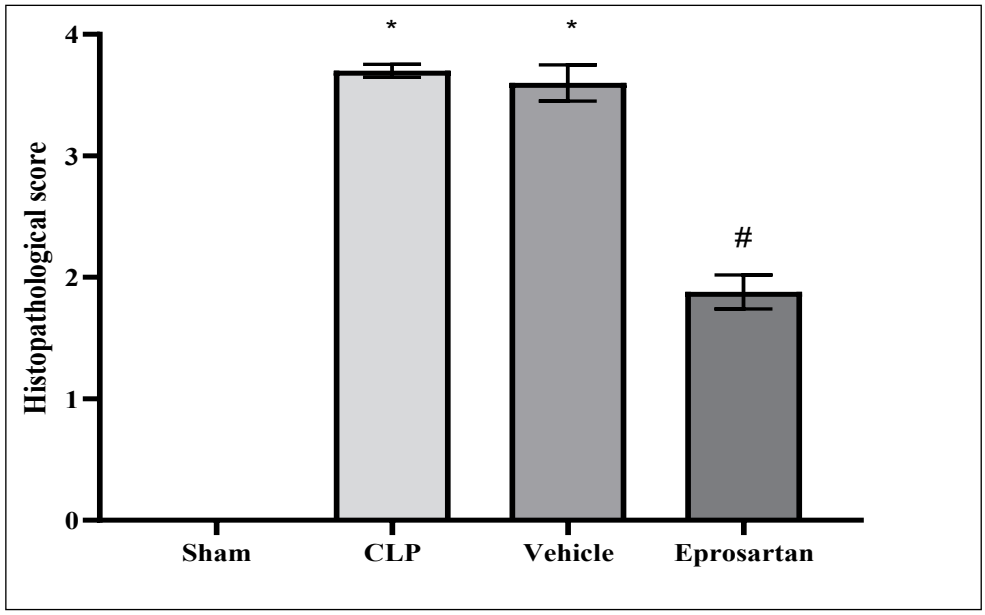

Fig. 7. Quantification of renal tissue damage scores of mice across all groups. *: $p < 0.001$, compared to Sham group; #: $p < 0.001$, compared to CLP or vehicle group. CLP: cecal ligation & puncture
Source: Own materials

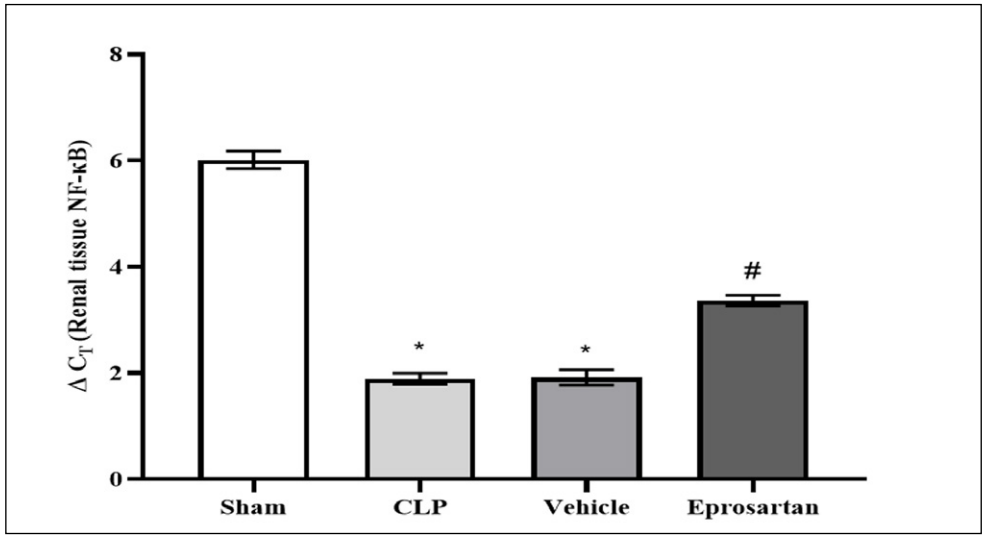

Fig. 8. Mean ± SEM NF-κB p65 mRNA expression levels in the experimental groups, *: $p<0.001$, *vs.* Sham group; #: $p <0.001$, *vs.* CLP or vehicle group. CLP: cecal ligation & puncture; ΔCT: delta cycle threshold; NF-κB: nuclear factor kappa B
Source: Own materials

to its inhibitory effect on ERK phosphorylation [32]. Collier and Schnellmann have elucidated that the proposed mechanism underlying acute renal injury encompasses the phosphorylation of STAT3 and (ERK1/2) [33]. Furthermore, the present investigation elucidated that the mRNA expression levels of NF-κB p65 exhibited a notable elevation in the CLP cohort in comparison to the sham cohort. This work corroborates findings from a preceding study, which indicated that the phosphorylation level of p65 within the renal tissue of the sham cohort was reduced, whereas the CLP cohort demonstrated a significant increase that was statistically significant when juxtaposed with the sham cohort [34]. Moreover, concerning the influence of Eprosartan on the expression levels of NF-κB, it markedly diminished p65 expression in renal tissues when compared to the CLP group. To our utmost knowledge, this investigation represents the inaugural demonstration of the impact of this pharmacological agent on renal NF-κB p65 expression within the CLP model of sepsis in murine subjects. The justification for such an observation may be ascribed to its anti-inflammatory properties and the reduction of IL-6 via the regulation of the upstream NF-κB signaling pathway. Eprosartan significantly reduces NF-κB expression, accompanied by downstream inflammatory cytokines in renal tissue samples [17]. Also, the CLP, along with vehicle groups, exhibited significant histopathological changes in comparison to the sham group. The renal tissue of the sham group mice had normal architecture, while the kidneys obtained from the mice in the CLP cohort exhibited signs of hemorrhage, severe inflammation, increased cytoplasmic eosinophilia, eosinophilic casts, and cytoplasmic vacuoles, as well as loss of brush border. These observations are consistent with those obtained by

[35] during their study on groups of CLP mice. They reported that CLP causes inflammation, necrosis, hemorrhage, and degeneration in kidney tissue. Notably, within the current investigation, the Eprosartan group reduced renal tissue injury when compared to the CLP. In a mouse model of sepsis for the Eprosartan group were arranged from no change to moderate changes, such as a marked decrease in inflammation, vascular congestion, cytoplasmic eosinophilia, and hemorrhage. As far as we know, this research was the first to show the protective effect of Eprosartan on the renal tissues in a mouse model of sepsis. This result may be attributed to eprosartan, which significantly plummeted the levels of inflammatory and apoptotic factors [17].

CONCLUSIONS

Findings revealed that Eprosartan preserves and improves renal function following sepsis by reducing NF-κB–driven inflammation and modulating apoptotic factors, particularly caspase-3, suggesting a potential therapeutic strategy for sepsis-related AKI.

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Ethics approval

In the present investigation, laboratory mice were employed as the experimental subjects. All sacrificial procedures were conducted under a combination of ketamine and xylazine-based anesthesia, with diligent efforts made to mitigate individual distress. The experimental methodologies and protocols employed in this study received approval on August 29, 2024, under reference number (20553) from the committee responsible for the ethics of laboratory animal care and use.

CONFLICT OF INTEREST

The Authors declare no conflict of interest

AUTHOR CONTRIBUTIONS

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