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*Original Article*

# Protective role of puerarin on LPS/D-Gal induced acute liver injury via restoring autophagy

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**Abstract:** Acute liver injury is a destructive liver disorder resulting from overwhelming liver inflammation, oxidative stress and hepatocyte death. Puerarin is a natural flavonoid compound isolated from the traditional Chinese herb radix puerariae. This study investigated the protective effects of puerarin against lipopolysaccharide (LPS)/D-galactosamine (D-Gal)-induced liver injury and the potential mechanisms in mice. Mice were given an intraperitoneal administration of puerarin 200 mg/kg 2 h prior to LPS (50 μg/kg)/D-Gal (400 mg/kg) injection and were sacrificed 6 h post LPS/D-Gal treatment. The results showed that administration of puerarin substantially alleviated LPS/D-Gal-induced acute liver injury in mice by increased survival rates, improved liver histopathology, reduced plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, alleviated production of pro-inflammatory cytokines, and suppressed hepatocyte apoptosis. Moreover, puerarin pretreatment activated autophagy by increased the ratio of LC3B-II/I and the protein levels of Beclin-1, decreased the levels of p62 protein expression. Taken together, these findings demonstrated that puerarin could prevent the LPS/D-Gal-induced liver injury in mice, and its mechanisms might be associated with the increments of autophagy and suppression of apoptosis.

**Keywords:** Puerarin, lipopolysaccharide (LPS), acute liver injury, autophagy, apoptosis

## Introduction

Acute liver injury is a dramatic clinical syndrome generally caused by drugs abuse, viruses infection, heavy alcohol consumption, as well as other reasons [1]. This highly destructive disorder is associated with a high mortality rate because there are no effective preventions or therapeutic strategies [2]. Therefore, the development of novel and effective hepatoprotective agents or activities is urgently needed. An animal model of acute liver injury, established by a combination of Lipopolysaccharide (LPS) and D-Galactosamine (D-Gal), has been commonly used as a mature platform to investigate the mechanisms underlying clinical liver disease and to develop effective hepatoprotective approaches [3]. LPS can stimulate Kupffer cells to produce inflammatory factors such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and monocyte chemotactic protein 1 (MCP-1), which subsequently induce hepatocyte apoptosis and necrosis. D-Gal is a hepatotoxic agent, which inhibits RNA and protein synthesis and enhances the hepatotoxicity of LPS [4, 5].

Autophagy is an evolutionarily conserved process in which long-lived proteins and damaged organelles are degraded in lysosomes [6]. Autophagy has been widely recognized as a crucial modulator of cell survival and homeostasis, while autophagy suppression can cause all kinds of pathological diseases in different tissues [7]. Accumulating evidences have demonstrated the critical association between autophagy and liver diseases, including acute liver injury [6, 8]. Importantly, activation of autophagy by pharmacotherapy can protect against acute liver injury induced by various stimulations [9]. Thus, strategies designed to resolve the dysfunction of autophagy seem to be beneficial for the treatment of acute liver injury.

Puerarin is a natural flavonoid compound that isolated from the traditional Chinese herb radix puerariae. Recent studies have revealed that puerarin possesses various pharmacological actions, including anti-oxidant, anti-inflammatory, cardioprotective, anti-cancer and anti-diabetic properties [10-12]. Researchers have reported that puerarin showed hepatoprotective effects in liver damage induced by alcohol, concanavalin A and carbon tetrachloride stimulation [13, 14]. However, the protective effect of puerarin against LPS/D-Gal-induced liver injury has not been reported. The aim of this study is to investigate the protective effects and potential mechanisms of puerarin on LPS/D-Gal-induced liver injury in mice.

## Materials and methods

### *Chemicals and reagents*

Puerarin, Lipopolysaccharide (LPS), D-galactosamine (D-Gal) and all other chemicals were purchased from Sigma-Aldrich (Shanghai, China). Superoxide Dismutase (SOD), Malondialdehyde (MDA), Alanine Transaminase (ALT) and Aspartate Transaminase (AST) commercial assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Antibodies against LC3B, Beclin-1, p62 and GAPDH were purchased from Cell Signaling Technology (Shanghai, China). HRP-conjugated goat anti-rabbit and goat anti-mouse antibodies were obtained from Thermo Fisher Scientific (Shanghai, China).

### *Animals and treatments*

Male C57BL/6 mice were obtained from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China). All animals were maintained under controlled environment (temperature of 22 ± 2°C, humidity at 58 ± 3%) and allowed to water ad libitum in 12 h light/dark cycles. All experimental protocols were performed in accordance with the guidelines approved by the Xiamen University Animal Research Committee to ensure the humane animal care and use. LPS/D-Gal-induced acute liver injury was performed in mice as previously reported [5]. Briefly, mice were intraperitoneally injected with LPS (50 μg/kg) and D-Gal (400 mg/kg) to induce acute liver injury or with saline in the control mice. The vehicle (10% Tween-80 + 10% PEG-400 + 80% saline) or a single dose of puerarin (200 mg/kg dissolved in vehicle) was administered 2 h prior to LPS/D-Gal injection. Mice were sacrificed 6 h after LPS/D-Gal injection. In the survival experiment, mice treated with LPS/D-Gal + puerarin or LPS/D-Gal + vehicle were monitored every 2 h basis for 36 h.

### *Liver histological analysis*

Fresh liver biopsy specimens were collected from mice and fixed in 4% paraformaldehyde, embedded in paraffin, sectioned into 5 μm thick slices using a sliding microtome (Leica, Shanghai, China), and stained with hematoxylin and eosin to examine the alteration of liver histological structures under standard light microscopy (Nikon, Shanghai, China).

### *TUNEL assay*

Apoptotic hepatocytes were identified using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay kit (Roche, Shanghai, China) according to the manufacturer's instructions, and followed by nuclear staining with 4'-6-diamidino-2-phenylindole (DAPI) (Vector Lab, Shenzhen, China). Stained areas were viewed and imaged under a fluorescence microscope (Olympus, Shanghai, China).

### *Plasma biochemistry analysis and hepatic oxidative stress assay*

Plasma ALT, AST concentrations and the levels of SOD, MDA in liver tissues were determined by a Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific, Shanghai, China) with commercial kits according to the manufacturer's instructions.

### *Western blotting*

Protein samples from liver homogenates were extracted using RIPA lysis buffer (Millipore, Beijing, China) containing protease inhibitor (Roche, Shanghai, China), and were quantified using the BCA protein assay kit (Thermo Fisher Scientific, Shanghai, China) based on the manufacturer's instructions. The proteins were separated by SDS-PAGE, transferred onto PVDF membranes, which were blocked and then incubated with primary antibodies at 4°C overnight. After washed thrice with Tris-buffered saline and Tween-20 buffer, the membranes were probed with the HRP-conjugated second-

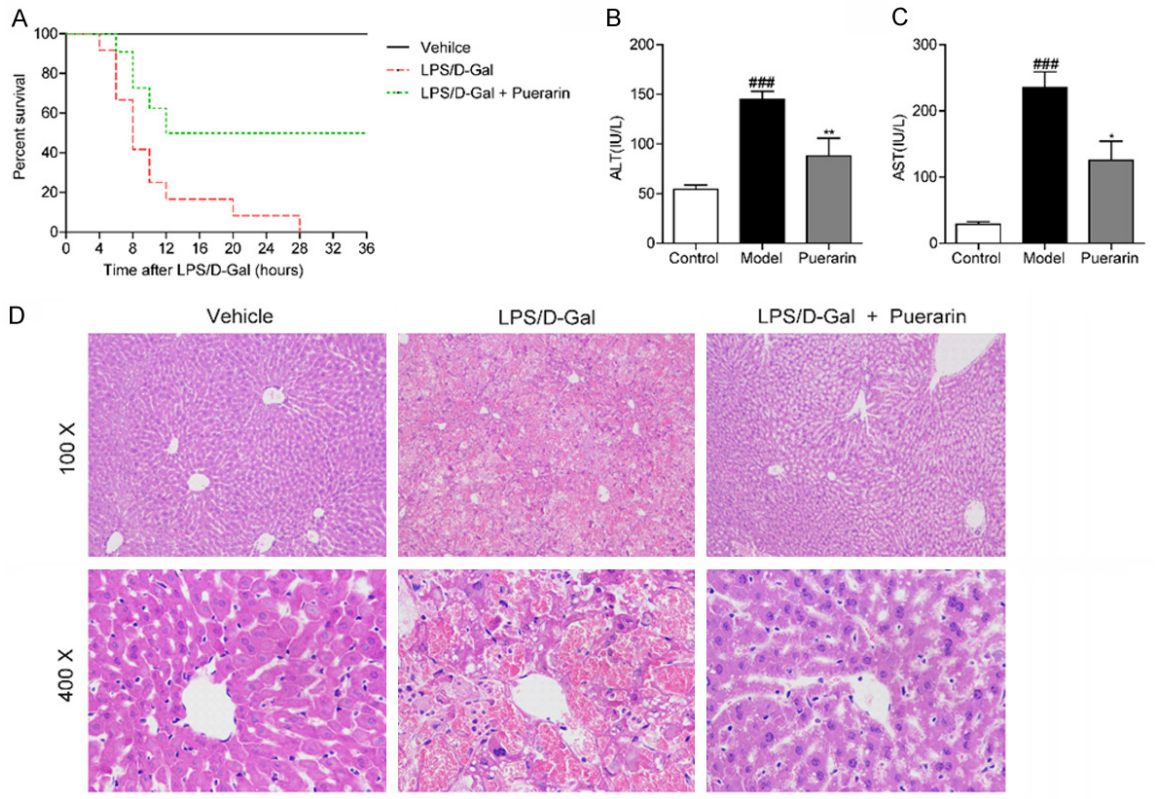


**Figure 1.** Puerarin attenuated LPS/D-Gal-induced acute liver injury. (A) Percent survival curve of mice following administration of LPS/D-Gal with or without puerarin treatment. mortality of mice was assessed every 2 h for 36 h. (B, C) Plasma ALT (A) and AST (B) levels in mice treated with Vehicle (Control group), LPS/D-Gal (Model group), LPS/D-Gal + puerarin (Puerarin group) for 6 h. (D) Hematoxylin-eosin (HE) staining (original magnifications, ×100, ×400) of liver tissues from mice treated with Vehicle, LPS/D-Gal, LPS/D-Gal + puerarin for 6 h. Data are shown as the mean ± SEM, n = 6-8 in each group. ###$P < 0.001$, *$P < 0.05$, **$P < 0.01$.

ary antibodies for 1 h at room temperature. Finally, protein bands were visualized by the enhanced chemiluminescence (ECL) detection kit and exposed to X-ray film (Kodak, Shanghai, China). The relative expression levels of target proteins were quantitated using Image J software and standardized to GAPDH protein levels.

*RNA isolation, cDNA synthesis and real time PCR*

The total RNA from liver tissues of mice was extracted using the TRIzol reagent (Invitrogen, Shanghai, China) following the manufacturer's protocol. cDNA was synthesized using a FastQuant RT kit (Tiangen, Beijing, China) according to the manufacturer's instructions. For Real time PCR, SYBR® Premix Ex Taq™ II (Takara, Dalian, China) was used and carried out on a Roche LightCycler 480 Real time PCR system, and mRNA levels were normalized relative to the levels of β-actin.

*Stasistical analysis*

GraphPad Prism version 5.0 software was used for all statistical calculations. Data were expressed as mean ± SEM. Statistical analysis was performed via one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test. $P < 0.05$ was accepted to be statistically significant.

## Results

*Puerarin exerted protective effects in LPS/D-Gal-induced acute liver injury mice*

To investigate the effect of puerarin on LPS/D-Gal-induced mortality, the survival rate was observed every 2 h within 36 h post LPS/D-Gal treatment. As shown in **Figure 1A**, the mice began to die 4 h after LPS/D-Gal injection, and the mice of the LPS/D-Gal group were all died at 28 h. However, the mice received puerarin pretreatment 2 h before LPS/D-Gal administra-

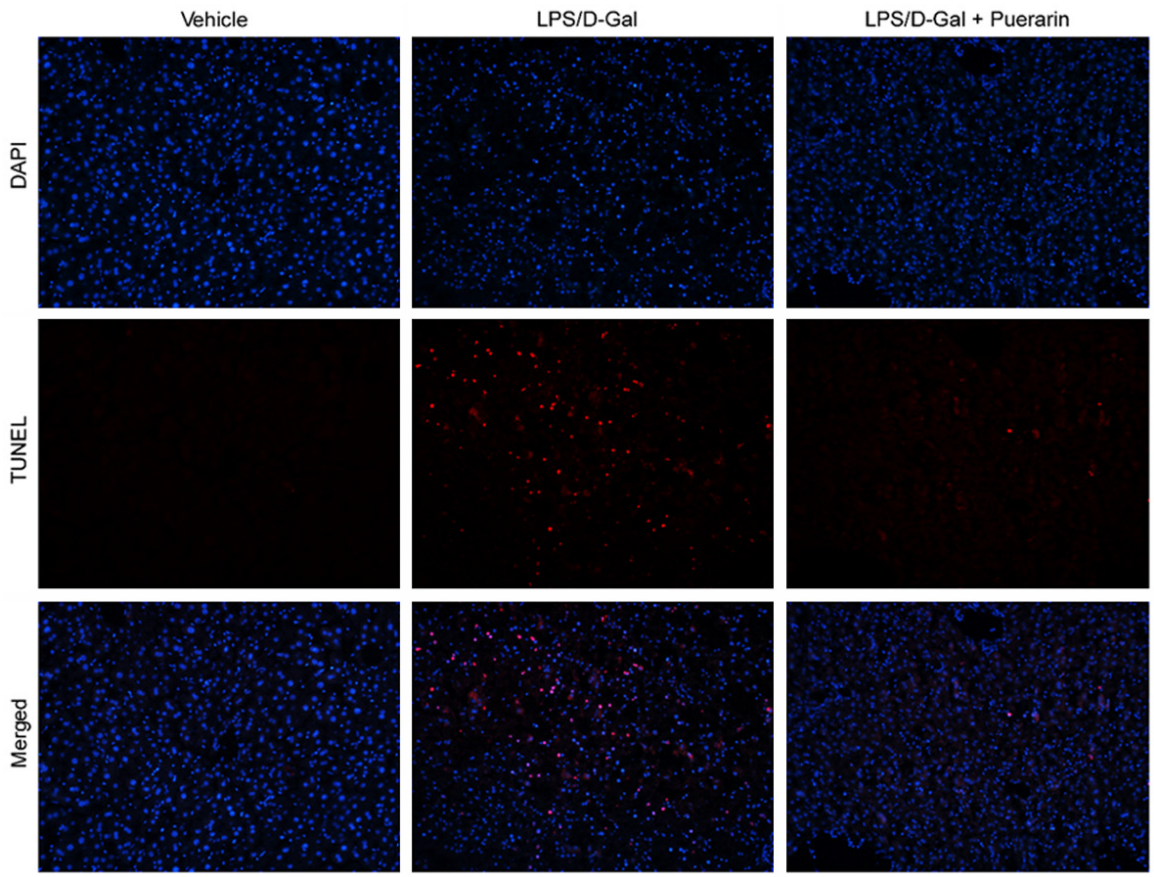


**Figure 2.** Puerarin pretreatment suppressed hepatic apoptosis upon LPS/D-Gal stimulation. TUNEL staining (original magnifications, ×200) of apoptotic hepatocytes counterstained with DAPI in liver tissue sections from mice treated with Vehicle (Control group), LPS/D-Gal (Model group), LPS/D-Gal + puerarin (Puerarin group) for 6 h.

tion had a significantly higher survival rate, and the survival rate was remained 58% at the end time point of this experiment. The results indicated that puerarin pretreatment could significantly improve the survival rate of LPS/D-Gal treated mice.

The plasma levels of ALT and AST activities, two important biochemical markers of liver failure, were determined to further confirm the protective effect of puerarin on LPS/D-Gal-induced liver injury [15]. As shown in **Figure 1B** and **1C**, plasma ALT and AST levels were dramatically increased in the LPS/D-Gal group compared with the control group ($P < 0.001$). However, puerarin pretreatment significantly reduced the plasma ALT ($P < 0.01$) and AST ($P < 0.05$) levels than in the LPS/D-Gal group.

To further evaluate the protective effects of puerarin on LPS/D-Gal-induced liver injury in mice, we assessed the histological changes 6 h post LPS/D-Gal stimulation. As shown in **Figure 1D**, liver tissues of the control group showed normal liver architecture. Liver tissues of LPS/D-Gal group showed abnormal pathological changes, including severe liver structure destruction, cytoplasmic vacuolization, extensive hemorrhage, and obvious inflammatory cell infiltration. However, puerarin treatment significantly mitigated the pathological process in the liver of LPS/D-Gal treated mice, as indicated by the well-organized hepatic lobular architecture and significantly decreased inflammatory cell infiltration. Those results above indicated that puerarin was effective in protecting mice against LPS/D-Gal-induced acute liver injury.

*Puerarin suppressed LPS/D-Gal-induced hepatocyte apoptosis in mice*

Hepatocyte apoptosis has been proved an important event during the development of acute liver injury [16]. To determine the effect

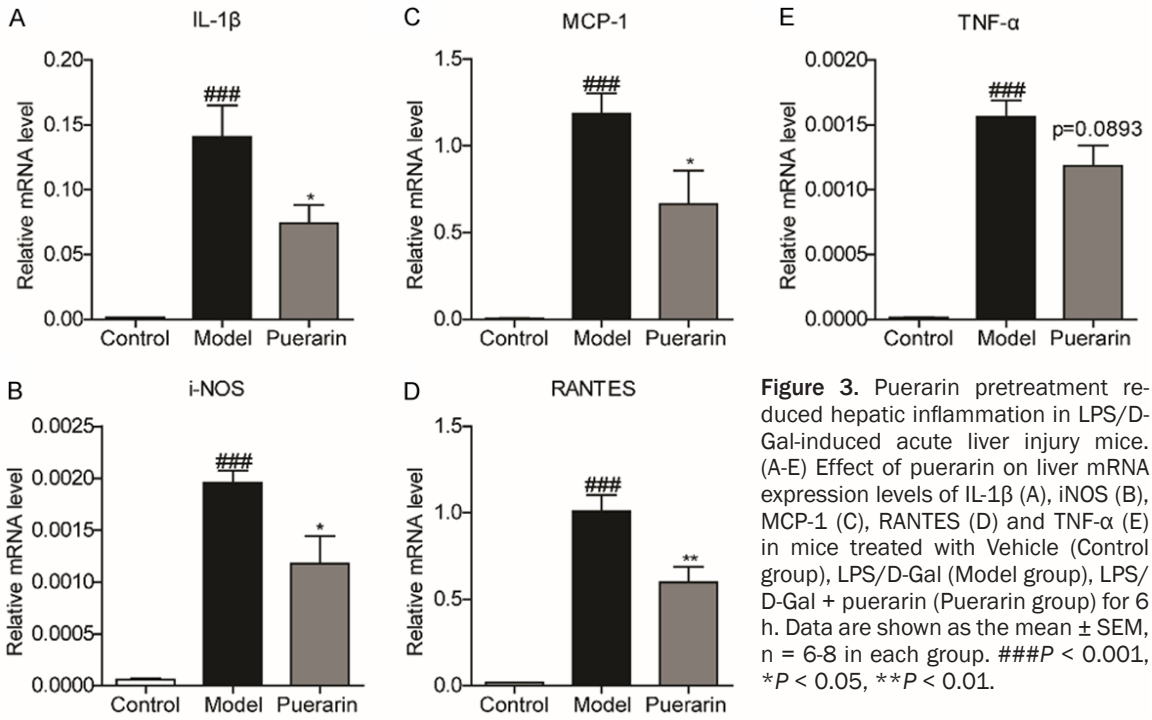


**Figure 3.** Puerarin pretreatment reduced hepatic inflammation in LPS/D-Gal-induced acute liver injury mice. (A-E) Effect of puerarin on liver mRNA expression levels of IL-1β (A), iNOS (B), MCP-1 (C), RANTES (D) and TNF-α (E) in mice treated with Vehicle (Control group), LPS/D-Gal (Model group), LPS/D-Gal + puerarin (Puerarin group) for 6 h. Data are shown as the mean ± SEM, n = 6-8 in each group. ###*P* < 0.001, **P* < 0.05, ***P* < 0.01.

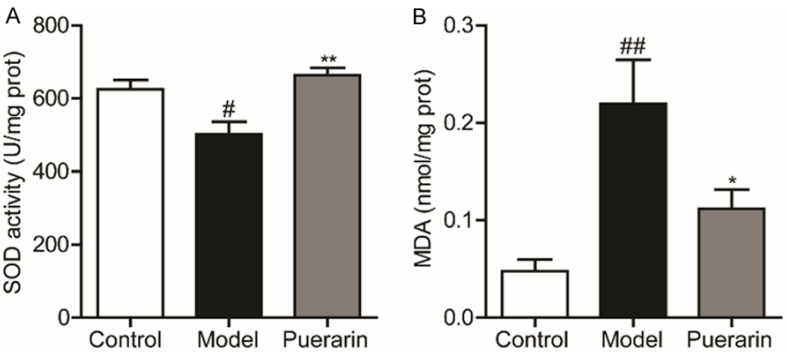


**Figure 4.** Puerarin ameliorated hepatic oxidative stress in LPS/D-Gal-induced acute liver injury mice. (A, B) Effect of puerarin on hepatic SOD activity (A) and MDA levels (B) in mice treated with Vehicle (Control group), LPS/D-Gal (Model group), LPS/D-Gal + puerarin (Puerarin group) for 6 h. Data are shown as the mean ± SEM, n = 6-8 in each group. #*P* < 0.05, ##*P* < 0.01, **P* < 0.05, ***P* < 0.01.

of puerarin on hepatocyte apoptosis induced by LPS/D-Gal, we performed TUNEL staining to locate and quantify the number of apoptotic cells in liver tissue sections. As shown in **Figure 2**, the number of TUNEL-positive hepatocytes in LPS/D-Gal group was significantly higher than the control group, while puerarin treatment effectively reduced the number of TUNEL-positive hepatocytes, indicating that puerarin significantly suppressed LPS/D-Gal induced hepatocyte apoptosis in mice.

*Puerarin inhibited hepatic inflammatory response induced by LPS/D-Gal injection in mice*

Hepatic inflammatory response plays critical roles in the progression and development of acute liver injury induced by LPS/D-Gal [5]. Here, we found that the expression of inflammatory mediators of IL-1β (**Figure 3A**), iNOS (**Figure 3B**), MCP-1 (**Figure 3C**) and RANTES (**Figure 3D**) in liver tissues were considerably higher in LPS/D-Gal group compare to the control group (*P* < 0.001). However, puerarin treatment dramatically reduced the expression of these inflammatory genes (*P* < 0.05, *P* < 0.05, *P* < 0.05, *P* < 0.01). Moreover, the hepatic expression of TNF-α (**Figure 3E**) was also upregulated post 6 h LPS/D-Gal treatment (*P* < 0.001), while no significant difference was observed in the puerarin treatment group compare to the LPS/D-Gal group (*P* = 0.0893). The results above indicated that puerarin could ameliorate LPS/D-Gal-induced hepatic inflammatory response.

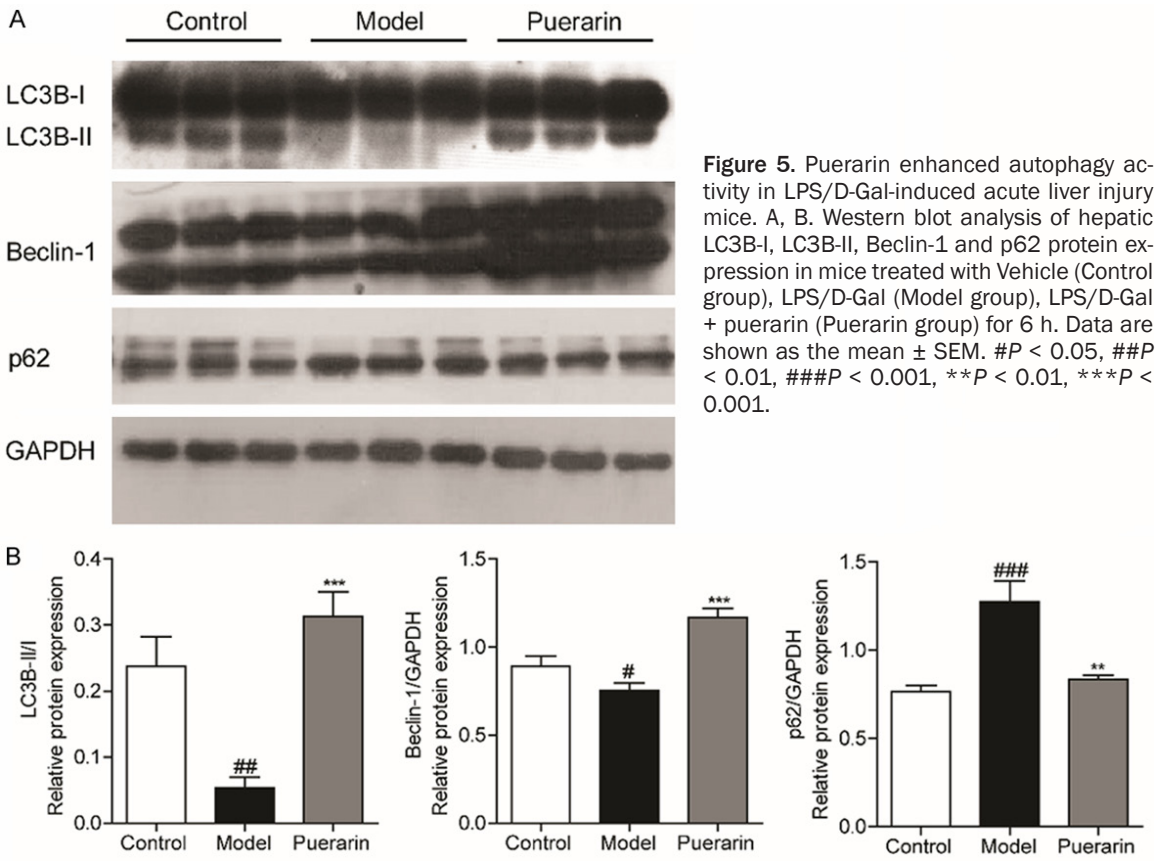


**Figure 5.** Puerarin enhanced autophagy activity in LPS/D-Gal-induced acute liver injury mice. A, B. Western blot analysis of hepatic LC3B-I, LC3B-II, Beclin-1 and p62 protein expression in mice treated with Vehicle (Control group), LPS/D-Gal (Model group), LPS/D-Gal + puerarin (Puerarin group) for 6 h. Data are shown as the mean ± SEM. #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001, *****P* < 0.01, ******P* < 0.001.

*Effects of puerarin on LPS/D-Gal induced oxidative stress*

Anti-oxidant system is suggested to be associated with pathological process of liver injury [17]. To investigate the effects of puerarin on LPS/D-Gal induced oxidative stress, liver SOD activities and MDA levels were detected at 6 h post LPS/D-Gal injection. As shown in **Figure 4A**, hepatic SOD activities significantly decreased in LPS/D-Gal group than in the control group, by contrast, LPS/D-Gal treatment resulted in a significant increase of hepatic MDA levels (**Figure 4B**). Additionally, puerarin pretreatment significantly reversed the decrease of SOD activities and the increase of MDA levels. These results suggested that puerarin could attenuate LPS/D-Gal-induced oxidative stress.

*Puerarin activated autophagy in LPS/D-Gal treated mice*

To explore the mechanism of puerarin on reducing LPS/D-Gal-induced acute liver injury, we detected the autophagy related protein levels in liver tissues. As shown in **Figure 5**, the ratio of LC3B-II/I expression and the protein levels of Beclin-1 were decreased in LPS/D-Gal group, while p62 protein levels were increased upon LPS/D-Gal treatment. However, puerarin treatment upregulated the levels of LC3II/I and Beclin-1, and down-regulated the expression of p62. Thus, the mechanism of puerarin protect against acute liver injury might associated with hepatocytes autophagy activation.

## Discussion

Previous studies have shown that puerarin has anti-oxidant and anti-inflammatory effects [18, 19]. In the present study, we found that puerarin treatment significantly attenuated LPS/D-Gal-induced high mortality, liver histological destructions, hepatic inflammatory response, as well as down-regulated ALT, AST levels. Furthermore, puerarin was found to promote autophagy and suppress hepatocyte apoptosis. These experimental results demonstrated the protective role of puerarin on LPS/D-Gal-induced acute liver injury in mice.

Inflammatory response has been reported as a major contributing factor, leading to liver injury under various stimulations [20]. LPS can acti-

vate Kupffer cells, leading to the release and accumulation of proinflammatory cytokines. Indeed, various reports showed that regulation of the inflammatory response is an attractive potential strategy in the treatment of acute liver injury [5, 21, 22]. Considering that puerarin has been proved anti-inflammation effect in some reports, and we have also reported the anti-inflammatory effect of puerarin in cultured endothelial cells, the possible molecular mechanisms regarding to inflammatory response were investigated in this study [12, 23, 24]. In line with previous studies, our results showed that LPS/D-Gal induced a significant increase of hepatic proinflammatory factors, including IL-1β, iNOS, MCP-1, RANTES and TNF-α. Puerarin treatment attenuated the increases of IL-1β, INOS, MCP-1 and RANTES but not TNF-α. TNF-α has been reported the most important inflammatory cytokine during the progress and development of acute liver injury [25]. However, in our study, no significant difference was observed between the LPS/D-Gal group and LPS/D-Gal + puerarin group, indicating that puerarin treatment attenuated liver injury might not due to regulation of TNF-α expression.

Oxidative stress is a pivotal activity to the onset of liver injury [26]. Previous studies have shown that LPS/D-Gal treatment leading to the impair of anti-oxidant capacity and resulted in elevated MDA, which is one of the most important biomarker of oxidative stress in liver [27]. In our study, similar alteration of hepatic MDA was observed in LPS/D-Gal treated mice, indicated severe oxidative stress in liver. SOD, an antioxidative enzyme, was widely known as the main constituent of protective enzyme system benefit for oxidative injury. Furthermore, we observed a significant decrease in SOD activity post LPS/D-Gal injection, which is in accordance with previously published reports [28, 29]. Our results showed that puerarin treatment significantly counteracted the LPS/D-Gal induced oxidative stress in liver, as characterized by suppressed MDA level and enhanced SOD activity.

Emerging evidences strongly suggest that the massive apoptosis of hepatocytes is a common symptom of acute liver injury induced by LPS/D-Gal [16, 30]. The anti-apoptotic effect of puerarin has been reported in neurocytes of a Parkinson's disease model [24]. In this study, a marked increase of TUNEL-positive hepatocytes was found in LPS/D-Gal treated mice, while puerarin pretreatment significantly reversed the LPS/D-Gal-induced apoptosis, indicating the anti-apoptotic effects of puerarin in hepatocytes of LPS/D-Gal-induced acute liver injury mice. It has been reported that the proinflammatory factor TNF-α played a key role in the induction of hepatocytes apoptosis during the progress of LPS/D-Gal-induced acute liver injury [31]. However, in the present study, we found no significant reduction of hepatic TNF-α expression in LPS/D-Gal-challenged mice pretreated with puerarin, indicating that anti-apoptotic effect of puerarin may not dependent on the regulation of TNF-α production.

Autophagy is an adaptive lysosomal degradation pathway through which damaged proteins and organelles are degraded and recycled [6]. Numerous manuscripts have reported the relationship between autophagy and cell death when the cells are subjected to stress [8, 32]. In recent years, accumulating evidences have indicated that autophagy plays an important role in the pathogenesis of acute liver injury, because autophagy suppression could promote the progression and development of acute liver injury [6, 8]. Moreover, it is reported that in the hepatocyte-specific autophagy related gene 5 (Atg5) or Atg7 knockout mice, LPS/D-Gal injection increased hepatocytes apoptosis and liver histological injury, suggesting that basal autophagy is critical to maintain hepatocytes survival and liver homeostasis [32, 33]. Most importantly, activation of autophagy could protect against LPS/D-Gal induced acute liver injury by suppressing apoptosis and inhibiting inflammatory response [9, 34]. The transformation from LC3B-I to LC3B-II has been reported the most important biomarker of autophagy activation. Autophagic flux can be assessed by combining measurement of the ratio of LCB-II/LC3B-I and the protein levels of Beclin-1 and p62 [9]. In the present study, we found that the ratio of LC3B-II/LC3B-I and the levels of Beclin-1 were decreased with the levels of p62 increased in LPS/D-Gal treated mice, which indicated an inhibition of autophagy. By contrast, western blot results demonstrated that puerarin treatment could activate autophagy through increase LC3B-II/LC3B-I conversion, Beclin-1 expression and p62 degradation. Those results in our study indicated that LPS/D-Gal inactivated the autophagic

response, and puerarin could reactivate autophagy in the progression of LPS/D-Gal induced acute liver injury. Therefore, the protective activity of puerarin against LPS/D-Gal induced acute liver injury, at least in part, by restoring the impaired autophagy.

In conclusion, the results of this study demonstrated that puerarin is effective in protecting the liver against LPS/D-Gal-induced acute liver injury. The mechanisms of the protection appeared to be activating autophagy, thus suppressing apoptosis and inhibiting inflammatory response. Hence, future study on mechanisms of how puerarin activate autophagy may help promote the development of an effective therapeutic agent against acute liver injury.

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## Disclosure of conflict of interest

None.

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