Ercao Qinggan decoction regulates apoptosis of hepatocytes in mice with acute liver failure via the TLR4-mediated PI3K/Akt/GSK3β signal pathway

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Abstract
Background: To clarify the inhibitory effect of Ercao Qinggan decoction (EQD) on acute liver failure (ALF) and its related mechanisms. Methods: HL-7702 hepatocytes were pretreated with TLR4 inhibitor CLI-095, glycogen synthase kinase 3 β (GSK3 β) inhibitor LiCl and different doses of EQD for 2 hours, and lipopolysaccharide (LPS) (10 μg/mL) for 24 hours. Cell apoptosis, TNF-α and IL-6 and GSK3β were detected by flow cytometry, immunofluorescence, quantitative polymerase chain reaction. After mice were gavaged with different concentrations of EQD for 12 days, ALF mouse models were established intraperitoneal injection of D-Gal/LPS. After 24 hours, the mice were euthanized and the liver tissue was stained with hematoxylin and eosin. Liver cell apoptosis, the serum levels of aspartate aminotransferase, alanine aminotransferase, TNF-α and IL-β were detected by terminal transferase-mediated dUTP nick end-labelling, enzyme linked immunosorbent assay, quantitative polymerase chain reaction, and Western blotting, respectively. These methods were also used to test the mRNA expression of Bax, Bcl-2 and the protein expression of GSK3β, p-Akt/Akt in livers. Results: CLI-095, LiCl, and EQD significantly inhibited apoptosis induced by LPS, the mRNA expression of IL-6, TNF-α and the nuclear translocation of GSK3β in HL-7702 hepatocytes. EQD dose-dependently inhibited hepatocyte apoptosis, the serum concentration of aspartate aminotransferase and ALT, the expression of TNF-α and IL-β, the ratio of p-GSK3β/GSK3β, p-Akt/Akt in alanine aminotransferase mice. Conclusion: EQD can inhibit hepatocyte apoptosis in ALF mice through regulating TLR4/PI3K/Akt/GSK3β signaling pathway.

Keywords: acute liver failure; Ercao Qinggan decoction; lipopolysaccharide; HL-7702; TLR4

ARTICLE

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Introduction

As a severe clinical syndrome, acute liver failure (ALF) usually accompanies large areas of hepatocyte necrosis. The main pathological change in ALF is the death of large numbers of hepatocytes, causing an acute episode of liver dysfunction. Recently, apoptosis, a key to ALF pathogenesis, has been regarded as one of the leading forms of hepatocyte death. Nevertheless, its mechanism in ALF remains unclear.

Phosphatidylinositol-3 kinase/serine threonine kinase (PI3K/Akt) pathway has various biological impacts and can modulate apoptosis and survival. It is a crucial signal pathway that mediates apoptosis [1]. Activated Akt promotes glycogen synthase kinase 3 β (GSK3β) phosphorylation and the downstream substrates. It was found that TLR4-mediated PI3K/Akt/GSK3β signaling pathway contributes to the apoptosis of BRL-3A rat hepatocytes [2].

Ercao Qianqiang deccion (EQD) is a traditional Chinese medicine developed by the Department of Liver Diseases of Wenzhou Hospital of Traditional Chinese Medicine for the treatment of acute and chronic hepatitis. EQD is prepared with Abri Herba and Sedl Herba, together with Artemisiae Scopariae Herba, Rhei Radix et Rhiizonz, Smilacis Glabrae Rhiizon, Alosmatis Rhiizon, Atractylidos Macrocephalae Rhiizon, Cnrti Reticulatae Pericarpium, Glycyrrhizae Radix. Among them, Abri Herba has the characteristics of mild and pungent taste, clearing heat and dampness, soothing liver and relieving depression. Sedl Herba has the characteristics of mild and slightly sour taste, clearing heat, detoxifying and diuretic functions in southern Zhejiang as the sovereign medicine, so as to achieve the therapeutic effect of eliminating heat and dampness, detoxifying and relieving jaundice. EQD has been used for quite a few years in clinical practice and it has a definite effect in improving liver function and clinical symptoms in patients. EQD can protect and repair the acute liver damage induced by lipopolysaccharide (LPS) [3]. In recent years, the role of toll-like receptors (TLRs) in various infectious diseases and relevant inflammatory injury due to nonpathogenic microbes has gradually gained attention. TLR4 can recognize LPS, the key component of Gram-negative bacteria cell wall. EQD can inhibit TLR4 and its downstream signal molecules in rat hepatocytes, and lessen the immune-related liver injury [4, 5]. However, whether it participates in EQD intervention on ALF mice or not is still unclear.

For investigating the role of EQD in apoptosis, LPS and LPS/D-Gal are utilized to induce apoptosis in HL-7702 hepatocytes and ALF in mice in this study, respectively. Besides, we determined the PI3K/Akt/GSK3β signaling pathway-related protein expression.

Materials and methods

The sources for HL-7702 hepatocytes (Wuhan Shangen Biotechnology Co., Ltd., Wuhan, China), experimental animals, and materials were introduced in Supplementary Table S1.

Cell treatment

To investigate the PI3K/Akt/GSK3β signal pathway, the HL-7702 hepatocytes were inoculated a density of 3000 cells per well to 96 well plates, divided into control, LPS (Beijing solarbio science & technology Co., Ltd., Beijing, China), LPS with CLI-095 (MedChemExpress, Shanghai, China) and LPS with LCLi (Shanghai YuanYe Biotechnology Co., Ltd., Shanghai, China) groups at random. After the cells fully adhered to the wall, 10 μg/mL CLI-095 or 10 μg/L LCLi was added. 2 hours later, 10 μg/mL LPS was added. Cells continued to cultivate for 24 hours.

To examine the effect of EQD, the LPS-stimulated HL-7702 hepatocytes were randomly assigned into control, LPS, LPS with low-dose EQD, LPS with medium-dose EQD and LPS with high-dose EQD groups. The HL-7702 hepatocytes were inoculated a density of 3000 cells per well to 96 well plates. After the cells fully adhered to the wall, medicated serum of EQD at different doses was added. 2 hours later, 10 μg/mL LPS was added. Cells were continued to cultivate for 24 hours.

Establishment of ALF model in mice

Animal experiments were in strict accordance with the Chinese Guidelines of Animal Care and Welfare, and this study was approved by Jiangxi Zhonghong Boyuan Biotechnology Co., Ltd. (Nanchang, China) (license num: 2021092801). BALB/C male mice (Hunan SJA Laboratory Animal Co., Ltd., Hunan, China) (age: 6 to 8 weeks, weight: 18–20 g) were raised in the laboratory at a temperature of 20–26 °C and a humidity of 40–70%. The mice were randomly allocated into control, LPS/D-GalN, LPS/D-GalN with high-dose EQD, LPS/D-GalN with medium-dose EQD, LPS/D-GalN with high-dose EQD groups (n = 6). The mice in the control group and LPS/D-GalN group were gavaged with 0.9% sodium chloride solution (3 mL/kg/d) once a day for 12 days. The mice in the EQD-treated groups were given intragastric administration of EQD at different doses (0.2 mL/d, 0.3 mL/d and 0.4 mL/d, respectively) once a day, for 12 days. After 4 hours of final administration, all mice except the control group were intraperitoneally injected with D-Gal (800 mg/g) and LPS (7.5 mg/kg) (Sigma-Aldrich Corp., St. Louis, MO, USA) to establish an ALF model. After 24 hours of injection of LPS, all mice were euthanized. The liver tissues and serum were collected for the following experiments.

Cell apoptosis by flow cytometry

Annexin V-FITC/PI apoptosis kit (Multisciences (Lianke) Biotech Co., Ltd., Hangzhou, China) was utilized for measuring cell apoptosis. Specifically, 1–3 × 10⁶ cells were rinsed twice using phosphate buffered saline (PBS). Later, 300 μL of pre-cooled 1 × binding buffer (diluted from 5 × binding buffer with double distilled water) was used for cell resuspension. Subsequently, 10 μL PI and 5 μL Annexin V-FITC were put into and slightly mixed with cells. Then, the incubation of the mixture was implemented at room temperature (RT) for 10 min under the condition of light avoidance. Next, the cells were mixed with 200 μL of pre-cooled 1 × binding buffer, and the analysis was performed using the NovoCyte™ Flow Cytometry (Acea Bio Co., Ltd., Hangzhou, China).

Immunofluorescence

After discarding the supernatant, the cells were washed in PBS for 3 times, each time for 3 minutes, and then they were fixed with 4% paraformaldehyde for 15 minutes. Subsequently, the cells were washed three times with PBS again, permeabilized by 0.5% Triton X-100 at room temperature for 20 minutes. Washed again with PBS, cells were incubated with 5% bovine serum albumin at 37 °C for 30 minutes. Later, another incubation was conducted with the GSK3β primary antibody (1:200) (Proteintech Group, Inc., Wuhan, China) at 4 °C overnight. Rinsed with PBS again, the mixtures were added with Cy3’s fluorescence secondary antibody (1:200) (Abclonal Biotech Co., Ltd., Wuhan, China) for 30 min of incubation at 37 °C. Next, the treated cells were counterstained via DAPI (Jiangsu Keygen Biotech Co., Ltd., Jiangsu, China) for 5 min in the dark. After adding 20% glyceral into cells, the cells were observed and analysed by the fluorescence microscope.

Polymerase chain reaction (PCR)

Total RNA was extracted by Trizion (Jiangsu Cown Biotech Co., Ltd., Yangzhou, China) reagent. And the concentration and purity of RNA were measured by UV-Vis spectrophotometer. Subsequently, reverse transcription and fluorescence quantitative PCR (Bio-Rad Laboratories Co., Ltd., Shanghai, China) detection were performed. The reaction system, steps and primer sequences were presented in Table 1 and Table 2, respectively. Besides, β-actin was set as the internal control, and the 2⁻ΔΔCt method was utilized to measure the mRNA expression levels.

Western blot

Added 100 μL cell lysis buffer to the cell, cooled on ice for 20 minutes, transferred to an eppendorf tube, and then centrifugated at 12000 rpm for 10 minutes, collected proteins from the supernatant. The
concentration of protein was determined using the bicinchoninic acid
method. After 1.5 hours of sodium dodecyl sulfate polyacrylamide gel
electrophoresis (SDS-PAGE, 300 mA), the protein was separated and
transferred to the membrane. Added the first and second antibodies to
the PVDF membrane and incubated them overnight at 4 °C and for 2
hours at room temperature, respectively. Washed the film, wet the
PVDF film with luminescent liquid, and placed it in the sample
placement area of the ultra-high sensitivity chemiluminescence
imaging system for imaging.

**Hematoxylin-eosin (HE) staining**
Paraffin sections were dewaxed to water, stained with hematoxylin
(Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China)
for nuclei, stained with eosin for cytoplasm, dehydrated and sealed,
examined under a microscope, and analyzed for image collection.

**Terminal transferase-mediated dUTP nick end-labelling (TUNEL)
test**
The tissue slices was dewaxed to water. Protease K working solution
was added and incubated at 37 °C for 30 minutes. Sufficient TUNEL
test solution was added dropwise and incubated at 42 °C in the dark
for 1 hour. After rinsing with PBS, DAPI was added dropwise and
incubated in the dark for 3 minutes. The sample was stained and the
excess DAPI was rinsed with PBS. Use absorbent paper to dry the
liquid on the glass slide, seal it with sealing solution containing anti-
fluorescence quenching agent, and then observe and collect images
under a fluorescence microscope.

**Enzyme linked immunosorbent assay (ELISA)**
Related assay kits were performed to test alanine aminotransferase
(ALT) and aspartate aminotransferase (AST) (Nanjing Jiancheng
Bioengineering Institute, Nanjing, China) in mice, and ELISA to
determine serum TNF-α and IL-1β. The wells were added with 50 μl
of samples or different concentration of standards, followed by 50 μl
of biotin-labeled antibody. Then, the plate was covered with sealing
film and incubated at 37 °C for 30 min. The liquid was discarded, and
each well was rinsed with 350 μl of washing solution for 5 times. The
samples were mixed with 100 μl of horseradish peroxidase
(HRP)-labeled antibody then incubated for 30 min at 37 °C. After
removing the liquid, each well was washed again with 350 μl of
washing solution for 5 times. Next, the mixtures were incubated with
50 μl of substrates A and B at 37 °C in the dark. 15 min later, 50 μl of
stop solution was supplemented. The optical density values were read
within 15 min at 450 nm.

**Statistical analysis**
The data was analyzed with Statistical Product and Service Solutions
20.0 software. All investigations were repeated three times. The
results were expressed as mean ± standard deviation. Quantitative

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**Table 1 Reaction system and steps of PCR**

<table>
<thead>
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<th>Reaction system</th>
<th>Volume</th>
<th>Reaction steps</th>
<th>Temperature</th>
<th>Time</th>
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<tbody>
<tr>
<td>2 × SYBR Green PCR Master Mix</td>
<td>10 μl</td>
<td>Pre-denaturation</td>
<td>95 °C</td>
<td>10 min</td>
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<tr>
<td>cDNA</td>
<td>1 μl</td>
<td>Denaturation</td>
<td>95 °C</td>
<td>10 sec</td>
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<tr>
<td>Upstream primer</td>
<td>0.4 μl</td>
<td>Annealing</td>
<td>58 °C</td>
<td>30 sec</td>
</tr>
<tr>
<td>Downstream primer</td>
<td>0.4 μl</td>
<td>Extension</td>
<td>72 °C</td>
<td>30 sec</td>
</tr>
<tr>
<td>RNase free dH2O</td>
<td>8.2 μl</td>
<td>Cycle</td>
<td>40 deg C</td>
<td></td>
</tr>
</tbody>
</table>

**PCR**, polymerase chain reaction.

**Table 2 Primer sequences and annealing temperature in qPCR**

<table>
<thead>
<tr>
<th>Primer sequences (5’-3’)</th>
<th>Product length (bp)</th>
<th>Annealing temperature (°C)</th>
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</thead>
<tbody>
<tr>
<td>Bax F</td>
<td>GACAGGGGCGCTTTTGTGCAC</td>
<td>187</td>
</tr>
<tr>
<td>Bax R</td>
<td>CAGCTGAGCAATCCACCTCCTGC</td>
<td>161</td>
</tr>
<tr>
<td>Bel-2 F</td>
<td>AGGATTTGGGCCGTCCCTTGA</td>
<td>161</td>
</tr>
<tr>
<td>Bel-2 R</td>
<td>ACAAAAGGATCCACCCAGGC</td>
<td>187</td>
</tr>
<tr>
<td>β-actin F</td>
<td>AGGGAAATGCCTGGCTGTC</td>
<td>192</td>
</tr>
<tr>
<td>β-actin R</td>
<td>CATACCCAGAAGGAAAGGCT</td>
<td>57.03</td>
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**qPCR**, quantitative polymerase chain reaction.

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Figure 1 Effect of TLR4 inhibitor and GSK3β inhibitor on the apoptosis of HL-7702 hepatocytes. *P < 0.05 vs. Control; †P < 0.05 vs. LPS. GSK3β, glycogen synthase kinase 3β; LPS, lipopolysaccharide; TLR4, toll-like receptor 4.

Figure 2 Effect of TLR4 inhibitor and GSK3β inhibitor on the mRNA relative expression of TNF-α and IL-6 in HL-7702 hepatocytes. *P < 0.05 vs. Control; †P < 0.05 vs. LPS. GSK3β, glycogen synthase kinase 3β; LPS, lipopolysaccharide; TLR4, toll-like receptor 4.

Figure 3 Effect of TLR4 inhibitor and GSK3β inhibitor on the nuclear translocation of GSK3β in HL-7702 hepatocytes. GSK3β, glycogen synthase kinase 3β; LPS, lipopolysaccharide; TLR4, toll-like receptor 4.
EQU inhibited LPS-induced GSK3β nuclear translocation in HL-7702 hepatocytes

Upon cell treatment using LPS and different doses of EQU, immunofluorescence detection showed that GSK3β in HL-7702 hepatocytes in the control group was mainly expressed in the cytoplasm. It seemed to be aggregated in the nucleus following LPS treatment. Treatment with different doses of EQU can significantly inhibit the nuclear translocation of GSK3β induced by LPS (Figure 6).

EQU improved the pathological changes of liver in ALF mice

The model of ALF mice was induced by LPS/D-GalN. The hepatocytes in the LPS/D-GaIn group were lightly stained, enlarged and arranged disorderly. The heterotypic hepatocytes were organized in a cord-like shape with abundant blood sinuses. A large number of small and deeply stained cell infiltration were existed in the liver tissues. The hepatocytes in the LPS/D-GalN + EQU-L and LPS/D-GalN + EQU-M groups arranged disorderly, and there was infiltration of inflammatory cells and a small amount of bleeding in the liver tissues. Bleeding and the infiltration of inflammatory cell were not seen in the LPS/D-GalN + EQU-H group (Figure 7).

EQU decreased serum AST and ALT in ALF mice

Compared with the control group, LPS/D-GalN treatment resulted a remarkable raise of AST and ALT in the mice serum, while EQU treatment resulted in the opposite (Figure 8).

EQU significantly decreased serum TNF-α and IL-1β in ALF mice

Compared with the control, the expression of TNF-α and IL-1β in serum markedly increased in the LPS/D-GalN group. However, EQU treatment can reverse above-mentioned results (Figure 9).

EQU decreased apoptosis in ALF mice liver tissues

LPS/D-GalN-induced ALF mice were treated with intragastric administration of low, medium or high doses of EQU. TUNEL assay analysis demonstrated an increase in apoptotic cells in liver tissues of the LPS/D-GalN group compared with the control group. However, EQU treatment can reverse above-mentioned results (Figure 10A).
Figure 6 Effect of the different doses of EQD on nuclear translocation of GSK3β in HL-7702 hepatocytes. GSK3β, glycogen synthase kinase 3 β; LPS, lipopolysaccharide; EQD, Ercao Qinggan decoction.

Figure 7 Effect of the different doses of EQD on pathological changes of liver tissues in ALF mice. Magnification, × 400. LPS, lipopolysaccharide; EQD, Ercao Qinggan decoction; ALF, acute liver failure; L, low dose; M, medium dose; H, high dose.
qPCR analysis showed a markedly increase in Bax and a significant reduction in Bcl-2 mRNA expression levels in the LPS/D-GalN group in comparison with the control. However, EQD treatment can reverse above-mentioned results (Figure 10B).

Effects of EQD on PI3K/Akt/GSK3β signal pathway in ALF mice

Western blot analysis showed a considerab decrease in p-GSK3β/GSK3β and p-Akt/Akt ratios in liver tissues in the LPS/D-GalN group compared with the control group. However, EQD treatment can reverse above-mentioned results (Figure 11).

Figure 8 Effect of the different doses of EQD on serum AST and ALT in ALF mice. *P < 0.05 vs. Control; †P < 0.05 vs. LPS/D-GalN. LPS, lipopolysaccharide; EQD, Ercao Qinggan decoction; ALF, acute liver failure; AST, aspartate aminotransferase; ALT, alanine aminotransferase; L, low dose; M, medium dose; H, high dose.

Figure 9 Effect of the different doses of EQD on TNF-α and IL-1β in the serum of ALF mice. *P < 0.05 vs. Control; †P < 0.05 vs. LPS/D-GalN. LPS, lipopolysaccharide; EQD, Ercao Qinggan decoction; ALF, acute liver failure; L, low dose; M, medium dose; H, high dose.

Figure 10 Effect of the different doses of EQD on apoptosis in the liver tissues of ALF mice. (A) TUNEL assay. (B) qPCR. *P < 0.05 vs. Control; †P < 0.05 vs. LPS/D-GalN. EQD, Ercao Qinggan decoction; ALF, acute liver failure; TUNEL, terminal transferase-mediated dUTP nick end-labelling; qPCR, quantitative polymerase chain reaction; LPS, lipopolysaccharide; L, low dose; M, medium dose; H, high dose.
Figure 11 Effect of the different doses of EQD on PI3K/Akt/GSK3β signal pathway in the liver tissues of ALF mice. *P < 0.05 vs. Control; **P < 0.05 vs. LPS/D-GaIN. EQD, Ercaio Qinggan decoction; GSK3β, glycosyln synthase kinase 3 β; LPS, lipopolysaccharide; ALF, acute liver failure; L, low dose; M, medium dose; H, high dose.

Discussion

It’s humid and hot in Wenzhou. Many famous experts in Lingnan use traditional Chinese medicine for clearing heat and promoting dampness in liver diseases, such as Professor Jian-Xing Qiu’s experience in chronic hepatitis B treatment, which is based on the damp-heat disease theory in Lingnan [6]. EQD is composed of Abri Herba and Sedi Herba, together with other heat-clearing and dampness-resolving drugs, and it has the therapeutic effects of clearing heat, promoting dampness, detoxifying and relieving jaundice. EQD can protect and repair acute liver injury induced by LPS. The gentleman medicine of EQD are Abri Herba and Sedi Herba. Both of them are genuine Chinese herbal medicine called “Wenbeiwei” in Wenzhou. Abri Herba is a dried whole plant of Acacia cantoniensis, a member of the genus Abrus, leguminosae. Its main components are flavonoids, alkaloids and saponins. They are effective in protecting the liver and treating hepatitis and cirrhosis [7]. Sedi Herba is the whole plant of Crassulaceae plant Sedum sarmentosum. Its main components are flavonoids and cyanidines. They have significant effects on the treatment of acute liver injury and liver failure [8].

Apoptosis, programmed cell death, is the main pathological characteristic of ALF [9]. Tissue homeostasis is secured by regulating cell proliferation and removing damaged, infected, or transformed cells. Hepatocyte apoptosis contributes to necrosis formation [10]. Excessive hepatocytes apoptosis causes the tissue destruction and ALF.

TLR4, a pattern recognition receptor, belongs to the TLR family. According to the recent study, an increased expression of TLR4 in the liver injury and ALF [11]. The PI3K/Akt signaling pathway, which mediates various biological effects, is crucial in the regulation of cell apoptosis and survival. PI3K, consists of regulatory (p85) and catalytic (p110) subunits, is a conserved signal transduction enzyme complex [12]. It regulates cell proliferation and inhibits apoptosis. PI3K activation can enhance phosphatidylinositol (3-5)-triphosphate formation, which is a secondary messenger, and activates Akt phosphorylation. Through activating or inhibiting the phosphorylation of its downstream substrates, such as GSK3β, activated Akt can influence cell proliferation, differentiation, apoptosis, and migration [13]. Akt can also inhibit cytokine c release by suppressing Bax activity and inhibit apoptosis by phosphorylating Ser136 of Bcl-2. The PI3K/Akt/GSK3β signaling pathway is the most important regulatory pathway for liver cell apoptosis [14]. Recent studies demonstrated that TLR4-mediated PI3K/Akt/GSK3β signaling pathway can regulate LPS-induced hepatocyte apoptosis. A study given by Chen, et al. [15] claimed that through the TLR4/PI3K/Akt/GSK3β signaling pathway, Patrinia can significantly inhibit apoptosis in LPS-induced BRL-3A hepatocytes, providing molecular mechanisms underlying the treatment of liver diseases. In summary, EQD can treat liver injury. But whether PI3K/Akt/GSK3β mediated by TLR4 has effect on cell apoptosis needs further verification.

To investigate how TLR4/PI3K/Akt/GSK3β affects cell apoptosis, the TLR4 inhibitor CLI-095 and the GSK3β inhibitor LiCl were used in this study to inhibit different sites of this pathway. Lithium can promote GSK3β Phosphorylation at serine-9. Phosphorylated GSK3β is an inactive form of GSK3β. Phosphorylated Akt makes GSK3β phosphorylation inhibit GSK3β-mediated apoptosis [16, 17]. The results in this study showed that the HL-7702 hepatocytes cell apoptosis rate and the mRNA expression of TNF-α and IL-6 were markedly increased with LPS treatment, which could be inhibited by TLR4 inhibitor CLI-095 and GSK3β inhibitor LiCl. GSK3β tended to accumulate in the nucleus following LPS treatment; however, CLI-095 and LiCl can inhibit the nuclear translocation of GSK3β. The results were consistent with the report in BRL-3A hepatocytes by Zhang, et al. [5], suggesting that TLR4-mediated PI3K/Akt/GSK3β signal pathway may be present in HL-7702 hepatocytes stimulated by LPS. In this study, the LPS-induced HL-7702 hepatocytes were treated with the low, medium and high doses of EQD. The findings showed significant inhibition of the cell apoptosis rate, the expression of TNF-α and IL-6 mRNA, and nuclear translocation of GSK3β in the EQD treatment groups compared with the LPS group, indicating that LPS-induced apoptosis in HL-7702 hepatocytes can be inhibited by EQD.

We further explored whether TLR4-mediated PI3K/Akt/GSK3β exists in EQD-treated ALF mice. ALF was induced in mice by intraperitoneal injection of LPS/D-GaIN [18]. LPS, the Gram-negative bacteria cell wall primary structural element, can cause activation of the intracellular signal, release of various inflammatory factors and cytotoxic substances, and induction of hepatocyte apoptosis and necrosis. Under LPS stimulation, TLR4 induces NF-kβ activation [19]. Activated NF-kβ transfers to the nucleus and regulates gene transcription related to inflammatory response, including TNF-α, IL-1β, IL-6, superoxide and nitric oxide, which in turn stimulates hepatocyte apoptosis and necrosis. ALT and AST are important indicators reflecting liver cell damage. They are significantly increase in ALF patients. Zhang [20] found that oxbymatrine pretreatment could improve the liver pathological changes of ALF rats, reduce serum...
aaminotransferases and protect hepatocytes. Additionally, it could suppress hepatocyte apoptosis and promote the p-GSK3β serine-9 and p-AKT Ser473 expression. This report indicated that EQD was able to relieve hepatocyte apoptosis via the TLR4/PI3K/Akt/GSK3β signal pathway. In this study, intraperitoneal injection of D-Gal/LPS markedly increased the expression of ALT, AST, TNF-α and IL-1β in the serum, and induced significant damage in liver tissues of mice, confirming the successful preparation of ALF mouse model. We found that EQD can effectively inhibit the excessively activated inflammatory response-induced by LPS/D-Gal, TNF-α and IL-1β secretion, serum ALT and AST levels, Bax mRNA expression levels, and hepatocyte apoptosis in liver tissues, and upregulate the Bcl-2 mRNA expression levels, exerting a protective effect on liver. Western blot showed that treatment with different doses of EQD increased the p-GSK3β and p-Akt expression levels in mice liver tissue compared with the ALF model group, with the high dose EQD group demonstrating the most significant effect. These findings indicated that EQD could suppress the TLR4/PI3K/Akt/GSK3β signal pathway.

In summary, EQD can suppress hepatocyte apoptosis through the TLR4/PI3K/Akt/GSK3β signal pathway. The findings of this study added to the experimental evidence on the influence of TLR4-mediated signal pathway on hepatocyte apoptosis in ALF, and contributed to the search for effective drugs in the treatment and prevention of ALF. However, this study was unable to investigate the main chemical components of EQD, and the main chemical components that inhibit liver cell apoptosis are unknown. Besides, further research on the specific target proteins and targets of EQD has not been conducted, resulting in insufficient evidence of the targets of EQD. The above questions will provide direction for our next research step.

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