Int J Clin Exp Med 2016;9(8):15935-15941
www.ijcem.com /ISSN:1940-5901/IJCEM0027815

Original Article

Paeoniflorin attenuates CLP-induced acute liver injury by activating Nrf2/γ-GCS in rats

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Received March 9, 2016; Accepted July 4, 2016; Epub August 15, 2016; Published August 30, 2016

Abstract: Objective: Acute liver injury (ALI) is a common and serious complication of sepsis. The imbalance between oxidation and anti-oxidation plays an important role in sepsis-induced ALI. Recent studies suggest that paeoniflorin (PF) may serve as an antioxidant. Nrf2-ARE signal pathway is known as an important anti-oxidative system. This study aimed to investigate the protective effects of PF on sepsis-induced ALI in which the role of Nrf2 was explored. Methods: Rats were randomly divided into 3 groups: sham group, sepsis group and PF group (n=12 per group). Sepsis was induced by cecal ligation and puncture (CLP). In PF group, rats were intraperitoneally rejected with PF at 90 mg/kg following CLP. The survival was determined within 48 h. At 6 h after treatment, the serum alanine aminotransferase (ALT), MDA content and SOD activity were measured and the liver was harvested for the Western blot assay and real time PCR of Nrf2 and γ-GCS expressions. Results: Our results indicated that the survival rate within 48 h increased significantly, the serum ALT and malondialdehyde (MDA) reduced markedly, the serum superoxide dismutase (SOD) activity increased remarkably and the mRNA and protein expressions of nucleus Nrf2 and γ-GCS in the liver were increased significantly in PF group as compared to sepsis group. Conclusion: Our findings suggest that PF is able to protect against sepsis induced ALI via modulating Nrf2 signaling pathway.

Keywords: Paeoniflorin, acute liver injury, cecal ligation and puncture, NF-E2-related factor 2

Introduction

Sepsis is defined as the acute systemic inflammatory response to infection by various pathogens, ranging from hemodynamic changes to multiple organ dysfunction syndrome and even death. Although the pathophysiology of sepsis has been studied extensively, the incidence and mortality of sepsis are still increasing over year, and thus sepsis has been a serious medical problem worldwide [1]. Liver is the second important organ affected by sepsis. The incidences of sepsis-induced liver dysfunction and liver failure range from 34% to 46% and 1.3% to 22%, respectively, and attenuating liver injury can lower the morbidity and mortality of septic patients [2]. To date, no effective strategies have been developed for the treatment of sepsis induced acute liver injury (ALI).

A variety of mechanisms have been proposed for the sepsis-induced ALI, including the uncontrolled systemic inflammation, hepatic ischemia and coagulopathy [3]. Oxidative stress also plays a crucial role in the pathology of sepsis-induced ALI. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that is able to promote the transcription of a battery of cytoprotective genes in response to oxidative and electrophilic stress [4]. Under normal conditions, Nrf2 is sequestered by kelch-like ECH associating protein 1 (Keap1) in the cytoplasm. In response to oxidative stress, Nrf2 is released from Keap1, translocates into the nucleus, then binds to antioxidant response element (ARE) on the promoter region of various genes, and induces an array of detoxifying and antioxidant defense genes to combat oxidative stress. Nrf2 target genes include antioxidant genes and phase II enzymes such as heme oxygenase-1 (HO-1), NAD(P)H quinone oxidoreductase1 (NQO1), γ-glutamyl cysteine synthethase (γ-GCS) and glutathione peroxidases. Glutathione (GSH) is an important intracellular antioxidant and has a key role in maintaining integrity and preventing oxidative damage in alveolar

epithelial cells. γ-GCS is the rate-limiting enzyme of GSH synthesis, and can regulate the intracellular GSH levels [5, 6]. Studies from animal models have indicated that the Nrf2-ARE pathway collectively exhibits diverse biological effects against viral hepatitis, nonalcoholic liver disease and fibrosis via inducing the expression of its target genes [7, 8]. A growing number of studies have shown that the Nrf2-ARE pathway plays a crucial role in the pathophysiology of sepsis [9, 10].

Paeoniflorin (PF), a monoterpene glycoside, is the main active compound extracted from the dried roots of *Paeonia* [11]. In liver diseases, PF consistently shows multiple pharmacological effects including anti-oxidative and anti-inflammatory activities, anti-hepatic fibrosis and hepatocyte regeneration promotion, with low toxicity [12]. Recent studies show that PF is able to attenuate the dimethylnitrosamine-induced liver fibrosis at least in part by decreasing tumor necrosis factor (TNF)-alpha in the serum and improving the anti-oxidative defense [13]. However, the protective effect of PF on sepsis induced ALI and the potential mechanism are still poorly understood.

This study aimed to investigate the protective effects of PF in a rat mode of sepsis induced ALI in which the role of Nrf2 pathway was evaluated.

Materials and methods

Reagents and animals

PF (purity >95%) was purchased from Xi'an Haoxuan Biotechnology Ltd Co. Sprague-Dawley (SD) rats (8-9 weeks old, weighing within 150~200 g) were purchased from Hunan Lai Ke Jing Da Experiment Animal Center All animals were housed under standard conditions and given *ad libitum* access to water and food. All the procedures were carried out according to the Guide for the Care and Use of Laboratory Animals.

Induction of sepsis

Sepsis was induced in rats by cecal ligation and puncture (CLP). Rats were completely anesthetized with chloral hydrate (3.5 ml/kg, i.p.) and a midline incision was made at the abdomen. The cecum was exposed and ligated 1.0 cm away from the end and punctured twice with a 16-gauge needle and gently squeezed to exude feces and to ensure that the two punctures did not close. The abdominal incision was then closed and saline was administered subcutaneously for fluid resuscitation (5 ml per 100 g body weight). In sham group, animals underwent laparotomy and bowel manipulation without ligation and perforation. All rats were given *ad libitum* access to food and water after recovery from anesthesia. Sixty-six adult male Sprague Dawley rats were randomly divided into three groups (n=22 per group). In sham group, rats were intraperitoneally treated with normal saline; in CLP group, rats were intraperitoneally injected with normal saline after CLP; in PF group, rats were intraperitoneally treated with PF at 90 mg/kg (15 mg/ml) after CLP [11].

Survival rate

Rats were observed for 48 h after CLP (n=12 per group) and the survival rate was calculated.

Plasma biochemistry

At 6 h after treatment, blood samples were taken from the eye or heart from survived rats. Serum was separated and processed for the measurement of alanine transaminase (ALT) with an Automated Chemical Analyzer.

Liver histopathology

At 6 h after CLP, the right lobe of the liver was rapidly harvested from survived after blood collection. Then, the liver was fixed in 10% formalin and embedded in paraffin. Tissues were cut into sections followed by HE staining. These sections were examined under a light microscope.

Measurement of MDA content and SOD activity

Liver was homogenized with saline at a weight-volume ratio of 1:9. The homogenate was centrifuged and the supernatant was collected for the detection of SOD activity and MDA content according to the manufacturer's instructions (Jiancheng Bioengineering Ltd, Nanjing, China). Protein content was measured with BCA method.

Western blot assay

The protein expressions of Nrf2 and γ-GCS were detected by western blot assay as

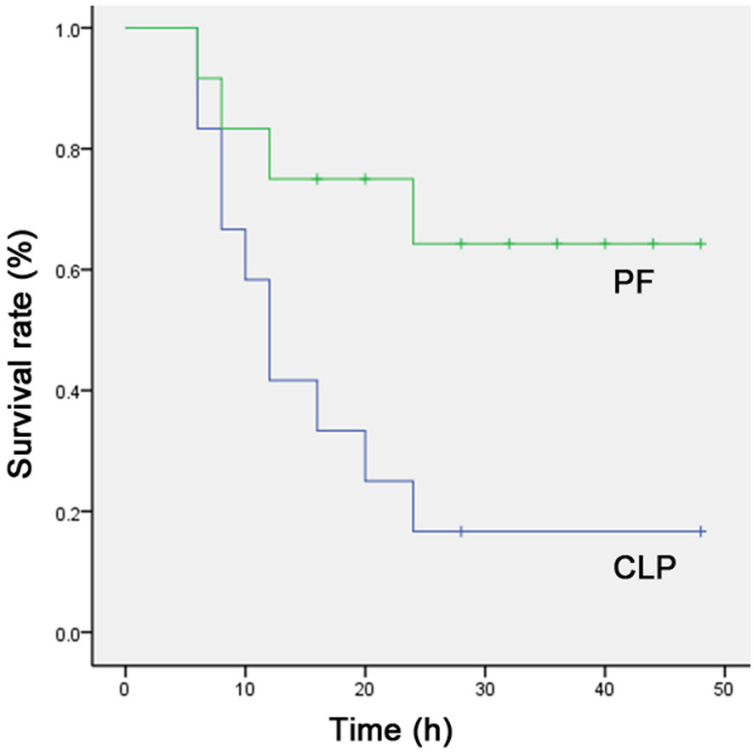

Figure 1. Survival rate in CLP group and CLP+PF group. The Kaplan-Meier method was used for the survival analysis. P<0.05: PF group vs. CLP group (log rank test).

Table 1. Serum ALT in sham group, CLP group and PF group

Group	n	ALT (U/L)
Sham	10	100.23±37.02
CLP	9	467.00±79.08$^{\Delta}$
PF	9	365.11±64.22*

Note: $^{\Delta}$P<0.01 vs. sham group; *P<0.01 vs. CLP group.

described previously [14]. In brief, equivalent amounts of protein were separated on by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto polyvinyl difluoride membranes (Millipore corporation). After blocking in normal saline, the membranes were independently incubated with primary antibodies: rabbit anti-Nrf2 (1:1000, Abcam) and anti-γ-GCS (1:5000, Abcam) at 4°C overnight. Then, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:5000, Abcam), followed by visualization with ECL detection reagent (Millipore Corporation). Gray intensity was analyzed with IPP6.0 image analysis software.

Real time polymerase chain reaction

Real time polymerase chain reaction (PCR) was performed as previously reported [15]. Total RNA was isolated by TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription into cDNA with total RNA was performed using the First-Strand cDNA synthesis kit (TAKARA). The PCR was conducted as follows: 95°C for 10 min followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. Primers used for PCR were as follows: Nrf2 forward: 5'-CCATTTGTAGATGACCATGAGTCGC-3', Reverse: 5'-ATCAGGGGTGGTGAAGACTG-3', γ-GCS forward: 5'-GAGCGAGATGCCGTCTTACA-3', Reverse: 5'-TTGCTACACCCATCCACCAC-3', and GADPH forward: 5'-GGCATCGTGGAAGGGCTCATG-3', Reverse: 5'-GCCAGTGAGCTTCCCGTTCAG-3'. The relative amount of mRNA was determined using the $2^{-\Delta\Delta CT}$ method. The mRNA expression was expressed as the fold change after normalization to GAPDH. PCR was performed in triplicate and at least repeated twice.

Statistical analysis

Statistical analysis was performed with SPSS Version 19.0 software. The Kaplan-Meier survival analysis was performed with the log rank test. Data are expressed as means ± standard deviation (SD). Comparisons among groups were conducted with one way analysis of variance (ANOVA). A value of $P<0.05$ was considered statistically significant.

Results

PF ameliorates CLP-induced ALI and improves survival

The survival rate was assessed with 48 h following CLP. As shown in **Figure 1**, the survival rate was 66.7% (8/12) in PF group and 16.7% in CLP group showing significant difference

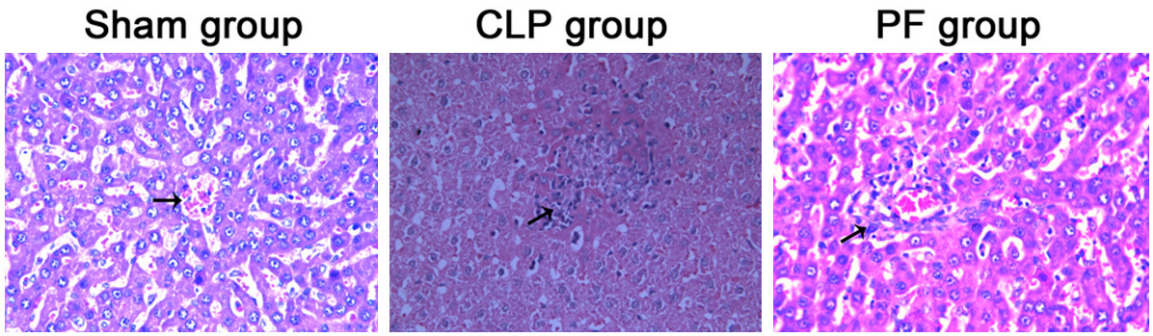

Figure 2. Pathological changes in the liver of sham group, CLP group and PF group. The liver was sampled at 6 h following CLP and processed for HE staining (original magnification ×400). In sham group, the hepatocytes were still normal and the sinusoids clear (arrow); In CLP group, a large amount of inflammatory cells infiltrated the liver, and large necrotic area was observed in the hepatic sinusoids (arrow). However, the liver injury was significantly relieved after PF treatment as compared to CLP group, only spotty necrotic area was found, and karyokinesis was noted in a fraction of hepatocytes (arrow).

Table 2. MDA content and SOD activity of the liver in sham group, CLP group and PF group

Group	n	MDA (nmol/mg prot)	SOD (U/mg prot)
Sham	10	1.33±0.13	3.50±0.22
CLP	8	2.60±0.27$^{\Delta}$	1.58±0.24$^{\Delta}$
PF	9	1.85±0.71*	2.65±0.11*

Note: $^{\Delta}$P<0.01 vs. Sham group; *P<0.01.

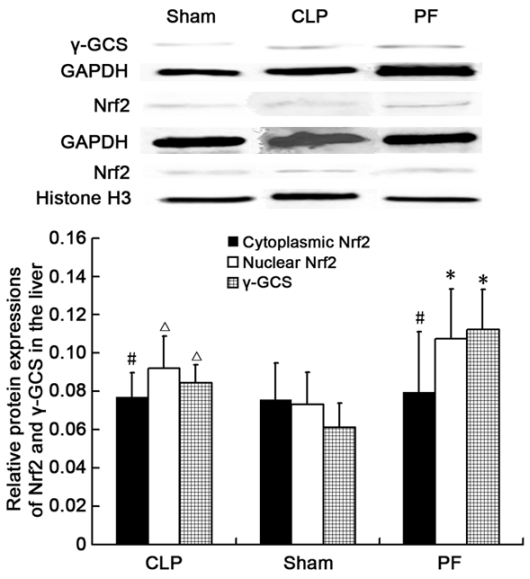

Figure 3. Protein expressions of Nrf2 and γ-GCS in the liver (Western blot assay). Data are expressed as mean value ± SD. $^{\Delta}$P<0.01 vs. sham group; *P<0.01 vs. PF group.

between them (P<0.05). As shown in **Table 1**, CLP-induced sepsis significantly increased the serum ALT (467.00±18.08 U/L) as compared to sham group (100.23±37.02 U/L). In PF group, the serum ALT was markedly lower than in CLP group (365.11±13.22 U/L vs. 467.00±18.08 U/L, P<0.01). In sham group, the hepatocytes were normal and the sinusoids clear (**Figure 2**). However, in CLP group, a large amount of inflammatory cells infiltrated in the liver and large necrotic area was observed in the hepatic sinusoids (**Figure 2**). However, the liver injury was relieved after PF treatment as compared to CLP group, only spotty necrotic area was observed and karyokinesis was only noted in a fraction of hepatocytes (**Figure 2**).

SOD activity and MDA content

As shown in **Table 2**, the septic rats exhibited a significant increase in the hepatic MDA content. Moreover, the MDA content in PF group was significantly lower than in CLP group (P<0.01). However, the hepatic SOD activity increased dramatically in PF group as compared to CLP group (P<0.01).

Effect of PF on the protein and mRNA expressions of Nrf2 and γ-GCS in the Liver

As shown in **Figure 3**, the protein expressions of Nrf2 and γ-GCS in the liver reduced at 6 h following CLP in CLP group as compared to sham group. After PF treatment, the protein expressions of Nrf2 and γ-GCS increased significantly when compared with CLP group (P<0.01).

The mRNA expressions of Nrf2 and γ-GCS increased markedly in PF group as compared to CLP group (Nrf2: 3.7758±0.557 vs. 0.7430±0.164, P<0.01; γ-GCS: 1.6001±0.279 vs.

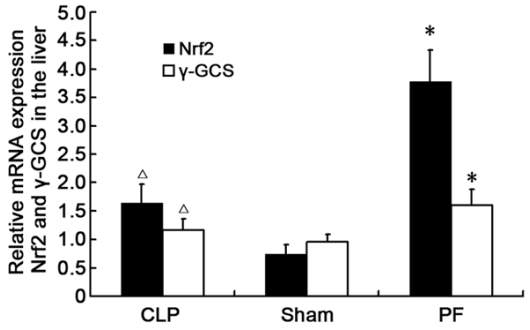

Figure 4. mRNA expressions of Nrf2 and γ-GCS (RT-PCR). Data are expressed as mean ± SD. $^{\Delta}$P<0.01 vs. sham group; *P<0.01 vs. PF group.

0.9646±0.126, P<0.01) (**Figure 4**). These results indicated that PF relieved the oxidative injury by activating Nrf2 and up-regulating γ-GCS in the liver of CLP rats.

Discussion

In recent decades, the morbidity and mortality of sepsis are still increasing despite the advances in the new therapeutic agents. The ALL secondary to sepsis is a common disease leading to death in the intensive care unit [2]. PF has been used widely as an antioxidant to protect cells against oxidative stress in the central nervous system diseases and in liver diseases [16-18]. This study was to examine the protective effects of PF on sepsis induced ALI and explores the potential role of Nrf2/γ-GCS in these effects in a CLP rat model.

Lipopolysaccharide (LPS) injection and CLP have been used in the establishment of sepsis models in animals, but CLP is the most widely used in rodents, in which an infection with mixed bacterial flora is induced and may cause ARDS and multiple organ dysfunction syndrome [19]. This technique involves the exteriorization of the caecum, ligation of the caecum distal to the ileocaecal valve and puncture of the ligated caecum. It may mimic the clinical profile and time course of abdominal peritonitis in humans: the hyperdynamic phase and metabolic phases in early phase sepsis and low systemic vascular resistance (SVR) in late phase [20]. The CLP model not only mimics the hemodynamic change but the septic response with respect to the immunoinflammatory characteristics as in humans. The systemic cytokine profile observed in CLP model is more reminiscent than that observed in septic patients, demonstrating both continuous and sustained increases [20]. Thus, the CLP model is viewed as a standard sepsis animal model.

Oxidative stress plays an important role in the pathogenesis of sepsis in which ROS overproduction is involved in the CLP-induced macrophage activation, leading to an excess inflammatory process and resulting in multiple organ dysfunction syndrome [21]. SOD catalyzes the dismutation of superoxide radical (O_2^-) to hydrogen peroxide (H_2O_2) and oxygen (O_2) [22]. As is well known to all, MDA is a lipid peroxidation product and has been used as a marker for tissue damage. In this study, results showed, when compared with CLP group, PF pretreatment significantly increased SOD activity. However, the liver MDA content was similar between sham group and PF group. In a rat model of sepsis created by CLP, MDA content of the liver significantly elevated in CLP group as compared to sham group, which was consistent with our findings [23]. It further confirmed that the oxidative stress was involved in the pathogenesis of sepsis induced acute liver injury. SOD is one of phase II enzyme induced by Nrf2. Nrf2 can regulate the transcriptional activation of antioxidant genes, exerting protective effect against oxidative damage [24-26]. Increasing studies show that Nrf2-knockout mice exhibit significant oxidative damage and are highly susceptible to hepatotoxicity [27]. Punicalagin, as an antioxidant, may induce the Nrf2/HO-1 expression via activating PI3K/AKT pathway, which inhibits LPS-induced oxidative stress in RAW264.7 macrophages [28]. Our previous findings have shown that as tragaloside-IV exhibits significant protective effect on D-GalN/LPS-induced acute liver failure in mice by up-regulating Nrf2 expression. GSH is the primary defense against ROS in living organisms and γ-GCS is the rate-limiting enzyme in the synthesis of GSH [29]. Furthermore, the transcription and expression of γ-GCS are regulated by Nrf2 [30]. Recent studies suggest that hydrogen gas can improve the survival and organ damage in rats with CLP-induced sepsis, which is associated with the regulation of oxidative stress, inflammatory response, and apoptosis through the Nrf2 signaling pathway [30]. To our knowledge, few studies have been conducted to investigate the protective effects of PF against CLP-induced ALI, and the role of Nrf2/γ-GCS in its protective effects. In the present study, results showed that PF treatment significantly increased the protein and mRNA expressions

of Nrf2 and its downstream γ-GCS in the liver of septic rats, accompanied by increase in SOD activity in the liver. Moreover, the survival was also significantly improved within 48 h following CLP in septic rats after PF treatment. These findings were consistent with those from the study of Kong et al, in which up-regulating the protein and mRNA expressions of Nrf2 was found to attenuate the liver injury in septic mice [31]. It has been reported that serum ALT and MDA were significantly lower and the expression of anti-oxidant enzyme γ-GCS was significantly higher in ischemia reperfusion injury rats with Nrf2 pathway activator pre-treatment as compared to the untreated rats, which partially attenuated the hepatic ischemia reperfusion injury in rats [32]. In addition, Jiang et al found that intravenous injection of PF alone or in combination with imipenem reduced CLP-induced mortality in rats by down-regulating TNF-α, IL-6 and high-mobility group-box 1 protein [33]. These are consistent with our findings that oxidative stress is involved in the pathophysiological of sepsis and activation of Nrf2 signaling pathway is able to maintain the oxidation/antioxidant balance, relieving sepsis and reducing mortality.

Taken together, PF is able to protect the liver against oxidative stress and the inflammation-induced liver damage. Our results indicate that the anti-oxidative activity of PF is, at least partly, attributed to secondary activation of Nrf2-induced antioxidant and phase II detoxification enzymes, such as γ-GCS, which protects against CLP-induced ALI. These findings provide new perspectives for the therapy of sepsis induced ALI with antioxidants and compounds against oxidative stress and excess inflammation.

In this study, only PF at one dose was used, and whether the protective effect of PF is dose dependent was not investigated to the serum ALT, and expressions of Nrf2 were not dynamically monitored in these rats. Further studies are required to elucidate whether other signaling pathways are involved in the pathogenesis of sepsis induced ALI in this model. In recent years, many studies have shown the mitochondrial dysfunction in sepsis, and demonstrate that sepsis may cause damage to the mitochondrial structure and functions, which in turn aggravates sepsis [34]. Thus, the interaction between mitochondrial dysfunction and sepsis induced ALI need to be further studied.

Conclusion

PF is able to alleviate CLP-induced ALI by activating Nrf2 signaling pathway, and may serve as a potential therapeutic agent for ALI in sepsis.

Acknowledgements

This work was supported by the National "Twelve-Five" Technological Supported Plan of China (2012BAI04B01).

Disclosure of conflict of interest

None.

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