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Nephroprotective Effect of Puerarin in Experimental Model of Endotoxemia

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Abstract
Objective: Endotoxemia-induced renal injury leads to the impairment of kidneys due to the presence of endotoxins, predominantly lipopolysaccharides (LPS), circulating within the bloodstream. We have studied the effect and mechanism of action of puerarin on kidney tissue by inserting the endotoxin with CLP process to stimulate inflammatory cytokines, oxidative stress, and apoptotic mediators. Puerarin is a natural compound used in traditional Chinese medicine, known for its medicinal properties.
Methods: Twenty-four adult male Swiss albino mice were randomly divided into four equal groups ($n = 6$): sham, CLP, vehicle puerarin (DMSO and corn oil the same amount of puerarin injected IP 1 hour after CLP) and puerarin group (injected IP (200 mg/kg) of puerarin one hour after CLP). Renal damage was assessed by statistical analysis and ELISA to measure IL-6, IL-1β, TNF-α, NF-κB, MDA, TAS, Caspase 3, and Bcl-2.
Results: Showed a significant decrease ($P < 0.05$) in all marks except TAS and Bcl-2, which showed a rise level ($P < 0.05$) in renal tissue.
Conclusion: Our results showed that puerarin can ameliorate the renal damage by suppressing TLR-4/NF-kB signaling pathway.
Keywords: Endotoxemia, sepsis, CLP, cytokines, oxidative stress, caspase 3, Bcl 2, puerarin

Introduction

Endotoxins, also known as lipopolysaccharides (LPS), possess a diverse biological property. Since 2018, research has unveiled their significance beyond their toxic effects. Endotoxins play an essential task in immune activation, initiating inflammatory responses through Toll-like receptor 4 (TLR4) signaling[1] which in turn activate NF-κB who is responsible to regulate inflammatory cytokine gene, apoptosis, and oncogenesis. They contribute to the pathogenesis of infectious diseases, modulate host immune responses, and impact conditions such as sepsis and inflammatory disorders. Understanding the biological properties of endotoxins is crucial for unraveling their role in immune regulation and disease pathogenesis.[2] Recent research has inspected the role of endotoxins in various diseases far from infection, including inflammatory bowel disease, sepsis, and metabolic disorders. They investigated the neutralizing antibodies and modulators of the host response. These progressions contribute to a better understanding of endotoxin biology and provide potential avenues for the development of novel therapies.[3] Cytokines are small proteins that act as chemical messengers, transmitting signals between cells to regulate immune responses, inflammation, and various biological processes.[4] It includes proinflammatory interleukins (IL-6, IL-1β, TNF-α), chemokines, and growth factors. Each type of cytokine has specific functions and can target specific cell types or receptors.[5] Sepsis emerges as a grave medical case starting when the body reacts to an infection. It manifests through an immune response that loses regulation in the face of infection, causing extensive inflammation, impaired organ function, and the potential for organ failure. The usual infection sources contain pneumonia, urinary tract infections, abdominal infections, and bloodstream infections.[6] When the immune system responds to the infection, it starts to release a large volume of cytokines into the bloodstream.[7] The collapse of the immune system due to continuous inflammation will impose harm for many organs, such as the cardiac system, pulmonary system, renal system, and liver tissues.[8]

Puerarin, may be consider the major bioactive compound separated from the roots of Pueraria lobata. Since then, comprehensive studies have been carried out to discover the pharmacological effects of puerarin.[9] Puerarin exhibits a diverse array of pharmacological properties and has found applications in various conditions management, including cardiac conditions and brain diseases, diabetes mellitus and its complications, osteonecrosis, Parkinson's disease, Alzheimer's disease, endometriosis, and tumor diseases.[10] Unfortunately, the clinical use of puerarin is still restricted in use and in narrow range, despite many pharmacological uses. Till now there are just two methods approved to administering. To our knowledge, few studies reported that puerarin has no side effect.[11] Recent studies reported that puerarin enhances the metformin activity in treatment of hyperglycemia.[12] They also concluded that the treatment with puerarin by intravenously causes change in pharmacokinetics of warfarin and decreases the $t_{1/2}$ in rat model.[13]

Materials and Methods

Study Design

Twenty-four mature male Swiss-albino mice, ranging from 8 to 12 weeks old and weighing between 20 to 30 g, were divided into four equally sized groups ($n = 6$). These groups consisted of a sham group (undergoing laparotomy without cecal ligation and puncture, or CLP), a sepsis group (undergoing laparotomy with CLP), a vehicle group (receiving an equivalent volume of DMSO and corn oil prior to CLP), a puerarin treatment group (receiving a 200 mg/kg intraperitoneal dose 1 hour after CLP). Urea and creatinine serum levels were measured using blood samples, while TNF-α, IL-6, IL-1β, caspase-3, Bcl-2, MDA, NF-κB, and TAS were examined in the kidney homogenate

to assess overall kidney health. Additionally, renal histopathology was also conducted for further analysis.

Experimental Procedure and Protocol

The animal facility located within the College of Sciences at the University of Kufa accommodated a population of 24 adult male mice, aged between 6 to 8 weeks and weighing 25 to 35 grams. Procured from the college's animal facility, these mice were maintained under a balanced regimen of twelve-hour light and twelve-hour darkness cycles, housed in ventilated cages within an appropriate environment and supplied with sufficient food. The study was carried out within a laboratory situated at the Department of Pharmacology and Therapeutics in the College of Medicine at Kufa University. The animals were randomly divided into 4groups, each consisting of six animals as follow:

Sham group: All specimens within this category underwent anesthesia and the laparotomy procedure; however, the CLP technique was not employed. These sham groups serve as the surgical control counterparts.

Cecal ligation and puncture operated group (CLP): In this group, each mouse experienced ligation and puncturing of the cecum. These mice subjected to CLP serve as a model of sepsis.

Vehicle group: before the CLP procedure, all mice inside this group were given an equal volume of vehicle (DMSO 10% + corn oil 90%) IP 1 hour after surgery for Puerarin.[14]

Puerarin treated group: all mice in this group were treated with (200 mg/kg) in 1 hour IP after the CLP process.[15] Each treatment was administered in the morning, and the survival of the animals was observed for duration of 24 hours. After euthanizing the mice, tissue samples were collected and preserved at a temperature of –80°C for future analysis.

Puerarin Preparation

Puerarin powder was acquired from Solarbio Company and formulated with a 10% diluted solution of DMSO in 90% corn oil. Subsequently, it was I.P administered at a dosage of 200 mg/kg, 1 hr. following the CLP procedure.[15–17]

Preparation of Samples

The aim of sample preparation is executed for measurement of the inflammatory, oxidative stress, and apoptosis mediator, including IL-6, IL-1β, TNF-α, NF-κB, TAS, MDA, CASPASE 3, BCL-2.

Blood Sample

Around 24 hours after CLP, blood was rapidly collected from each mouse's heart. The collected blood samples were then placed into gel tubes and kept at 37°C for 30 minutes without the addition of an anticoagulant. Proper labeling was done for each gel tube, and they were arranged on a rack. Following this, the samples were subjected to centrifugation at approximately 3000 rpm for duration of 10 minutes, resulting in the extraction of serum. This serum was utilized for measuring Urea and Creatinine levels.[18]

Tissue Preparation for ELISA and Histopathology Examination

The kidney tissue was prepared by homogenizing it using a high-intensity ultrasonic liquid processor in a solution consisting of 0.1 M phosphate buffered saline (PBS) with a pH of 7.4, at a ratio of 1:10 (weight/volume). This solution also contained 0.5% Triton X-100 and a protease inhibitor cocktail. Prior to homogenization, the kidney tissue was washed with ice-cold saline to remove any red cells or clots. After homogenization, the resulting mixtures were centrifuged at 5,000 rpm for 5 minutes at a temperature of 4°C. The supernatants were then collected and stored at –80°C for later use in the ELISA procedure to detect specific markers.[19] A segment of the kidney underwent a series of preparatory steps, including drying and cleaning, before being preserved in paraffin. Subsequently, it was precision-sliced into 5 mm-thick sections using a rotary microtome. These kidney sections were then mounted onto slides, subjected to staining with H&E staining dye, and securely covered with glass slides, rendering them suitable for examination under a microscope. The evaluation of renal tissue injury was carried out by two experienced pathologists in a blind fashion. Six randomly selected areas were assessed, utilizing a scoring system to gauge the extent of renal injuries. This scoring system considered various factors such as cellular swelling, heightened cytoplasmic Eosinophilia, inflammation, red blood cell extravasation, vascular congestion, interstitial edema, and the percentage of damage. Features of damage that are dependent include: cellular swelling, cytoplasmic eosinophilia, inflammation, vascular congestion, interstitial edema, extravasation of RBCs, staining by Eosin and Hematoxylin. The results of damaged were scored as follows:[20]

- Score 0: Normal texture without any damage.
- Score 1: Damage below 25% (mild).
- Score 2: Damage between 25% and 50% (moderate).
- Score 3: Damage between 50% and 75% (severe).
- Score 4: Damage between 75% and 100% (highly severe).

Analytical Statistics

In the process of statistical analysis, the software SPSS version 26–2019 was used. The assessment of data normality was conducted using both the Kolmogorov-Smirnov and Shapiro tests within the SPSS program. For data that exhibited a normal distribution (parametric data), the T-test and ANOVA were applied at a significance level of ($P \leq 0.05$).[21]

Results

Effect of Sepsis and Puerarin on Renal Function Tests

In this experiment, we found that the blood urea and creatinine levels were notably lower in the sham group than the CLP group, with statistical significance ($P \leq 0.05$). The blood levels of urea and creatinine in the puerarin treated group were significantly ($P \leq 0.05$) lower than those in the CLP group as seen in the Figures 1 and 2.

Effect of Sepsis and Puerarin on Inflammatory Cytokines

The levels of IL-6, IL-1β, TNF-α, and NF-κB in renal tissue are higher in sepsis and vehicle group than in sham group, while these levels of cytokine decreased significantly ($P < 0.05$) with induction of puerarin treatment group as in Figures 3–6.

Effect of Sepsis and Puerarin on Oxidative Stress

The level of MDA in renal tissue is higher in sepsis and vehicle group than in the sham group. In contrary to TAS

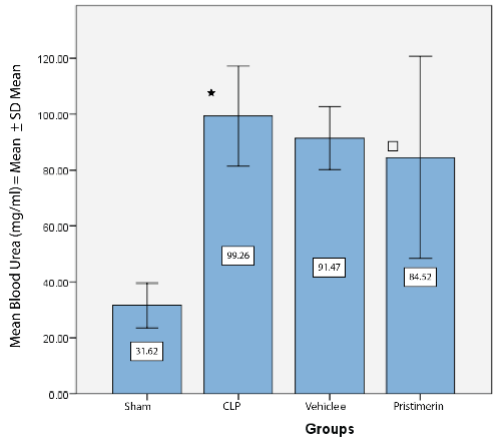

Fig. 1 **Difference of mean blood urea among study groups ($P \leq 0.05$). *Significantly higher than the sham group. □Significantly higher than clp group.**

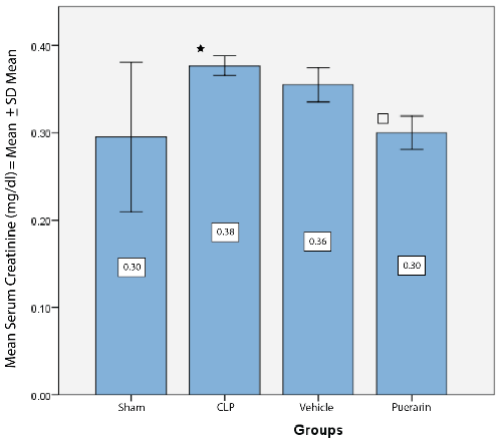

Fig. 2 **Difference of mean serum creatinine among study groups (*$P \leq 0.05$). *Significantly higher than the sham group. □Significantly higher than clp group.**

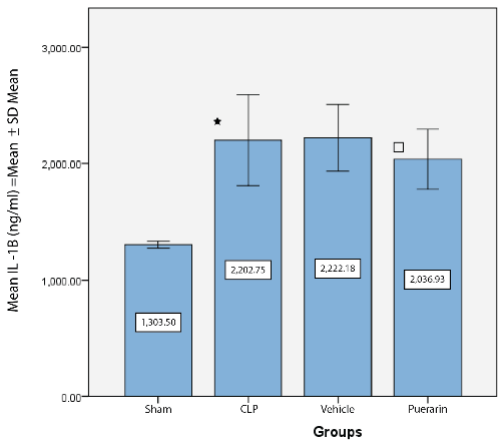

Fig. 3 **Mean of IL-1β in renal tissue (*$P \leq 0.05$).**

level which decreases in sepsis and vehicle group. After puerarin induction, the level of MDA decreased significantly ($P \leq 0.05$), while the TAS levels was increased as seen in Figures 7 and 8.

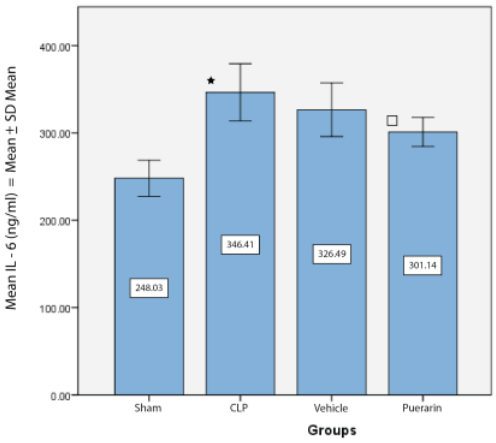

Fig. 4 **Mean of IL-6 in renal tissue (*$P \leq 0.05$).**

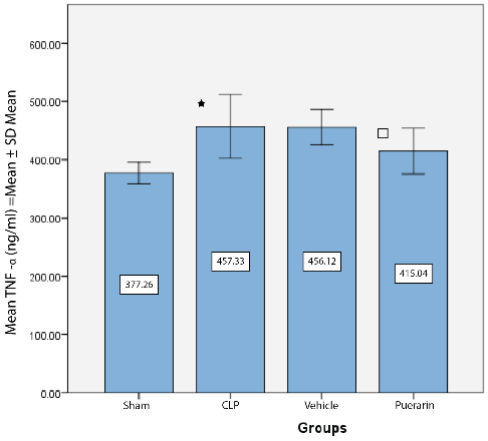

Fig. 5 **Mean of TNFα in renal tissue (*$P \leq 0.05$). *Significantly higher than the sham group. □Significantly higher than clp group.**

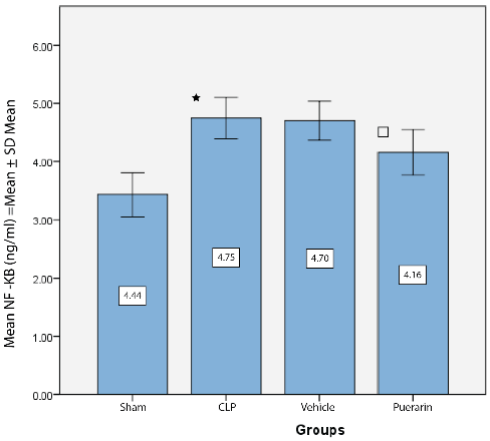

Fig. 6 **Mean of NF-κB in renal tissue (*$P \leq 0.05$). *Significantly higher than the sham group. □Significantly higher than clp group.**

Effect of Sepsis and Puerarin on Apoptosis Mediator

The levels of caspase 3 in renal tissue are higher in sepsis and vehicle group than in sham group, while levels of caspase 3 decreased significantly ($P \leq 0.05$) with induction of puerarin treatment group as seen in Figure 9. On contrary to caspase 3, the levels of Bcl-2 in renal tissue are lower in sepsis and vehicle

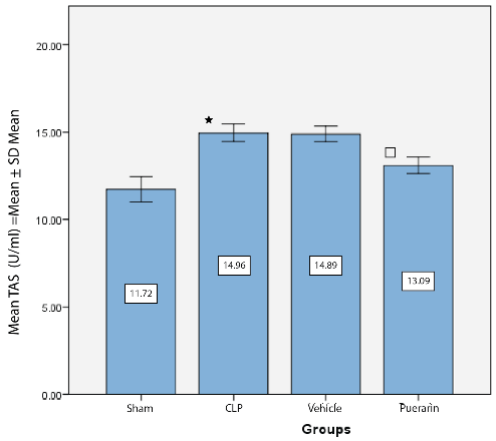

Fig. 7 **Mean of TAS in renal tissue(*$P \leq 0.05$). *Significantly higher than the sham group. □Significantly higher than clp group.**

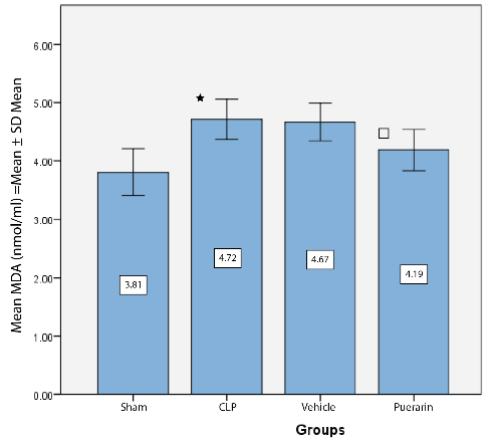

Fig. 8 **Mean of MDA in renal tissue (*$P \leq 0.05$).**

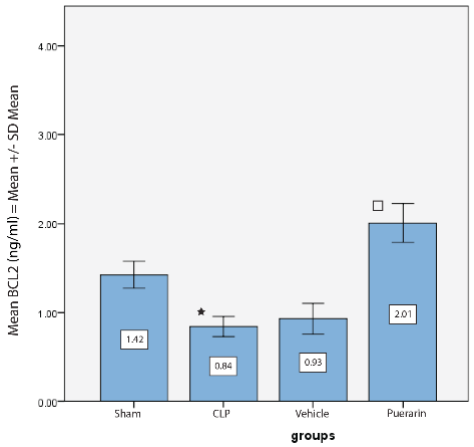

Fig. 9 **Mean of Bcl-2 in renal tissue ($P \leq 0.05$). *Significantly higher than the sham group. □Significantly higher than clp group.**

group than in sham group, while levels of Bcl-2 increased significantly ($P \leq 0.05$) with induction of puerarin treatment group as seen in Figure 10.

Effect of Sepsis and Puerarin on Renal Histopathology

The cross-section of the renal tissue in sham group exhibited a normal kidney structure, including normal glomeruli

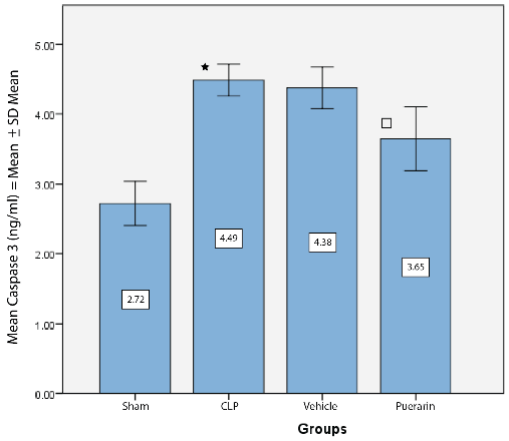

Fig. 10 **Mean of caspase 3 in renal tissue ($P \leq 0.05$).**

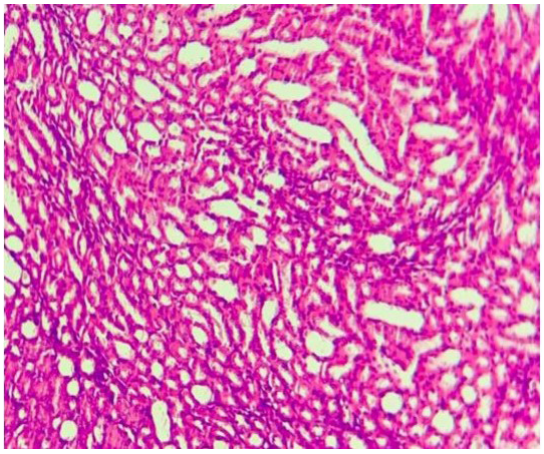

Fig. 11 **Sham Group H&E X100.**

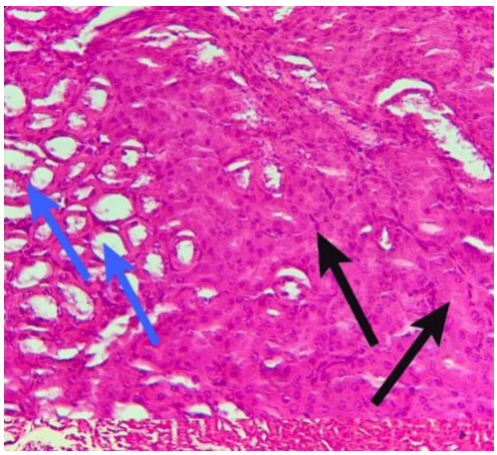

Fig. 12 **CLP group H&E X400.**

and renal tubules, as observed in Figure 11 conclusion of our examination. In CLP group renal tissue exhibited significant renal damage, marked by cellular swelling, increased cytoplasmic Eosinophilia, inflammation, red blood cell extravasation, vascular obstruction, and interstitial edema. Additionally, the renal tissue contained macrophages and neutrophils, as shown in Figure 12. The renal tissue exposed

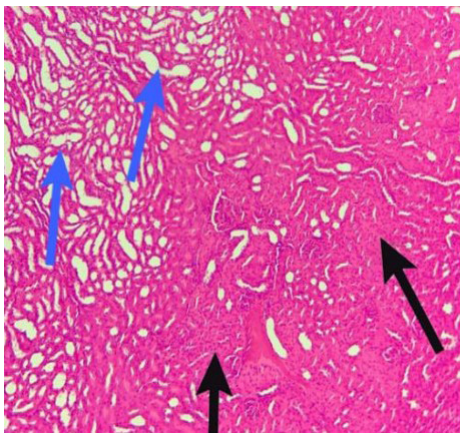

Fig. 13 **Vehicle group H&E X100.**

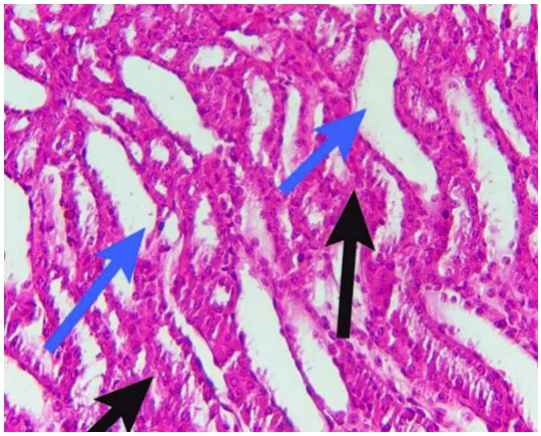

Fig. 14 **Puerarin treatment group H&E X400.**

to the vehicle group exhibited renal damage, characterized by the presence of cellular edema, elevated inflammation, vascular obstruction, cytoplasmic eosinophilia, red blood cell extravasation, and interstitial edema as shown in Figure 13, providing further insights into these histopathological alterations. Notably, this group exhibited a significantly higher histopathological damage score compared to normal renal tissue.

Puerarin Groups: In our experimental study, a prominent distinction ($P \leq 0.05$) emerged between the treated group and the untreated CLP and vehicle group notably, the CLP and vehicle group displayed abnormal kidney structure and marked renal injury, while the cross-sectional analysis of renal tissue from this group revealed only moderate alterations in renal structure. This confirmed the nephroprotective efficacy of puerarin during sepsis, as evidenced by a severity score 3 which represents a 60% score for damaged renal tubules. Figure 14 provides a visual representation of the renal structure and the severity score mean for the puerarin-treated group.

Figure 11 graphical image of Kidney segment for sham group demonstrates normal histology. Renal tubules with score 4 damage involving 95% of examined tubules as seen in Figure 12 of CLP group. Cellular swelling and increased cytoplasmic eosinophilia (black arrows refer to Cellular swelling and cytoplasmic eosinophilia), and (blue arrows normal tubules).

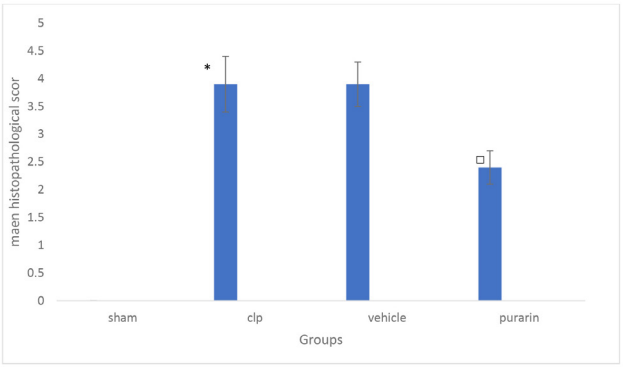

Fig. 15 **Severity score mean of renal tissue histopathology ($P \leq 0.05$).**

Vehicle group showed renal tubules with score 4 damage involving 90% of examined tubules as shown in Figure 13. Black arrows refer to cellular swelling and increased cytoplasmic eosinophilia and blue arrow refer to normal tubules. Puerarin treatment group showed renal tubules with score 3 damage which represents 60% score for damaged renal tubules, (Figure 14). Black arrows refer to cellular swelling and increases cytoplasmic eosinophilia and blue arrows refer to normal tubules Figure 15.

Discussion

Sepsis, an intense and absolutely existence contamination, is related to the body's response to an infection.[22] It is a chief purpose of kidney failure, inflicting a rapid and extreme decline in kidney function.[23] Sepsis has many signs and symptoms, and relying on which part of the body is affected, the quantity of those signs and symptoms can be varies. One organ system that is especially at risk of sepsis is the kidneys. The kidney injury caused by sepsis will show signs and symptoms which includes reduced urine output, modifications in kidney function which leads to renal failure.[24]

We found a noticeable rise in blood urea nitrogen and serum creatinine levels among both the sepsis and vehicle groups when compared to the sham group.[25]

The effect of sepsis on BUN and S. creatinine may be due to increase the activity of immune system, hemodynamic imbalance, and direct effect of infection on renal tissues. According to the study results, administering puerarin after inducing sepsis has a large effect on lowering levels of urea and creatinine in contrast to the CLP group.[26]

Puerarin shows an effect on reducing BUN and Creatinine level due to antioxidative stress and inhibition inflammatory cytokine like IL-6 and TNF-α.

Interleukin-6 level in kidney tissue after sepsis induction by cecal ligation and Puncture showed a significant elevation in comparison to the sham group.[26,27] IL-6 is an influential cytokine in acute phase reaction in case of inflammation and sepsis induction.[28] In present research, we found the real role of puerarin management after sepsis induction by CLP. Our findings revealed a significant drop in IL-6 levels in renal tissue in comparison to the CLP group.[29] The dropping of IL-6 level in renal tissue can be attributed to puerarin's anti-inflammatory effect, particularly its potential to target macrophages and prevent their activation, alleviating the systemic inflammatory response.

The CLP group showed a real increase in IL-1β levels noticed in kidney tissue.[30] TLR4 is the first target of the endotoxin, so this binding of TLR4 with endotoxin will activate the inflammatory cytokine IL-1β. Through this experimental study, we found that the administering of puerarin after inducing sepsis ended in a highly decrease in IL-1β level inside renal tissue in comparison to the CLP group.[31] Puerarin successfully alleviates sepsis that causes AKI in mice may be due to modulating the systemic inflammation response index (SIRI) and NF-κB pathway.[32]

Our study showed that the elevations of TNF-α level in kidney tissue in CLP group when compared to the sham group.[4] TNF-α is an important inflammatory cytokine in the immune system. Its response is due to its early and inevitable role in the release of other cytokines, as well as its direct functional implications in septic shock.[33] The administering of puerarin after inducing sepsis resulted in a significant decrease in TNF-α level in renal tissue, in contrast to the CLP group.[31] The effect of puerarin is due to its ability to suppress the NF-κB signaling pathway.[27] Observations of this study showed high NF-κB levels in kidney tissue in CLP group in a comparison with sham group.[34] NF-κB is activated by any disturbances in immune systemic homeostasis in replying to sepsis and inflammation. This study exhibits the ability of puerarin as a treatment for sepsis, as it substantially reduced the levels of NF-κB in renal tissue when administered after sepsis induction.[35] This effect of puerarin may be attributed to its capability to suppress the phosphorylation and degradation of the NF-κB signaling pathway. During the experimental work of this study, we observed a high increase in the level of MDA in kidney tissue in the CLP group when making a comparison to the sham group.[36] The MDA level is elevated when lipid peroxidation increased, which is related to sepsis induction. The use of puerarin treatment after sepsis induction showed a noteworthy lower in MDA levels in renal tissue, as compared to the CLP group.[18,37] These findings explain the puerarin antioxidant effect by its effect on Nrf2 pathway.

Through this study, we noticed that the mice group that subjected to CLP operation shows a decreasing in TAS level in the kidney tissue in comparison to the sham group, the increasing of TAS Due to the imbalance between cellular antioxidant and oxidative states result in sepsis. The present work showed that treatment with puerarin can successfully increase the levels of TAS in renal tissue as compared to the CLP group, with a significant difference.[38] The antioxidant effect of puerarin may be due to activation of Nrf2 which is responsible for the regulation of antioxidative stress.

Induction of sepsis reveal a clear elevation in caspase 3 level in the sepsis group in contrast to the sham group.[2] Endotoxins stimulate sepsis and in turn, stimulate the apoptotic pathway by expression of caspase 9 and caspase 3. Administration of puerarin after sepsis induction considerably decreases caspase-3 levels in renal tissue.[17,29] This may be due to decreasing the cleaved caspase 3 and Bax/Bcl2 expression.

On the other hand, the sepsis induced by CLP showed a significant decrease in Bcl-2 levels in kidney tissue. One of the major pathophysiological processes in sepsis is the increase of programmed cell death of the immune system in turn it decreases the level of Bcl2. After puerarin induction, we noticed an increase in levels in Bcl-2 in renal tissue in contrast to the CLP group.[39] These results can be attributed to puerarin's ability to protect the damaged renal tissue by inhibiting the NF-κB signaling pathway, led to a reduction of Bax expression, and multiplied Bcl-2 levels.

We demonstrate in our study a noticeable elevation in the mean severity of kidney damage in both CLP and vehicle groups when compared to the sham.[40] Histological evaluation revealed accelerated cytoplasmic staining, inflammatory response, interstitial swelling, red blood cell leakage, vascular blockages, and overall damage. The effect of sepsis on histopathology of kidney tissues related to potent pro-inflammatory mediators' induction (IL-6, IL-1b and TNF-α). Through this experimental study, it has been found that puerarin significantly reduce the overall renal damage that resulted from of CLP. Analysis of the severity rating for the puerarin treatment group showed that there was only mild to moderate renal damage.[29] Puerarin effect on renal histopathology may be due to inhibiting oxidative stress and alleviated TLR-4 expressions, and activation for SOD.

Conclusion

The inhibition of the TLR-4/NF-κB signaling with its downstream signaling pathways by puerarin proves to be a promising approach to mitigate kidney injury in mice during sepsis induced by CLP. These compounds show significant protective effects, exemplifying their potential in preventing the onset of acute renal dysfunction.

Conflict of Interest

None.

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