Efficacy of decoction from Jieduan Niwan formula on rat model of acute-on-chronic liver failure induced by porcine serum

Li Jinxia, Zhang Qiuyun, Gao Lianyin, Du Yuqiong, Chen Yu

OBJECTIVE: To dynamically observe the efficacy of Jieduan Niwan formula (JDNW) on a rat model of acute-on-chronic liver failure (ACLF).

METHODS: Seventy Wistar rats were divided into control group (6 rats), model group (22 rats), JDNW group (21 rats), and SP600125 group (21 rats). 13 weeks’ porcine serum injection followed with D-galactosamine and lipopolysaccharide joint acute attack was used to establish ACLF model. Rats in JDNW group were orally given JDNW formula for 3 days before acute attack; rats in SP600125 group were injected with SP600125 30 min ahead of acute attack. Rats were sacrificed respectively at 4, 8 and 12 h after model established. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), Creatinine (CR), blood urea nitrogen (BUN), prothrombin activity (PTA) were examined by biochemical process, Tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10), transformed growth factor-beta 1 (TGF-β1), High mobility group box-1 (HMGB-1), CD3, CD4, CD8 were analyzed by enzyme-linked immunosorbent assay, apoptotic index (AI) was detected by terminal-deoxyxynucleotidyl transferase mediated nick end labeling staining, expression of Bad, phosphorylated Jun N-terminal kinases (p-JNK) and Cytochrome C (Cyt C) were detected by immunohistochemical analysis, Bax and Bid were detected by Western blot analysis.

RESULTS: In model group, the levels of ALT, AST, TBIL, CR, BUN, IL-1β, IL-6, IL-10, TGF-β1 and HMGB-1 remarkably increased and PTA decreased compared with control group (P<0.05), as time goes on, ALT, AST, TBIL, CR, BUN, continued to grow, while IL-1β, IL-6, IL-10, HMGB-1, TGF-β1 and PTA gradually decreased; massive necrosis could be seen; the levels of TNF-α, CD3, CD4, CD8, AI, p-JNK, Bax, Bad, Bid and Cyt C increased at 4 h and peaked at 8 h, but decreased at 12 h (P<0.05). JDNW group, by contrast, showed less pathological injury, increased PTA level, and reduced ALT, AST, TBIL, TNF-α, IL-1β, IL-6, IL-10, TGF-β1, HMGB-1, CD3, CD4 and CD8 levels (P<0.05), moreover, the AI and expression of p-JNK, Bax, Bad, Bid and Cyt C were lower than model group at 4 and 8 h but were higher at 12 h (P<0.05). Similar results were observed in SP600125 group.
CONCLUSION: An ACLF rat model with low mortality can be established by porcine serum joint with D-galactosamine + lipopolysaccharide induction; JDNW decoction can effectively suppress the inflammatory reaction, improve the immune system, and protect the liver of ACLF rats, the mechanism might involve the inhibition of the JNK-induced mitochondrial apoptotic pathway.

Keywords: Acute-on-chronic liver failure; JNK mitogen-activated protein kinases; Apoptosis; Jieduan Niwan formula

INTRODUCTION

Acute-on-chronic liver failure (ACLF) is a common type of liver failure in China. There is no consensus definition amongst the Asia-Pacific Association for the Study of Liver Disease, the European Association for the Study of the Liver and the American Association for the Study of Liver Disease, yet they both emphasize the pathological process of acute hepatic insult on the basis of pre-existing chronic liver diseases. In China, compensated and decompensated hepatitis B-related cirrhosis account for 70% of the primary diseases; the reactivation of hepatitis B virus is the main inducement that causes the rapid deterioration of liver function and the subsequent multi-organ failure within a few weeks. The main performances of ACLF are progressive jaundice, coagulopathy, ascites, hepatic encephalopathy, and other signs of multi-organ dysfunction. As a disease with poor prognosis, ACLF still lacks effective treatment, patients can only count on antiviral, artificial liver, liver transplantation and symptomatic treatment.

In recent years, Traditional Chinese Medicine (TCM) has been used to reduce the complications and mortality of ACLF, as well as improving the patients’ life quality. A multicenter and randomized controlled study demonstrated that the mortality of patients treated with basic + TCM treatment is significantly lower than who undergo basic therapies. According to TCM theory, the pathogenesis of ACLF is mostly composed of dampness, toxicity and stasis, which ultimately result in the imbalance of Yin and yang, therefore, the treatment should be combined with dehumidifying, detoxifying, removing stasis, and adjusting Yin and Yang. Based on this acknowledgment, TCM master Qian Ying creates the Jieduan Niwan formula (JDNW). Clinical experiments showed that: Combined treatments of basic + JDNW formula can significantly reduce the symptoms, protect liver function, prevent complications and improve the prognosis of ACLF patients. In former studies, human serum albumin (HSA) accompanied with D-galactosamine (D-GalN) and lipopolysaccharide (LPS) were used to establish an ACLF rat model, but the mortality rates as high as 58% which caused a huge loss of resources and capitals, therefore, we replaced the HSA with a 13-week intraperitoneal injection of porcine serum, an ACLF rat model with a lower mortality as 4% has been successfully established. By using this model, we have confirmed that the equivalent dose of JDNW has a better effect on ACLF rats compared with large and small doses. The subsequent experiments all adopt the equivalent dose, namely 21.7 g·kg⁻¹·d⁻¹, and found that JDNW can prolong the survival time of ACLF rats, and reduce their liver injury and hepatocyte apoptosis, but the mechanism remains unknown.

Apoptosis is one of the main pathogenesis of ACLF. C-Jun N-terminal kinase (JNK) plays an important role in the initiation and execution of apoptosis. Studies have found that mitochondria is a primary target of pro-apoptotic signaling by JNK; phosphorylated-JNK (p-JNK) can be translocated to the mitochondrial membrane, phosphorylate Bcl-2 superfamily and activate caspase cascade, by which means mediate apoptosis via the mitochondrial pathway. Therefore, we propose a hypothesis: JNK-induced mitochondrial apoptotic pathway may be a potential target of JDNW to protect liver cells and prevent ACLF. In this study, using porcine serum joint D-GalN + LPS to try to establish ACLF rat model and verify its repeatability, we aimed to evaluate the efficacy of JDNW on the JNK-induced mitochondrial apoptotic pathway, and to reveal the possible mechanism behind the action.

MATERIALS AND METHODS

Reagents

Porcine serum: Beijing Baidai Co., Ltd. (Beijing, China). D-GalN, LPS, and SP600125: Sigma-Aldrich Co. (St. Louis, MO, USA). Monoclonal mouse anti-p-JNK: Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Monoclonal rabbit anti-Bax and anti-Bid: Abcam Trading Co., Ltd. (Cambridge, MA, USA). Monoclonal rabbit anti-cytochrome c (Cyt C) and anti-β-actin: Cell Signaling Technology, Inc. (Danvers, MA, USA). Polink-1 HRP DAB detection system for rabbit and mouse primary antibody: Golden Bridge Biotech Co., Ltd. (Beijing, China). DyLight 680 goat anti-rabbit IgG: EarthOx Life Sciences (Millbrae, CA, USA). In Situ Cell Apoptosis Detection Kit II (AP) and monoclonal rabbit anti-Bad: Boster Co., Ltd. (Wuhan, China). Rat IL-1β, IL-6, IL-10 ELISA Kit: eBioScience (San Diego, CA, USA); TGF-β, HMGB-1, CD3, CD4, CD8 ELISA Kit: Xitang Co., Ltd. (Shanghai, China).

Preparation of JDNW

The composition of JDNW is: Yexiazhu (Herba Phylanthi Urinariae) 30 g, Huangqi (Radix Astragali Mon-
golici) 30 g, Gualou (Fructus et Semen Trichosanthis) 30 g, Jinqiancao (Herba Lysimachiae) 30 g, Huisheng (Herba Visci Colorati) 30 g, Sanqi (Radix Notoginseng) 6 g, Ezhu (Rhizoma Curcumae Phaeocaulis) 6 g, Danshen (Radix Salviae Miltiorrhizae) 20 g, Dihuang (Radix Rehmanniae) 20 g, Fuzi (Radix Aconiti Lateralis Preparata) 15 g. All medical plants were purchased from Beijing Tong Ren Tang Group Co., Ltd. (Beijing, China). Plant authenticity was identified twice morphologically and anatomically. All herbs were soaked in distilled water for 1 h before heating. Fuzi (Radix Aconiti Lateralis Preparata) was boiled in 1000 mL water for 30 min first to remove toxicity. The rest of the ingredients were then boiled for another hour. The plants were filtered out and heated in 500 mL water for second extraction under the same condition. Filtered suspensions were mixed and concentrated to 50 mL (with a density of 4.34 g/mL). The aqueous formula was stored at 4 °C and heated to 37 °C in water bath before use.

**Animal experiment**

Seventy male Wistar rats of SPF grade weighing 180-200 g were provided by the Vital River Laboratories (Beijing, China). Animals were housed in a SPF-grade environment, fed with normal water and food. All procedures were under the approval of the Animal Experiments and Experimental Animal Welfare Committee of Capital Medical University (Registration No. AEEI-2014-039).

According to the stochastic averaging principle, the rats were divided into control group (n = 6), model group (n = 22), JDNW group (n = 21), and SP600125 group (n = 21). Except for the control group, all rats were intraperitoneally injected with porcine serum at 0.5 mL/rat twice a week. The model was verified by hematoxylin and eosin (HE) staining at the end of 13th week. Before the acute insult, rats in JDNW group were orally given 21.7 g·kg⁻¹·d⁻¹ JDNW decoction twice a day for 3 d. Rats in SP600125 group were given 15 mg/kg SP600125 through one-off intraperitoneal injection at 30 min ahead of acute attack. After treatments, rats were given 800 mg/kg D-GalN and 100 μg/kg LPS intraperitoneal injection to cause acute liver damage. Rats in control group were given the same volume of normal saline instead. Each group of rats was randomly divided into three even subgroups and sacrificed respectively at 4, 8 and 12 h after D-GalN + LPS attack. Under anesthesia by intraperitoneal injection of chloral hydrate (350 mg/ kg), blood sample was collected from abdominal aorta and liver was removed. The right lobe was fixed in 10% neutral-buffered formalin, and other lobes were diced and frozen in liquid nitrogen for further proteome study.

**Blood sample examination**

Blood sample was centrifuged at 1509.3 × g for 4 °C for 15 min to isolate the serum and the plasma. Alanine transaminase (ALT), aspartate transaminase (AST), total bilirubin (TBIL), urea nitrogen (BUN) and creatinine (CR) were detected under biochemical methods by Hitachi 7600 Automatic Analyzer (Hitachi, Inc., Tokyo, Japan). Plasma prothrombin activity (PTA) was detected by Beckman Coulter ACL-TOP700 blood coagulation analyzer (Beckman Coulter, Inc., Brea, CA, USA).

**ELISA analysis of inflammatory factors**

Tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10), transformed growth factor-beta 1 (TGF-B1), High mobility group box-1 (HMGB-1), CD3, CD4, CD8 was examined by corresponding enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer’s protocol, OD value was detected by Multiskan MK3 enzyme marker (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

**Liver histology observation**

Formalin-fixed liver tissues were embedded in paraffin and stained with HE. The slices were observed and captured by Nikon Eclipse 80i microscope (Nikon, Inc., Tokyo, Japan) under × 200 magnification. A semi-quantitative scoring criterion was used to assess the degree of liver injury: Normal hepatic structure scored 0; visible hepatocyte degeneration, +1; spotty necrosis in hepatic lobule, +2; focal necrosis in less than 1/3 lobule, +3; patchy necrosis in 1/3 - 2/3 lobule, +4; necrosis in more than 2/3 lobule, +5.

**TUNEL assay and Bad immunohistochemical double staining**

Apoptosis was detected by Terminal-deoxynucleotidyl Transferase Mediated Nick End Labeling (TUNEL) staining according to the manufacturer’s protocol. Apoptotic cells were stained as dark blue. After coloration, slides were rinsed thrice with phosphate buffered saline and blocked with 10% goat serum at 37 °C for 30 min. The slides were then incubated with anti-Bad antibody (1:50) at 4 °C overnight. The Polink-1 HRP DAB detection system was performed at 37 °C for 1 h. As a result, Bad was stained as brown. Five visual fields were randomly selected from each slide and captured by Nikon Eclipse 80i microscope under × 400 magnification. The integral optical density (IOD) of Bad expression was analyzed by NIKON NIS-Elements BR software (Nikon, Inc., Tokyo, Japan). Apoptosis cells were counted by Image Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA), AI was calculated, AI = apoptosis cell number/total cell number.

**Immunohistochemical analysis of p-JNK and Cyt C**

Tissue sections were routinely dewaxed and hydrated. After the heat-mediated antigen retrieval, the sections were blocked with 10% goat serum at 37 °C for 1 h; then incubated with anti-p-JNK antibody (1:100) or anti-Cyt C antibody (1:200) at 4 °C overnight. Polink-1 horseradish peroxidase diaminobenzidine
Western blot analysis of Bax and Bid
Liver tissues were disrupted in lysis buffer. Supernatants were collected after centrifugation. The protein lysates were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis for 1.5 h at 120 V, and electrophoretically transferred onto 0.22-μm NC membranes. After blocking with 5% nonfat milk for 1 h, the membranes were incubated with anti-Bax antibody (1: 1000) and anti-Bid antibody (1: 1000) respectively at 4 °C overnight; then incubated the membranes with DyLight 680 goat anti-rabbit IgG for 1 h at room temperature. Scanned the membranes with Odyssey infrared fluorescent scanner (LI-COR Co., Lincoln, NE, USA) and detected IOD by ImageJ software (GraphPad Software, Inc., San Diego, CA, USA). The relative expression of the protein is calculated by dividing the β-actin expression level.

Statistical analysis
Statistical analysis was performed by SPSS 19.0 (IBM Corp. Released 2010. IBM SPSS Statistics for Windows, Version 19.0, Armonk, NY, USA). Continuous data were presented as mean ± standard deviation ( ± ). One-way analysis of variance was performed to test the differences between groups. Least-significant difference method was applied when the variance was equal, whereas Tamhane’s T2 test was performed as long as the variance was not equal. Pearson’s correlation method was used for the correlation analysis. A P < 0.05 was considered to be statistically significant.

RESULTS
Effect of JDNW on liver, renal and coagulation function
Compared to control group, ALT, AST, and TBIL levels in model group were significantly increased at 4 h and continued to grow over time, at 12 h, values reached 10 times in the control group (Figure 1A-1C); in contrast, PTA gradually decreased (Figure 1D), and dropped to 23.6% at 12 h. Compared to the corresponding time point model group, ALT, AST, and TBIL levels in JDNW and SP600125 group distinctly decreased, and PTA increased, the differences were statistically significant at 4 and 8 h (P < 0.05). In terms of renal function (Figure 1E, 1F), CR and BUN in model group were higher than normal group (P < 0.01), whereas JDNW and SP600125 group has lower CR and BUN than model group in each time point (P > 0.05).
**Effect of JDNW on inflammatory factors and immune response**

Levels of TNF-α, IL-1β, IL-6, IL-10, HMGB-1, TGF-β, CD3, CD4, CD8 in model group were significantly higher compared to normal group, \( P < 0.01 \), the contents of TNF-α, CD3, CD4, CD8 have peaked in 8 h and declined in 12 h, whereas IL-1β, IL-6, IL-10, HMGB-1 and TGF-β continued to decrease as time goes on. Compared with model group in each time point, TNF-α, IL-1β, IL-6, IL-10, HMGB-1, TGF-β, CD3, CD4, CD8 levels in JDNW and SP600125 group were lower, the differences were statistically significant at 4 h \( P < 0.05 \) (Figure 2).

**Figure 2** Inflammatory factors and immune response in different groups

A: TNF-α; B: IL-1β; C: IL-6; D: IL-10; E: HMGB-1; F: TGF-β; G: CD3; H: CD4; I: CD8. 13 weeks’ porcine serum injection followed with D-galactosamine and lipopolysaccharide joint acute attack was used to establish ACLF model. Control and Model group were orally given normal saline, JDNW group was orally given JDNW formula \((112.6 \pm 30.1 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1})\) for 3 d before acute attack; SP600125 group was injected with SP600125 \((15 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1})\) 30 min ahead of acute attack. Rats were sacrificed respectively at 4, 8 and 12 h after model established. TNF-α: Tumor necrosis factor-alpha; IL-1β: interleukin-1β; IL-6: interleukin-6; IL-10: interleukin-10; HMGB-1: High mobility group box-1; TGF-β: transformed growth factor-beta 1; JDNW: Jieduan Niwan formula; ACLF: acute-on-chronic liver failure. Data were presented as mean ± standard deviation \((n = 6)\). \(^* P < 0.05\); \(^{*} P < 0.01\), vs control group; \(^{*} P < 0.05\); \(^{*} P < 0.01\), vs model group.

**Effect of JDNW on liver histology**

In normal group, the hepatic lobule was intact structure; the hepatocytes were arranged radially from the central vein to the surrounding area. The morphology of hepatocytes was normal and has no inflammatory cell infiltration. In model 4 h group, on the basis of liver fibrosis, disordered arrangement of liver cell, hepatocyte swelling, focal acidophilic degeneration, apoptotic body formation, a small amount of inflammatory cell infiltration and central venous congestion could be seen. With the extension of time, the degree of liver cell necrosis gradually deepened, cell degeneration and infiltration of inflammatory cells expanded, at the end of the 12th h, massive or sub massive necrosis formed.
a large number of inflammatory cells infiltration and the collapse of liver tissue structure has been observed. Compared with the corresponding time point model group, the degree of hepatic tissue necrosis and inflammatory cell infiltration in the group of JDNW and SP600125 were relatively lighter, and certain hepatic cord structures could be seen in the lobule (Figure 3).

**Effect of JDNW on AI, p-JNK, Cyt C, and Bad expression**

As shown in Figure 4, the nuclei of apoptotic cells were stained as dark blue, while Bad in cytoplasm was brown. In Figure 5, brownish p-JNK and Cyt C can be detected in nucleus and cytoplasm respectively. By analyzing the IOD values we can see: the expressions of p-JNK, Cyt C and Bad in model 4 h group were apparently higher than control group, as time went on, they continued to grow and peaked at 8 h, but reduced at 12 h (P < 0.01). At 4 and 8 h, the expressions of p-JNK, Cyt C and Bad in both JDNW and SP600125 group were lower than model group (P < 0.01), but higher at 12 h (P > 0.05), it can be seen that the expressions of these three proteins in the group of JDNW and SP600125 group showed a gradually increasing trend over time, there has no process of rising and falling back. AI in each group showed the same tendency as above.

**Effect of JDNW on Bax and Bid expression**

The relative ratio of Bax and Bid expression (IOD of Bax/IOD of β-actin) in model 4 h group significantly increased compared to control group (P < 0.05), and it peaked at 8 h, decreased at 12 h. On the other hand, Bad and Bid in JDNW and SP600125 group were lower than model group at 4 and 8 h (P < 0.05) but higher at 12 h (Figure 7).

**Correlation analysis of the effect of JDNW and SP600125**

The Pearson correlation analysis found that, in 4, 8, 12 h, the effect of JDNW and SP600125 on p-JNK, Cyt C, Bid expression and AI were correlated (P < 0.01). At 8, 12 h, the effect of the two drugs on Bax and Bad expression were consistent (P < 0.01), but no significant correlation in 4 h. The correlation analysis coefficients and scatter diagrams are shown in Table 1 and Figure 8 respectively.

**DISCUSSION**

In this study we introduced D-GalN + LPS acute insult, an ACLF model with low mortality has been successfully established. In order to verify the repeatability, this experiment continues to use this method to build with SP600125 (15 mg·kg−1·d−1) 30 min ahead of acute attack. Rats were sacrificed respectively at 4, 8 and 12 h after model established. JDNW: Jieduan Niwan formula; ACLF: acute-on-chronic liver failure; HE: hematoxylin and eosin. Data were presented as mean ± standard deviation (n = 6). *P < 0.01, vs control group; †P < 0.05 vs model group.
Inflammatory infiltration is one of the main mechanisms of ACLF. In this experiment, TNF-α, IL-1β, IL-6, IL-10, HMGB-1 and TGF-β, in model rats significantly raised at 4 h, indicates severe inflammatory infiltrates has occurred, accordingly, CD3, CD4, CD8 increased, prompted an immune response. However, from 8-12 h, inflammation factors gradually reduced, and after 8 h’s peak, the immune system became paralysis, manifested as CD3, CD4, CD8 declined, indicates that the organ function and immune system gradually failed in models rats. By contrast, at 4, 8 h, inflammatory factors in JDNW group were all lower than model group, CD3, CD4, CD8 correspondingly reduced, and the contents of alexines showed a trend of increase over time, illustrate that JDNW can effectively restrain the inflammatory reaction, and improve their immune function to a certain extent in ACLF rats.

The liver protective effect of JDNW is notable. At each time point, the levels of ALT, AST, TBIL were correspondingly reduced, and the contents of alexines showed a trend of increase over time, illustrate that JDNW can effectively improve ACLF rat’s liver function and coagulation function, and protect their liver from continuous pathological damage. This conclusion is consistent with our previous study in HAS-induced ACLF rats. In addition, the JNK inhibitor SP600125 showed the same liver protective effect,
13 weeks’ porcine serum injection followed with D-galactosamine and lipopolysaccharide joint acute attack was used to establish ACLF model. Control and model group were orally given normal saline, JDNW group was orally given JDNW formula (21.7 g·kg⁻¹·d⁻¹) for 3 d before acute attack; SP600125 group was injected with SP600125 (15 mg·kg⁻¹·d⁻¹) 30 min ahead of acute attack. Rats were sacrificed respectively at 4, 8 and 12 h after model established. JDNW: Jieduan Niwan formula; ACLF: acute-on-chronic liver failure; IOD: integral optical density; p-JNK: phosphorylated Jun N-terminal kinases. Data were presented as mean ± standard deviation (n = 6). aP < 0.01, vs control group; bP < 0.01, vs model group.

Figure 5 Expression of p-JNK in each group
A-J: liver immunohistochemical double staining, ×200; A: control; B: model at 4 h; C: JDNW at 4 h; D: SP600125 at 4 h; E: model at 8 h; F: JDNW at 8 h; G: SP600125 at 8 h; H: model at 12 h; I: JDNW at 12 h; J: SP600125 at 12 h; K: IOD of p-JNK expression. 13 weeks’ porcine serum injection followed with D-galactosamine and lipopolysaccharide joint acute attack was used to establish ACLF model. Control and model group were orally given normal saline, JDNW group was orally given JDNW formula (21.7 g·kg⁻¹·d⁻¹) for 3 d before acute attack; SP600125 group was injected with SP600125 (15 mg·kg⁻¹·d⁻¹) 30 min ahead of acute attack. Rats were sacrificed respectively at 4, 8 and 12 h after model established. JDNW: Jieduan Niwan formula; ACLF: acute-on-chronic liver failure; IOD: integral optical density; p-JNK: phosphorylated Jun N-terminal kinases. Data were presented as mean ± standard deviation (n = 6). aP < 0.01, vs control group; bP < 0.01, vs model group.

Figure 6 Expression of Cyt-C in each group
A-J: liver immunohistochemical double staining, ×200; A: control; B: model at 4 h; C: JDNW at 4 h; D: SP600125 at 4 h; E: model at 8 h; F: JDNW at 8 h; G: SP600125 at 8 h; H: model at 12 h; I: JDNW at 12 h; J: SP600125 at 12 h; K: IOD of Cytochrome C expression. 13 weeks’ porcine serum injection followed with D-galactosamine and lipopolysaccharide joint acute attack was used to establish ACLF model. Control and model group were orally given normal saline, JDNW group was orally given JDNW formula (21.7 g·kg⁻¹·d⁻¹) for 3 d before acute attack; SP600125 group was injected with SP600125 (15 mg·kg⁻¹·d⁻¹) 30 min ahead of acute attack. Rats were sacrificed respectively at 4, 8 and 12 h after model established. JDNW: Jieduan Niwan formula; ACLF: acute-on-chronic liver failure; IOD: integral optical density; Cyt C: Cytochrome C. Data were presented as mean ± standard deviation (n = 6). aP < 0.01, vs control group; bP < 0.01, vs model group.
Figure 7 Expression of Bax and Bid
A1, A2: Bax relative expression; B1, B2: Bid relative expression. 1: model at 4 h; 2: JDNW at 4 h; 3: SP600125 at 4 h; 4: model at 8 h; 5: JDNW at 8 h; 6: SP600125 at 8 h; 7: model at 12 h; 8: JDNW at 12 h; 9: SP600125 at 12 h; 10: control. 13 weeks' porcine serum injection followed with D-galactosamine and lipopolysaccharide joint acute attack was used to establish ACLF model. Control and Model group were orally given normal saline, JDNW group was orally given JDNW formula (21.7 g·kg⁻¹·d⁻¹) for 3 d before acute attack; SP600125 group was injected with SP600125 (15 mg·kg⁻¹·d⁻¹) 30 min ahead of acute attack. Rats were sacrificed respectively at 4, 8 and 12 h after model established. JDNW: Jieduan Niwan formula; ACLF: acute-on-chronic liver failure. Data were presented as mean ± standard deviation (n = 5). *P < 0.05, vs control group. **P < 0.05, vs model group.

Figure 8 Correlation analysis of the effects of Jieduan Niwan formula (JDNW) and SP600125
A: p-JNK; B: Bax; C: Cyt C; D: Bad; E: Apoptosis Index; F: Bid. p-JNK: phosphorylated Jun N-terminal kinases; Cyt C: Cytochrome C.

Table 1: Correlation analysis of the effect of Jieduan Niwan formula and SP600125

<table>
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<tr>
<th>Item</th>
<th>4 h</th>
<th>8 h</th>
<th>12 h</th>
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<tr>
<td></td>
<td>R value</td>
<td>P value</td>
<td>R value</td>
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<tr>
<td>p-JNK</td>
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<td>0.00</td>
<td>0.88</td>
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<tr>
<td>Cyt C</td>
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<td>0.00</td>
<td>0.88</td>
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<tr>
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<td>0.03</td>
<td>−0.83</td>
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<tr>
<td>Bax</td>
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<td>0.08</td>
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<tr>
<td>Bad</td>
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<td>0.93</td>
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<tr>
<td>Bid</td>
<td>0.95</td>
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Notes: p-JNK: phosphorylated Jun N-terminal kinases; Cyt C: cytochrome C; AI: apoptosis index.
suggesting that inhibiting the activation of JNK might be the possible mechanism of JDNW to protect the liver. JNK is a member of the mitogen-activated protein kinase superfamily. JNK can be phosphorylated by multiple environmental stresses and cytokines, such as TNF-α, IL-1β, IL-6 and TGF-β.  

P-JNK can either activate intranuclear transcription factors like c-Jun, p53, or translocate to the mitochondrial membrane to phosphorylate the members of Bcl-2 superfamily, and consequently mediate mitochondrial apoptotic pathway.  

According to research, Bax, Bid and Bad are the main objectives regulated by JNK; they can act on the outer membrane of the mitochondria, cause electrophoretic mobility shift and permeability transition, release the Cyt C, and leads to apoptotic body formation and caspase cascade, programmed cell death occurs eventually. In previous study, we have confirmed that JDNW can significantly downregulate Caspase3 and Caspase8 expression and inhibit apoptosis in ACLF rats, but the mechanism remains unclear. In this study, p-JNK, Bax, Bid, Bad, Cyt C expression and AI significantly increased in model group, they continuously increased from 4 to 8 h but dropped at 12 h, which suggest that JNK-induced mitochondrial apoptotic pathway has been activated and apoptosis occurred in ACLF rats; the apoptosis activity gradually increased and reached a peak at 8 h; but largely reduced at 12 h. According to Majno G’s article, this collapse in AI and cytokine expression may due to the dominance of cell necrosis in the late stage of ACLF; in this period, A large number of hepatocytes died, causing apoptosis activity decreased correspondingly. In contrast, at 4 and 8 h, the AI and the expression of p-JNK, Bax, Bid and Cyt C in JDNW group were distinctly lower than model group, suggests the apoptosis inhibition effect of JDNW is realized through inhibition of JNK-induced mitochondrial apoptotic pathway; at 12 h, AI and the above cytokines in JDNW group were slightly higher than model group, and from 4h to 12h, the levels showed a gradually increasing trend, indicates that JDNW has delayed the progression of ACLF and postponed the progress of cell necrosis and cell death. Same outcomes were found in SP600125 group. The efficacy of JDNW and SP600125 on p-JNK, Bax, Bid, Bad, Cyt C and AI were correlated, which further suggests that interdiction of JNK-induced mitochondrial apoptotic pathways might be the possible mechanism.

In conclusion, ACLF rat model with low mortality can be successfully established by porcine serum joint with D-GalN + LPS acute insult. JDNW decoction can effectively suppress the inflammatory reaction, improve the immune system, and protect the liver of ACLF rats. The underlying mechanism might involve the inhibition of the JNK-induced mitochondrial apoptotic pathway.


