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Research Article

A Study on the Therapeutic Mechanism of Liquiritin on Rats with Acute Chronic Liver Failure

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Abstract

Background and Objective: Natural drug extracts are safe and useful in some disease treatment. Liquiritin is a triterpene saponin extracted from the root of *Glycyrrhiza uralensis*. However, It has been unclear liquiritin's treatment in ACLF. To investigate the effect of liquiritin on liver injury in ACLF rats and preliminarily explore the mechanism. **Materials and Methods:** The ACLF model was induced by intraperitoneal injection of D-galactose solution after ligation of the bile duct. The AST, ALT, TBil, PA, endotoxin, IL -6, IL-8 and TNF- α in serum were detected. The pathological morphology of rat liver tissue were observed and the expression of TLR4/NF- κ B (p65) pathway related proteins in rat liver tissue was detected. The apoptosis cell number of liver tissues from different groups was evaluated by TUNEL. **Results:** The AST, ALT, TBil, endotoxin, IL-6, IL-8, TNF- α in serum, apoptosis cell number and the protein expression levels of TLR4, p-NF- κ B (p65) /NF- κ B (p65) in liver tissues were significantly increased in low, middle and high dose groups ($p < 0.05$, respectively) while, the level of PA in serum was significantly decreased ($p < 0.05$) and the pathological damage of liver tissue was reduced. With the increase of liquiritin dosage the levels of AST, ALT, TBil, endotoxin, IL-6, IL-8 and TNF- α in serum, apoptosis cell number and the protein expression levels of TLR4, NF- κ B (p65)/NF- κ B (p65) in liver tissues of ACLF rats were increased gradually ($p < 0.05$, respectively) while the level of PA in serum was decreased gradually ($p < 0.05$) and the pathological damage of liver tissue was also gradually reduced. **Conclusion:** Liquiritin can alleviate liver injury in ACLF rats via TLR4/NF- κ B(p65) pathway.

Key words: Liquiritin, acute-on-chronic liver failure, rats, liver injury, toll-like receptor 4/nuclear factor- κ B (p65) pathway

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The term acute phase of chronic liver failure (ACLF) is defined as an acute or subacute decompensated syndrome of liver function on the basis of chronic liver diseases such as liver cirrhosis, chronic hepatitis and chronic cholestatic liver disease. ACLF is commonly characterized by one or multiple organ failures, rapid disease progression and high mortality^{1,2}. Antiviral therapy, liver transplantation and artificial liver are common medical choices for the treatment of ACLF clinically, resulting in significantly reduced mortality, which, however, still exhibits an unsatisfied overall therapeutic effects³. It has been demonstrated that in the process of liver failure, the liver tissue is subjected to the triple lethal whammy of immune injury, hypoxic-ischemic injury and endotoxemia and the inflammatory response is the central link in the process of pathological progression⁴. Toll-like receptor 4 (TLR4) is an intracellular pattern recognition receptor that can activate the nuclear factors- κ B (NF- κ B) signaling pathway, which can induce an inflammatory cascade and participate in the inflammatory injury process of multiple diseases^{5,6}. Prior research has reported that TLR4/NF- κ B signaling pathway can be involved in the process of liver failure⁷. Furthermore, liquiritin is a triterpene saponin extracted from the root of *Glycyrrhiza uralensis*⁸. According to modern studies, liquiritin has pharmacological effects such as antioxidation, anti-allergy, enhancing immunity, bacteriostasis and neuroprotection⁹. With respect to the above, the present study was carried out to observe the alleviating effect of liquiritin on liver injury in ACLF rats and explore its mechanism from the aspect of inflammatory response.

MATERIALS AND METHODS

Study area: This study was done in Nanjing Yuhua Hospital, Yuhua Branch of Nanjing First Hospital from 12-05-2020 to 06-05-2022.

Experimental animals: About 45 SD rats, weighing 200-230 g, were purchased from Shanghai Lingchang Biotechnology Co., Ltd., (production license No.: SCXK (Shanghai)2018-0003). The experimental rats were raised with free access to drinking and diet in a condition at 22-26°C with a relative humidity of 40-70% and a 12-12 hrs light-dark cycle.

Main reagents: Liquiritin control (Chengdu MUST Bio-technology Co., Ltd., Batch No.: MUST-16082811), lactulose (Item No.: L77521, Shanghai Acme Biochemical Technology Co., Ltd.), rat Enzyme Linked Immunosorbent Assay (ELISA) kits

for endotoxin, interleukin (IL)-6 and IL-8, tumor necrosis factor- α (TNF- α) (Item No.: HL20392, HL20064, HL20700 and HL20040, Shanghai Haling Biotechnology Co., Ltd.), HE staining kit (Item No.: RS3390-3, G-clone (Beijing) Biotechnology Co., Ltd.), TLR4 antibody, NF- κ B p-p65 antibody, NF- κ B p65 antibody, GAPDH antibody, goat anti-rabbit IgG (Item No.: ab13867, ab239882, ab16502, ab186930 and ab133470, Abcam, USA).

Main instruments: The AU5800 automatic biochemical analyzer (Beckman, USA), MK3 Microplate Reader (Thermo, USA), CKX41SF microscope (Olympus, Japan), GS-800 gel scanning imaging system (Bio-Rad, USA).

Methods

Experimental modeling, grouping and administration: After adaptive feeding for 1 week, rats were used to establish the ACLF model by referring to prior literature¹⁰, which was divided into two stages:

- **Construction of the model of cholestatic cirrhosis in rats:** After fasting for 12 hrs, the rats underwent common bile duct ligation, followed by anesthesia by intraperitoneal injection of 4% chloral hydrate (0.7 mL/100 g). After abdominal skin preparation and aseptic disinfection, the abdominal cavity of the experimental rats was cut open with the separation of the common bile duct, followed by ligation with 4-0 silk thread and cutting off the common bile duct from the middle section of the ligation. The abdominal cavity was rinsed with normal saline, followed by bile duct ligation. The model of cholestatic cirrhosis was successfully established in rats that survived 4 weeks after bile duct ligation
- **Construction of ACLF model in rats:** The rats that had been successfully modeled with cholestatic cirrhosis were fasted for 12 hrs and were given 0.2 g mL⁻¹ D-galactosamine solution at the rate of 0.2 g kg⁻¹ through intraperitoneal injection. After 24 hrs, acute liver injury could be induced on the basis of liver cirrhosis and the rat ACLF model was established eventually

About 45 rats with the successful establishment of the ACLF model were divided into Model group as well as low-, medium- and high-dose liquiritin (low, middle and high) groups, with 9 rats in each group. Meanwhile, another 9 rats without modeling were regarded as the normal group. Rats in low, middle and high groups were provided with 1, 2 and

10 mg g⁻¹ liquiritin through gavage. While, those in the normal group and model group were given the same amount of normal saline by gavage, once a day for 2 weeks.

Determination of liver function in rats: Rats were anesthetized by intraperitoneal injection of 4% chloral hydrate (7mL/100 g) 24 hrs after the last administration. The rats were fixed on the anatomical table in the supine position and 3-5 mL of inferior vena cava blood was collected by laparotomy. After standing at room temperature for 30 min, the collected blood sample was centrifuged at 3,000 r/min for 15 min, with the separation of the supernatant was divided into two parts. One part of the serum was used for detecting the levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (TBil) and prealbumin (PA) by the biochemical analyzer.

Detection of the expressions of serum endotoxin and inflammation-related factors by ELISA: By using the ELISA kit, the other part of the serum was included for the detection of endotoxin, IL-6, IL-8 and TNF- α . The levels of endotoxin, IL-6, IL-8 and TNF- α in serum were detected according to the operating steps of the kit instructions.

Histopathological observation of rat liver: After the inferior vena cava blood was collected by laparotomy, the liver tissue of the same liver lobe was resected, washed with normal saline and divided into two parts. One was used for Western blot detection. The other part was fixed in a 10% neutral formaldehyde solution, embedded in paraffin, sectioned and dewaxed for HE, TUNEL and immunohistochemical staining. The histopathologic characteristics of liver tissue were observed under a microscope.

Detection of cell apoptosis in liver tissue of rats in each group by TUNEL staining: The rat liver tissue (10%) was taken out from the refrigerator, fixed by conventional methods, embedded in paraffin, soaked in xylene twice and gradient soaked for 5 min. After air drying, the tissues were soaked in 3% hydrogen peroxide-methanol for 10 min and rinsed with PBS for 3 min each time. Then, the following operations were carried out on pre-cooled ethanol at 4°C: 0.1% TritonX-100, 0.1% buffer for 2 min and PBS rinsing for 3 times (3 min each time). Subsequently, the TUNEL reaction mixture was added for reaction in a wet box for 1 hr in dark at 37°C after sealing with sealing membrane. The membrane was sealed with neutral resin following another PBS rinsing, gradient ethanol dehydration and xylene for transparent processing. The results

were observed by microscope and the absorbance values of each group were analyzed by Image J software.

Detection of the levels of TLR4 and p-NF- κ B (p65) in liver tissue of rats in each group by immunohistochemistry:

Another part of rat liver tissue (20%) was taken out from the refrigerator and routinely fixed, dehydrated, waxed, embedded and sliced. After PBS washing and sealing with egg white at 37°C, the primary antibody (1:100) was added for incubation overnight at 4 and overnight incubation by adding the secondary antibody (1:200). With another PBS washing, hematoxylin was used for re-staining and the next step was sealing and fixation. The results were observed by microscope and ImagePro Plus8.0 software was used for statistical analysis.

Detection of the expressions of TLR4/NF- κ B signaling pathway related proteins in rat liver by Western blot:

The remaining liver tissues were lysed on ice to extract total protein and the protein concentration was determined by the BCA method. After the protein sample was denatured, SDS-PAGE gel electrophoresis was performed, the protein was transferred to the PVDF membrane, followed by sealing using non-fat milk powder for 1 hr. The primary antibodies (TLR4 antibody, p-NF- κ B (p65) antibody, NF- κ B (p65) antibody and GAPDH antibody, 1:1 000) were added for incubation overnight. After washing with TBST, goat anti-rabbit IgG secondary antibody (horseradish peroxidase labeled, 1:2 000) was added for 1 hr of incubation. Following development using ECL, the gel scanning imaging system was used to scan the image and analyze the grayscale.

Statistical analysis: The data were analyzed by SPSS 21.0 software. The experimental data were expressed by Mean $\pm$ Standard deviation (Mean $\pm$ SD). One-way Analysis of Variance (ANOVA) was performed for the comparison among multiple groups and SNK-q was performed for the pairwise comparison among multiple groups. The $p < 0.05$ was used to indicate the statistically significant difference.

RESULTS

Liver function indexes of rats in each group: Compared with the normal group, the model group showed a significant increase in the levels of AST, ALT and TBil in rat plasma ($p < 0.001$, respectively, Table 1) but obviously decrease in the level of PA ($p < 0.001$, Table 1), while, compared with model group, liquiritin intervention groups (low, middle and high) had remarkably down regulated levels of AST, ALT and TBil in

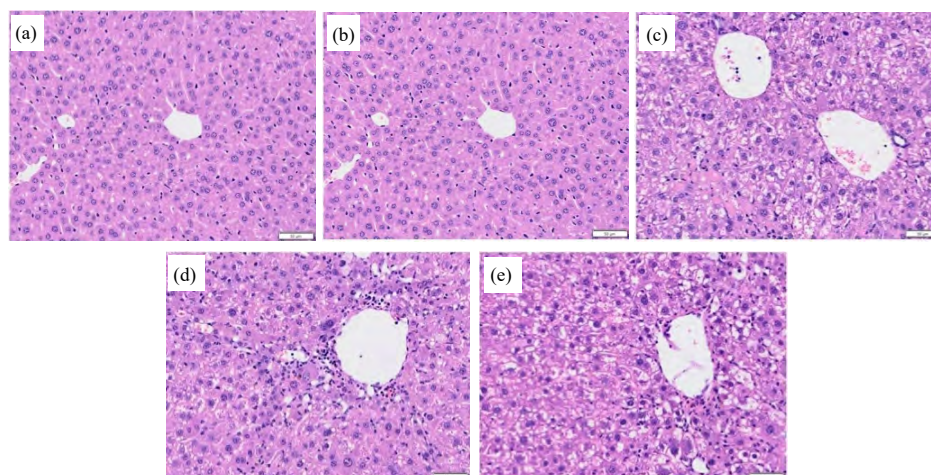


Fig. 1(a-e): Pathological morphology of liver tissue of rats in each group (H&E staining, $\times 200$), (a) Normal group, (b) ACLF model group, (c) ACLF model rats treated with low-dose liquiritin (1 mg g^{-1}), (d) ACLF model rats treated with middle-dose liquiritin (2 mg g^{-1}) and (e) ACLF model rats treated with high-dose liquiritin (10 mg g^{-1})

Table 1: Comparison of serum AST, ALT, Tbil and PA levels in rats (Mean $\pm$ SD, $n = 9$)

Group	AST (U L^{-1})	ALT (U L^{-1})	Tbil ($\mu\text{mol L}^{-1}$)	PA (mg L^{-1})
Normal	81.79 ± 5.86	32.20 ± 1.84	5.47 ± 0.15	330.55 ± 12.88
Model	$319.60 \pm 6.47^{***}$	$238.56 \pm 9.85^{***}$	$175.30 \pm 11.40^{***}$	$185.08 \pm 11.91^{***}$
Low	$215.03 \pm 7.03^{\#}$	$161.13 \pm 7.36^{\#}$	$98.60 \pm 4.67^{\#}$	$215.82 \pm 14.48^{\#}$
Middle	$177.98 \pm 4.98^{\#\$,}$	$99.17 \pm 2.75^{\#\$,}$	$60.34 \pm 3.26^{\#\$,}$	$252.88 \pm 14.87^{\#\$,}$
High	$153.43 \pm 8.38^{\#\$, \&}$	$60.95 \pm 2.78^{\#\$, \&}$	$34.21 \pm 3.26^{\#\$, \&}$	$301.77 \pm 12.63^{\#\$, \&}$

*** $p < 0.001$ vs. Normal, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. Model, $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ vs. Low, $^{\&}p < 0.05$ vs. Middle

Table 2: Comparison of serum endotoxin and IL-6, IL-8, TNF- α levels in rats (Mean $\pm$ SD, $n = 9$)

Group	Endotoxin (EU mL^{-1})	IL-6 (pg mL^{-1})	IL-8 (pg mL^{-1})	TNF- α (pg mL^{-1})
Normal	0.22 ± 0.03	84.96 ± 5.87	174.21 ± 5.41	16.33 ± 2.18
Model	$3.44 \pm 0.11^{***}$	$316.61 \pm 16.46^{***}$	$372.12 \pm 18.18^{***}$	$68.37 \pm 4.91^{***}$
Low	$1.35 \pm 0.09^{\#}$	$220.38 \pm 8.79^{\#}$	$285.72 \pm 10.69^{\#}$	$45.48 \pm 3.25^{\#}$
Middle	$1.02 \pm 0.06^{\#\$,}$	$175.93 \pm 6.43^{\#\$,}$	$250.69 \pm 7.93^{\#\$,}$	$36.01 \pm 2.13^{\#\$,}$
High	$0.52 \pm 0.04^{\#\$, \&}$	$122.83 \pm 5.66^{\#\$, \&}$	$214.29 \pm 6.68^{\#\$, \&}$	$27.44 \pm 1.81^{\#\$, \&}$

*** $p < 0.001$ vs. Normal, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. Model, $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ vs. Low, $^{\&}p < 0.05$ vs. Middle

rat plasma ($p < 0.05$, respectively, Table 1) but highly upregulated PA level ($p < 0.05$, Table 1). Besides, there were significant differences in the levels of AST, ALT, Tbil and PA among different liquiritin intervention groups ($p < 0.05$, respectively, Table 1).

Expressions of serum endotoxin and inflammation related factors in rats of each group: Compared with the normal group, the model group had significantly increased endotoxin, IL-6, IL-8 and TNF- α levels in rat plasma ($p < 0.001$, respectively, Table 2), furthermore, relative to the model group, there were obviously reduced endotoxin, IL-6, IL-8 and TNF- α levels in liquiritin intervention groups ($p < 0.05$, respectively, Table 2). Moreover, evident differences were observed in the levels of endotoxin, IL-6, IL-8 and TNF- α among different liquiritin intervention groups ($p < 0.05$, respectively, Table 2).

Pathological morphology of rat liver tissue in each group:

The cells in the liver tissue of the normal group were arranged orderly, without degeneration, necrosis or inflammatory cell infiltration (Fig. 1a). In the model group, hepatocytes showed patchy necrosis, with visible inflammatory cell infiltration and fibrous tissue proliferation (Fig. 1b). The pathological injury of liver tissue in the low (Fig. 1c), middle (Fig. 1d) and high (Fig. 1e) groups was reduced to varying degrees when compared with the model group (Fig. 1).

Cell apoptosis of rat liver tissue in each group:

According to the results of TUNEL staining, there was a highly increased number of apoptotic cells in the model group than that in the normal group ($p < 0.001$, Fig. 2a and b). After intervening with liquiritin, the number of apoptotic cells in liver tissue decreased significantly in the low, middle and high groups

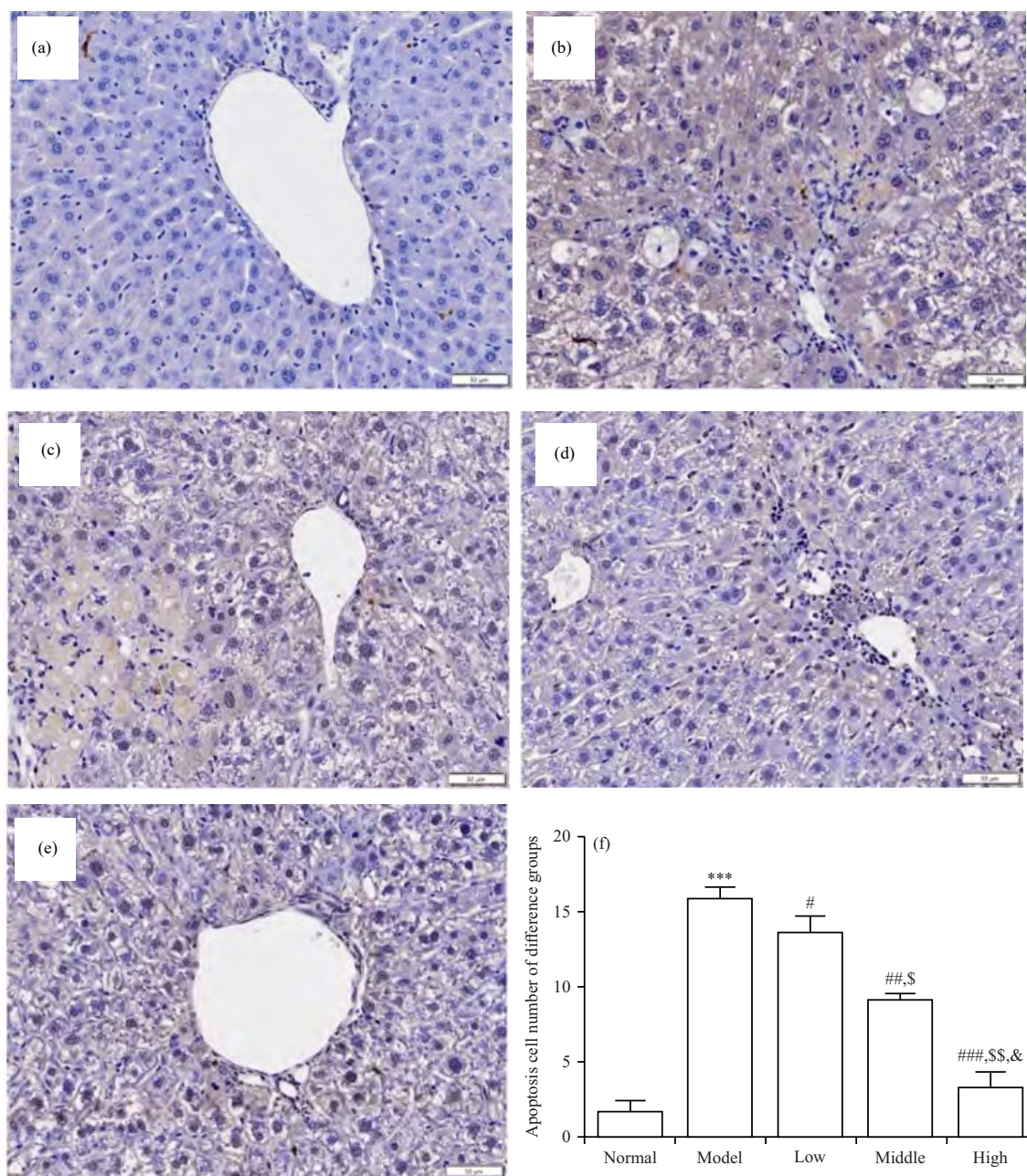


Fig. 2(a-e): Apoptosis cell number by TUNEL assay ($\times 200$), (a) Normal group, (b) ACLF model group, (c) ACLF model rats treated with low-dose liquiritin (1 mg g^{-1}), (d) ACLF model rats treated with middle-dose liquiritin (2 mg g^{-1}) and (e) ACLF model rats treated with high-dose liquiritin (10 mg g^{-1})

*** $p < 0.001$, compared with normal, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with model, $^{\circ}p < 0.05$, $^{ss}p < 0.01$, compared with low and $^{\&p} < 0.05$ compared with middle

than that in the model group ($p < 0.05$, respectively, Fig. 2c-e). Besides, there were significant differences in the number of apoptotic cells among different liquiritin intervention groups ($p < 0.05$, respectively, Fig. 2).

Protein expressions of TLR4 and p-NF- κ B(p65) in liver tissue of rats in each group by immunohistochemistry: There was a significant increase in the protein expressions of TLR4 and p-NF- κ B(p65) in the liver tissue of rats from the model group

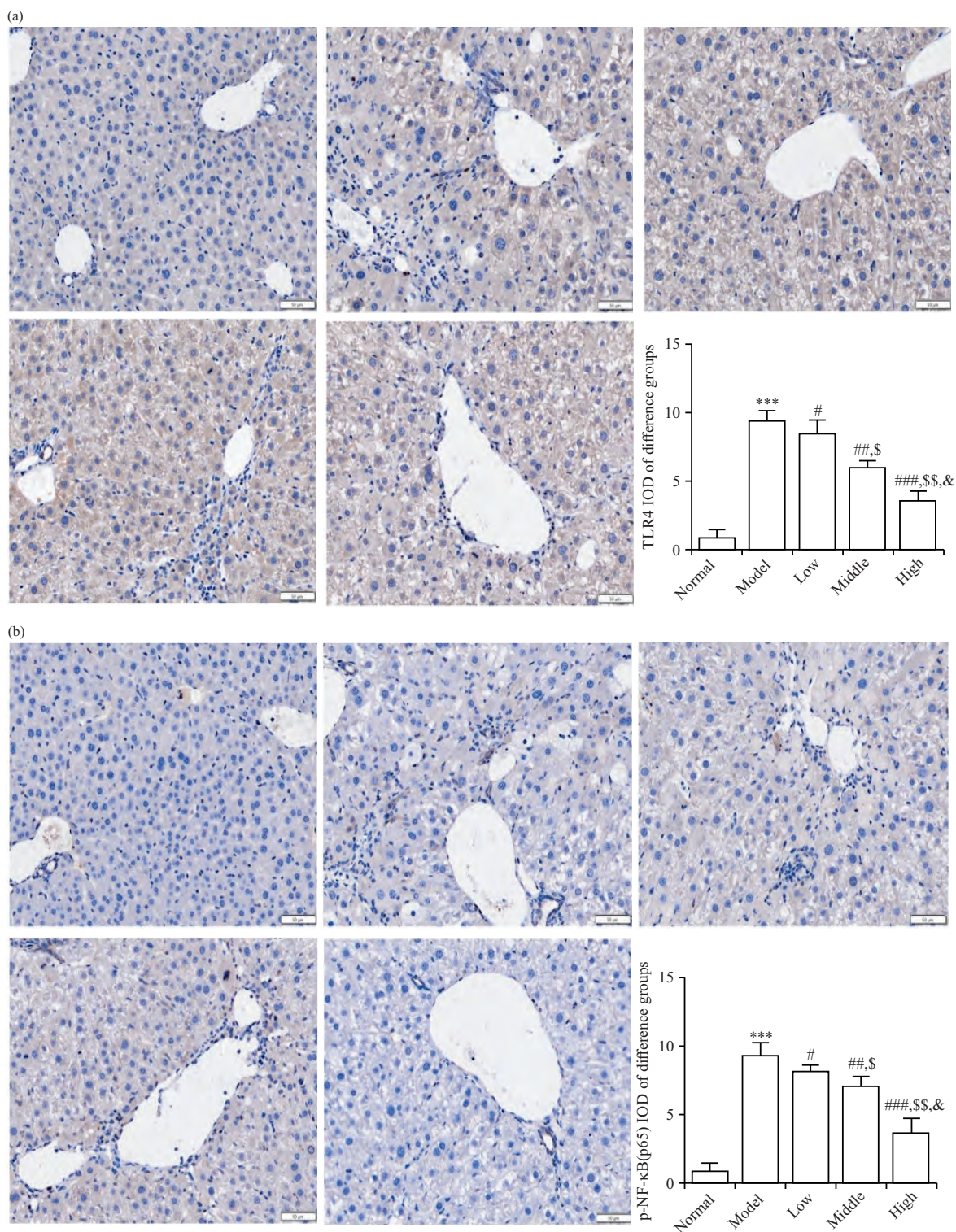


Fig. 3(a-b): TLR4 and p-NF-κB(p65) proteins expression by IHC in liver tissues ($\times 200$), Normal: Normal group, Model: ACLF model group, Low: ACLF model rats treated with low-dose liquiritin (1 mg g^{-1}), Middle: ACLF model rats treated with middle-dose liquiritin (2 mg g^{-1}), High: ACLF model rats treated with high-dose liquiritin (10 mg g^{-1}), (a) TLR4 protein expression in liver tissues ($\times 200$) and (b) p-NF-κB(p65) protein expression in liver tissues ($\times 200$)

*** $p < 0.001$, compared with normal, * $p < 0.05$, # $p < 0.01$, ### $p < 0.001$, compared with model, $^{\circ}p < 0.05$, $^{ss}p < 0.01$, compared with low and $^{\&}p < 0.05$ compared with middle

than those in the normal group ($p < 0.001$, respectively, Fig. 3a and b). Furthermore, compared with the model group, a significantly reduced trend was found in the protein expressions of TLR4 and p-NF- κ B (p65) in the low, middle and

high groups ($p < 0.05$, respectively, Fig. 3a and b). In addition, obvious differences were noticed in the protein expressions of TLR4 and p-NF- κ B(p65) among different liquiritin intervention groups ($p < 0.05$, respectively, Fig. 3a and b).

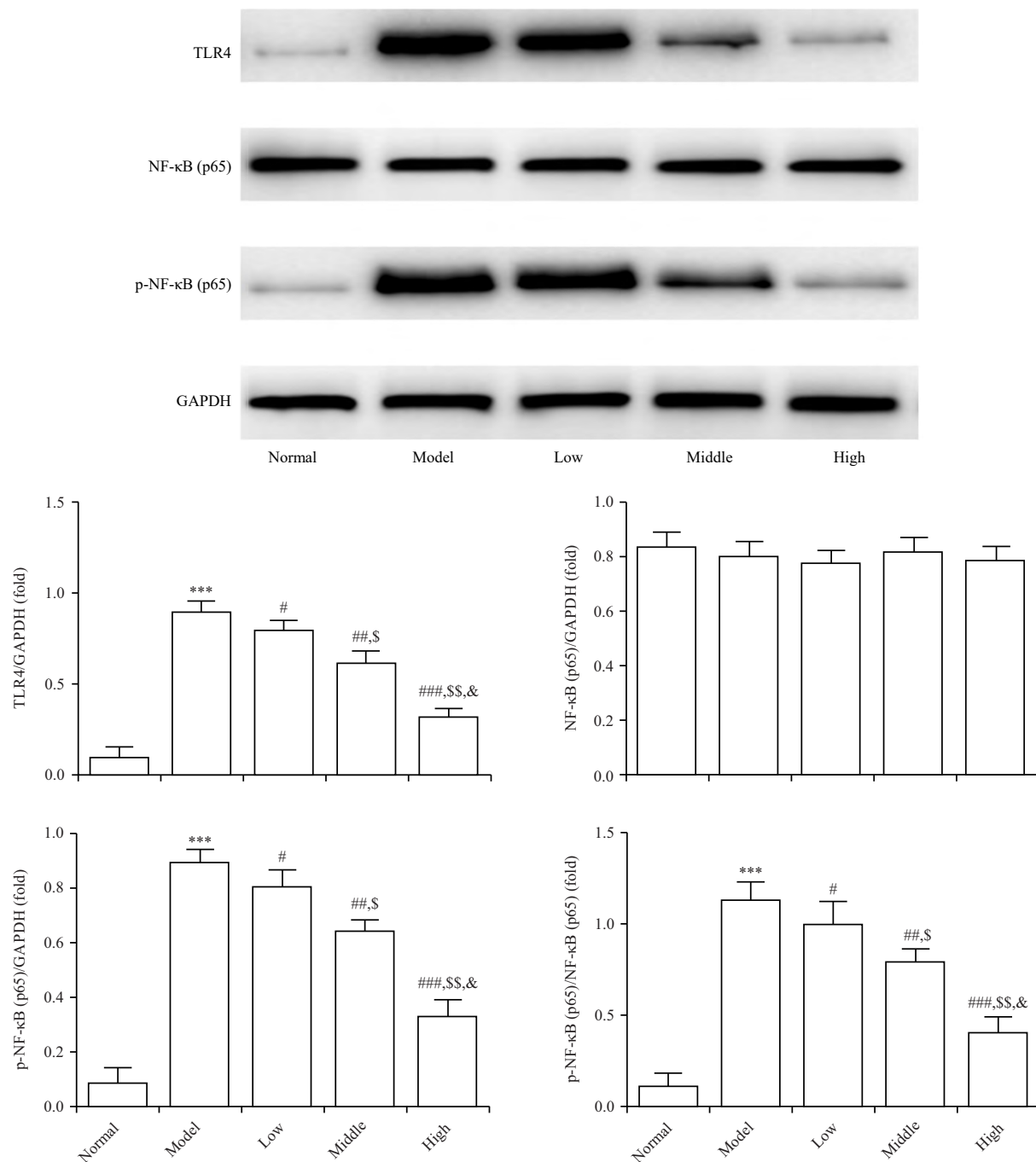


Fig. 4: TLR4 and p-NF- κ B(p65) proteins expression by WB assay in liver tissues, Normal: Normal group, Model: ACLF model group, Low: ACLF model rats treated with low-dose liquiritin (1 mg g^{-1}), Middle: ACLF model rats treated with middle-dose liquiritin (2 mg g^{-1}), High: ACLF model rats treated with high-dose liquiritin (10 mg g^{-1})

*** $p < 0.001$, compared with normal, * $p < 0.05$, # $p < 0.01$, ### $p < 0.001$, compared with model, \$ $p < 0.05$, \$ $p < 0.01$, compared with low and & $p < 0.05$, compared with middle

Protein expressions of TLR4 and p-NF- κ B(p65) in liver tissue of rats in each group by Western blot: In relative to the normal group, the model group showed significantly upregulated protein expressions of TLR4 and p-NF- κ B (p65) in the liver tissue of rats ($p < 0.001$, respectively, Fig. 4). Compared with the model group, there were much lower protein expressions of TLR4 and p-NF- κ B (p65) in the low, middle and high groups ($p < 0.05$, respectively, Fig. 4). Besides, the protein expressions of TLR4 and p-NF- κ B (p65) exhibited significant differences among different liquiritin intervention groups ($p < 0.05$, respectively, Fig. 4).

DISCUSSION

The ACLF is defined as a clinical syndrome of acute or subacute decompensation of liver function that develops within the short term in patients with chronic liver disease. The major clinical manifestations of ACLF are extreme fatigue, rapid exacerbation of jaundice, bleeding tendency, obvious gastrointestinal symptoms and decompensated ascites, etc. The disease can progress rapidly and the 3-month mortality can be as high as 50%¹¹. Due to the lack of matched liver, liver transplantation shows an obvious limitation in the treatment of ACLF and it is still managed by medication at present. Therefore, much attention has been paid to the identification of efficient therapeutic drugs for ACLF. Furthermore, Yu *et al.*⁹ established an additional ACLF modeling method, which is simple, rapid, effective and convenient for clinical drug research when compared with other modeling approaches. Therefore, this study used this modeling method to establish the ACLF model. In this study, according to the results of HE staining, there was patchy necrosis of hepatocytes, infiltration of inflammatory cells and proliferation of fibrous tissue in the liver tissue of rats from the Model group, TUNEL staining revealed that the model group had a significantly increased number of apoptotic hepatocytes, moreover, serum detection indicated that there were highly increase in the levels of AST, ALT, TBil, endotoxin and inflammatory factors IL-6, IL-8 and TNF- α , while, a much lower level of PA. All these results suggested aggravated inflammatory response in rats, obviously deteriorated liver function of rats and notably decreased inactivation ability of the liver to endotoxin.

Liquiritin is one of the main ingredients in licorice, which has been demonstrated to have extensive pharmacological activities, such as antitumor, antioxidation, anti-inflammation and protective effects on the heart and brain¹². For instance, Zhao *et al.*¹³ found in their research that liquiritin can effectively prevent and treat *Escherichia coli* related tumors

by inhibiting the polarization of M2 macrophages. Denzer *et al.*¹⁴ has proven in their experiment that liquiritin can improve mitochondrial function in an oxidative/nitrosative stress cell model. While in our study, after treating ACLF rats with liquiritin supplement, there were alleviated pathological damage of liver tissue, significantly reduced levels of serum AST, ALT, TBil, endotoxin, IL-6, IL-8 and TNF- α and obviously increased level of PA in rat serum. Moreover, an improvement in the therapeutic effect was observed with the increase in dosage. All these findings indicate that liquiritin can reduce the level of inflammation in ACLF rats, improve liver injury, enhance liver function and strengthen the inactivation ability of the liver to endotoxin.

Furthermore, TLR4/NF- κ B is an important signaling pathway to induce an inflammatory response. Inhibiting its activation has been discovered to inhibit liver fibrosis and hence improve liver function¹⁵⁻¹⁷. Du *et al.*¹⁸ have reported that the mechanism of Kaempferol protecting hepatocytes from the hepatotoxicity of Acetaminophen can be explained by the inhibition of the TLR4/NF- κ B signaling pathway. In the present study, TLR4 and NF- κ B p-p(65)/NF- κ B p65 protein expressions were significantly upregulated in the liver tissue of rats from the model group when compared with those in the normal group. It may suggested that TLR4/NF- κ B signaling pathway may be involved in the pathological changes and cell apoptosis of liver tissue in ACLF rats. Significantly, with liquiritin supplement, the protein expressions of TLR4 and p-NF- κ B(p65)/NF- κ B(p65) decreased significantly in the liver tissue of ACLF rats. Accordingly, the mechanism of liquiritin improving ACLF liver injury may be attributed partially to the inhibition of the TLR4/NF- κ B signal pathway.

CONCLUSION

To sum up, liquiritin can improve liver injury, reduce inflammatory response and hepatocyte apoptosis and improve liver function in ACLF rats. Findings in this research revealed that its mechanism may be associated with the inhibition of the TLR4/NF- κ B signaling pathway.

SIGNIFICANCE STATEMENT

Acute chronic liver failure (ACLF) can cause multiple organ failure, however, the treatment drugs were limited, natural drug extracts are safe and useful in some disease treatments. Liquiritin is a triterpene saponin extracted from the root of *Glycyrrhiza uralensis*. Our present study could support liquiritin treatment effects in ACLF.

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