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Original Article

Xijiao Dihuang Decoction (犀角地黄汤) and *Rehmannia glutinosa* Libosch. Protect Mice against Lipopolysaccharide and Tumor Necrosis Factor Alpha-Induced Acute Liver Failure*

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ABSTRACT **Objective**: To investigate the hepatoprotective effect of Xijiao Dihuang Decoction (犀角地黄汤, XJDHD) on lipopolysaccharide (LPS)- and tumor necrosis factor alpha (TNF-α)-induced acute liver failure (ALF) as well as the underlying mechanism of action, and to clarify the key herbs and components of XJDHD. **Methods**: LPS/D-galactosamine (D-GalN) or TNF-α/D-GalN were intraperitoneally injected into C57BL/6J mice to induce ALF. Simultaneously, XJDHD or its individual herbs and components were orally administered. Survival rates, transaminase levels in serum, and hepatic histology were examined to evaluate the effects of XJDHD. The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay and real-time polymerase chain reaction were additionally performed to expound the mechanism underlying the anti-apoptotic activity of XJDHD. **Results**: Oral administration of XJDHD protected mice from lethal liver failure induced by LPS and TNF-α, with notable amelioration of liver injury in histology and a significant decrease in transaminase levels in serum. XJDHD significantly inhibited apoptosis of hepatocytes and enhanced expression of the anti-apoptosis genes, c-Flip, Iap1, Gadd45b and A20 (all $P<0.05$). In addition, *Rehmannia glutinosa* Libosch. was identified as the key herb of XJDHD and galactose as the effective component of *Rehmannia glutinosa* Libosch. that protects against ALF. **Conclusions**: XJDHD inhibits TNF-α-induced apoptosis of hepatocytes by promoting the expression of nuclear factor κB-regulated anti-apoptotic genes. *Rehmannia glutinosa* Libosch. may be the most effective herb of XJDHD and galactose is an active component in this protection.

KEYWORDS Xijiao Dihuang Decoction, Chinese medicine, *Rehmannia glutinosa* Libosch., acute liver failure, lipopolysaccharides, tumor necrosis factor alpha

Acute liver failure (ALF) is a complex and progressive disease with rapid onset of hepatocyte death or dysfunction due to several etiologies, including viruses, drugs, toxins and other genetic and autoimmune conditions.[1] Although liver transplantation is an effective life-saving therapy, mortality remains high owing to shortage of donors and extremely high medical costs. Imminent identification of more therapeutic drugs to prevent ALF is therefore an urgent necessity.

The lipopolysaccharide (LPS)/D-galactosamine (D-GalN)-induced ALF model has been accepted to screen hepatoprotective drugs and investigate their underlying mechanisms. In this model, multiple cytokines are induced by LPS, such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-10 and IL-6. Among these, TNF-α is initially released and promotes the production of other cytokines that play a key role in apoptosis of hepatocytes.[2] D-GalN, a sensitizing agent, amplifies the toxicity of LPS and TNF-α specifically to the liver with no damage to other organs or tissues.[3]

Recently, Chinese medicine (CM) has attracted increasing attention worldwide for its potential value in identifying new medicinal component(s). A number of CM prescriptions and herbs are reported to prevent against ALF induced by LPS/D-GalN in animals, such as Liangxue Huayu Recipe (凉血化瘀方),[4,5] garlic, sedum sarmentosum and echinacea alkamide.[6-8] Xijiao Dihuang Decoction (犀角地黄汤, XJDHD) was first recorded in *Valuable Prescriptions for Emergency*

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(Bei Ji Qian Jin Yao Fang). With the effects of clearing heat and detoxifying, as well as cooling blood and inducing resuscitation, XJDHD was used to treat allergic purpura, uremia, psoriasis and liver diseases. Recently, we founded that XJDHD increases the survival rate of LPS/D-GalN-injected mice.[9] These findings prompted us to further explore the efficacious herbs in XJDHD and underlying mechanism of action in liver protection.

XJDHD consists of the following ingredients: *Cornu Bubali* 30 g, *Rehmannia glutinosa* Libosch. 24 g, *Paeonia lactiflora* Pall. 12 g, and *Paeonia suffruticosa* Andr. 9 g. The water extract of *Cornu Bubali* has antipyretic and antioxidant properties and fragmented keratins prepared from *Cornu Bubali* may act as a drug carrier.[10,11] Thus, *Cornu Bubali* is proposed to alleviate liver injury or enhance the effects of other herbs. *Rehmannia glutinosa* Libosch. is widely used in East Asia for its putative medicinal value in treating fever, bleeding and hormonal disorders in the clinic, and further reported to promote diabetic wound healing, suppress immune responses, ameliorate liver inflammation and fibrosis in an experimental study.[12-14] *Paeonia lactiflora* Pall. has been shown to attenuate ALF induced by carbon tetrachloride (CCl_4), and *Paeonia suffruticosa* Andr. to alleviate inflammation and protect against acute hepatotoxicity induced by CCl_4, D-GalN and α-naphthylisothiocyanate.[15-20] Moreover, the *Paeonia suffruticosa* Andr. extract appears to play a protective role in acetaminophen-induced hepatotoxicity.[21] However, the mechanism of action of XJDHD and the effective constituent herbs that prevent LPS-induced ALF remain to be established. Data from the present study have shown that XJDHD protects against TNF-α/D-GalN-induced liver failure in mice by promoting the expression of anti-apoptotic genes and inhibiting TNF-α-induced apoptosis of hepatocytes. Moreover, *Rehmannia glutinosa* Libosch. and *Radix rehmannia praeparata* are the key medicinal herbs of XJDHD, with galactose as the common efficacious component.

METHODS

Reagents

LPS (from *E. coli*, 0111:B4), D-GalN, D-galactose, stachyose and verbascose were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Recombinant TNF-α was obtained from Sinobio Co. (Shanghai, China), catalpol was from Merck Co. (Darmstadt, Germany), and raffinose was from Amresco Co. (Solon, USA).

Herbs and Extract Preparation

Cornu Bubali, *Rehmannia glutinosa* Libosch., *Radix rehmannia praeparata*, *Paeonia lactiflora* Pall., and *Paeonia suffruticosa* Andr. were purchased from Beijing Tong-Ren-Tang Drug Store (Beijing, China). XJDHD contains 0.2 g/mL *Cornu Bubali*, 0.16 g/mL *Rehmannia glutinosa* Libosch., 0.08 g/mL *Paeonia lactiflora* Pall. and 0.06 g/mL *Paeonia suffruticosa* Andr. The single herb decoction was provided at doses of 5, 4, 2 or 1.5 g/kg, respectively.

To obtain crude extracts of the *Rehmannia glutinosa* Libosch. decoction, 750 mL of 95% aqueous ethanol was slowly added to 250 mL *Rehmannia glutinosa* Libosch. (or *Radix rehmannia praeparata*) decoction. The mixture was stirred thoroughly, incubated overnight at 4 ℃, and separated into two parts after centrifugation at 4,000 r/min for 20 min. The supernatant (ethanol extract) was evaporated to eliminate ethanol, and finally dissolved to 250 mL with double distilled water (ddH_2O). The precipitate (water extract) was dissolved to 250 mL with ddH_2O.

Animals and Treatments

Seven hundred and forty-two male C57BL/6J mice (6–8 weeks old) were purchased from the Institute of Zoology, Chinese Academy of Sciences [SCXK (Beijing) 2009-0007], housed under a 12-h light-dark cycle and provided standard laboratory chow. Temperature and relative humidity were maintained at 25±2 ℃ and 55%±5%, respectively. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Capital Medical University, China.

To induce ALF, LPS/D-GalN and TNF-α/D-GalN models were used in the present study. LPS and D-GalN were co-injected intraperitoneally into mice at the doses of 0.25 and 400 mg/kg, respectively. In the TNF-α/D-GalN model, 30 μg/kg TNF-α and 700 mg/kg D-GalN were injected intraperitoneally into mice. To identify the constituent herb(s) of XJDHD effective in protection against liver failure, mice were treated with decoction of *Cornu Bubali*, *Rehmannia glutinosa* Libosch., *Paeonia lactiflora* Pall., or *Paeonia suffruticosa* Andr., *Radix rehmannia praeparata*. According to the content of them in *Rehmannia glutinosa*

Libosch., stachyose, mannotriose, raffinose, galactose and catalpol were administered at the doses of 2000, 1000, 500, 100 and 100 mg/kg, respectively.[22,23]

Mice were randomly divided into several groups as indicated in the experiments. For drug treatment, the decoction, herb extract, catalpol or saccharide was orally administered immediately after D-GalN injection. Control group mice were given equal volume of ddH_2O. To calculate the survival percentage, mice were observed for 24 h. To evaluate transaminase activity in serum and pathological injury in liver, mice were sacrificed 5 h after D-GalN injection.

Determination of Transaminases Activity

Activities of serum alanine transaminase (ALT) and aspartate transaminase (AST) were determined with an automated procedure in the automatic analyzer (Model 7600 Series, Hitachi, Japan).

Multiplex Cytokine Assay

For serum cytokine measurement, the BD cytometric beads array (CBA) mouse inflammation kit (BD Biosciences, San Jose, CA, USA) was used to determine IL-12p70, TNF-α, interferon-γ (IFN-γ), Monocyte chemoattractant protein -1 (MCP-1), IL-10 and IL-6 levels. Data were acquired on a BD™ FACS Calibur flow cytometer (BD, USA). Measurements were performed according to the manufacturer's instructions.

Hematoxylin and Eosin Staining and TUNEL Assay

Liver tissues were fixed in 10% neutral buffered formalin, dehydrated, embedded in paraffin and sliced in 5 μm sections. Hematoxylin and eosin (HE) staining was performed to evaluate histopathology damage. Apoptotic hepatocytes were detected using the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay with the in situ cell death detection kit (Merck, Darmstadt, Germany), according to the manufacturer's instructions.

RNA Isolation and Real-Time Polymerase Chain Reaction Assay

During the process of TNF-α-induced apoptosis, nuclear factor-kappa B (NF-κB) plays a pivotal role in cell survival through promoting the transcription of a variety of anti-apoptotic genes. Accordingly, the expression levels of NF-κB downstream genes, including inhibitor of apoptosis protein 1 (IAP-1), cellular FADD-like-IL-1β-converting-enzyme (FLICE)-like inhibitory protein (c-Flip), growth arrest and DNA-damage-inducible 45b (Gadd45b) and A20 were examined.[24] Total RNA from mouse liver was extracted with TRIzol. Reverse-transcription polymerase chain reaction (PCR) was performed using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). The mRNA levels of c-FLIP, c-IAP1, Gadd45b, A20 and β-actin were determined with the ABI Prism 7500 real-time PCR system (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was performed using FastStart Universal SYBR Green Master (Roche, Mannheim, Germany). The primers used were as follows: c-Flip, 5'-GCG ACC GCC CGG TAG TGT CT-3' (forward) and 5'-CCA CAG CAG CCA GGT TCT CGT-3' (reverse); c-IAP1, 5'-TGT GGC CTG ATG TTG GAT AAC (forward)-3' and 5'-GGT GAC GAA TGT GCA AAT CTA CT (reverse)-3' Gadd45b, 5'-CAA CGC GGT TCA GA A GAT GC-3' (forward) and 5'-GGT CCA CAT TCA TCA GTT TGG C-3' (reverse); A20, 5'-CCC CTG GTG ACC CTG AAG GAC A-3' (forward) and 5'-CCG TGG TCC CAG CCT TGC AC-3' (reverse); β-actin, 5'-GGC TGT ATT CCC CTC CAT CG-3' (forward) and 5'-CCA GTT GGT AAC AAT GCC ATG T-3' (reverse). Relative mRNA levels for specific genes were normalized to β-actin.

Statistical Analysis

All values are presented as mean ± standard deviation, and analyzed using SPSS 16.0 software. The Log-rank test (for survival), *t*-test (for cytokines and transaminases) and one-way ANOVA (for mRNA) were used to compare differences. *P* values less than 0.05 were taken as statistically significant.

RESULTS

XJDHD Protects Mice from LPS/D-GalN-Induced ALF without Affecting Cytokine Production

Similar to our previous findings, XJDHD induced a significant increase in the survival rates of LPS/D-GalN challenged mice (Figure 1A). Concurrently, XJDHD decreased transaminase activity in serum ($P<0.05$, Figure 1B) and ameliorated pathological damage in the liver (Figure 1C). It brought little influence in transaminases and liver damage to mice after treatment with XJDHD alone (data not shown).

To further explore the protective mechanism of XJDHD, cytokine secretion in serum was detected.

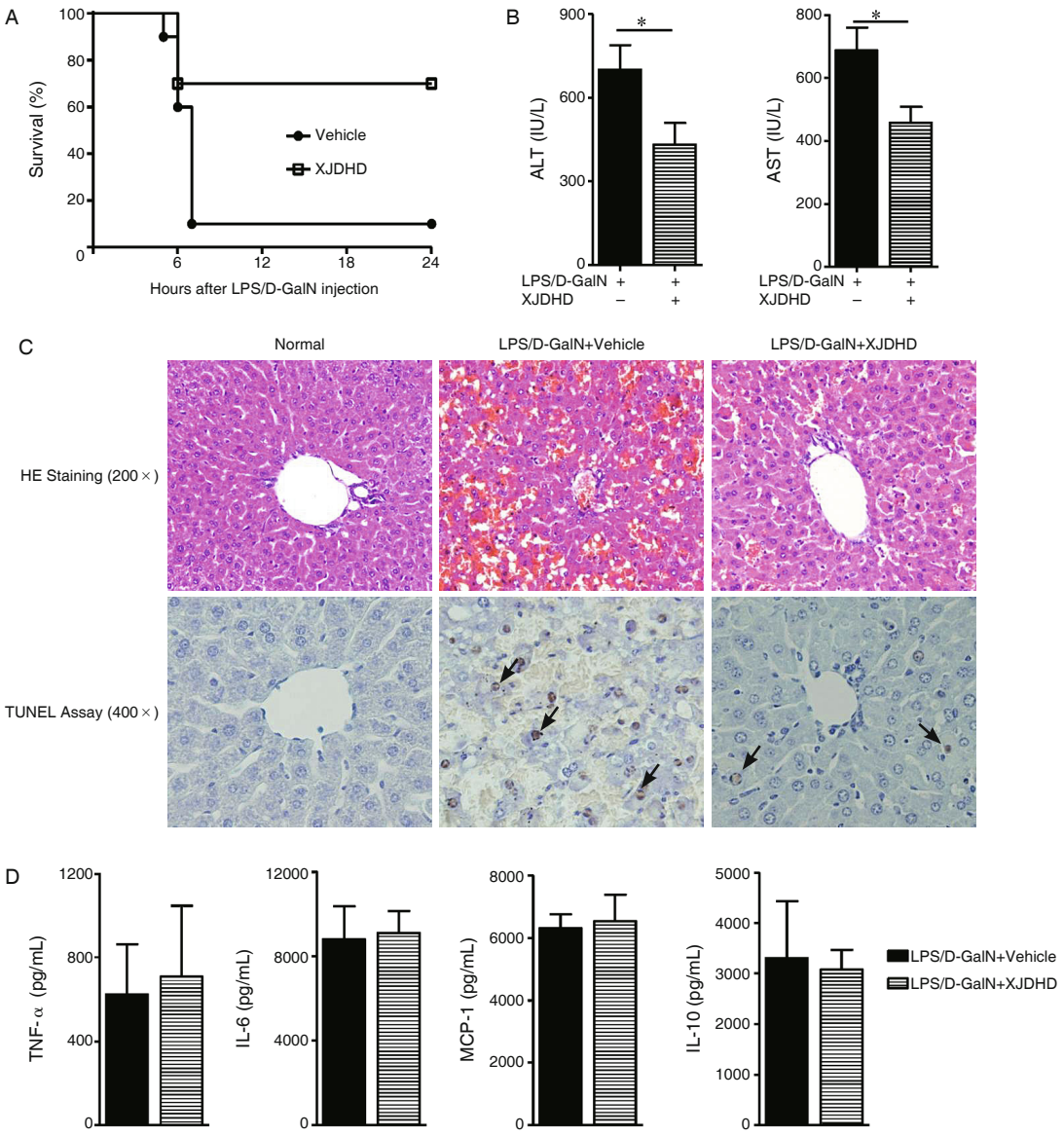

Figure 1. XJDHD Protects Mice from LPS/D-GalN-Induced ALF without Affecting Cytokine Production

Notes: A: *n*=10. B: Activities of ALT and AST in serum were measured 5 h after LPS/D-GalN injection (*n*=10), $^{*}P<0.05$. C: Liver histopathology with HE staining (upper panel) and apoptosis detection with TUNEL assay (lower panel); mice were treated as for Figure 1B, *n*=3; arrows showed apoptotic cells. D: Cytokine levels in serum at 3 h after LPS/D-GalN challenge were detected with CBA technology (*n*=10).

However, neither the pro- nor anti-inflammatory cytokines examined were affected by XJDHD, including TNF-α, IL-6, IL-10, and MCP-1 ($P>0.05$, Figure 1D).

XJDHD and *Rehmannia glutinosa* Libosch. Inhibit TNF-α-Induced Apoptosis of Hepatocytes

TNF-α is the core pro-inflammatory cytokine and the major cause of lethality in LPS/D-GalN mice. Since XJDHD protected mice from LPS/D-GalN-induced ALF without affecting TNF-α production, we further investigated whether XJDHD protects against TNF-α/D-GalN-induced liver injury. As shown in Figure 2A, XJDHD enhanced the survival rate of mice injected with TNF-α/D-GalN by 85.7%. The protective role of XJDHD was further confirmed by significant decreased ALT and AST levels in serum ($P<0.05$, Figure 2B), alleviated histomorphologic changes in liver (Figure 2C, upper panel) and reduced frequency of apoptotic hepatocytes (Figure 2C, lower panel) in TNF-α/D-GalN injected mice.

Notably, expression levels of these anti-apoptotic genes, c-Flip, IAP-1, Gadd45b and A20, were enhanced after XJDHD treatment, compared to that in control samples ($P<0.05$, Figure 3).

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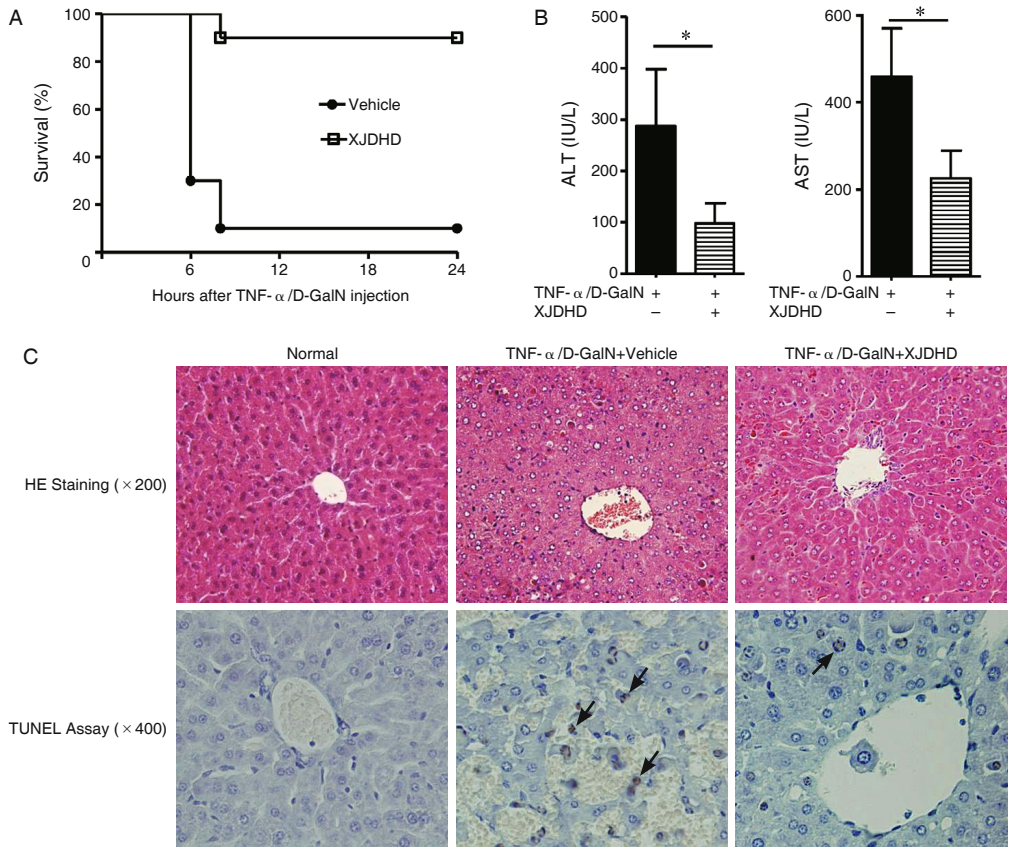

Figure 2. Protective Effects of XJDHD in TNF-α/D-GalN Injected Mice

Notes: A: *n*=10. B: Activities of ALT and AST in serum were measured 5 h after TNF-α/D-GalN injection (*n*=8), $^{*}P<0.01$. C: Liver histopathology with HE staining (upper panel) and apoptosis detection with TUNEL assay (lower panel), the apoptotic hepatocytes were dark stained (arrow); Mice were treated as for Figure 2B.

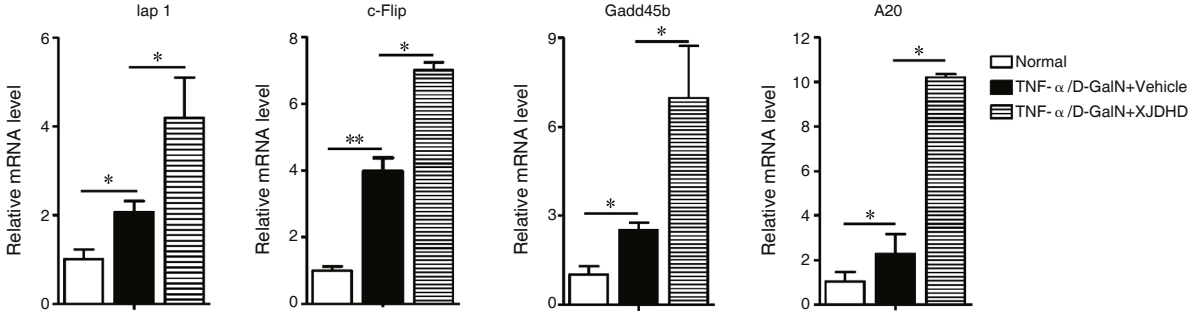

Figure 3. XJDHD Enhances the Expression of NF-κB-Induced Anti-apoptotic Genes ($\bar{x} \pm s$, *n*=3)

Notes: The mRNA levels of Iap1, c-Flip, Gadd45b and A20 in liver were examined using real-time PCR, mice were treated as for Figure 2B, and total RNA in liver was isolated after 3 h, $^{*}P<0.05$, $^{***}P<0.01$.

Galactose is Efficacious Component of *Rehmannia glutinosa* Libosch. and *Radix rehmannia praeparata* that Prevents TNF-α-Evoked Liver Failure

To identify the effective constituent herb(s) of XJDHD in protection against liver failure, mice were treated with decoctions of the four individual herbs. As shown in Figure 4A, *Rehmannia glutinosa* Libosch. enhanced the survival rate by 80%, while the other three herbs failed to protect TNF-α/D-GalN-injected mice.

Radix rehmannia praeparata, the steamed root of *Rehmannia glutinosa* Libosch., is similar to *Rehmannia glutinosa* Libosch. in terms of the main ingredients, but differs in component concentrations.[25] Compared with *Rehmannia glutinosa* Libosch., *Radix rehmannia praeparata* exhibited a similar protective effect on the TNF-α/D-GalN model (Figure 4B), implying that the efficacious component(s) in *Rehmannia glutinosa*

Libosch. and *Radix rehmannia praeparata* is stable in structure and (or) sufficient in content during processing. In addition, post-treatment of TNF-α/D-GalN-injected mice with *Radix rehmannia praeparata* led to identical protective activity within 15 min comparable with that observed upon simultaneous treatment (Figure 4C).

The ethanol extract (EE) and water extract (WE) from *Rehmannia glutinosa* Libosch. and *Radix rehmannia* decoction were prepared to further establish the efficacious component(s). EE of *Rehmannia glutinosa* Libosch. and *Radix rehmannia praeparata* clearly enhanced the survival rate of TNF-α/D-GalN model mice, while the water extract (WE) had no protective effects (Figure 4D).

We further investigated the hepatoprotective monomers of *Rehmannia glutinosa* Libosch. and *Radix rehmannia praeparata*, including iridoids, saccharides, glycosides, etc.[25] Catalpol, the most representative component of iridoids, failed to protect against ALF in TNF-α/D-GalN-injected mice (Figure 4C). Stachyose, raffinose and verbascose, the main oligosaccharides of *Rehmannia glutinosa* Libosch. and *Radix rehmannia praeparata*, did not reduce the mortality of TNF-α/D-GalN mice, while galactose markedly increased the survival rate of mice by 50% (Figure 4E). Furthermore, galactose decreased transaminases level in serum and apoptotic hepatocytes, and ameliorated liver injury (data not shown). In view of these findings, we propose that galactose is the hepatoprotective monomer in *Rehmannia glutinosa* Libosch. and *Radix rehmannia praeparata*.

DISCUSSION

Challenge of mice with LPS and D-GalN leads to fatal liver damage, the core mechanism involving the production of abundant TNF-α and subsequent induction of hepatocyte apoptosis via TNFR1.[26] In the present study, we concluded that XJDHD protects mice against LPS- and TNF-α-induced ALF by inhibiting TNF-α-evoked hepatocyte apoptosis. Notably, XJDHD afforded more comprehensive protection in the TNF-α/D-GalN model than the LPS/D-GalN model. One possible explanation is that in addition to TNF-α, LPS triggers the production of inflammatory mediators, such as IL-1β, IL-6, and IL-12, which aggravate liver injury in the LPS/D-GalN model.

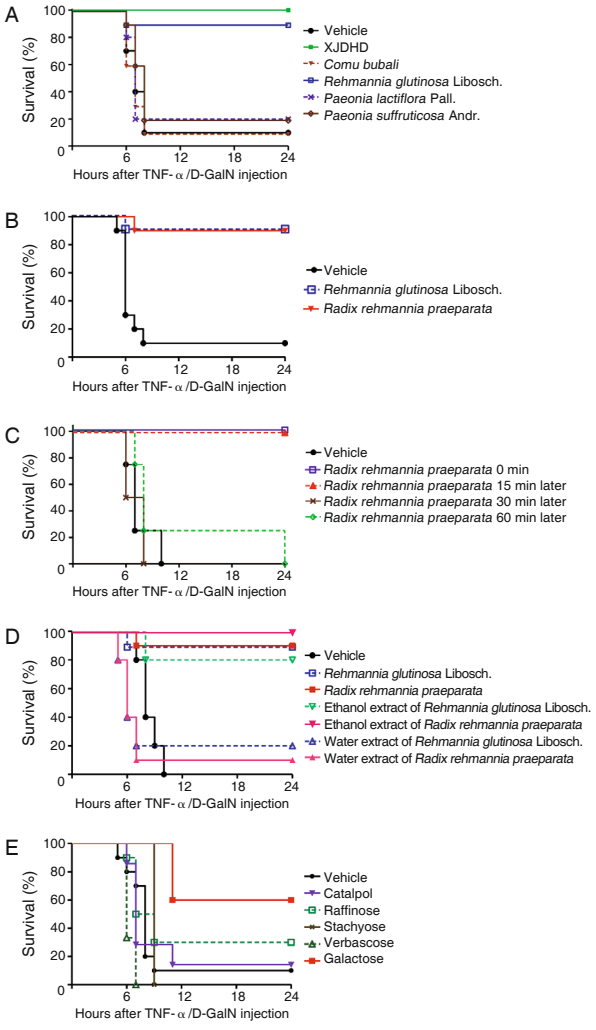

Figure 4. *Rehmannia glutinosa* Libosch. Is the Key Constituent of XJDHD Inducing Anti-apoptotic Activity, with Galactose as the Efficacious Monomer

Notes: A, B, D: *n*=10. C: Mice were treated with *Radix rehmannia praeparata* at different time-points after TNF-α/D-GalN injection (*n*=8). E: *n*=6–10.

Since XJDHD did not appear to affect serum levels of TNF-α in the LPS/D-GalN model, we examined whether this decoction acts on TNF-α-induced liver injury. XJDHD protected mice against TNF-α/D-GalN-induced mortality. This finding was further supported by data from an in situ TUNEL assay showing that XJDHD inhibits TNF-α-induced apoptosis of hepatocytes.

In hepatocytes, TNF-α-induced cell apoptosis was mainly regulated by NF-κB pathway. NF-κB is a key regulator in protecting hepatocytes from apoptosis by up-regulating the expression of anti-apoptotic genes, such as c-Flip, IAP-1, Gadd45b, and A20. NF-κB-dependent genes prone to have anti-apoptotic effects. The c-Flip protein can inhibit apoptosis

through interfering with the catalytical activity of caspase-8. The IAP-1 protein directly bind effector caspases and block apoptosis induced by TNF-α. Gadd45β may prevent the mitochondria-dependent pathway.[27] The mechanism by which A20 inhibits apoptosis and regulates cell survival in specific cell type remains to be clarified but may be accociated with its de-ubiquitylating activity.[28,29] In the study, we detected elevated expression of a cluster of NF-κB-regulated genes known to antagonize hepatocyte apoptosis. Thus, XJDHD may play an anti-apoptotic role by acting on a signaling pathway downstream of TNF-α/TNFR1.

XJDHD is composed of four herbs: *Cornu Bubali*, *Rehmannia glutinosa* Libosch., *Paeonia lactiflora* Pall. and *Paeonia suffruticosa* Andr. In our experiments, only *Rehmannia glutinosa* Libosch. was capable of preventing ALF in the TNF-α/D-GalN mouse model. *Rehmannia glutinosa* Libosch. mainly contains iridoids, saccharides and trace amounts of amino acids and inorganic ions.[25] Iridoids and polysaccharides are more sensitive to heat and pH and generally water-soluble, while oligosaccharides and monosaccharides are more stable to heat and insoluble in ethanol.[30] Since *Rehmannia glutinosa* Libosch. and its processed drug, *Radix rehmannia praeparata*, are also able to protect against TNF-α/D-GalN-induced ALF in mice, the effective constituents should be heat-stable. Moreover, ethanol extract, rather than water extract, of *Rehmannia glutinosa* Libosch. and *Radix rehmannia praeparata* exerted optimal protective effects on ALF mice, indicating that the effective constituents are both ethanol soluble and heat-stable. Based on these findings, we further screened the oligosaccharides and monosaccharides in *Rehmannia glutinosa* Libosch. and *Radix rehmannia praeparata* and consequently identified galactose as a key effective ingredient. In contrast, catalpol, a representative component of iridoids with unstable properties,[31] exerted no protective effects on TNF-α/D-GalN mice.

In summary, we present evidence demonstrating that *Rehmannia glutinosa* Libosch. in XJDHD plays a protective role in TNF-α-induced ALF, and galactose is a key effective ingredient. It indicates *Rehmannia glutinosa* Libosch. and galactose may be promising components for the treatment and prevention of ALF. The mechanisms by which these components specifically inhibit apoptosis of hepatocytes warrant investigation in future studies.

Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

Wang XB, Zeng H and Zhu LL were responsible for the conception and design of the study. Liu YM, Li R, Zhang JL and Yao SS contributed to the animal experiment, herbs and extract preparation, HE staining and TUNEL assay of the study. The transaminases activity detection, multiplex cytokine assay, and real-time PCR assay were attributed to Zhu LL, Liu YM and Zhou XB. Liu YM and Zhu LL were responsible for statistical analysis of all the data and drafted the manuscript. Wang XB and Zeng H revised and commented on the draft. All authors participated in interpretation of the findings and approved the final version of the manuscript.

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