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Geniposide protected hepatocytes from acetaminophen hepatotoxicity by down-regulating CYP 2E1 expression and inhibiting TLR 4/NF-κB signaling pathway

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ABSTRACT

Acetaminophen (APAP) is a widely used over-the-counter drug for antipyretic and analgesic, but an overdose will induce acute liver injury. APAP hepatotoxicity has been the most common cause of acute liver failure in western countries with high morbidity and mortality. Geniposide (GP), an iridoid glycoside extracted from the fruit of *Gardenia jasminoides*, has been reported to exert a profound anti-inflammatory activity on acute and chronic diseases. However, it is never demonstrated whether GP can protect hepatocytes from APAP hepatotoxicity. In this study, we investigated the protective effect and underlying mechanism of GP against AILI. The results showed that GP pretreatment reduced the levels of ALT and AST in a dose-dependent manner and alleviated hepatocyte necrosis and apoptosis in mice exposed at APAP. Moreover, it suppressed the expression of CYP 2E1 and attenuated the exhaustion of GSH and accumulation of MDA in the liver. Furthermore, GP remarkably inhibited inflammatory cells infiltration and mitigated the release of IL-1β and TNF-α, and inhibited Toll-like receptor 4 (TLR4) expression and nuclear factor kappa (NF-κB) activation. These data suggested that GP could effectively protect hepatocytes from APAP hepatotoxicity through the down-regulation of CYP 2E1 expression and the inhibition of TLR4/NF-κB signaling pathway.

1. Introduction

Acetaminophen (APAP) is a widely used antipyretic and analgesic drug used to relieve symptoms such as pain and fever at the safe therapeutic doses [1]. However, excessive intake of APAP can lead to severe liver injury, which has become the most frequent cause of drug-induced acute liver injury In the USA and much of western Europe [2,3]. What is worse, the limited therapeutic strategy used for treatment is frequently subject to the unsatisfactory effect. Currently, *N*-acetyl-cysteine (NAC) is the optimum approach to treating the APAP-induced liver injury in the clinic but the efficacy is uncertain for the obscure phage of APAP hepatotoxicity and a part of patients still gradually turn to liver failure [4]. Undoubtedly, it is indeed required to distinguish new drugs with excellent security and effectiveness to render those patients to recover and avoid liver failure.

Normally, the major part of APAP is eliminated by UDP-glucuronosyltransferase (UGT) and sulfotransferase (SULT) in the form of phenolic glucuronide and sulfate conjugations which are subsequently excreted into urine and bile, while a small proportion of APAP is oxidized by cytochrome P450 (CYP450) enzymes to *N*-acetyl-p-benzoquinone imine (NAPQI), an oxidizing intermediate, which reacts with glutathione (GSH) under the catalyzation of glutathione transferase for detoxification [5,6]. The metabolism of APAP can be in a state of imbalance after APAP overdose, which leads to the predomination of CYP450 in APAP biotransformation and overproduction of NAPQI [6]. It is worthy to note that CYP 2E1, one of CYP450 family member, is the most active in metabolism from APAP to the toxic intermediate, and the upregulation of CYP 2E1 appears after APAP overdose [7]. NAPQI overload not only exhausts GSH in liver but also has a covalent combination of cellular proteins especially mitochondrial proteins, which

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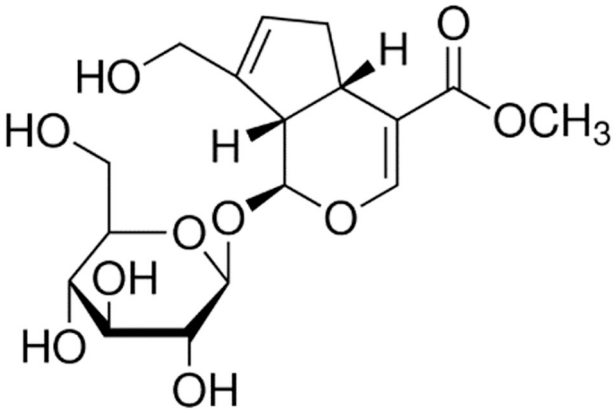

Fig. 1. The chemical structure of GP.

makes mitochondria dysfunction, ATP depletion, the extensive formation of reactive oxygen species (ROS), and hepatocyte necrosis and apoptosis, ultimately leading to acute liver injury [7,8]. The damage and death of hepatocytes induced by metabolism imbalance are initial events in APAP hepatotoxicity, which lead to local and small-scale injury to the liver. However, the secondary attack is a more fatal phase in APAP-induced liver injury, which is stimulated by damage-associated molecular patterns (DAMPs) released by lysed hepatocytes, bonding and activating pattern recognition receptors (PRRs) on immune cells, like Toll-like receptors (TLRs), and then evoking the fulminant inflammation via cascade amplification [9]. Among the TLRs, TLR4 is the crucial sensor to transmit an inflammatory signal, which provokes the release of inflammatory mediators and initiate the migration and infiltration of inflammatory cells to the lesion [10]. Previous studies have reported that TLR4 knockout and utilizing antagonist to TLR4 can alleviate acute liver injury by APAP exposure [11,12], which indicates that weakening TLR4 signaling pathway is an effective target to protect the liver, and it plays a critical role in the model of APAP hepatotoxicity.

Geniposide (GP, Fig. 1), an iridoid glycoside purified from the fruit of *Gardenia jasminoides Ellis*, is known to have a wide spectrum of anti-inflammatory, anti-oxidative and anti-tumor effects [13–16]. GP has been demonstrated that it inhibits the inflammatory responses in TNBS-induced experimental rat colitis [17]. In addition, GP alleviates carbon tetrachloride-induced acute liver injury by suppressing MeCP2 in mice with and LPS-treated THP-1 cells [18]. Moreover, GP has inhibition on the TLR4/NF-κB signaling pathway and production of TNF-α, IL-1β and IL-6 in LPS-induced lung injury [19]. However, whether it is effective for APAP hepatotoxicity and the underlying mechanisms of these effects on the inflammation remain unclear. Thus, we investigated the protective efficacy of GP on APAP-induced liver injury by assessing the alleviation of hepatocytes necrosis and apoptosis, the expression of CYP 2E1, oxidative stress, inflammatory cytokines level, the infiltration of inflammatory cells, and the TLR4/NF-κB signaling pathway. Ultimately, our study revealed that GP alleviated APAP-induced liver injury and the protective effect was related to the suppression of the CYP 2E1 expression and oxidative stress, and the inhibition of TLR4/NF-κB signaling pathway appearing as the amelioration of inflammatory cells infiltration and cytokines release.

2. Materials and methods

2.1. Chemicals and reagents

Geniposide (C17H24O10, MW: 388.37, purity: > 98%) was purchased from Aladdin Industrial Corporation (Shanghai, China). APAP was obtained from Sangon Biotech (Shanghai, China). Kits for measurement of alanine aminotransferase (ALT), aspartate aminotransferase (AST), Glutathione (GSH) and Malondialdehyde (MDA) were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Caspase 3 colorimetric assay kit was purchased from Beyotime Institute of Biotechnology (Jiangsu, China). The enzyme-linked immunosorbent assay (ELISA) kits for detecting TNF-α and IL-1β were obtained from Bender Med Systems (Vienna, Austria). Rabbit anti-mouse TLR4 and CYP 2E1 antibodies were purchased from Abcam (Cambridge, UK). Rabbit anti-mouse phospho–IRAK1, phospho–IκB, and phospho-NFκB p65 were purchased from Cell Signaling Technology (Boston, MA, USA). Mouse anti-GAPDH was supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Bicinchoninic acid (BCA) protein assay kit, horseradish peroxidase-conjugated goat anti-rabbit antibody and enhanced chemiluminescent (ECL) reagents were obtained from Pierce Biotechnology (Rockford, IL, USA).

2.2. Animals experiments

Male C57 mice, aged 6–8 weeks, weighing 18–22 g, were supplied by the Animal Center of Chongqing Medical University (Chongqing, China). The experimental animals were maintained under standard conditions of 20 to 25 °C temperature, 55% humidity and 12 h of a light/dark cycle and provided with regular feeding and free drinking. All experimental operations involving animals followed the procedures approved by the animal care and use committee of Chongqing medical university.

Mice fasted overnight but free to drink before the operation. APAP was dissolved in warm saline and intraperitoneally injected (350 mg/kg body weight) to induce acute liver injury. Within 24 h before APAP injection, GP (10, 30 or 100 mg/kg dissolved in 0.5% carboxymethylcellulose sodium) or vehicle was orally administered every 8 h, a total of 3 times. 18 h after APAP exposure, the mice were all sacrificed, and the blood was collected and centrifugated at 3000 RPM for 5 min to obtain serum. Liver tissues were fixed in 4% paraformaldehyde or saved under 80 °C for further analysis.

2.3. Serum aminotransferase activity measurement

ALT and AST activities in serum as the biomarkers of hepatocellular injury were measured with detection kits according to the manufacturer's instruction.

2.4. Histological analysis

The paraformaldehyde-fixed and paraffin-embedded liver slices were stained with hematoxylin and eosin and then observe the injury degree under light microscopy (Nikon, Tokyo, Japan).

2.5. Caspase 3 activity assay

The liver tissue was homogenized in cell lysis buffer and centrifuged at 10,000*g* for 1 min to collect the supernatant (100 g protein). The supernatant was incubated with Ac-DEVD-pNA substrate and reaction buffer at 37 °C for 90 min. The caspase 3 activity of every group was assayed at 405 nm, as well as normalized by the total protein concentration of the same sample.

2.6. Measurement of GSH and MDA

The content of GSH and MDA in liver tissues was detected to assess oxidative stress using detection kits following the manufacturer's instruction.

2.7. Western blot analysis

We homogenated the liver samples in the RIPA lysis buffer (Beyotime, Shanghai, China) and gain the supernatant by centrifuging

it at 12,000*g*, 4 °C for 10 min. Then the concentration of total protein was measured by the BCA protein assay kit (Pierce Chemical, Rockford, IL). Afterward, 40 μg total proteins were separated in 10% SDS-PAGE and transferred to nitrocellulose membrane. The membranes were blocked by 5% fat-free milk dissolved in Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBST) for 1 h at room temperature and then were incubated with primary antibody at 4 °C overnight. Subsequently, the followed incubation was operated with the HRP-conjugated secondary antibody for 1 h at room temperature. Finally, these were detected by an ECL chemiluminescent system and Image Lab software (Biorad, USA).

2.8. ELISA for cytokines

Liver tissues were collected to determine the concentration of TNF-α and IL-1β levels by ELISA kits according to the instructions of the manufacturer.

2.9. RT-qPCR

The extraction of total RNA was operated by homogenized liver tissues and RNA extraction reagent (Takara, Japan), according to the manufacturer's protocol. The complementary DNA (cDNA) samples of CYP 2E1 were synthesized for Real-Time PCR reaction. The sequences of CYP 2E1 primers were 5′-CGT TGC CTT GCT TGT CTG GA-3′ (sense) and 5′-AAG AAA GGA ATT GGG AAA GGT CC-3′ (antisense) and GAPDH primers were 5′-AGG TCG GTG TGA ACG GAT TTG-3′ (sense) and 5′-TGT AGA CCA TGT AGT TGA GGT CA-3′ (antisense), for the internal standard. At last, we adopt the $2-\Delta\Delta Ct$ method to calculate the relative expression level of CYP 2E1.

2.10. Flow cytometry for macrophages and neutrophils

Liver tissue of each group was ground and hydrolyzed with 0.02% IV collagenase at 37 °C to prepare a highly active cell suspension, which was filtered and centrifuged at 50*g* for 5 min to collect the supernatant. The cell precipitates were resuspended by phosphate buffer and incubated with PE-labeled anti-Ly6G or F4/80 antibody and FITC-labeled anti-CD45 or CD11b antibody in the condition of 4 °C and darkness. Eventually, the infiltration of macrophages and neutrophils was determined by Flow cytometry.

2.11. Statistical analysis

All data were expressed as means ± standard deviations (SD) and were assessed by one-way analysis of variance (ANOVA) and student's test. A P-value was < 0.05 to be considered statistically significant.

3. Results

3.1. GP alleviated the liver injury in mice exposed to APAP overdose

To investigate the protective effects of GP against APAP hepatotoxicity, the levels of serum ALT and AST were measured as biochemical markers to reflect the degree of liver injury. As shown in Fig. 2A and B, the activities of ALT and AST in the APAP group presented significant increases, compared to the control group ($P < 0.05$). However, GP (10, 30, or 100 mg/kg) pretreatment markedly declined the increases of ALT and AST in a dose-dependent manner, compared with the APAP group. Accordingly, these data demonstrated that GP pretreatment alleviated APAP-induced the liver injury caused by APAP. For the most visible improvement of GP at a dose of 100 mg/kg, we selected the dose to explore the further mechanism of GP.

3.2. GP dampened APAP-induced hepatic necrosis and apoptosis

To more comprehensively evaluate the protective effects of GP, we performed a histopathological approach and measured the activity of caspase 3 to assess the necrosis and apoptosis of hepatocytes. From the histological analysis by HE staining (Fig. 3A), the liver of the control group had normal lobular architecture and cell structure, while extensive hepatocellular necrosis and nuclear fragmentation occurred in the APAP group. As expected, GP pretreatment attenuated the pathological lesion in the liver of mice induced by APAP overdose. Moreover, as an important effector in the apoptotic cascade, caspase 3 activity was evaluated. As shown in Fig. 3B, APAP induced a robust increase in the caspase 3 activity of the liver, which was dramatically inhibited by GP pretreatment ($P < 0.01$).

3.3. GP suppressed the upregulation of the expression of CYP 2E1 in the liver of APAP-exposed mice

The expression of CYP 2E1 was detected by Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and Western blot. In control and GP groups, the expression of CYP 2E1 mRNA and protein was detected at low levels. When mice upon APAP overdose, there is a significant upregulation of CYP 2E1 expression in the liver. However, this upregulation was markedly inhibited by GP pretreatment (Fig. 4A and B).

3.4. GP refreshed GSH and attenuated MDA in the liver of APAP-exposed mice

To investigate GSH depletion and lipid peroxidation in the liver, the contents of GSH and MDA in the liver were measured. From Fig. 5A and B, the content of GSH was decreased after APAP administration, while GP pretreatment restored this decrease. In a meanwhile, APAP induced a significant elevation in the content of MDA, which was remarkedly diminished by GP pretreatment.

3.5. GP mitigated the release of pro-inflammatory cytokines in APAP-induced liver injury

IL-1β and TNF-α are the main cytokines to trigger and aggravate inflammation in APAP hepatotoxicity, the level of which in the liver were analyzed by ELISA kits. As shown in Fig. 6A and B, IL-1β and TNF-α levels in the APAP group were conspicuously augmented compared to the control group ($P < 0.01$). However, both of them were declined in GP pretreatment group when compared to the APAP group ($P < 0.01$).

3.6. GP ameliorated the infiltration of macrophages and neutrophils in APAP-induced liver injury

The trigger and amplification of inflammation in APAP hepatotoxicity are closely associated with recurrence and infiltration of inflammatory cells in the liver, especially macrophages and neutrophils, which is considered one of the most crucial events in liver injury. Data determined by Flow cytometry shown in Fig. 7A and B, $CD11b^+F4/80^+$ macrophages and $CD45^+Ly6G^+$ neutrophils were markedly increased in the liver of APAP-exposed mice compared to non-APAP-challenged animals. However, pretreatment with GP ameliorated the infiltration of macrophages and neutrophils into APAP-damaged liver.

3.7. GP inhibited the TLR4/NF-κB signaling activation in APAP-induced liver injury

TLR4/NF-κB signaling pathway plays a vital role in the cascade amplification of inflammation in acetaminophen hepatotoxicity, and its consistent activation is bound to lead to intense inflammatory reactions and severe injuries. Therefore, we investigated the situation of the

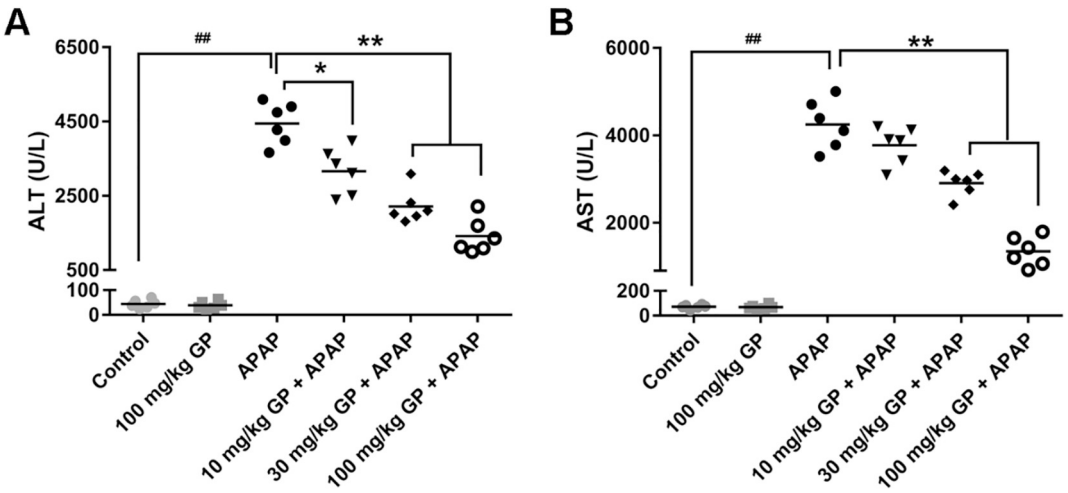

Fig. 2. GP alleviated the liver injury in mice exposed to APAP overdose. The activities of serum ALT (A) and AST (B) were determined at 18 h after APAP exposure. Data were expressed as mean ± SD, n = 6, $^{\#\#}P < 0.01$ compared with the control group, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with APAP group.

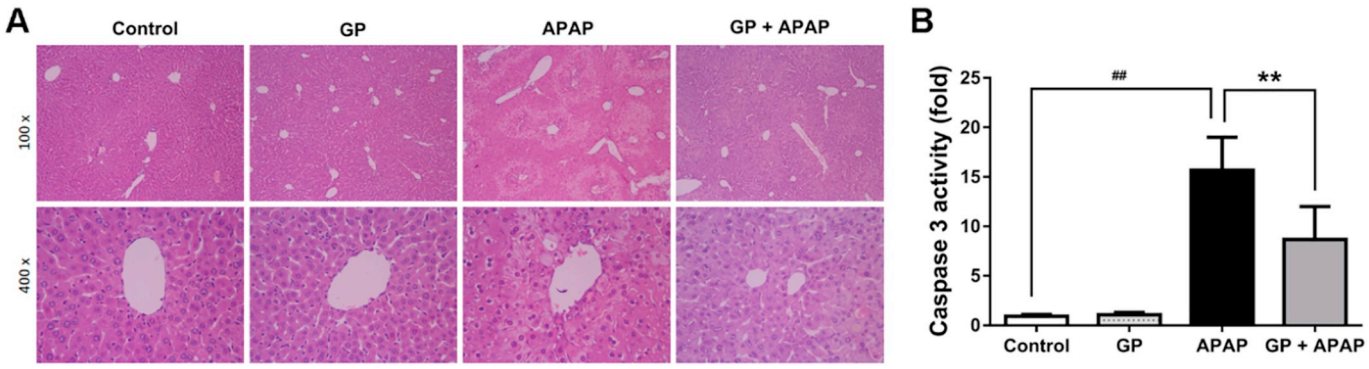

Fig. 3. GP dampened APAP-induced hepatic necrosis and apoptosis. (A) The liver pathological changes were evaluated by hematoxylin-eosin staining and observed under a microscope (200× magnifications). (B) The caspase 3 activity was determined. Data were expressed as mean ± SD, n = 6, $^{\#\#}P < 0.01$ compared with the control group, $^{**}P < 0.01$ compared with APAP group.

TLR4/NF-κB signaling pathway by Western blot. As shown in Fig. 8A and B, in the both control group and GP group, hepatic TLR4 protein expressed at a low level, but the activated downstream signal molecules of TLR4 such as p-IRAK1, p-IκB, and p-NF-κB (p-p65) were undetectable. Upon APAP overdose, as expected, TLR4 and its downstream signal molecules activation in the liver were significantly upregulated in mice compared to the control group. However, GP pretreatment markedly inhibited TLR4, p-IRAK1, p-IκB, and p-NF-κB expression and activation in the liver of mice challenged by APAP exposure. Notably, total NF-κB (p65) expression was unchanged in four different groups.

4. Discussion

Drug-induced liver failure is a global health problem and the treatment remains a great challenge, which poses a threat to human life [20]. What's more, APAP overdose has been the dominant cause of drug-induced liver injury, resulting in high morbidity and mortality

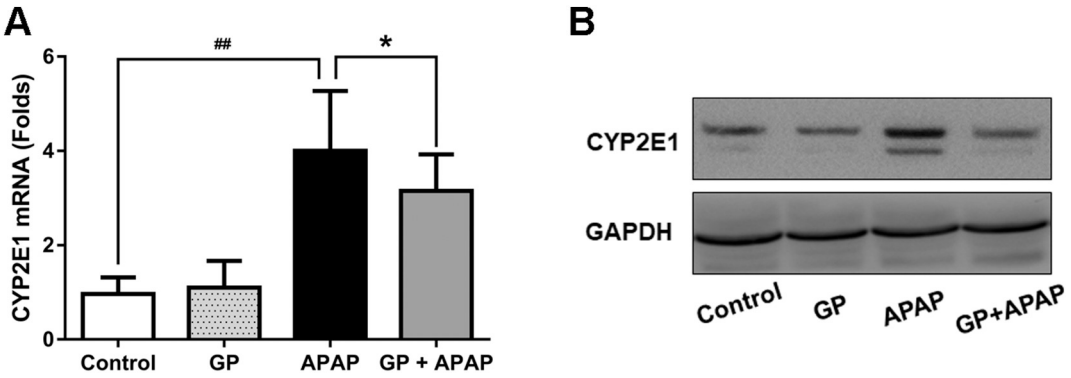

Fig. 4. GP suppressed the upregulation of the expression of CYP 2E1 in AILI mice. The mRNA of CYP 2E1 in the liver was evaluated by qRT-PCR (A) and the protein of CYP 2E1 was measured western blot (B). Data were expressed as mean ± SD, n = 6, $^{\#\#}P < 0.01$ compared with the control group, $^{*}P < 0.05$ compared with APAP group.

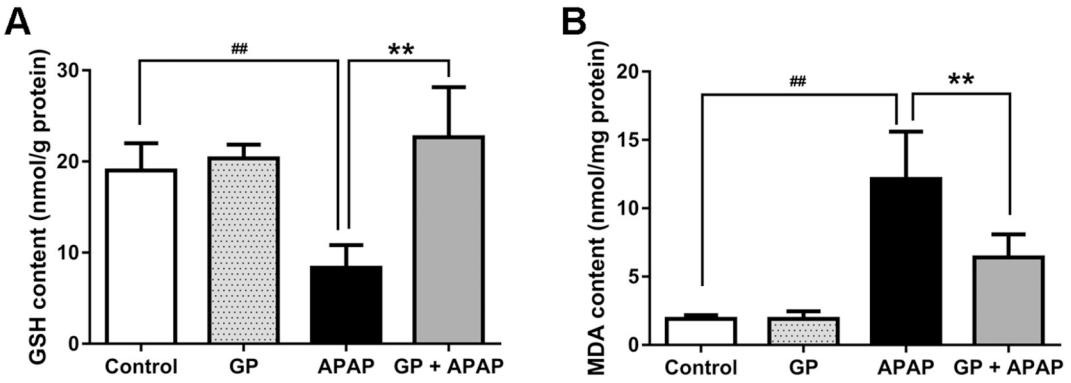

Fig. 5. GP refreshed GSH and attenuated MDA in APAP exposed mice. Hepatic GSH contents (A) and MDA (B) were determined at 18 h after APAP administration. Data were expressed as mean ± SD, n = 6, $^{\#\#}P < 0.01$ compared with the control group, $^{**}P < 0.01$ compared with APAP group.

through triggering acute liver injury (ALI) or even acute liver failure (ALF). Limitation on therapeutic strategies and deficiency of early diagnostic biomarkers hinder the breakthrough in the treatment of APAP-induced liver injury [1]. At present, the booming exploration of natural products has increasingly attached importance to herbal medicine in the treatment of APAP-induced liver injury. Accordingly, we investigated whether the GP pretreatment could protect the liver from APAP hepatotoxicity in mice. The results demonstrated that GP significantly reduced the activities of ALT and AST in serum, and markedly alleviated hepatic pathological damage in mice after APAP overdose, featuring as the diminution of necrosis and apoptosis of hepatocytes. In term of the mechanism, GP suppressed the upregulation of the CYP 2E1 expression, abated the oxidative stress and pro-inflammatory cytokines production, and mitigated the infiltration of macrophages and neutrophils in the liver of mice exposed APAP. Furthermore, this protective effect was evidently related to the inhibition of the TLR4/NF-κB signaling pathway.

Normally, APAP is metabolized by the drug metabolizing enzymes in parenchymal cells of the liver, depending on the UGT and SULT pathway. Excessive APAP saturates physiological metabolic pathways and turns to abnormal metabolism, causing the metabolic imbalance of APAP. The accumulation of intermediate NAPQI is provoked by the hyperactivity of the abnormal metabolic pathway, which is equal to a trigger event for the early injury. In an aspect, this toxic product requires GSH to scavenge as a combination form, resulting in its large-scale depletion, in another respect, the formation of protein adducts via reacting with sulfhydryl groups leads to mitochondrial dysfunction and cell death, as well as modulation of the innate immune system, which has been considered the major damage mechanism of APAP overdose [21]. Due to APAP-induced liver injury is dose-dependent, the output of NAPQI can be regarded as one of the decisive factors leading to liver damage [22]. Worthy to mention, CYPs are a group of enzymes mainly involving in the abnormal transformation from APAP to NAPQI, among which CYP 2E1 is the most active enzyme in producing of NAPQI. Moreover, an increase in CYP 2E1 expression was observed after APAP overdose in several studies [23,24], which was similarly proved by our results. The up-regulation of its expression accelerates the generation and accumulation of NQPQI, serving as an imperative factor in the promotion of APAP-induced liver injury. Interestingly, GP pretreatment impeded the increase of CYP 2E1 expression at mRNA and protein levels in the liver parenchymal cells. The obstacle to NAPQI overproduction exerts a crucial effect on alleviating the early injury of APAP hepatotoxicity, which is the embodiment of GP preventing APAP hepatotoxicity from the source.

After NAPQI overload, the covalent binding between intercellular protein and NAPQI, leads to mitochondrial dysfunction and ROS overproduction, inducing the first attack to hepatocytes. Meanwhile, the critical member of the antioxidant system, GSH is massively undermined after NAPQI overproduce, causing the deficiency of ROS clearance [8]. Accordingly, the accumulation of oxidative stress exhausts the antioxidant defense system and damages cellular macromolecules, such as lipids, DNA and proteins followed by the necrosis and apoptosis of hepatocytes [25]. Reducing the production of NAPQI can alleviate oxidative stress to prevent further injury of hepatocytes [26]. In our results, attributed to the reduced NAPQI generation by down-regulating CYP 2E1, GP significantly suppressed the oxidative stress, which was demonstrated by the restoration of GSH and diminution of MDA. In total, the prevention of GP in the metabolism imbalance phase depended on down-regulating CYP 2E1 expression in hepatocytes to lessen the production of NAPQI, causing the attenuation of oxidative stress.

(Since APAP is metabolized in liver parenchymal cells and GP inhibited the metabolism by CYP 2E1, GP can alleviate the generation of intercellular oxidative stress leading to necrosis and apoptosis)

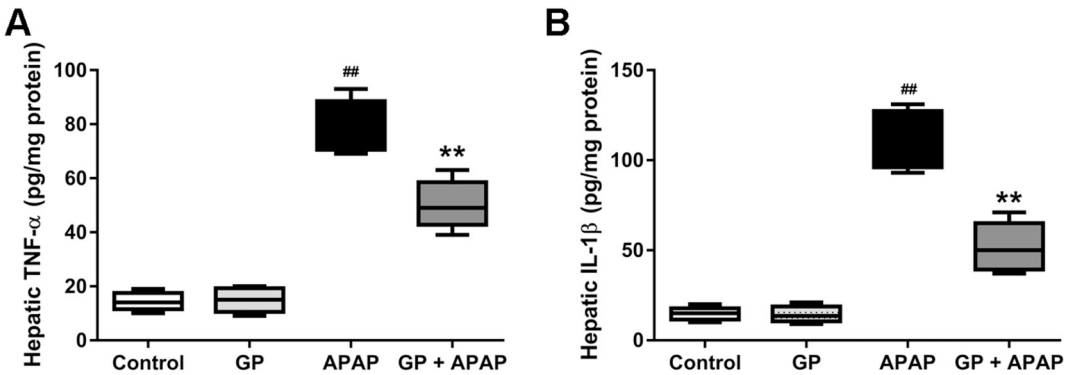

Fig. 6. GP mitigated the release of pro-inflammatory cytokines in APAP-induced liver injury. The serum TNF-α (A) and 1L-1β (B) were detected by ELISA. Data were expressed as mean ± SD, n = 6, $^{\#\#}P < 0.01$ compared with the control group, $^{**}P < 0.01$ compared with APAP group.

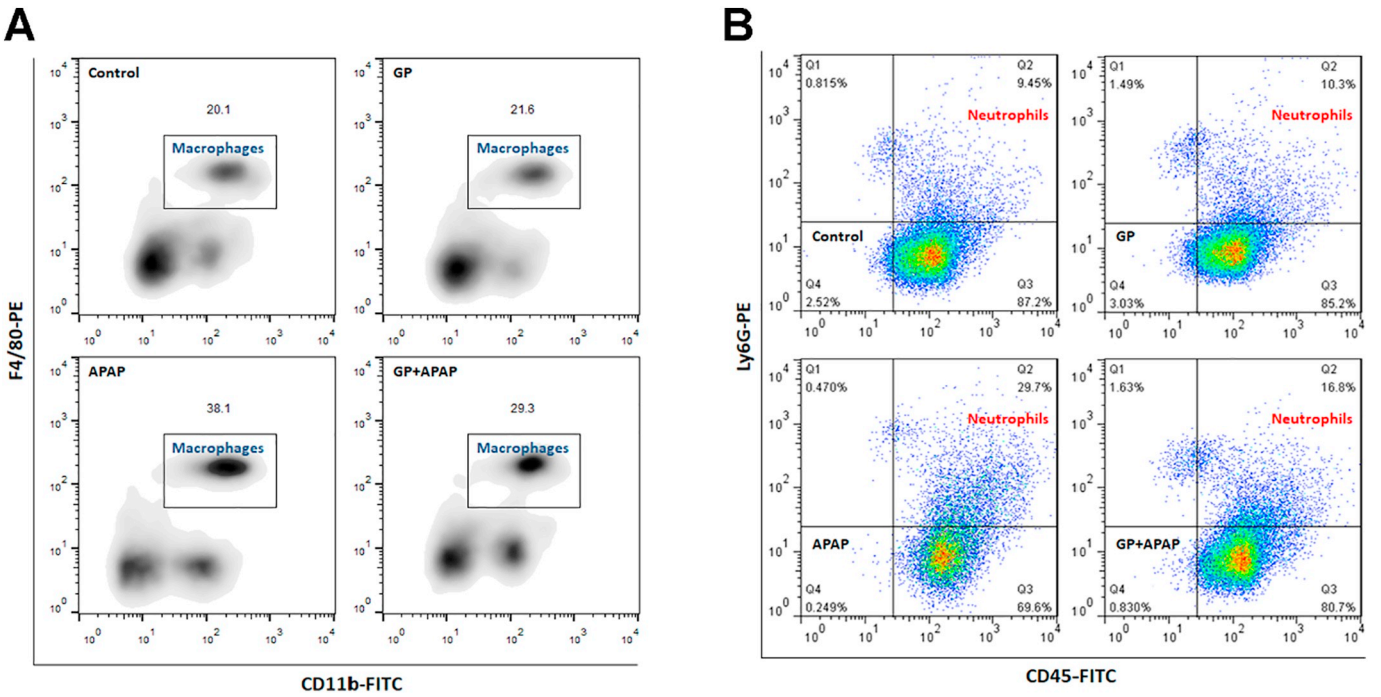

Fig. 7. GP ameliorated the infiltration of macrophages and neutrophils in APAP-induced liver injury. The infiltration of hepatic macrophages (A) and neutrophils (B) were detected by flow cytometry.

It is well known that hepatocyte death and subsequent inflammatory responses play critical roles in APAP hepatotoxicity [27]. Endogenous DAMPs such as HMGB1, DNA fragments, heat shock proteins, which are the cellular contents that are usually separated from the extracellular environment, are released from necrotic hepatocytes in response to APAP overdoses. They subsequently bind and activate pattern recognition receptors (PPRs) of the innate immune cells, triggering intense non-specific immune responses and severe sterile inflammation [28]. Therefore, hepatocyte necrosis, as the initiation of inflammation induced by APAP toxicity, is the key to the secondary injury. In our study, it is remarkable that GP alleviated the liver injury supported by decreased the levels of ALT and AST in serum, and lessened the necrosis of hepatocytes. Apart from necrosis, the apoptosis of hepatocyte is a critical event in the pathophysiology of APAP hepatotoxicity, although its role remains controversial. Data from previous studies suggest that necrosis rather than apoptosis is predominant in APAP-induced liver injury both in vivo and in vitro models, which is the mainstream view on AILI [29]. Previous studies demonstrated that typical necrotic morphology on the central region of the hepatic lobules, including vacuolation of cells, swelling of cells and organelles and nuclear fragmentation, but not typical histological features of apoptosis such as cell contraction, chromatin condensation, and formation of apoptotic bodies, are observed in the liver of APAP-exposed mice [30]. Besides, some studies have reported that the alteration of caspase 3 activity, an indicator of apoptosis, was undetectable in their APAP overdose models [31]. However, an increasing number of studies have elucidated the key role of apoptosis on APAP-induced liver injury [32]. Moreover, there were conspicuous changes of caspase 3 activity in our results, and GP pretreatment markedly mitigated APAP-induced the apoptosis with the decline of caspase 3 activity in the liver, which supported the view. In term of the discrepancy, present studies consider the difference in diet and nutrition as a major cause and propose a hypothesis related to ATP depletion [33,34]. Above all, the inhibition of necrosis and apoptosis could dampen the release of DAMPs, which was one of the underlying mechanisms that GP protect hepatocytes against the damage by APAP poisoning.

The occurrence of severe liver injury or even failure has a close correlation with the uncontrolled amplification of cascade inflammation, which consecutively is stimulated by diverse inflammatory activators [9]. The activators also called DAMPs and released from necrotic and apoptotic hepatocytes, are recognized by PRRs on the membrane of immune cells and activate them through distinct inflammatory signaling pathway to release various inflammatory mediators [28]. The activation of PRRs can have multiple effects, including the activation of inflammation signaling, the release of cytokines and chemokines [10]. Among the PRRs involving in the APAP hepatotoxicity, TLR4 plays a critical role in trigger action of cascade inflammation. TLR4 actuates and regulates innate immune inflammation by MyD88-dependent and TRIF-dependent pathways. In APAP hepatotoxicity, the intracellular region of activated TLR4 receptor TIR binds to the carboxyl terminus of MyD88, while the amino terminus of MyD88 binds to the amino terminus of IRAK to activate IKK complex, prompting the phosphorylation and ubiquitination of IκB [26,35]. NF-κB, a crucial nuclear transcription factor, consisting of p50, p52, p65, c-Rel, RelB, combines with IκB as a dimer in resting state. The degradation of IκB dissociates NFκB to and enters the nucleus to initiate the transcription of inflammation mediators [36]. It has been reported that the knockout or blockade of TLR4 can alleviate inflammatory response to protect the liver from APAP hepatotoxicity [11,12]. In the present study, a decline in the number of TLR4 and the phosphorylation of IRAK1, IκB and p65 and was observed, implicating that GP can protect the liver from APAP hepatotoxicity by inhibiting the activation of TLR4/NF-κB signaling pathway after APAP overdose exposure.

As the dominant pro-inflammatory cytokines in APAP-primed TLR4/NF-κB signaling pathway, TNF-α and IL-1β exert multiple pathological effects including the activation of immune cells, aggravation of inflammatory response, and exaggeration of liver injury [22]. In fact, TNF-α and IL-1β can also cause the synthesis and release of chemokines, which recruit specific types of innate immune cells to infiltrate damaged areas, such as macrophages and neutrophils, further amplifying the inflammatory response and exacerbating hepatocyte injury [9,37]. Recruitment and infiltration play a key role in the transition

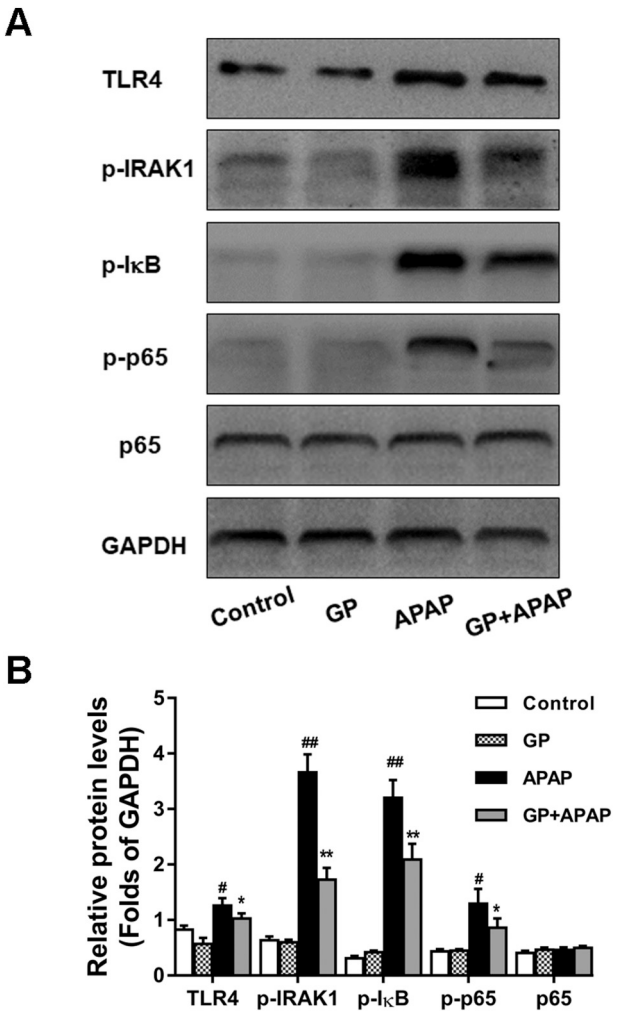

Fig. 8. GP inhibited the TLR4/NF-κB signaling pathway in APAP-induced liver injury. (A) The protein levels of TLR4, p-IRAK1, p-IκB, p-p65 in liver tissue were determined by Western blot analysis. (B) Quantitative results of indicated proteins by Western blotting analysis. Data were expressed as mean ± SD, n = 3, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the control group, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with APAP group.

from the metabolism imbalance to the secondary inflammatory cascade. Restriction on the infiltration of inflammatory cells can effectively alleviate the inflammation after APAP overdose [38,39]. In addition, in fact, considerable literature has illuminated that the Kupffer cells, a type of tissue-resided macrophages in the liver, are involved in the recruitment of monocytes and neutrophils in circulation to amplify inflammation, owing to repeatedly release pro-inflammatory factors and chemokines [40]. Besides, the activation of Kupffer cells depends on the TLR4/NF-κB signaling pathway. Therefore, GP pretreatment mitigated the levels of TNF-α and IL-1β and attenuated the infiltration of neutrophils and macrophages in the liver of APAP overdose by inhibiting the TLR4/NF-κB signaling pathway, which was the protective effect of GP in the late phase of APAP hepatotoxicity.

In conclusion, this study demonstrated that GP exerted the profound protective effect against APAP-induced liver injury. In the phase of metabolism imbalance, GP down-regulated CYP2E1 expression to reduce the production of NAPQI and ameliorate oxidative stress as well as restore GSH content, which attenuated hepatic pathological damage induced by APAP. In the phase of inflammation cascade, GP suppressed the release of inflammatory cytokines and alleviated the infiltration hepatic macrophages and neutrophils, which was closely associated with the inhibition of TLR4/NF-κB signaling pathway. However, the multiple effects of GP on oxidative stress, inflammation, and metabolic factors need to be further investigated. Taken together, these findings suggested that GP could protect the liver from APAP hepatotoxicity and was a potential candidate against for APAP overdose.

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Declaration of Competing Interest

The authors declare no conflict of interest to disclose.

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