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Rosmarinic acid protects mice from lipopolysaccharide/D-galactosamine-induced acute liver injury by inhibiting MAPKs/NF-κB and activating Nrf2/HO-1 signaling pathways

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ABSTRACT

Rosmarinic acid (RA) has antioxidation, anticancer, antibacterial, anti-inflammatory and various biological functions. In our study, we aim to evaluate effects of RA on acute liver injury caused by LPS and D-galactosamine (D-GalN) and its underlying molecular mechanism in mice. Our findings showed that RA could protect C57BL/6 mice from LPS/D-GalN-induced acute liver injury, which not only reflected on declining aspartate aminotransferase (AST) and alanine aminotransferase (ALT) of the serum, but also restrained the phosphorylation of nuclear factor-kappa B (NF-κB), extracellular signal-regulated kinase (ERK1/2) and p38 protein expression and the content of tissue myeloperoxidase (MPO) elevation. Moreover, RA could enhance the level of glutathione-dependent peroxidase (GSH-PX). Furthermore, RA promoted that nuclear factor erythroid-2-related factor 2 (Nrf2) transported into nucleus, and then up-regulated heme oxygenase 1 (HO-1), glutamate-cysteine ligase catalytic (GCLC), glutamate cysteine ligase modifier (GCLM) and quinone oxidoreductase (NQO1). These results indicated that RA could protect the mice from acute liver injury induced by LPS/D-GalN.

1. Introduction

Acute liver injury, which can lead to rapid loss of liver function, is a syndrome caused by different pathogenesis (including hepatic encephalopathy, as well as the injury of kidney and lung) [1]. Drug-induced liver injury is a main reason of acute liver injury [2]. In spite of rare morbidity, acute liver failure has high mortality without liver transplantation. The common reasons of death are multiorgan failure, hemorrhage, infection and cerebral edema [1]. It is widely considered that inflammation and oxidative stress play important roles in acute liver injury [3,4]. However, its pathogenesis is unclear, and there is no specific medicine for acute liver injury. Thus, searching for the effective drug becomes increasingly imperative. It has been reported that LPS can exacerbate liver fibrosis [5], and D-galactosamine is a sensitinogen that can lead to the lethal effects of tumor necrosis factor (TNF) [6], which is associated with liver failure [7]. Mice are injected with LPS/D-GalN which results in acute liver injury, and will die in 5–9 h [8]. The AST and ALT in serum, the important biomarker, normally can be used to reflect the liver injury induced by drug toxicity, viral infection, alcohol abuse and fatty liver [9]. Although the aminotransferase has less relation with liver injury, it is acceptable that ALT and AST were released to blood after the hepatocyte membrane injured [10,11].

MPO, an enzyme in heme peroxidase family, can release to extracellular fluid under the stimulation of inflammation. Despite the MPO has ability to eliminate pathogenic microorganism, superfluous MPO can induce oxidative injury of DNA and protein [12]. Abnormally expressing and releasing, MPO can aggravate inflammation and tissue injury and lead to tissue damage [13,14]. Therefore, the improvement of MPO can be a signal of inflammation and oxidative stress in normal diseases (such as systemic inflammation and localized intestinal inflammation) [15,16]. Lots of herbal medicines have been discovered that they can ameliorate the traditional remedies of liver injury [17]. In addition, GSH-PX is an important enzyme in metabolism of GSH in oxidative stress and can catalyze the conversion of peroxide. To some extent, GSH-PX can reflect the capacity of antioxidant [18].

After the LPS combines with the Toll-like receptor 4 (TLR4), IKK and mitogen-activated protein kinases (MAPKs) are activated with inflammatory factor releasing in abundance [19]. One of the pathways to

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respond LPS need the participation of myd88 and TLR4 [20] then NF-κB activated. Existing in all cell types, NF-κB is significant in adjusting inflammation of the gene expression [21]. Besides, NF-κB plays an important role in adjusting the pathway of inflammation in liver. It has been reported that the regulatory network of NF-κB is relevant MAPKs, while though the interaction of NF-κB and MAPKs they can adjust death of cell [22]. MAPKs family is one of the most ancient signal transduction pathways, and eucaryon has numbers of MAPKs pathway to participate in gene expression, mitosis, metabolism, motility, survival, apoptosis, and differentiation [23]. MAPKs have three conventional members, which are ERK1/2, c-Jun amino (N)-terminal kinases 1/2/3 (JNK1/2/3) and p38 isoforms (α, β, γ, and δ). JNK1/2 has 10 subtypes, and they respond various cell reaction varying with different stimulations [24]. ERK1/2 can activate target protein in cytoplasm or accelerate the expression of transcription factor in nucleus [25]. Irritated by pro-inflammatory cytokines and LPS, P38 adjusts caspase cascade pathway to initiate cell apoptosis [26,27].

Nrf2, which adjusts > 200 gene of protection to reflect oxidative stress, is a mean regulatory factor in antioxidant defense system [28]. Nrf2 is ubiquity in cell with keap1, and it transports to nucleus while high oxidative stress comes. Nrf2 induces the antioxidative gene to express, such as GCLM, GCLC [29]. Numbers of protein and enzymes induced by Nrf2 locate in specific cellular compartment to adjust oxidative stress. Glutathione (GSH) shows nucleophilic, antioxidant and detoxifying properties, while GCLC and GCLM formed a heterodimer is a rate-limiting step in the synthesis of glutathione (GSH). HO-1 takes effects on cell and tissue injury and reacts in anti-inflammatory action [30]. NQO1 participates in cytophylaxis though rejecting oxidative stress [31]. Meanwhile, it is reported that Nrf2 has anti-inflammatory. Nrf2 KO mice have a tendency of inflammation in multiple organs [32,33]. Studies show that the anti-inflammation of Nrf2 is associated with NF-κB [34].

RA is one of the extract of *Rosmarinus officinalis* Linn, and a recent research has found that RA has plenty of biological activity. *Rosmarinus officinalis* Linn, a normal household plant, has well used in flavouring food, drink, as well as in cosmetics. The essence of *Rosmarinus officinalis* Linn has been authorized as an antioxidant for food preservation by European Union [35]. RA can interact with enzyme translocator activated by other medicine in vitro experiment. While, expect verifying the ability of antioxidation and antibacterial, RA has reported it has well biological activity in anti-inflammation, anticancer and tissue repair in vivo experimentation. RA prevents lung and kidney through suppressing oxidative stress and the releasing inflammatory cytokine [36,37]. However, there is rare study reported the protection of RA in mice acute liver acute.

2. Materials and methods

2.1. Materials

RA was purchased from Chengdu Pufei De Biotech co., Ltd. (Chengdu, China). We purchased Dimethylsulfoxide (DMSO) and LPS (*Escherichia coli* lipopolysaccharide, 055:B5) from Sigma-Aldrich (St. Louis, MO, USA). D-Galactosamine hydrochloride was bought from Aladdin Industrial Corporation (Shanghai, China). AST, ALT, GSH-PX and MPO detection kits were acquired by the Jiancheng Bioengineering Institute of Nanjing (Nanjing, Jiangsu, China). Anti-bodies against GCLC, GCLM, NQO1, phosphorylation-ERK1/2 (p-ERK1/2), ERK1/2, phosphorylation-JNK1/2 (p-JNK1/2), JNK1/2, phosphorylation-p-38 (p-p38), p38, IκBα, phosphorylation-IκBα (p-IκBα), NF-κB and phosphorylation-NF-κB (p-NF-κB) were obtained from Cell signal technology (Boston, MA, USA). Anti-HO-1 and anti-Nrf2 monoclonal antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-Lamin B, anti-β-actin monoclonal antibodies were purchased from Proteintech Group Inc. (Boston, MA, USA). HRP-conjugated goat anti-rabbit and goat anti-mouse antibodies were provided by Boster Biological Technology (Wuhan, Hubei, China). All other chemicals were of reagent grade.

2.2. Animals

Male C57BL/6 mice (6–8 weeks old, weighting approximately 18 to 22 g each) were purchased from the Liaoning Changsheng Biotechnology (Liaoning, China). Given adequate food and water, the mice were housed in cages with the temperature of 24 ± 1 °C and a relative humidity of 55 ± 10%.

2.3. Method

To induce acute liver injury, we treated the mice with intraperitoneal (i.p.) injection of 400 μL sterile phosphate-buffered saline (PBS) solution containing LPS (30 μg/kg) and D-GalN (600 mg/kg). After 3 h, we collected the blood from retro-orbital venous plexus. And the liver tissues were removed for histopathological examination and protein extraction. The mice of the control group were injected PBS. In therapeutic groups, mice were intraperitoneally injected by RA (25 mg/kg, 50 mg/kg or 100 mg/kg) for twice (the first time interval for 12 h), and at 1 h after the second dose of RA, followed by exposure to LPS (30 μg/kg) and D-GalN (600 mg/kg). As a negative control, RA (100 mg/kg) was injected into the mice. All of the RA dissolved in 5% DMSO.

2.4. Histopathological evaluation

The left lateral lobe of liver was isolated after 3 h stimulation of LPS/D-GalN. Using normal 10% neutral buffered formalin, the liver tissues fixed for 48 h. Dehydrated by a series of graded ethanol, the part of liver was embedded by paraffin wax. We stained 5-μm-thick sections with hematoxylin and eosin (H&E) to assess the injury degree.

2.5. ALT and AST level in blood

Placing in room temperature for 16 h, we centrifugated the sample of blood with 3000 rpm (10 min, 4 °C). The serum was detected with kits purchased by Jiancheng Bioengineering Institute of Nanjing. Then, the expression of ALT and AST was measured under the instruction.

2.6. MPO and GSH-PX analyses

The fresh liver tissue was homogenized and dissolved in normal saline, and then the compound was used to detect the MPO with the instruction. Then the absorbancy was measured at 460 nm and the activity of MPO was obtained though the formula. With the instruction of the product, the liver tissue was homogenized and dissolved in special buffer solution. After disposing, we acquired the level of GSH-PX though measuring the absorbancy at 412 nm.

2.7. Western blot

The liver tissue of mice was lysed by Radio Immunoprecipitation Assay (RIPA) with 1 mM phenylmethanesulfonyl fluoride (PMSF) to get protein. In addition, extracted nuclear and cytoplasmic protein was under the guidance of the instruction. Before separating the compound protein by 12% SDS-polyacrylamide gel, we measured the protein concentration using BCA protein assay kit (Thermo, America). And then, a sample of protein (20 μg) transfer onto polyvinylidene fluoride (PVDF) membrane. Blocked by 5% (w/v) nonfat dry milk for 2 h, PVDF membrane was incubated overnight at 4 °C in particular primary antibody. After washed by Tris-buffered Saline with Tween 20 (TBST) for three times, PVDF membrane was incubated in HRP-conjugated secondary antibody (1:5000) for 2 h in room temperature. We used ECL to help us detect the signal, after that we analyzed all results by means of

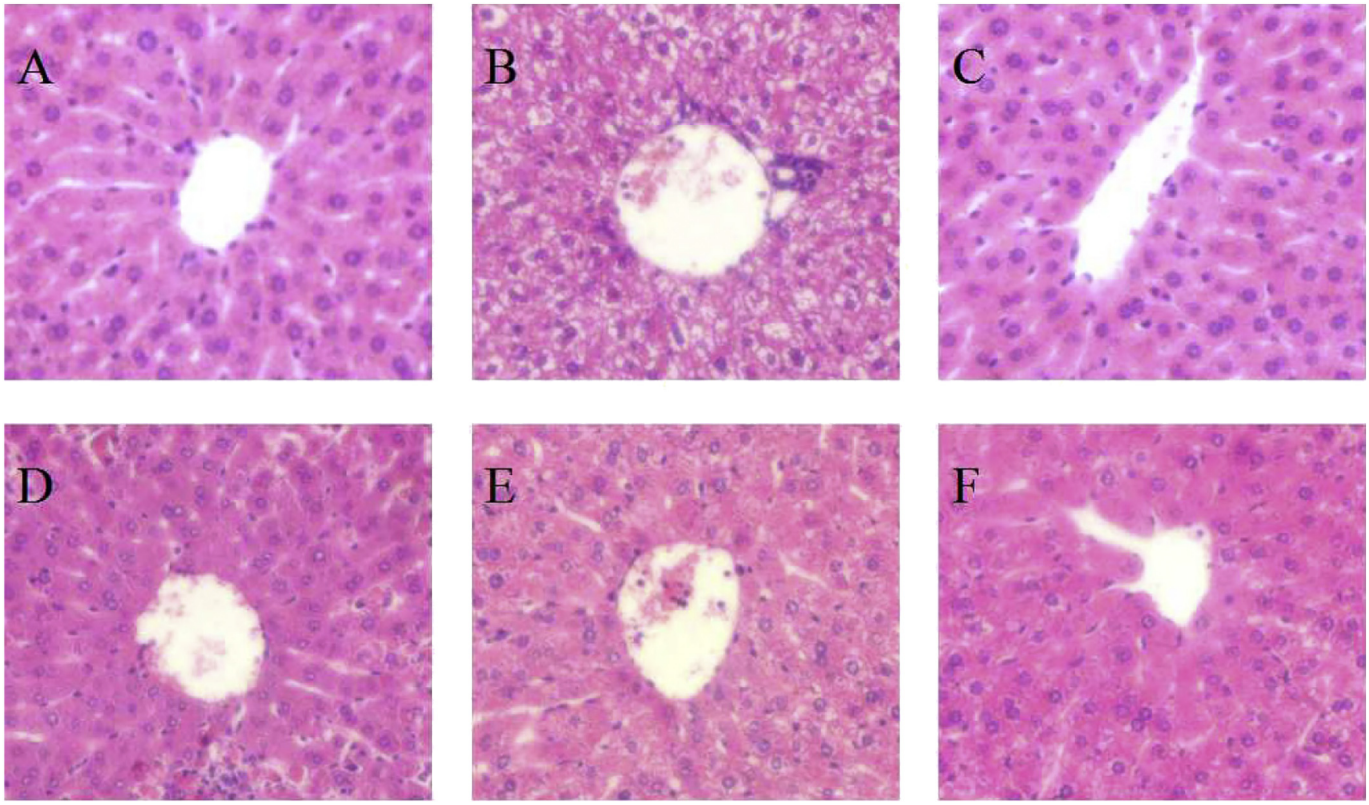

Fig. 1. Effects of SHK in hepatotoxicity on LPS/D-GalN-induced acute liver injury.
Mice were given LPS/D-GalN, before this, RA was injected twice (12 h and 1 h before the LPS/D-GalN). Three hours later, the liver tissue was fixed and then stained with hematoxylin and eosin. Panel A is the control group, and B shows the LPS/D-GalN group. Then D, E and F are the RA (25 mg/kg, 50 mg/kg and 100 mg/kg) with LPS/D-GalN group. Panel C is the only RA (100 mg/kg) treatment group.

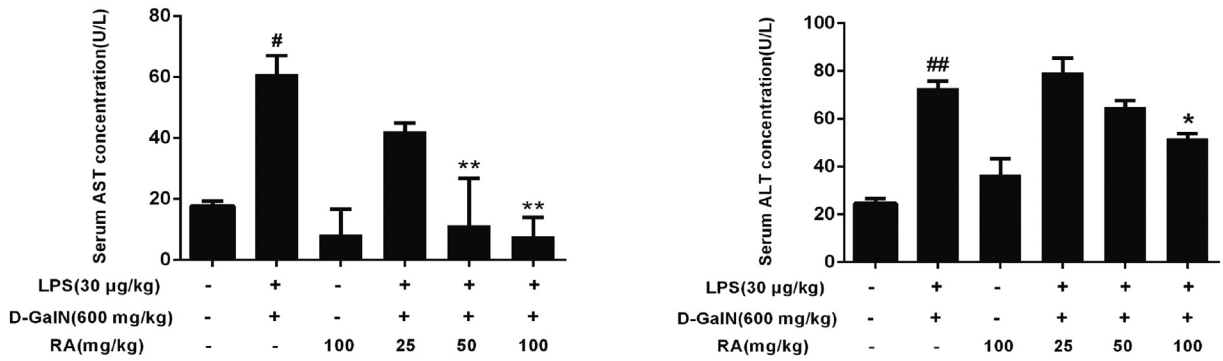

Fig. 2. Effects of RA on mice acute liver injury induced by LPS/D-GalN in AST, ALT level.
RA was given to mice at 12 h and 1 h before the stimulation, and then LPS/D-GalN was injected to mice. Three hours later, we used the blood to detect the serum AST and ALT. The data was demonstrated as means ± SD and had three independent experiments. $^{\#}p < 0.05$ vs. the control group; $^{\#\#}p < 0.01$ vs. the control group; $^{*}p < 0.05$ vs. the LPS/D-GalN group; $^{**}p < 0.01$ vs. the LPS/D-GalN group.

Image J gel analysis software.

2.8. Statistical analysis

All data was expressed as mean ± SD. The statistical analysis was performed by the one-way ANOVA using the GraphPad Prism 6.0 software. Statistical significance was accepted at $P < 0.05$ or $P < 0.01$.

3. Result

3.1. Effects of SHK in hepatotoxicity on LPS/D-GalN-induced acute liver injury

To improve specificity and visibility, the liver tissue was stained 5-μm-thick sections with H & E. Fig. 1A is the control group. As is shown in Fig. 1B, injected by LPS/D-GalN, liver tissue had severe intrahepatic hemorrhage and necrosis. And with the treatment of RA (25 mg/kg, 50 mg/kg and 100 mg/kg), the intrahepatic hemorrhage and cell necrosis was alleviated in Fig. 1D, E and F. The only RA (100 mg/kg)-treated mice group was not found toxic.

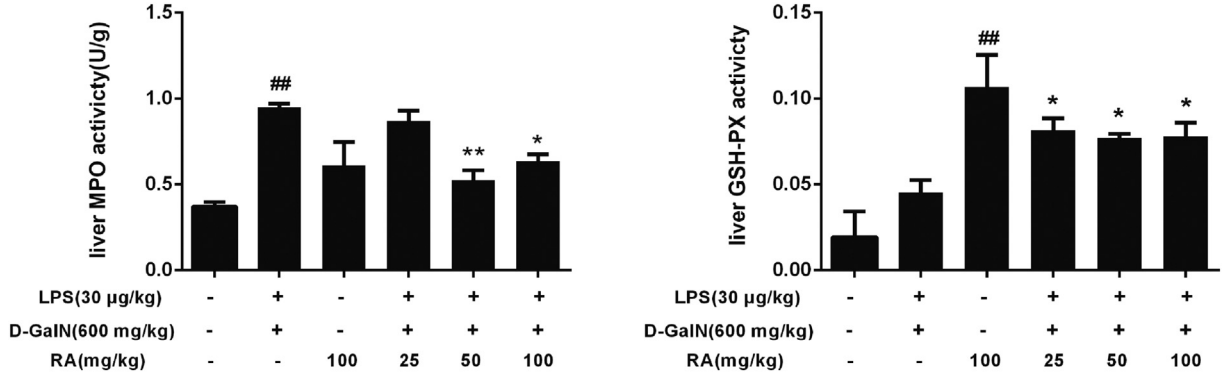

Fig. 3. Effects of RA on mice acute liver injury induced by LPS/D-GalN in MPO and GSH-PX level.
Before the administration of LPS/D-GalN, we gave RA to mice for 12 h and 1 h. Three hours later, we took the liver tissue to measure MPO and GSH-PX. All of the data was collected three independent experiments and was shown as means ± SD. $^{\#\#}p < 0.01$ vs. the control group; $^{*}p < 0.05$ vs. the LPS/D-GalN group; $^{**}p < 0.01$ vs. the LPS/D-GalN group.

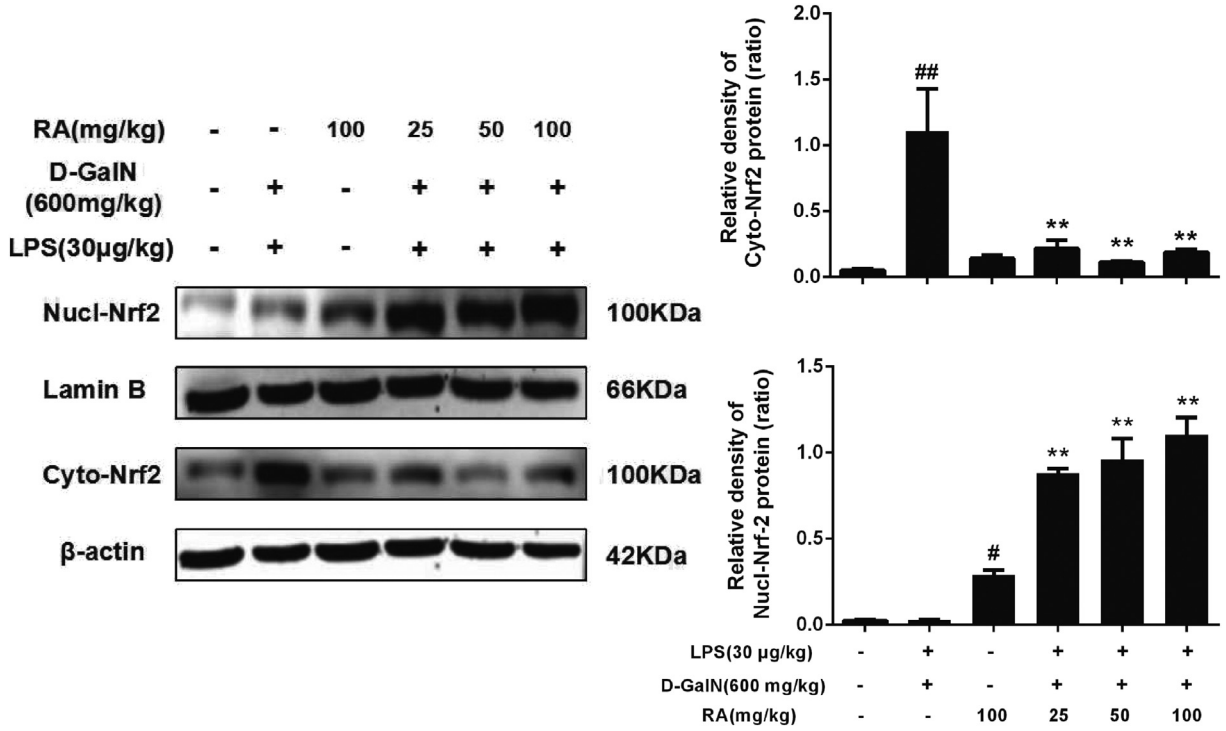

Fig. 4. Effects of RA on mice acute liver injury induced by LPS/D-GalN in Nrf2 expression.
Mice were injected RA 12 h and 1 h before the LPS/D-GalN stimulated. After the injection of LPS/D-GalN for 3 h, liver of mice was lysed to detect the protein by western blot. The level of Nucl-Nrf2 was compared with lamin B and the Cyto-Nrf2 was compared with β-actin. Three independent experiments were used for collecting for the data shown as means ± SD. $^{\#}p < 0.05$ vs. the control group; $^{\#\#}p < 0.01$ vs. the control group; $^{**}p < 0.01$ vs. the LPS/D-GalN group.

3.2. Effects of RA on ALT and AST levels in C57BL/6 mice induced by LPS/D-GalN

To investigate the effect of RA, we detected AST and ALT in serum of mice. It is shown in Fig. 2 that RA (100 mg/kg) has no effect on AST and ALT compared with control group. While LPS/D-GalN up-regulated the AST, RA (50 mg/kg and 100 mg/kg) down-regulated it. However, the ALT in serum was decreased in group of high dose (100 mg/kg).

3.3. Effects of RA on mice acute liver injury induced by LPS/D-GalN in MPO and GSH-PX level

Then, we detected the antioxidant capacity of RA. Fig. 3 illustrated that treated the RA (50 mg/kg and 100 mg/kg), the MPO in the therapeutic group was lower than the LPS/D-GalN group, and the only treatment of RA (100 mg/kg) group had no effects on MPO compared with the control group. And the level of GSH-PX increased, while the LPS/D-GalN group had no different with the control group.

3.4. Effects of RA on mice acute liver injury induced by LPS/D-GalN in Nrf2 expression

For Nrf2 is one of the regulatory proteins in cell protecting, we investigated the expression of it by western blot. As it is shown in Fig. 4, the expression of Nrf2 in cell nucleus enhanced after treatment of RA (25 mg/kg, 50 mg/kg and 100 mg/kg) for LPS/D-GalN-induced acute liver injury in mice. And in cytoplasm, we found that LPS/D-GalN group had high expression of Nrf2.

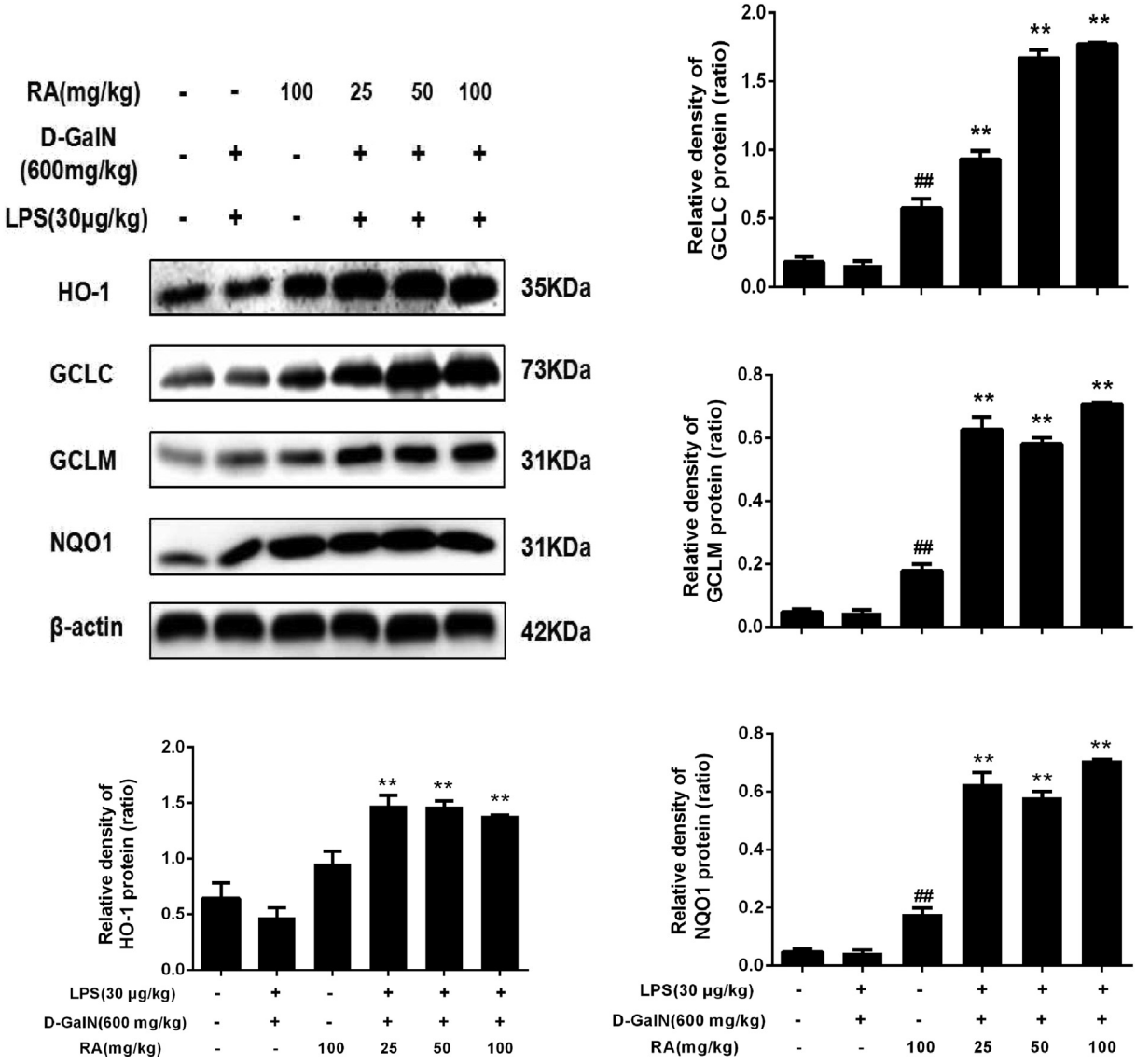

Fig. 5. Effects of RA on mice acute liver injury induced by LPS/D-GalN in HO-1, GCLC, GCLM and NQO1 expression.
RA (25 mg/kg, 50 mg/kg and 100 mg/kg) was given to mice for twice (12 h and 1 before given LPS/D-GalN), and then injected LPS (30 μg/kg) and D-GalN (600 mg/kg) for 3 h. The level of GCLC, GCLM, NQO1, and HO-1 were detected by western blot with the compared with the internal control (β-actin). All of the data was presented as means ± SD of three independent experiments. $^{\#\#}p < 0.01$ vs. the control group; $^{**}p < 0.01$ vs. the LPS/D-GalN group.

3.5. Effects of RA on acute liver injury induced by LPS/D-GalN in HO-1, GCLC, GCLM and NQO1 expression

After detected the expression of Nrf2, we took investigation on its downstream protein, such as GCLC and GCLM. Fig. 5 demonstrated the levels of GCLC, GCLM, NQO1, and HO-1 with LPS/D-GalN group were higher than it in LPS/D-GalN group after treated by RA (25 mg/kg, 50 mg/kg and 100 mg/kg). In addition, the expression of GCLC, GCLM and NQO1 in the only treatment of RA (100 mg/kg) group was enhanced obviously compared with the control group (shown in Fig. 5).

3.6. Effects of RA on acute liver injury induced by LPS/D-GalN in NF-κB pathway

To investigate the anti-inflammatory ability of RA, we checked the NF-κB and IκBα by western blot. As the result is shown in Fig. 6, RA (25 mg/kg, 50 mg/kg and 100 mg/kg) could decrease the expression of p-NF-κB and p-IκBα in LPS/D-GalN-induced acute liver injury. And RA (100 mg/kg) did not improve the level of p-NF-κB and p-IκBα.

3.7. Effects of RA on acute liver injury induced by LPS/D-GalN in MAPKs pathway

MAPKs family has multiple complex functions in cellular processes, thus we investigated the levels of JNK1/2, ERK1/2 and p38 phosphorylation in liver. Fig. 7 presents that treated by RA, the expression of p-ERK1/2 decreased by the dosage dependent. But only the high dosage (RA 100 mg/kg) reduced the expression of p-p38. However, RA has no effect on p-JNK1/2 in LPS/D-GalN-induced acute liver injury. Besides, the only treatment of RA (100 mg/kg) group could not influence the expression of p-JNK1/2, p-ERK1/2 and p-p-38.

4. Discussion

Acute liver injury is a syndrome caused by lots of pathogenesis. The damage cell may lead to senescence, apoptosis or necrosis [38]. There are multitudinous models of mice to investigate the pathogeny, and LPS/D-GalN-induced acute liver injury is a universal model [1]. Previous studies shows that several extract of plants could prevent liver

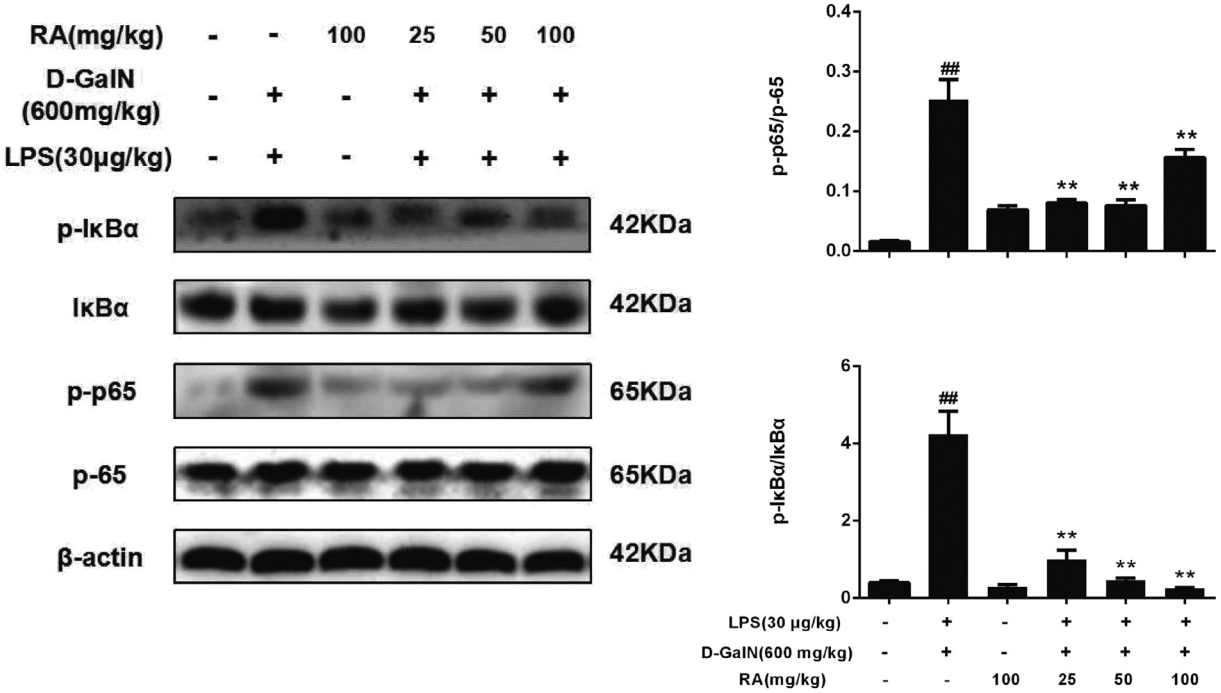

Fig. 6. Effects of RA on mice acute liver injury induced by LPS/D-GalN in NF- κB pathway.
The mice were given twice treatment (12 h and 1 h before given LPS/D-GalN), and then they were injected stimulant (LPS/D-GalN) by intraperitoneal injection. The protein was detected by western bolt, and the result was compared with the internal control (β-actin). Three independent experiments were collected for data and they were shown as means ± SD of three independent experiments. $^{\#\#}p < 0.01$ vs. the control group; $^{**}p < 0.01$ vs. the LPS/D-GalN group.

injury induced inflammation and oxidative stress [39,40]. Consistent with previous studies, we observed the oxidation resistance of RA on LPS/D-GalN induced acute liver injury and possible molecule mechanism. We found that RA can protect the mice form LPS/D-GalN-induced acute liver injury.

AST and ALT are the most common used liver injury biomarkers. In this study, we found that RA can decrease the level of AST and ALT induced by LPS/D-GalN. Since 1961, AST and ALT has been used as liver injury biomarkers, which could be increased by cell inducing plasma membrane disruption. The result showed that AST and ALT increased in LPS/D-GalN-induced acute liver injury. Besides, RA conspicuously reduced the AST and ALT of serum in this model. Moreover, pathological section showed that RA could preserve hepatocyte and alleviate congestion of liver. These results suggested that RA could protect acute liver injury of mice induced by LPS/D-GalN.

Studies shows that, except neutrophils releasing MPO, stellate macrophages and Kupffer cell in liver can also produce MPO. These activated cells local release oxidizing agent and cytokine during hepatic fibrosis [41]. LPS/D-GalN can increase the expression of MPO. In our experiment, MPO was obviously decreased after the treatment of RA. Besides, GSH-PX, a well-known intracellular enzyme, can catalyze reduction of peroxide to protect structure and function of cytomembrane. These results showed that RA could effectively enhance the expression of GSH-PX. The results of this experiment indicated that RA played a positive role in LPS/D-GalN-induced acute liver injury of mice.

Nrf2 is a nuclear transcription factor that can induce numbers of cytoprotective gene to express. Because the half life of Nrf2 is 20 min, it cannot be detected at resting state [42]. After transporting to nucleus, Nrf2 can induce the expression of detoxification enzyme and antioxidant enzyme to relieve the oxidative stress. In this study, we found RA could improve the Nrf2 expression in nucleus. Meanwhile, HO-1 is an enzyme catalyzing substrate to carbon monoxide (CO) and biliverdin (it can transform into bilirubin though enzyme catalyzing), which make sense in antioxidant and anti-inflammatory [43]. NQO1 has been proved that it is assistance in cytophylaxis and removes superoxide [44]. GCLC and GCLM are significant in compounding GSH. It has reported the increasing expression of GCLC and GCLM is relevant to the activation of Nrf2. The result indicated that the increasing of Nrf2 in nucleus induced by RA, the expression of HO-1, NQO1, GCLC and GCLM were higher than LPS/D-GalN group.

NF-κB, a nuclear transcription factor, has effects on lots of biological phenomenon, such as inflammation and immunoreaction [45]. Normally, NF-κB combined with IκB has not biological activity. Activated by TNF-α and IL-1β, IκB separates with NF-κB by phosphorylated, and then NF-κB transports to nucleus to induced transcription of target gene [45]. Though our experiment, we found that RA inhibited the phosphorylation of NF-κB and IκB. In addition, NF-κB can affect the level of Keap1 in nucleus, and high level of Keap1 in nucleus decreases the transduction of Nrf2-ARE [46]. However, HO-1 has essential effects on restraining NF-κB though Nrf2 pathway. HO-1 can restrain the expression of inflammatory factor and cell adhesion molecules. For the NF-κB activated is in need of pro-inflammatory gene, this mechanism of anti-inflammatory may make effects though restraining the phosphorylation of NF-κB [47]. Results demonstrated that the phosphorylation of NF-κB and IκBα decreased after the treatment of RA, which indicated that RA could protect the LPS/D-GalN-induced acute liver injury. However, the effects of RA in Nrf2 and NF-κB remain to be further studies.

MAPKs are important transporters of signal from cytomembrane to cell nucleus and have a group of serine-threonine protein kinase activated by various irritants (such as, inflammation and oxidative stress). Western blot results showed that RA could decrease the expression of p-ERK and p-p38, while p-JNK had not significant change. JNK has complex regulatory network, and plays an important role in programmed cell death and apoptosis. ERK, which can be activated by stimuli compound such as growth factors and cytokines, can accelerate proliferation of tumor cell. P38 pathway is significant in cell to respond numbers of extracellular stimulation (cytokines and oxidative stress) [48]. It has reported that hepatoma was related to liver injury [49]. Therefore relieving the phosphorylation of MAPKs is in urgently

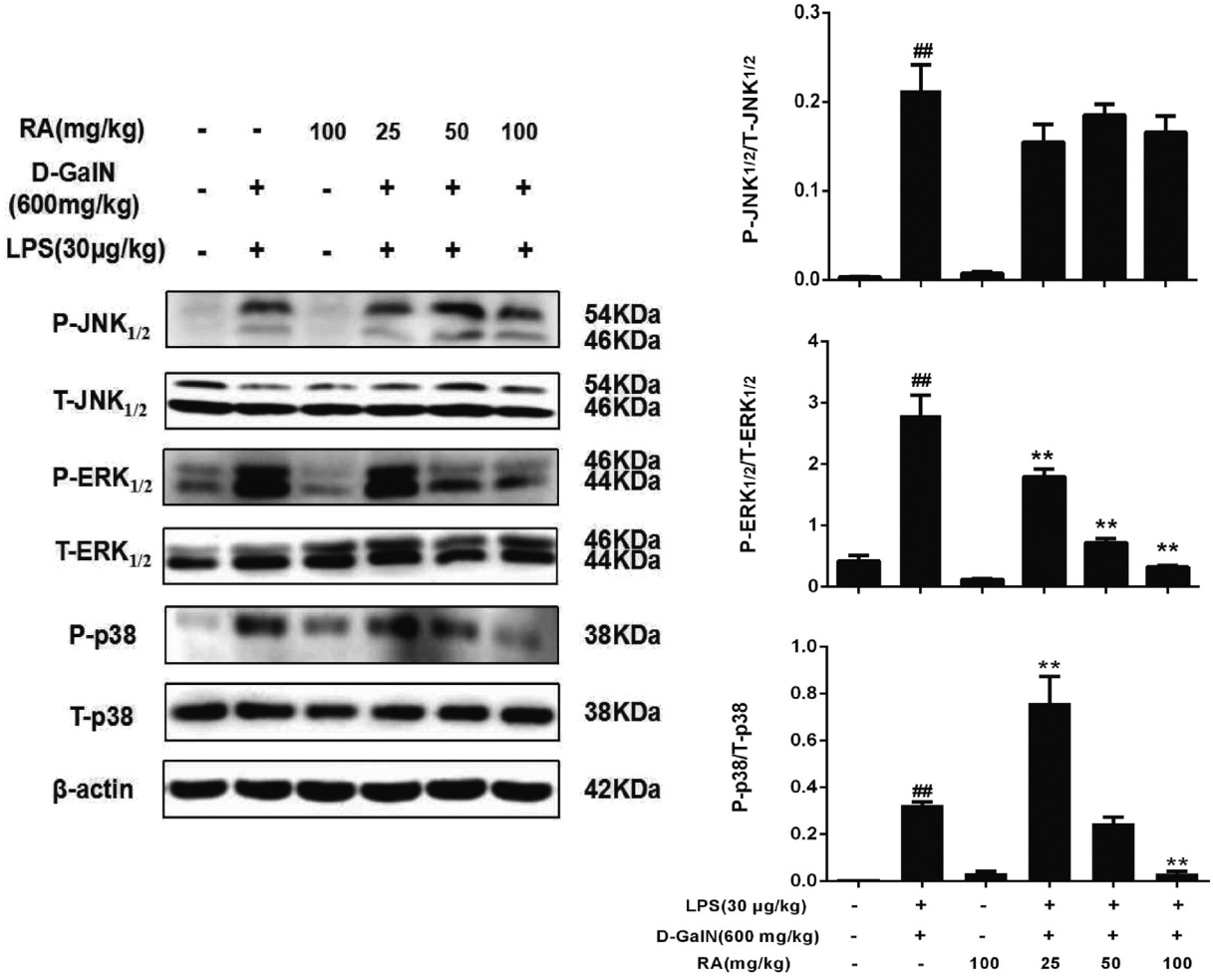

Fig. 7. Effects of RA on mice acute liver injury induced by LPS/D-GalN in MAPK pathway.
LPS/D-GalN was injected to mice and effecting for 3 h, before this RA (25 mg/kg, 50 mg/kg and 100 mg/kg) was given to mice for twice (12 h and 1 h before the administration of LPS/D-GalN). Western blot was used to detect the expression of p-JNK, p-ERK and p-p38. The data has three independent experiments and was demonstrated as means ± SD. $^{\#\#}p < 0.01$ vs. the control group; $^{**}p < 0.01$ vs. the LPS/D-GalN group.

needed. Meanwhile, it has been reported that the increased HO-1 can reduce p38α, which suggests that these protein may have the mutual adjustment in apoptosis [50]. Results indicated that RA could preserve acute liver injury of mice induced by LPS/D-GalN though MAPKs pathway.

In this study, we investigated the effects of RA in LPS/D-GalN-induced acute liver injury on mice. RA could boost the Nrf2 nucleus transport, and then it improved the expression of HO-1, NQO1, GCLC and GCLM. RA could decrease the expression of p-ERK, p-p38, p-NF-κB, and p-IκBα. Meanwhile, RA decreased the expression of MPO and increased the level of GSH-PX. As *Rosemarinus officinalis* L. is wide used in food, drink and cosmetics, RA may be a potential drug in liver injury for it security and antioxidant.

Conflict of interest

The authors declare no competing financial interest.

Authors' contributions

Z.L., G.W.L. and H.H.F. contributed to the design. Y.W., Z.L. L.W. and Q.L.Z. did the data collection. Z.L., Y.T., G.W.L. and M.Y.J. did the analysis. Z.L. did the writing of the article. B.Y.S., T.Y., H.H.F. did the revisions of the article.

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