

Pachymic acid ameliorates sepsis-induced acute kidney injury by suppressing inflammation and activating the Nrf2/HO-1 pathway in rats

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Abstract. – OBJECTIVE: To investigate the protective effects and underlying mechanisms of pachymic acid (PA) on sepsis-induced acute kidney injury (AKI).

MATERIALS AND METHODS: Sepsis-induced AKI model was made by cecal ligation and puncture (CLP) surgery in SD rats. Animals were randomly divided into 5 groups: a sham group, a CLP group, and three PA-treated groups, which received intraperitoneal injection of PA at the dosage of 5, 20 and 50 mg/kg.bw, respectively. Kidney index, Cre and BUN contents were determined to evaluate the renal function. Pathological changes of kidney tissue were observed by HE staining. Levels of inflammatory mediators (TNF- α , IL-6) were measured to assess the inflammation in renal tissue. Moreover, the expression levels of iNOS, Nuclear factor E2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1) were studied by Real-time PCR and Western blot.

RESULTS: PA treatment can significantly decrease the kidney index, and notably drop the contents of Cre and BUN. Renal pathological damage was also found to be effectively improved by PA in a dose-dependent manner. PA treatment was observed to inhibit the renal inflammation by reducing the TNF- α and IL-6 levels. Besides, PA treatment significantly decreased the expression levels of iNOS, and enhanced the expression of Nrf2 and HO-1 in the kidney.

CONCLUSIONS: PA had potential therapeutic effects on sepsis-induced AKI in rats, and the activity may be associated with the anti-inflammatory function and antioxidant effect via activating Nrf2/HO-1 pathway.

Key Words:

Acute kidney injury, Pachymic acid, Cecal ligation and puncture, Sepsis, anti-inflammation, Nrf2/HO-1 signaling pathway.

Introduction

As a common complication of burn, trauma, hypoxia, and post-surgery, sepsis is a systemic inflammatory response syndrome, leading to multi-organ dysfunction. It is reported to affect 50-100/100,000 people in the developed countries¹. As one of the most common ramifications of sepsis, acute kidney injury (AKI), characterized by hypouricemia, azotemia and renal tubular necrosis, contributes significantly to the morbidity and mortality of hospitalized patients². However, the exact pathological mechanisms of AKI in sepsis is still unclear. The current treatment strategies for AKI, such as fluid resuscitation and supportive care, improve renal damage in some degree, but the mortality rates of AKI remain unacceptably high at 30%³. The widespread application and promotion of kidney transplant are also limited for the reasons of scarcity of donor, high cost and immune rejection. Therefore, searching and developing new strategies that can effectively prevent and attenuate AKI caused by sepsis is imperative. Emerging studies^{4,5} consistently indicate that traditional Chinese medicine could prevent and manage AKI. *Poria cocos*, which is also known as Fu Ling, is one of the most important traditional Chinese medicines for its diuretic and sedative effects. As a lanostane-type triterpenoid from *Poria cocos*, pachymic acid (PA) was found to possess a wide range of biological activities, such as anti-inflammatory effect, antioxidant activity, anti-cancer properties and so on^{6,7}. Previous researches^{8,9} revealed that PA inhibited tumor growth by suppressing NF- κ B signaling pathway. Another investigation¹⁰ demonstrated that PA had the scavenging effect on free-radicals. Moreover,

PA was also found to reduce postprandial blood glucose levels via enhanced insulin sensitivity irrespective of PPAR- γ and stimulate glucose uptake by improving the expression and trans-location of glucose transporter type 4^{11,12}. Despite the diverse studies conducted to investigate the biological activities of PA, the potential of PA against kidney injury is poorly defined. This work was designed to investigate the renal protective effects and underlying mechanisms of PA on sepsis-induced AKI rats.

Materials and Methods

Reagents and Animals

White crystalline powder of PA used in the study was obtained from Shanghai Chembest chemical technology Co. Ltd (Shanghai, China) and stored away from light at 4°C. Male Sprague-Dawley rats, weighing between 250 and 280 g, were provided by Shanghai Slac Laboratory Animal Co. Ltd (Shanghai, China). Animals were housed in the specific pathogen-free animal room with the temperature keeping 20-26°C and relative humidity 40-70%. They were allowed free access to chow and water throughout the study. All animal experiment operations were conducted according to nursing and Use Guidance for Animal Experiment Operation of National Institutes of Health.

Animal Groups

One hundred and twenty rats were randomly distributed into the following five groups (n=24 per group): a sham group, a CLP control group, a low-dose PA (L-PA) group, a mid-dose PA (M-PA) group and a high-dose PA (H-PA) group. Animals of the three experimental treatment groups received intraperitoneal injection with PA at the dosages of 5, 20 and 50 mg/kg.bw, respectively. The sham and CLP control group were treated with normal saline (1 ml/100 mg) of the same volume.

AKI Modeling

Undergoing a 3-day adaptation and a 12-h deprivation of food but not water, all animals, except those in sham group, were manufactured as classic sepsis-induced AKI models by the method of cecal ligation and puncture (CLP). After the anesthetized with chloral hydrate anesthesia (0.3 ml/100 g.bw) and skin sterilization, a midline abdominal incision about 2-3 cm was

made to expose the cecum which was then ligated between the terminal and ileocecal valve. Then, an 18-gauge needle was used to puncture through the central segment of ligation, and a small amount of cecal contents was squeezed out through the puncture wound. Finally, the cecum was restored into the abdominal cavity and the surgical incision was sutured layer by layer. Comparatively, ceca of rats in the sham group were exposed and massaged as described above, but they were not ligated or punctured.

Specimens Collection

At 6, 12 and 24 h after PA administration, blood samples of 2 ml were obtained from aorta abdominals for biochemical analysis. Each time, one-third of the rats (n=8) were taken out from each group randomly. After the collection of blood, cervical dislocation was carried out, and both sides of kidneys were harvested and weighted to calculate the organ indexes. Then, the left kidneys were stored in 10% formaldehyde solution for two weeks and then transferred and kept in 80% ethyl alcohol until histopathological analyses. At the same time the right kidneys were quickly stored at -80°C.

Measurement of Blood Parameters and Cytokines

Using the fully automatic biochemical analyzer TBA-40FR (Toshiba, Minato, Tokyo, Japan), the contents of creatinine (Cre) and blood urea nitrogen (BUN) were measured by reagent kits according to the manufacturer's protocols (BioSino, Beijing, China). The levels of tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) were detected using enzyme-linked immunosorbent assay kits specific for rats (Boster, Wuhan, China).

Histopathological Examination

At 24 h post-CLP, portions of the kidney tissues were fixed in 10% neutral-buffered formalin and processed routinely by embedding in paraffin. Then paraffin-fixed tissue specimens were sliced into 4 μ m-thick sections, which were mounted on glass slides and stained with Hematoxylin and Eosin (HE staining). A light microscopy BX51 (Olympus, Tokyo, Japan) was used to examine the renal histopathological changes.

Real-time Quantitative RT-PCR Analysis

Total RNA was extracted from kidneys of different groups, and the cDNA was synthesized using PrimeScript RT Reagent Kit with

gDNA Eraser (TaKaRa, Dalian, China). Relative levels of the target genes were determined by Real-time PCR using the Ultra SYBR Mixture (TaKaRa, Dalian, China). Primer sequences were as follows: inducible nitric oxide synthase (iNOS), sense 5'-ACATCAGGTCGGCCATCACT-3', antisense 5'-CGTACCGGATGAGCTGTGAATT-3'; Nuclear factor E2-related factor 2 (Nrf2), sense 5'-GGACCTAAAGCACAGCAACACAT-3', antisense 5'-TCGGCTTGAATGTTTGTCTTTTGTG-3'; heme oxygenase-1 (HO-1), sense 5'-CTTTTTTCACCTTCCCGAGCATC-3', antisense 5'-GGTCTTAGCCTCTTCTGTCACCCTGT-3'. GAPDH, used as an internal control, was amplified by the use of sense 5'-TCAAGAAGGTGGTGAAGCAG-3' and antisense, 5'-AGGTGGAAGAATGGGAGTTG' primers. Thermal cycling conditions included an initial denaturation step at 95°C for 5 min then 40 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s, followed by final extension at 72°C for 5 min. For each sample, reactions were set up in triplicate to ensure the reproducibility of the results. The quantification of the relative transcript levels was performed using the comparative threshold (Ct) method.

Western Blot Analysis

The total protein samples were extracted from the kidney tissues using RIPA cell lysis buffer containing proteinase inhibitor cocktail, and the protein concentrations were determined by the method of bicinchoninic acid assay. An equivalent amount of each protein sample (40 µg) was loaded and separated through sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene difluoride membranes (PVDF) (Millipore, Billerica, MA, USA). The membranes were then sealed with 5% skimmed milk powder, incubated with primary antibodies against iNOS (1:500, Bioss), Nrf2 (1:500, Abcam, Cambridge, MA, USA) and HO-1 (1:1000, Abcam Cambridge, MA, USA) overnight at 4°C and washed. Then, membranes reacted with corresponding horse radish peroxidase (HRP)-labeled secondary antibody (1:5000, Beyotime, Shanghai, China) for a further 1 h at 37°C. After washing the membrane, the electroluminescence (ECL) reagent was added. X-ray film exposure, developing and fixing were performed. Image J 1.46 analysis software was used to visualize the Western blotted strip. The protein expression levels were expressed as a ratio to the endogenous control, GAPDH.

Statistical Analysis

Data statistic analysis was performed by SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) and all data were expressed as means±SD. Changes between samples were compared by Student's *t*-test, and differences between groups were compared by the method of one-way ANOVA. LSD test was used to post-hoc test ANOVA. $p < 0.05$ was considered statistically significant.

Results

PA Reduced Renal Injury in Rats With Sepsis

Firstly, kidney coefficients of each group at three time points (6, 12 and 24 h) were calculated to reflect the renal functional state from the macroscopic aspect. The result showed that (Figure 1A), kidney coefficients of CLP and the three drug treatment groups were all significantly higher ($p < 0.05$) than those of the sham group, indicating that sepsis-induced AKI models were successfully established and their kidneys were injured in different extent. High-dose of PA administration was found to significantly decrease the kidney coefficients ($p < 0.05$). The change of Cre and BUN, as the important blood indexes representing renal function, were also detected. According to the results (Figure 1B, C), PA administration significantly reduced the CLP-enhanced Cre and BUN contents in the serum ($p < 0.05$), in a dose-dependent manner. Morphological changes in the rat kidneys at 24 h post-treatment were determined by H&E staining (Figure 1D). We can found that, the kidney tubules and glomerulus in sham group were morphologically normal. In contrast, CLP group showed series degenerative changes in the renal tissues, appearing expansion, hyperemia in the glomerular capillary, edema, vacuolar degeneration, and significant narrowing down in renal tubular cavity. When talking about the PA treatment groups, all of the above degenerative changes of renal tissues in the three groups were gradually reduced, presenting significantly dose-dependent improvement effects.

PA Decreased the Pro-Inflammatory Cytokine Expression in Rats With Sepsis

The levels of inflammatory factors such as TNF- α and IL-6 at different time points were detected in this study to represent the inflammation in kidney injury. As demonstrated in Figure 2 A-B, kidney tissues of the CLP and PA treatment

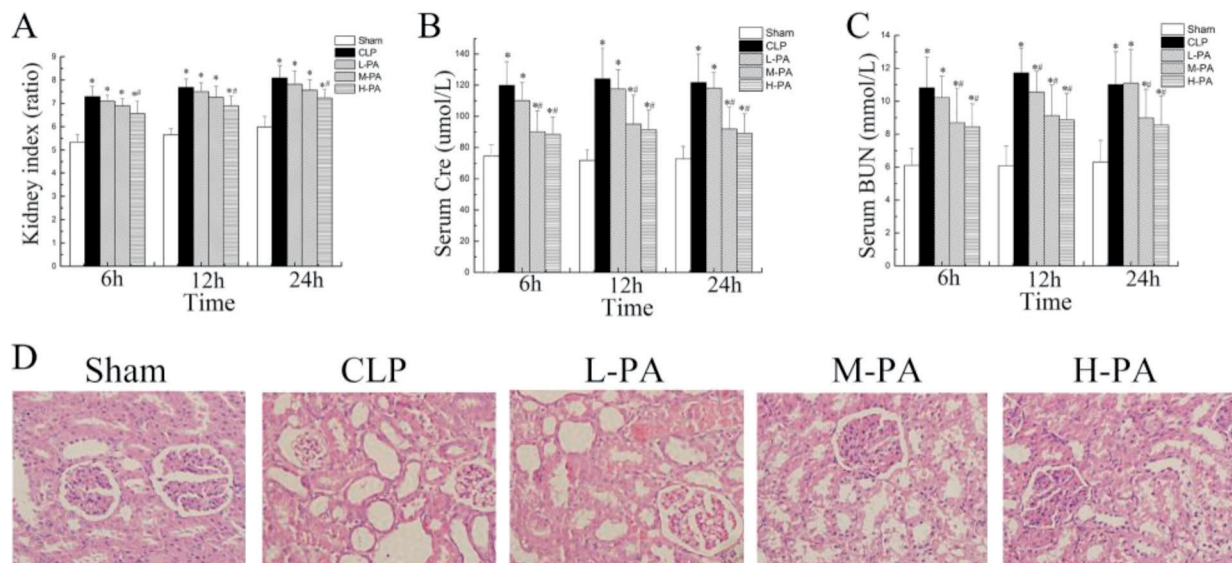


Figure 1. Effects of PA on kidney function in a rat model of sepsis-induced AKI. **(A)** The change of kidney indexes; **(B)** serum levels of Cre; **(C)** serum levels of BUN; **(D)** HE staining of kidney tissue. *Indicates significant difference from the sham group at $p < 0.05$; #indicates significant difference from the CLP group at $p < 0.05$ ($n=8$ per group).

groups all exhibited obvious inflammation, showing significant increase ($p < 0.05$) in the levels of TNF- α and IL-6. When it came to the drug treatment groups, the levels of inflammatory factors in the M-PA and H-PA group were markedly lower than ($p < 0.05$) those of the CLP model group; however, the levels of TNF- α and IL-6 between the L-PA and CLP group were not significantly different ($p > 0.05$). What's more, PA treatment was founded to be time-dependent, characterizing as highest inflammatory factors levels at 6 h post-treatment, and showing gradually relative lower levels at 12 and 24 h post-treatment.

PA Inhibited Renal iNOS Expression in Rats With Sepsis

We evaluated whether the expression levels of iNOS at 24 h post-CLP surgery were affected by the CLP procedure using Real-time quantitative RT-PCR analysis and Western blot analysis. Our results revealed that the transcription of iNOS (Figure 3A) was significantly increased in the pyemic rats ($p < 0.05$), whereas it was suppressed by middle and high dose of PA treatment ($p < 0.05$). The protein expression of iNOS (Figure 3B) in different groups had the same results: showing significantly higher in

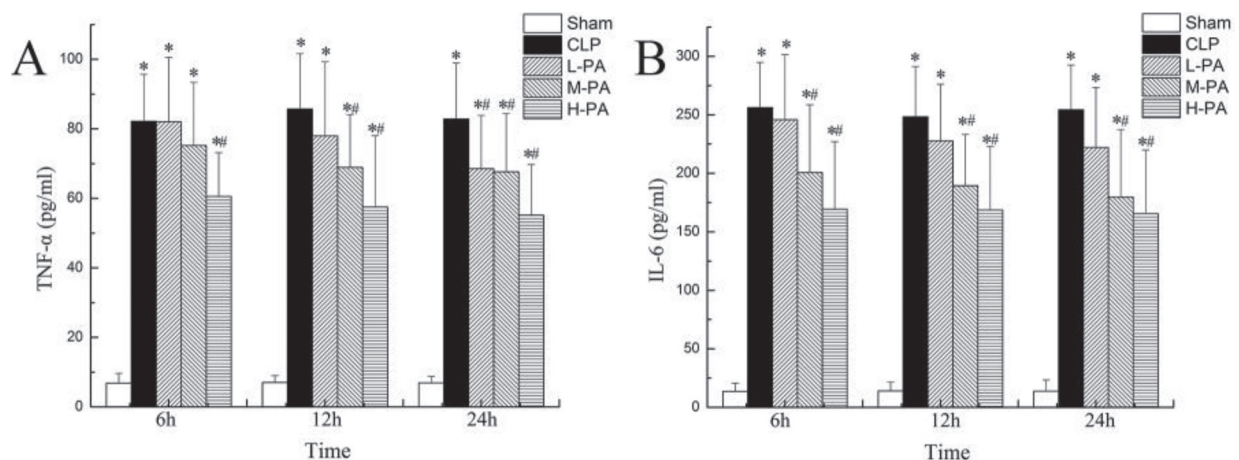


Figure 2. Effects of PA on serum protein levels of TNF- α **(A)** and IL-6 **(B)** in a rat model of sepsis-induced AKI. *indicates significant difference from the sham group at $p < 0.05$; #indicates significant difference from the CLP group at $p < 0.05$ ($n=8$ per group).

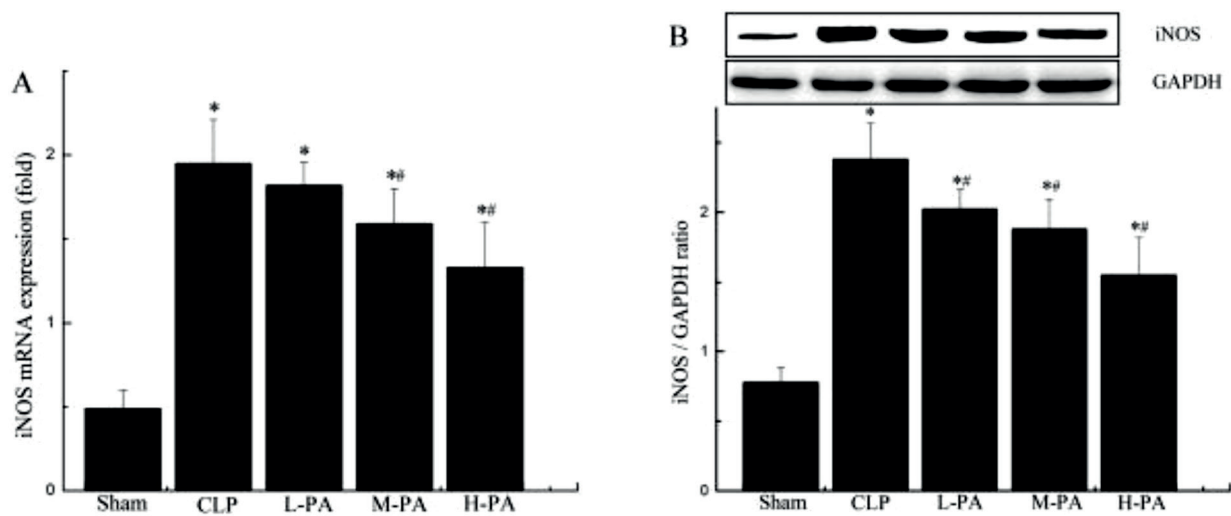


Figure 3. Effects of PA on the expression of iNOS in rat kidney tissues at 24 h post-CLP procedure. **(A)** Renal iNOS mRNA expression was detected by Real-time quantitative RT-PCR. **(B)** Renal iNOS protein expression was detected by Western blot. *Indicates significant difference from the sham group at $p < 0.05$; #Indicates significant difference from the CLP group at $p < 0.05$ ($n=8$ per group).

the CLP group ($p < 0.05$) and markedly lower in the three PA treatment groups ($p < 0.05$).

PA Promoted the Activation of Nrf2/HO-1 Signaling Pathway in Rats With Sepsis

To investigate the effect of PA on Nrf2/HO-1 signaling pathway, mRNA and protein levels of Nrf2 and HO-1 at 24 h post-surgery were assessed. We noted that (Figure 4 A-B) CLP induced a marked decrease in the Nrf2 mRNA expression ($p < 0.05$), while the mRNA level of HO-1 in the CLP group was not significantly different from the sham group ($p > 0.05$). However, a remarkable increase of both the Nrf2 and HO-1 mRNA levels was observed in the PA-treated group ($p < 0.05$). Consistently, PA treatment was also found to increase the protein levels of Nrf2 and HO-1 (Figure 4C, D) in a dose-dependent manner, when compared with the CLP group ($p < 0.05$). Our data thus suggest that the administration of PA promoted the activation of Nrf2/HO-1 signaling pathway.

Discussion

In the present study, septic acute kidney injury model was made by CLP surgery in SD rats to investigate the renoprotective effects of PA. In evaluating these effects, we found that the CLP group showed significantly higher kidney index-

es and biomarkers of kidney injury (Cre, BUN) in comparison to the sham group. Moreover, histological examination of the kidney of rats undergoing a CLP operation was characterized by glomerular degeneration, tubular lumen dilation and cellular infiltration. The administration of PA markedly reversed all these changes in a dose-dependent manner, indicating a protective role in treating sepsis-related kidney injury. Since inflammatory response participates in the onset and development of early AKI^{13,14}, we then focused on the anti-inflammatory effects of PA in septic AKI. Emerging researches consistently indicate that various of negative factors, such as ischemia/reperfusion injury, bacterial infection and lipopolysaccharide (LPS) exposure will irritate infiltration of inflammatory cells in the renal parenchyma¹⁵. Chemokines and cytokines secreted by the aberrant immune cells, will directly cause renal damage as well as give rise to inflammatory cascade reactions by activating Toll-like receptor-4 (TLR-4) and nuclear factor- κ B (NF- κ B) signaling pathways, ultimately leading to systemic inflammatory response syndrome. Therefore, controlling abnormal immune responses in the kidney might prevent AKI and improve the clinical outlook in sepsis. In fact, depleting macrophages and reducing the release of TNF- α and/or IL-1 β in septic models have been proven to be an effective therapy to ameliorate sepsis¹⁶⁻¹⁸. PA has been confirmed to possess the strong ability of anti-inflammation. Li et

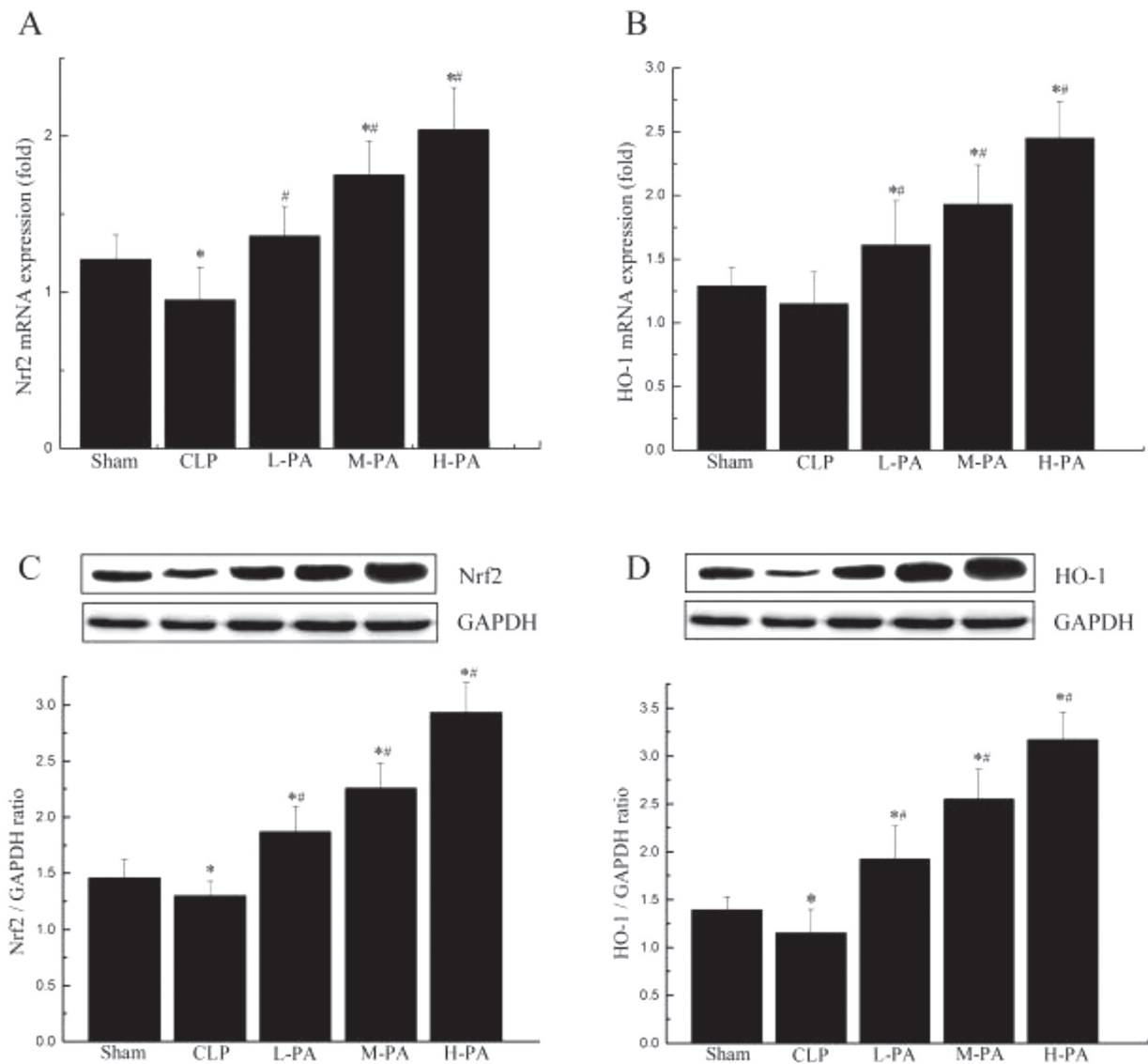


Figure 4. Effects of PA on the expression of Nrf2 and HO-1 in rat kidney tissues at 24 h post-CLP procedure. **(A-B)** renal Nrf2 and HO-1 mRNA expression was detected by Real-time quantitative RT-PCR. **(C-D)** Renal Nrf2 and HO-1 protein levels were detected by Western blot. *Indicates significant difference from the sham group at $p < 0.05$; #Indicates significant difference from the CLP group at $p < 0.05$ ($n=8$ per group).

al¹⁹ revealed that PA could protect cardiomyocytes away from LPS-induced inflammation by attenuating the level of TNF- α , IL-1 and IL-6. Consistently, Kim et al²⁰ also found that PA suppressed the aberrant release of TNF- α and IL-1 β , as well as inhibited the NF- κ B translocation in oral inflammation. In this study, the septic rats were detected with a significant increase of TNF- α and IL-6 concentrations, which were then reduced by PA dose and time dependently. The results suggest that the improving effect of PA on AKI may be partially due to its inflammatory inhibition potency. The overproduction of

pro-inflammatory mediators can accelerate the release of reactive nitrogen/oxygen species, such as nitric oxide (NO). Among the three isoforms of NOS, cytotoxic and pathologic NO of high levels are mostly produced by iNOS. It has been reported²¹ that excessive levels of NO and iNOS aggravate the ischemia reperfusion injury in the kidney. Pathak et al^{22,23} proved that both NO antagonists and NOS inhibitors, as well as knockout techniques of iNOS gene, could act as the potential novel treatment for sepsis-induced AKI. Interestingly, Cai et al²⁴ had discovered that PA isolated from the dried sclerotia of *Poria cocos*

had the inhibitory activity on NO production and iNOS expression from LPS-induced RAW 264.7 cells. From our findings, PA was found to notably reduce the CLP-induced renal iNOS over-expression in rats, indicating that by decreasing the iNOS induced NO production, PA can effectively alleviate the sepsis-induced nephrotoxicity. Recent studies^{25,26} have highlighted the key role of oxidative stress caused by increased production of ROS in the development of renal dysfunction followed by AKI or other diseases. Nrf2, as the 'master regulator' of antioxidant responses, could act with antioxidant response element (ARE), and then induce the expression of a series' detoxifying enzymes and antioxidant proteins, such as HO-1 and SOD²⁷. Accumulating evidence has confirmed that the activation of Nrf2 could effectively inhibit the development and progression of AKI^{28,29}. In the present paper, we discovered that the Nrf2 and HO-1 expression in the rats with pyemic renal injury were significantly increased. Nevertheless, PA treatment was found to markedly inhibit the CLP-enhanced renal Nrf2 and HO-1 expression. Our data agree with the research of Lee et al³⁰ that PA improved pulpal inflammation and promoted odontogenesis via increasing HO-1 expression and inducing Nrf2 translocation into nucleus. Therefore, the ameliorative effects of PA on sepsis-related renal damage may also benefit from its activation function on Nrf2/HO-1 pathway against oxidative stress.

Conclusions

In this study, the PA showed anti-inflammatory function and antioxidant effect via activating Nrf2/HO-1 pathway in the rats with pyemic renal injury, suggesting the therapeutic potential to attenuate sepsis-induced AKI.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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