

## Original Research Article

# Puerarin mitigates acute liver injury in septic rats by regulating proinflammatory factors and oxidative stress levels

Jinan Zheng<sup>1\*</sup>, Qing Huang<sup>1</sup>, Jingjing Fang<sup>2</sup>

<sup>1</sup>Department of Emergency, <sup>2</sup>Department of Intensive Care Unit, Affiliated Hospital of Medical College of Ningbo University, Ningbo 315000, Zhejiang Province, China

\*For correspondence: **Email:** [wutv74@163.com](mailto:wutv74@163.com)

Sent for review: 30 June 2021

Revised accepted: 22 October 2021

### Abstract

**Purpose:** To determine the protective effect of puerarin against acute liver injury in septic rats, and the mechanism involved

**Methods:** Eighty-seven Sprague-Dawley (SD) rats were assigned to control, sepsis and puerarin groups (each having 29 rats). Serum levels of NF- $\kappa$ B, TNF- $\alpha$ , IL-1 $\beta$ , IL-6, ALT and AST were assayed. Liver lesions and levels of NO, SOD, iNOS and malondialdehyde (MDA) were measured using standard procedures.

**Results:** Compared with the control group, the levels of NF- $\kappa$ B, TNF- $\alpha$ , IL-1 $\beta$ , IL-6, AST, ALT, NO, MDA and iNOS significantly increased in the sepsis group, while SOD level decreased significantly. In contrast, there were marked decreases in NF- $\kappa$ B, TNF- $\alpha$ , IL-1 $\beta$ , AST, ALT, NO, MDA and iNOS in puerarin group, relative to the sepsis group, while SOD expression level was significantly increased ( $p < 0.05$ ). The level of p-p38 in liver of septic rats was up-regulated, relative to control rats, while Nrf2 significantly decreased ( $p < 0.05$ ). The expression level of p-p38 in the puerarin group was significantly decreased, relative to the sepsis group, while the expression level of Nrf2 significantly increased ( $p < 0.05$ ).

**Conclusion:** Puerarin mitigates acute liver injury in septic rats by inhibiting NF- $\kappa$ B and p38 signaling pathway, down-regulating proinflammatory factors, and suppressing oxidative stress. Thus, puerarin may be developed for use in the treatment liver injury.

**Keywords:** Puerarin, Proinflammatory factors, Oxidative stress, Sepsis, Acute liver injury

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## INTRODUCTION

Sepsis, a systemic inflammatory response syndrome, is one of the serious complications of life-threatening diseases such as severe infection, shock and trauma. It is a disease diagnosed frequently in hospital intensive care

units. Sepsis is of sudden onset and rapid development, and it is associated with increasing morbidity and mortality [1]. Advances in medical diagnosis have led to improvements in early diagnosis of sepsis and effective treatment of sepsis patients. However, sepsis-related

mortality is still high, a situation which seriously threatens the lives and health of patients.

The liver is an important organ for metabolism of nutrients [2]. Moreover, it is the largest endothelial phagocytic system which activates and releases a variety of cytokines, and plays an important role in initiating multi-organ failure due to sepsis. Studies have confirmed that the liver is one of the most affected organs in the septic state. Sepsis causes early liver injury, decreased liver function and liver cell damage which result in multiple organ failure [3]. However, the mechanism involved in development of sepsis-related liver injury is not yet clearly understood.

Puerarin is an isoflavone which has been shown to improve microcirculation and protection against *myocardial ischemia* [4]. Studies have found that puerarin exerts hepatoprotective effect by lowering inflammatory reactions and reducing oxidation-induced damage [5]. This research was carried out to investigate the influence of puerarin on acute liver injury in rats with sepsis.

## EXPERIMENTAL

### Animals

A total of 87 healthy male SD rats with mean body weight of  $204 \pm 18$  g were randomly selected. The rats were obtained from Zhuhai Baixiantong Biotechnology Co. Ltd [production license = SCXK (Guangdong) 2020-005; use license = SYXK (Guangdong) 2020-0229]. They were fed adaptively for 1 week at laboratory temperature of  $23 \pm 4$  °C and humidity of  $50 \pm 12$  %, in an environment with 12-h light/12-h dark photoperiod. This study received approval from the Animal Ethics Committee of Affiliated Hospital of Medical College of Ningbo University, and was performed in line with "Principles of Laboratory Animal Care" [6].

### Main equipment and reagents used

The major instruments and reagents used, and their sources (in brackets) were: low-temperature, high-speed centrifuge (Yancheng Kaite Experimental Instrument Co. Ltd, model TGL18M); electronic balance (Shanghai Lichen Instrument Technology Co. Ltd, model CP124C); -80 °C ultra-low temperature refrigerator (Jinan Zhuolong Biotechnology Co. Ltd., model: BDF-86H458); paraffin slicing machine (Shenyang Hengsong Technology Co. Ltd., model: HS-S7220-B); H & E staining kit (Solaport Technology Co. Ltd.); electron microscope (Beijing Jingkeluida Technology Co. Ltd., model: EM208S eyepiece), and puerarin (Jiangxi

Zhongshan Pharmaceutical Co. Ltd., batch No. : 20184384, specification: 2 mL, equivalent to 100 mg puerarin).

### Establishment of rat model of sepsis

Three groups of 29 rats were used: control, sepsis and puerarin groups. A longitudinal cut was made along the main central axis of the lower abdomen of each rat in the supine position under anesthesia. The abdominal cavity was opened to expose the cecum. The lower blood vessels of the cecum were separated and ligated. The center of the cecum was pierced with a needle, and the contents of the cecum were gently squeezed to overflow into the abdominal cavity. Then, the cecum incision was sutured layer by layer. In postoperative rats, drowsiness, decreased feed intake or refusal to eat, low urine output, lethargy, abscess in the abdominal intestinal duct, bleeding and necrosis of the cecal wall, were evidence of successful establishment of sepsis. Rats in sham group had their abdomen opened only, without ligation and perforation. Rats in the puerarin group received the drug at a dose of 80 mg/kg, while rats in sham and sepsis groups were given normal saline in place of drug.

Rat cardiac blood (3 mL) was taken at 12, 24 and 48 h, and the serum samples obtained after centrifugation were placed in cryogenic refrigerator at 80 °C prior to analysis.

### ELISA

Expressions of TNF- $\alpha$ , NF- $\kappa$ B, IL-1 $\beta$  and IL-6 were determined using ELISA.

### Biochemical analysis

Serum levels of alanine aminotransferase (ALT) and aspartate (AST) were determined using automatic biochemical analyzer (Indiko™, Thermo Scientific, USA).

### Histopathological examination

Histopathological changes in liver were determined with hematoxylin and eosin (H & E) staining. Following sacrifice of the rats, the liver tissues were excised and routinely processed into paraffin sections, dewaxed with xylene, hydrated with gradient alcohol, and subjected to hematoxylin staining for 15 min, followed by staining with eosin dye for 3 min. The stained sections were rinsed with phosphate buffer, dried at room temperature, dehydrated, cleared, sealed with neutral gum, and observed and recorded under a light microscope.

**Table 1:** Serum inflammatory factors in the two groups (mean  $\pm$  SD)

Group	Time	NF-kB (ng/mL)	TNF- $\alpha$ (pg/mL)	IL-1 $\beta$ (pg/mL)	IL-6 (pg/mL)
Control	12 h	6.07 $\pm$ 0.36	6.34 $\pm$ 1.61	3.40 $\pm$ 0.58	2.40 $\pm$ 0.77
	24 h	6.11 $\pm$ 0.40	6.91 $\pm$ 1.27	3.35 $\pm$ 0.55	2.41 $\pm$ 0.63
	48 h	6.15 $\pm$ 0.43	7.02 $\pm$ 1.16	3.39 $\pm$ 0.61	2.43 $\pm$ 0.68
Sepsis	12 h	8.58 $\pm$ 0.47 <sup>a</sup>	37.42 $\pm$ 4.57 <sup>a</sup>	55.74 $\pm$ 2.99 <sup>a</sup>	30.54 $\pm$ 2.50 <sup>a</sup>
	24 h	11.10 $\pm$ 0.45 <sup>ab</sup>	24.35 $\pm$ 6.02 <sup>ab</sup>	39.06 $\pm$ 3.78 <sup>ab</sup>	24.66 $\pm$ 2.03 <sup>ab</sup>
	48 h	14.36 $\pm$ 0.46 <sup>abc</sup>	18.57 $\pm$ 2.33 <sup>abc</sup>	17.27 $\pm$ 2.64 <sup>abc</sup>	17.27 $\pm$ 2.64 <sup>abc</sup>
Puerarin	12 h	6.87 $\pm$ 0.49 <sup>abcd</sup>	29.47 $\pm$ 4.54 <sup>abcd</sup>	46.71 $\pm$ 1.09 <sup>abcd</sup>	25.13 $\pm$ 1.75 <sup>abcd</sup>
	24 h	8.79 $\pm$ 0.49 <sup>abcde</sup>	15.39 $\pm$ 4.51 <sup>abcde</sup>	20.95 $\pm$ 2.51 <sup>abcde</sup>	17.56 $\pm$ 2.11 <sup>abcde</sup>
	48 h	11.58 $\pm$ 0.51 <sup>abcdef</sup>	9.25 $\pm$ 1.28 <sup>abcdef</sup>	11.83 $\pm$ 2.27 <sup>abcdef</sup>	10.43 $\pm$ 1.29 <sup>abcdef</sup>

<sup>a</sup> $P < 0.05$ , vs the control at 12 h (<sup>a</sup>); vs the control at 24 h (<sup>b</sup>); vs the control at 48 h (<sup>c</sup>); vs septic group at 12 h (<sup>d</sup>); 24 h (<sup>e</sup>) and 48 h (<sup>f</sup>)

### Assessment of oxidative stress indicators

Oxidative stress indicators were also determined. The levels of NO and SOD in liver tissues of rats were determined with nitrate reductase and xanthine oxidase, respectively, while iNOS and malondialdehyde (MDA) levels were determined with thiobarbituric acid method.

### Determination of protein expressions

The protein expression levels of p-p38 and nuclear factor E2 p45-related factor2 (NRF2) in liver tissues of each group were determined using Western blotting. Total protein was extracted from liver tissue, and protein level of the lysate was determined with BCA method. Then, the protein was resolved with SDS-polyacrylamide gel electrophoresis PAGE and transferred to PVDF membrane. The membrane was incubated overnight at 4 °C with primary antibodies for p-p38 and NRF2, followed by incubation with HRP-linked secondary antibody for 1 h at room temperature. The bands were subjected to ECL, and images were acquired and stored using a gel imaging system.

### Statistical analysis

The SPSS20.0 software package was used for statistical analysis. Differences between two groups with respect to measurement data for serum inflammatory factors, oxidative stress, biochemical indices and other indices, were statistically evaluated with independent sample *t*-test. Results of statistical analysis were considered significant at  $p < 0.5$ .

## RESULTS

### Serum inflammatory factors

As shown in Table 1, compared with the control group at all periods, pro-inflammatory factors in septic rats were markedly up-regulated, but the expressions of these factors were markedly

reduced in puerarin group at all periods, relative to the sepsis group.

### Serum AST and ALT levels

Compared with the control group at each time point, the levels of AST and ALT in the sepsis group were significantly increased ( $p < 0.05$ ). However, AST and ALT levels in the puerarin group were significantly decreased at all periods, when compared with septic group (Table 2).

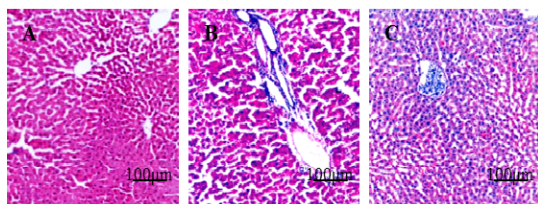
**Table 2:** Levels of AST and ALT in the two groups (mean  $\pm$  SD)

Group	Time (h)	AST (ng/mL)	ALT (U/L)
Control	12	30.20 $\pm$ 1.57	49.78 $\pm$ 5.19
	24	29.91 $\pm$ 1.97	49.97 $\pm$ 5.05
	48	30.03 $\pm$ 1.28	50.03 $\pm$ 5.52
Sepsis	12	55.39 $\pm$ 1.03 <sup>a</sup>	121.77 $\pm$ 11.70 <sup>a</sup>
	24	60.17 $\pm$ 1.19 <sup>ab</sup>	107.85 $\pm$ 6.59 <sup>ab</sup>
	48	74.65 $\pm$ 3.67 <sup>abc</sup>	98.63 $\pm$ 6.60 <sup>abc</sup>
Puerarin	12	47.57 $\pm$ 5.68 <sup>abcd</sup>	101.08 $\pm$ 7.86 <sup>abcd</sup>
	24	50.76 $\pm$ 1.59 <sup>abcde</sup>	82.14 $\pm$ 7.48 <sup>abcde</sup>
	48	63.65 $\pm$ 2.49 <sup>abcdef</sup>	72.42 $\pm$ 6.65 <sup>abcdef</sup>

$P < 0.05$ , vs the control at 12 h (<sup>a</sup>); vs the control at 24 h (<sup>b</sup>); vs the control at 48 h (<sup>c</sup>); vs septic group at 12 h (<sup>d</sup>), 24 h (<sup>e</sup>) and 48 h (<sup>f</sup>)

### Histopathological changes in rat liver

The liver tissues subjected to histological analysis were only those from rats treated for 48 h. The hepatocytes of the control group were intact and orderly, with centered nuclei, and nucleoli clearly visible. No fibrosis or inflammatory exudation was observed. In contrast, the structure of liver cells from sepsis rats was disorganized, with evidence of diffuse vacuolar degeneration, massive infiltration of inflammatory cells, and focal necrosis. Compared with sepsis group, the structures of hepatocytes from the puerarin group were improved significantly. These results are shown in Figure 1.



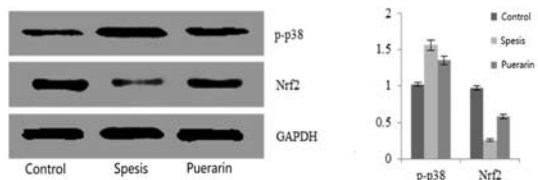
**Figure 1:** Histopathological features of liver tissue in each group. A: control group; B: sepsis group; C: puerarin group. (H & E; ×200)

**Oxidative stress**

The amounts of NO, MDA and iNOS in septic rats were significantly raised at each time point, while SOD levels of SOD were markedly decreased, relative to control rats. However, the levels of NO, MDA and iNOS in puerarin group at each time were significantly decreased, while that of SOD was significantly increased, relative to the sepsis group ( $p < 0.05$ ). The results are presented in Table 3.

**P-p38 and Nrf2 expression levels in liver tissue of rats**

As shown in Figure 2, the expression levels of p-p38 and Nrf2 in liver tissue of rats in sepsis group were significantly increased, relative to the control group ( $p < 0.05$ ). However, the expression levels of p-p38 and Nrf2 in puerarin group were markedly decreased, relative to septic group.



**Figure 2:** Comparison of the expression levels of p-p38 and Nrf2 in liver tissue of rats amongst the groups

**Table 3:** Comparison of oxidative stress indices of rats (mean ± SD)

Group	Time (h)	NO (µmol/g)	MDA (nmol/mg)	iNOS (U/mg)	SOD (U/mg)
Control	12	2.78±0.81	4.84±0.06	0.87±0.16	145.04±5.08
	24	2.67±0.77	4.94±0.09	0.85±0.17	145.61±5.08
	48	2.63±0.78	4.95±0.08	0.84±0.18	149.07±6.11
Sepsis	12	8.40±1.04 <sup>a</sup>	9.05±0.09 <sup>a</sup>	2.16±0.69 <sup>a</sup>	107.23±5.97 <sup>a</sup>
	24	6.62±1.03 <sup>ab</sup>	11.04±0.08 <sup>ab</sup>	1.63±0.35 <sup>ab</sup>	94.19±6.17 <sup>ab</sup>
	48	5.02±1.17 <sup>abc</sup>	14.18±0.09 <sup>abc</sup>	1.12±0.42 <sup>abc</sup>	89.52±5.83 <sup>abc</sup>
Puerarin	12	7.20±1.05 <sup>abcd</sup>	7.17±0.22 <sup>abcd</sup>	1.78±0.61 <sup>abcd</sup>	116.86±6.66 <sup>abcd</sup>
	24	6.08±1.12 <sup>abcde</sup>	8.71±0.30 <sup>abcde</sup>	0.94±0.27 <sup>abcde</sup>	99.63±4.36 <sup>abcde</sup>
	48	4.57±0.86 <sup>abcdef</sup>	9.16±0.23 <sup>abcdef</sup>	0.84±0.22 <sup>abcdef</sup>	94.41±3.45 <sup>abcdef</sup>

$P < 0.05$ , vs the control at 12 h (<sup>a</sup>); vs the control at 24 h (<sup>b</sup>); vs the control at 48 h (<sup>c</sup>); vs septic group at 12 h (<sup>d</sup>), 24 h (<sup>e</sup>) and 48 h (<sup>f</sup>)

**DISCUSSION**

Sepsis is a systemic inflammatory reaction which is the basis of multiple organ dysfunction syndrome, and it has been included amongst top ten reasons for death in patients. The liver is an important detoxification organ of the human body. Studies have found that the deterioration of liver function can be used as a predictor of severe symptoms and poor prognosis in critically ill sepsis patients. In addition, septic liver failure induces multiple organ dysfunction syndrome, which seriously threatens the lives of patients [7,8]. Therefore, early intervention in acute sepsis liver injury plays an important role in delaying the occurrence of liver failure and improving the prognosis of patients.

Traditional Chinese medicine (TCM) has a unique approach to the treatment of sepsis, bleeding and coagulation. The important treatment methods in TCM involve *clearing away heat* and detoxification, promoting blood circulation and *removing blood stasis* [9]. Puerarin is an isoflavone which dilates coronary artery, reduces myocardial oxygen consumption and improves microcirculation. In addition, some studies have found that puerarin inhibits platelet aggregation and scavenges oxygen free radicals [10,11]. In this study, puerarin was used to treat septic rats, with the aim of studying its effect on acute liver injury, and the mechanism involved.

It has been reported that sepsis may cause liver cell injury because bacterial endotoxins stimulate macrophages to induce formation of TNF-α, IL-6 and other pro-inflammatory cytokines during sepsis, thereby inducing liver cell injury [12]. When sepsis occurs, inflammatory coagulation processes are activated, and the two promote each other, thereby causing deficiency of blood and oxygen supply in the microcirculation.

It has been shown that TNF- $\alpha$  is one of the most important proinflammatory cytokines with the fastest response, earliest release and the most extensive cytotoxic effects, while NF- $\kappa$ B is a transcription factor widely found in various cells in the body. Sepsis enhances innate or adaptive immune responses by activating NF- $\kappa$ B and p38 signaling pathways, thereby mediating increases in TNF- $\alpha$ , IL-6 and IL-1 $\beta$  [13]. In addition, sepsis induces the release of other inflammatory factors, leading to systemic multiple organ dysfunction. The transaminases AST and ALT are important indicators of liver function impairment. The results of this study showed that puerarin significantly mitigated liver injury caused by sepsis in rats, due to inhibition of pro-inflammatory cytokines. Increases in levels of oxygen free radicals trigger oxidation-induced lesions. Changes in levels of NO, MDA, iNOS and SOD are important indices that reflect the level of oxygen free radicals *in vivo*. Studies have found that sepsis is often associated with dysfunctions in multiple organs, including heart, liver, kidney and lungs. Oxidation-induced injury may be crucial in multiple organ dysfunction [14]. The production of NO is catalyzed by NOS which exists in three forms: nNOS, iNOS and eNOS. Nitric oxide (NO) is a free radical with strong reactivity. It has been reported that in sepsis, NO inhibited the synthesis of total protein and glycogen in liver cells, causing a direct impact on liver metabolic function [15]. Some scholars have reported that sepsis significantly increased the level of MDA due to damage to the integrity of membranes and impairment of the function of membrane proteins, leading to disorders in energy metabolism [16]. Superoxide dismutase (SOD) is an antioxidant enzyme, the level of which is negatively correlated with the level of oxidative stress, while NRF2 is a receptor of oxidative stress and a core transcriptional regulator of the endogenous antioxidant system. The results of this study suggest that the puerarin-induced mitigation of acute liver injury in sepsis rats is linked to the inhibition of oxidative damage.

## CONCLUSION

Puerarin alleviates acute liver injury in sepsis rats by inhibiting NF- $\kappa$ B/p38 signaling pathway, and suppression of the levels of pro-inflammatory factors and oxidative stress. Thus, puerarin may be used to treat liver injury.

## DECLARATIONS

### Conflict of Interest

No conflict of interest associated with this work.

## Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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