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Protective effects of levo-tetrahydropalmatine on hepatic ischemia/reperfusion injury are mediated by inhibition of the ERK/NF-κB pathway

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ABSTRACT

Background: Hepatic ischemia/reperfusion (IR) injury is a common medical phenomenon that occurs during a number of clinical conditions, such as liver transplantation, severe injuries, and shock. In our study, we determined the protective functions of levo-tetrahydropalmatine (L-THP) on hepatic IR injury in mice by inhibiting the ERK/NF-κB signaling pathway.

Method: BALB/c mice were randomly divided into six groups as follows: normal control (NC); sham; L-THP (40 mg/kg); IR; L-THP (20 mg/kg) + IR; and L-THP (40 mg/kg) + IR. Liver tissues and sera were collected at three time points after reperfusion (2, 8, and 24 h). The liver enzyme, inflammatory factor, and other protein levels in the serum and liver tissues were detected.

Results: L-THP pretreatment alleviated hepatocyte injury caused by IR and reduced the production of proinflammatory cytokines, such as IL-6 and TNF-α. Furthermore, L-THP could inhibit the ERK/NF-κB signaling pathway to attenuate hepatocyte apoptosis and autophagy. And the protective effect of L-THP is positively correlated with its dose.

Conclusion: L-THP protects the liver from IR injury by inhibiting the release of inflammatory factors and alleviating liver cell apoptosis and autophagy. The protective functions of L-THP may be partly based on the downregulation of the ERK/NF-κB pathway.

1. Introduction

Hepatic ischemia/reperfusion (IR) injury, which is characterized by interruption of the hepatic blood supply and subsequent re-establishment of blood flow, is a common consequence of various clinical situations, such as liver transplantation, trauma, shock, and resection of intrahepatic tumors. Hepatic IR can cause graft dysfunction, poor function, or even graft failure after liver transplantation [1,2]. Furthermore, hepatic IR injury also causes injury in the distant organs. For example, acute kidney injury has been observed in rats with hepatic IR injury [3]. Hence, an effective measure against hepatic IR injury is needed.

The mechanism of hepatic IR injury is complex, and a variety of factors underlie the mechanism. It has been commonly considered that the hepatic IR injury is triggered by the activation of Kupffer cells. The Kupffer cells are activated, then produce a variety of inflammatory factors, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, interferon (IFN)-γ, and reactive oxygen species (ROS) [4]. Activated Kupffer cells also accumulate and activate neutrophils, thereby aggravating the inflammatory hepatic injury. As a result, proteins involved in cell apoptosis are activated and hepatocytes die during hepatic IR.

Apoptosis, type I programmed cell death, is considered to be involved in the pathogenesis of various liver diseases, such as autoimmune hepatitis [5–10] and hepatocellular carcinoma [11,12]. Apoptosis is activated by a variety of extrinsic or intrinsic pathways factors. Previous research has shown that hepatocyte necrosis and apoptosis occur during hepatic IR injury [4,13]. Furthermore, it has been shown that downregulation of hepatocyte apoptosis exerts a protective effect on hepatic IR injury [1].

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Autophagy is also called type II programmed cell death [14]. Autophagy is an evolutionarily-conserved, self-digesting process which promotes cell survival by lysosomal digestion [15]; however, over-activated cell autophagy results in cell death. Autophagy is involved in the pathologic processes of a number of diseases, involving hepatic IR injury. Wu et al. [1] found that quercetin significantly attenuates hepatic IR injury by inhibiting autophagy.

Levo-tetrahydropalmatine (L-THP) is an active component of the traditional Chinese medicine, *Corydalis yanhusuo*. L-THP has been used in China for many years as an anxiolytic and sedative in clinics [16].

In recent years, L-THP has shown the potential to treat organ IR injury. Han et al. [17] reported that L-THP could exert protective functions on myocardial IR injury by inhibiting the production of proinflammatory cytokines, including TNF-α and myeloperoxidase (MPO), and alleviating cardiomyocyte apoptosis. Mao et al. [18] showed that L-THP attenuates blood-brain barrier injuries in mice focal cerebral IR injury model; however, the effects of L-THP on hepatic IR injury remain unknown.

In our study, we explored the functions of L-THP on hepatic IR injury in mice. And we further explored the possible mechanisms of its protective effects. Based on the results, we concluded that L-THP administration reduces inflammatory factors, hepatic apoptosis, and autophagy induced by hepatic IR. And these protective effects are partly based on the inhibition of the ERK/NF-κB signaling pathway.

2. Materials and methods

2.1. Reagents

L-THP and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The aspartate aminotransferase (AST) and alanine aminotransferase (ALT) microplate test kits were bought from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Enzyme-linked immunosorbent assay (ELISA) kits for TNF-α and IL-6 were purchased from Shanghai Lianshuo Biological Technology Co., Ltd. (Shanghai, China). The primary antibodies used in the present study were anti-β-actin, anti-TNF-α, anti-IL-6, anti-Bax, anti-Bcl-2, anti-caspase 3, anti-caspase 9, anti-LC3, anti-Beclin-1 (Proteintech, Chicago, IL, USA), anti-NF-κB (p65) (Cell Signaling Technology, Danvers, MA, USA), anti-ERK, and anti-p-ERK (Epitomics, Burlingame, CA, USA). PCR kits were acquired from Takara (Dalian, China).

2.2. Animals

A total of 84 healthy male BALB/c mice (23 ± 2 g, 6–8 weeks old) were acquired from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). The mice were kept in disinfected plastic cages. The animal house temperature was controlled at 24 °C ± 2 °C and under a 12 h light/dark cycle. We raised the mice with clean mouse feed and drinking water. All of the animal experiments were designed in compliance with the National Institutes of Health Guidelines. All of the experiments in the present study were approved by the Animal Care and Use Committee of Shanghai Tongji University (Shanghai, China).

2.3. Establishment of a hepatic IR injury mouse model

A mouse model with 70% hepatic ischemia was built by the method of previous studies [19,20]. Before surgery, the mice were fasted for approximately 24 h, but they could drink clean water. Then, to anesthetize the mice, we administrated the mice with of 1.25% sodium pentobarbital (Nembutal, St. Louis, MO, USA) by intraperitoneal injection. After the response to pain stimulation disappeared, the mice were placed on an experimental table. Then, each mouse underwent a midline laparotomy. To achieve 70% hepatic ischemia, a metal microvascular clamp was used to clip the hepatic artery, portal vein, and bile duct for approximately 45 min. By removing the clamp, the blood flow was re-established and reperfusion was achieved. Then, the abdominal cavity was closed. An electric blanket was used to keep the mice warm before awakening.

2.4. Experimental design

L-THP was diluted with 1% DMSO to 20 mg/ml and 40 mg/ml, and the solution was administrated by intragastric injection once a day for 5 days before surgery. Eighty-four male BALB/c mice were randomly divided into the six groups as follows:

1. Normal control (NC) (n = 6), no treatment;
2. Sham (n = 18), mice underwent sham surgical procedures without IR;
3. L-THP (n = 6), mice underwent intragastric administration of 40 mg/kg L-THP once daily for 5 d;
4. IR (n = 18), mice underwent ischemia and reperfusion;
5. L-THP(20) + IR (n = 18), mice underwent intragastric administration of 20 mg/kg once daily for 5 d before IR; and.
6. L-THP(40) + IR (n = 18), mice underwent intragastric administration of 40 mg/kg once daily for 5 d before IR.

Mice in the NC and L-THP groups were killed after 5 days. For the other 4 groups, 6 mice in each group were randomly chosen and killed at 2, 8, or 24 h after IR. We collected mice orbital blood and tissues of liver lobes immediately for further biochemical analysis.

2.5. Biochemical assays

Before the mice were killed, we removed the mice eyeballs with tweezers and collected orbital blood samples, then placed the samples at 4 °C for 4 h. The samples were then centrifugated at 2000 × g at 4 °C for 10 min, the serum was isolated and stored at −80 °C for further research. Then, we used an automated chemical analyzer (Olympus AU1000; Olympus, Tokyo, Japan) to determine the levels of serum ALT and ALT. The serum levels of TNF-α and IL-6 were detected by using the ELISA kits following the guidelines.

2.6. Histopathology

A part of the largest liver lobe was removed after the mouse was sacrificed, then fixed with 4% paraformaldehyde for 24 h. The tissues were dehydrated with ethanol and embedded in paraffin. The liver tissues were then cut into 5-μm sections and stained with hematoxylin and eosin (H&E). Finally, light microscopy was used to determine the severity of injury in the tissue sections.

2.7. Immunohistochemistry

The prepared paraffin-embedded 5-μm liver tissue sections were heated at 60 °C for 20 min. Then, the sections were dewaxed with xylene and dehydrated in different concentrations of alcohol. After the antigens were recovered, the liver sections were incubated in 3% hydrogen peroxide (H_2O_2) solution for 20 min at 37 °C to block endogenous peroxidase activity. Then, 5% bovine serum albumin (BSA) was added to the sections to block non-specific proteins. The liver sections were then incubated at 4 °C overnight with the following primary antibodies: anti-TNF-α; anti-Bax; anti-Bcl-2; anti-caspase-3; anti-Beclin-1; anti-LC3; anti-NF-κB; and anti-p-ERK. Then, the primary antibodies in the liver sections were visualized with secondary antibodies using a diaminobenzidine (DAB) kit. Finally, antibody binding in the sections was observed using a light microscope.

2.8. TUNEL assay

We performed TUNEL assay to determine the apoptosis of liver cells.

The prepared 5-μm sections dewaxed and then dehydrated. Then, 20 μg/ml proteinase K was used for the digestion of the sections. After washed for 4 times, the sections were added with TUNEL reaction buffer. Finally, the positive areas were observed by the light microscope.

2.9. Western blot analysis

The liver tissue samples were collected after the mice were sacrificed and stored at −80 °C. After the total protein in the liver tissues was extracted with radioimmunoprecipitation assay lysis buffer, a bicinchoninic acid assay (BCA) was used to determine the protein concentration. Then, the protein samples were electrophoresed on 7.5%, 10%, or 12.5% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE).

The proteins were then transferred onto nitrocellulose (NC) or polyvinylidene difluoride (PVDF) membranes. Then, the membranes were blocked with 5% non-fat milk or 5% bovine serum albumin (BSA) for at least 1 h. The membranes were incubated with primary antibodies at 4 °C overnight. On the second day, the membranes were thrice-washed with phosphate-buffered saline containing 0.1% Tween (PBST). The membranes were incubated with the secondary antibodies for 1 h at 37 °C, then thrice-washed using PBST again. Finally, the expression of specific protein was detected using the Odyssey two-color infrared laser imaging system (Licor, Lincoln, NE, USA).

2.10. Reverse transcription PCR (RT-PCR) and quantitative real-time PCR (qPCR)

TRIzol reagent was used to isolate total RNA from the frozen liver tissues according to the guidelines. Total RNA was then reverse-transcribed into cDNA according to the manufacturer's instructions (TaKaRa Biotechnology, China). Then, SYBR Premix EX Taq (TaKaRa Biotechnology, China) was used to perform real-time PCR with the resulting cDNA. Finally, expression of the target gene was determined using a 7900HT Fast PCR System (Applied Biosystems, Foster City, CA, USA). The primer sequences used in the qPCR are provided in Table 1.

2.11. Statistical analysis

All experimental data in the present study are expressed as the mean ± standard deviation (SD). All the statistical comparisons were analyzed by Student's *t*-test and *P*-values < 0.05 were considered statistically significant. All the figures for statistical analyses were drawn using GraphPad Prism (v6.0).

Table 1
Sequences of primers used for qPCR.

Gene	DNA strand	Primer sequence (5′–3′)
β-Actin	Forward	GGCTGTATTCCCCTCCATCG
	Reverse	CCAGTTGGTAACAATGCCATGT
TNF-α	Forward	CAGGCGGTGCCTATGTCTC
	Reverse	CGATCACCCCGAAGTTCAGTAG
IL-6	Forward	CTGCAAGAGACTTCCATCCAG
	Reverse	AGTGGTATAGACAGGTCTGTTGG
Bax	Forward	AGACAGGGGCCTTTTTGCTAC
	Reverse	AATTCGCCGGAGACACTCG
Beclin-1	Forward	ATGGAGGGGTCTAAGGCGTC
	Reverse	TGGGCTGTGGTAAGTAATGGA
Bcl-2	Forward	GCTACCGTCGTCGTGACTTCGC
	Reverse	CCCCACCGAACTCAAAGAAGG
LC3	Forward	GACCGCTGTAAGGAGGTGC
	Reverse	AGAAGCCGAAGGTTTCTTGGG
NF-κB	Forward	ATGGCAGACGATGATCCCTAC
	Reverse	CGGATCGAAATCCCCTCTGTT

3. Results

3.1. L-THP and laparotomy did not affect normal liver function

Previous studies have shown that 40 mg/kg of L-THP and performing a laparotomy do not affect normal liver tissues [4,21]. To verify these findings, we detected the effects of 40 mg/kg L-THP and laparotomy on the mice serum levels of ALT, AST, and inflammatory cytokines (TNF-α and IL-6) in the NC group, sham group and L-THP group. Fig. 1A & B showed that no significant differences in serum liver enzyme levels and serum inflammatory factor levels were detected among the three groups. Furthermore, no obvious liver cell necrosis was detected in the H&*E*-stained sections from these three groups (Fig. 1C). Thus, the mice in the sham group were chosen as the controls in the present study.

3.2. L-THP pretreatment attenuated mice liver injury induced by IR

The serum levels of ALT and AST indicated the extent of liver injury. We determined the levels of liver enzymes in the mice serum 2, 8, 24 h after hepatic reperfusion. As shown in Fig. 2A, the liver enzymes levels in the IR group were significantly higher than the sham group. And the ALT and AST levels could be reduced by the L-THP pretreatment. And 40 mg/kg L-THP showed a stronger effect on reducing the liver enzymes levels than 20 mg/kg L-THP. Furthermore, pathological changes in the liver sections were observed (Fig. 2B). No necrosis areas were identified in the sham group, while hepatocyte necrosis was observed in the IR group. L-THP pretreatment significantly attenuated liver cell necrosis compared to the IR group; 40 mg/kg of L-THP had a stronger protective effect than 20 mg/kg of L-THP. Based on these results, we could conclude that L-THP exerted a protective effect on hepatic IR injury, and this protective effect is positive-correlated with the dosage of L-THP.

3.3. L-THP pretreatment inhibited the release of inflammatory factors in hepatic IR injury

Proinflammatory cytokines, including TNF-α and IL-6, play a key role in the pathologic processes of hepatic IR injury. In the present study, ELISA, western blotting, immunohistochemistry and qRT-PCR were applied to detect the functions of L-THP pretreatment on inflammatory reactions during hepatic IR. The ELISA results indicated that the serum levels of TNF-α and IL-6 were increased at three time points in the IR group. The pretreatment with L-THP reduced the serum levels of these inflammatory cytokines, especially the higher dose (Fig. 3A). The results of western blotting and qRT-PCR (Fig. 3B & C) showed that L-THP pre-treatment reduced mRNA and protein expression of these inflammatory factors in the liver tissues, which were further confirmed by the results of immunohistochemistry (Fig. 3D). And this effect is dependent on the dosage of L-THP. These results showed that the inflammatory reactions were attenuated by the L-THP pretreatment during hepatic IR in mice.

3.4. L-THP administration alleviated hepatic IR-induced hepatocyte apoptosis and autophagy

Bax, caspase 3, and caspase 9 are all proapoptotic proteins, while Bcl-2 reduces cell apoptosis. Belin-1 and LC3 play key roles in cell autophagy. Both hepatocyte apoptosis and autophagy take part in the pathologic process of hepatic IR injury. In the present study, western blotting and qRT-PCR were used to determine the functions of L-THP pretreatment on protein and mRNA levels of the markers of cell apoptosis and autophagy. The protein and mRNA levels of Bax, caspase 3, caspase 9, Beclin-1, and LC3 in the IR group increased significantly. The elevation of these markers was alleviated by L-THP administration. And this protective effect of L-THP was dose-dependent. Bcl-2 expression was downregulated by hepatic IR, while Bcl-2 expression was

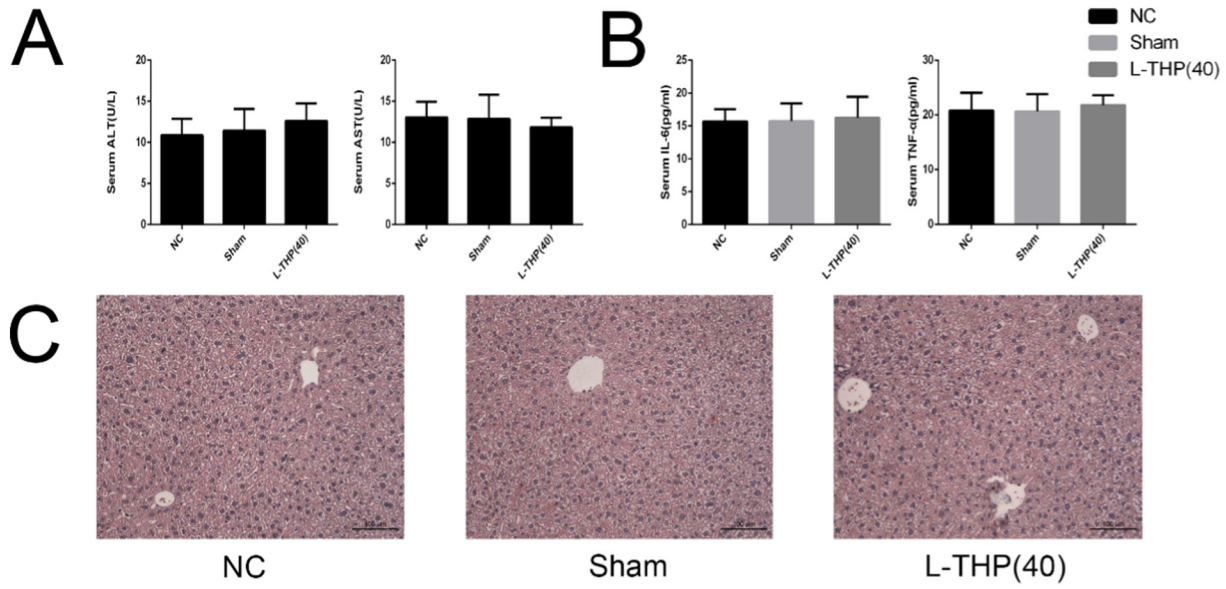

Fig. 1. Effects of L-THP (40 mg/kg) and laparotomy on normal liver tissue. (A) Serum AST and ALT levels were expressed as the mean ± SD ($n = 6$, $P > 0.05$). (B) Serum level of TNF-a and IL-6 detected by ELISA; data are the mean ± SD ($n = 6$, $P > 0.05$). (C) Representative H&E staining in sections of the liver (original magnification, ×200).

upregulated by L-THP pretreatment (Fig. 4A & B). The results of immunohistochemistry and TUNEL assay further confirmed the results of western blotting and qRT-PCR (Fig. 4C&D). Based on the above results, we could conclude that hepatic IR-induced hepatocyte apoptosis and autophagy were alleviated by the L-THP administration.

3.5. L-THP administration prevented the ERK/NF-κB pathway activation during hepatic IR injury

Based on the above results, we concluded that L-THP administration reduced the release of inflammatory factors and attenuated hepatocyte apoptosis and autophagy during hepatic IR injury in mice; however, the underlying mechanism is unknown and needs further research. The ERK/NF-κB pathway takes part in hepatocyte apoptosis and autophagy during hepatic IR. Hence, we performed qRT-PCR, western blotting, and immunohistochemistry staining to detect the functions of L-THP on the ERK/NF-κB signaling pathway during hepatic IR (Fig. 5A & B). No difference of total ERK levels could be found among the sham, IR, L-THP(20) + IR, and L-THP(40) + IR groups. Phosphorylated ERK (p-ERK) is the activated form of ERK. The protein and mRNA levels of p-ERK and NF-κB were significantly higher in the IR group than that in the sham group, which was downregulated by the L-THP administration. These findings were further confirmed by the results of immunohistochemistry staining (Fig. 5C). All of these results showed that the protective functions of L-THP on hepatic IR injury were partly via prevention of the ERK/NF-κB signaling pathway activation.

4. Discussion

Hepatic IR injury is a pathophysiologic process that occurs in a variety of common clinical situations, which leads to the high mortality of patients who have undergone liver transplantation or resection of intrahepatic tumors. Furthermore, previous studies have shown that hepatic IR injury also leads to renal and myocardial injury [22,23]. Hence, the mechanism(s) underlying hepatic IR injury needs investigation and effective strategies for hepatic IR injury should be identified. L-THP is the main active component of the traditional Chinese medicine, *Corydalis yanhusuo*. Recent studies have shown that L-THP exerts protective effects on IR injuries involving the brain and heart [17,18,24] by reducing the release of proinflammatory cytokines and alleviating cell apoptosis. In our study, we confirmed that normal liver function isn't affected by the L-THP administration and explored the functions of L-THP pretreatment on hepatic IR injury in mice and its possible mechanism.

ALT and AST are liver enzymes whose serum levels increase when hepatocytes die. The serum levels of these two liver enzymes indicate the severity of liver injury [25,26]. It has been found that these two liver enzymes levels of mice in the IR group were significantly higher than that in the sham group. L-THP pretreatment downregulated the liver enzymes, and this protective effect was dependent on the dosage of L-THP. These results are further confirmed by the results of pathological changes, indicating that L-THP could alleviate the severity of liver injury induced by hepatic IR.

The pathophysiologic processes taking part in hepatic IR injury are complex. In the initial period, the oxidative phosphorylation levels of liver cells decrease due to oxygen deficiency, thus influencing the production of adenosine triphosphate (ATP) [27]. With the depletion of ATP, the swollen hepatocytes produce reactive oxygen species (ROS). Kupffer cells are activated by ROS, then release a variety of proinflammatory cytokines, such as TNF-α and IL-6. Neutrophils and T cells accumulate and are activated by the Kupffer cells, then produce more inflammatory factors, which further aggravates the inflammatory injury in the liver. In our study, we performed ELISA, western blotting, qRT-PCR and immunohistochemistry to detect the inflammatory factors levels in the serum and liver tissues. It has been found that L-THP administration inhibited hepatic IR-induced release of TNF-α and IL-6, and this anti-inflammatory effect is dependent on the dose of L-THP. TNF-α has been previously shown to play a key role in various signal pathways, which could lead to cell apoptosis during hepatic IR injury [1,28].

NF-κB is a family of protein mediators that regulate various innate and adaptive immune responses [29,30]. The NF-κB family consists of the following five proteins: c-Rel (Rel); p65 (RelA); RelB; p50(NF-κB1); and p52(NF-κB2). It has been confirmed that NF-κB is activated by TNF family cytokines, such as TNF-α, to regulate cell proliferation, differentiation, and survival [31]. NF-κB has been confirmed to take part in the pathological mechanism underlying hepatic IR injury; activated NF-κB, in turn, leads to hepatocyte apoptosis and autophagy [32]. Sherif et al. [33] reported that vildagliptin exerts a protective effect on hepatic IR injury, and this effect is related to inhibition of the NF-κB signaling

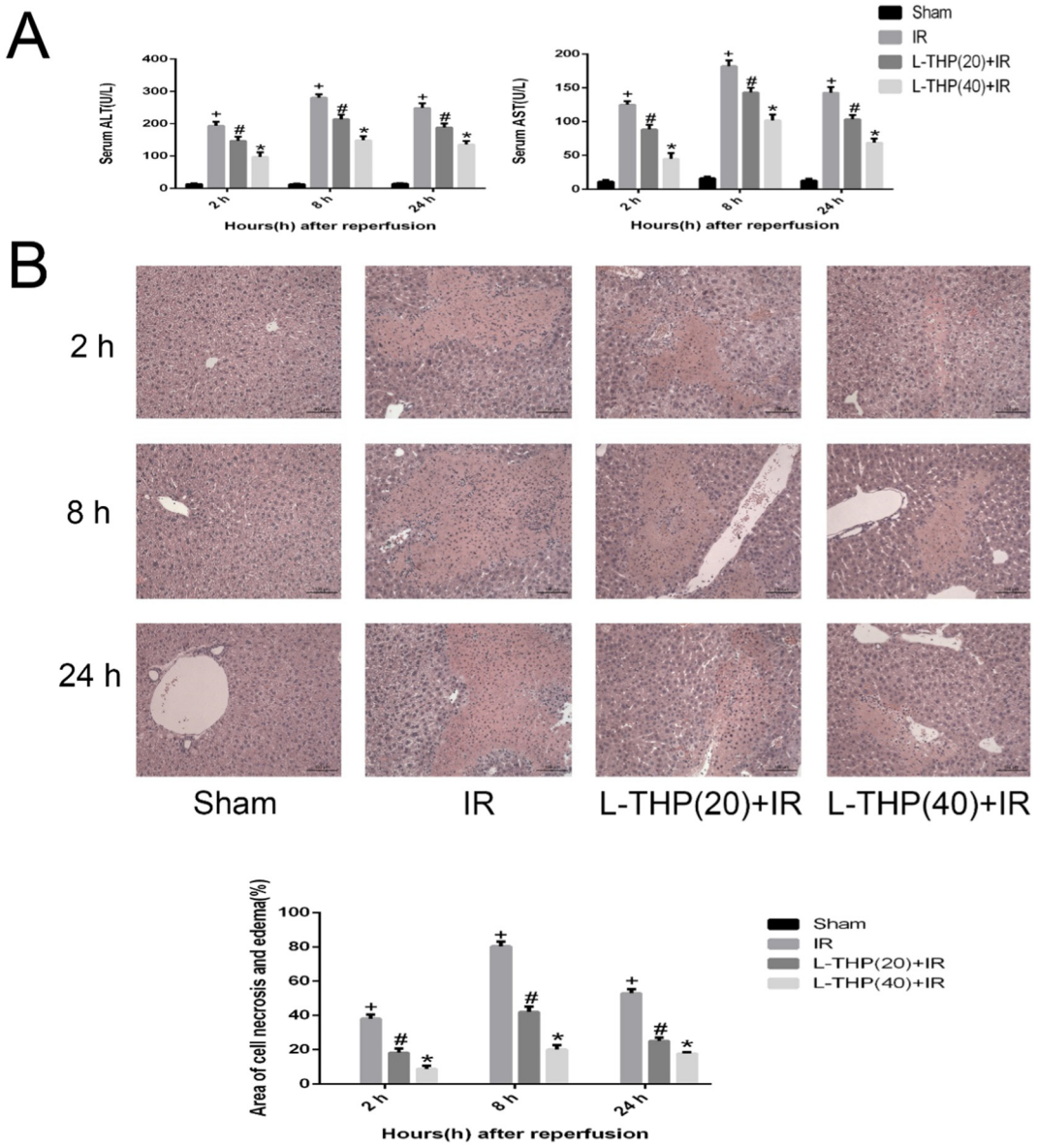

Fig. 2. Effects of L-THP on the liver function and histopathology of hepatic IR mice. (A) The levels of serum ALT and AST changed at three time points, depending on the L-THP dose, 20 mg/kg or 40 mg/kg. Data are given as means ± SD ($n = 6$, $^{+}P < 0.05$ for Sham versus IR, $^{\#}P < 0.05$ for L-THP(20) + IR versus IR, and $^{*}P < 0.05$ for L-THP(20) + IR versus L-THP(40) + IR). (B) The necrotic area stained with H&E was analyzed with Image-Pro Plus 6.0 (magnification, ×200). The results showed statistically significant differences. ($n = 6$, $^{+}P < 0.05$ for Sham versus IR, $^{\#}P < 0.05$ for L-THP(20) + IR versus IR, and $^{*}P < 0.05$ for L-THP (20) + IR versus L-THP(40) + IR).

pathway. Qu et al. [34] showed that L-THP alleviates neuroinflammation by reducing the expression of NF-κB. Thus, we detected the levels of NF-κB protein and mRNA in liver tissues in our study. The results indicated that hepatic IR induced NF-κB expression, which was inhibited by L-THP pretreatment in a dosage-dependent manner. Extracellular signal-regulated kinase (ERK) is a member of the mitogen-activated protein kinases (MAPKs) signaling mediator family. ERK is considered a significant protein mediator that regulates cell proliferation and differentiation [35]. ERK is activated to phosphorylated-ERK (p-ERK) by a variety of stimuli, such as proinflammatory cytokines and drugs [36]. It has been confirm by previous studies that phosphorylation of ERK take part in liver disorders, including liver fibrosis [37], hepatocellular carcinoma [38], and hepatic IR injury [39]. Furthermore, previous studies have shown that ERK activates NF-κB [40,41]. Wu et al. [1] reported that quercetin alleviates hepatic IR injury by inhibiting the TNF-α-mediated ERK/NF-κB pathway. The results of our study showed that during hepatic IR injury, L-THP pretreatment inhibits ERK phosphorylation by reducing the release of TNF-α, and subsequently inhibiting the activation of NF-κB.

Activation of NF-κB induces hepatocyte apoptosis and autophagy during liver injury induced by IR [42,43]. The extent of apoptosis and autophagy were detected to better understand how L-THP pretreatment reduced liver injury during hepatic IR. Bax and Bcl-2 are members of the Bcl-2 protein family. Bax belongs to a pro-apoptotic signaling mediator. Once activated, cytochrome C (cyto C) is produced into the cytoplasm [44] and activates caspase 3 and 9, leading to cell apoptosis [45], while Bcl-2 belongs to the anti-apoptotic mediators that reduces the release of cyto C. The Bcl-2:Bax ratio indicates the extent of cell apoptosis [46]. In our research, the results of western blotting, qRT-PCR and immunohistochemistry demonstrated that L-THP pretreatments upregulate Bcl-2 expression, while reducing these three proapoptotic proteins expression. The above results indicated that L-THP pretreatment alleviated hepatocyte apoptosis during hepatic IR injury by inhibiting NF-κB activation.

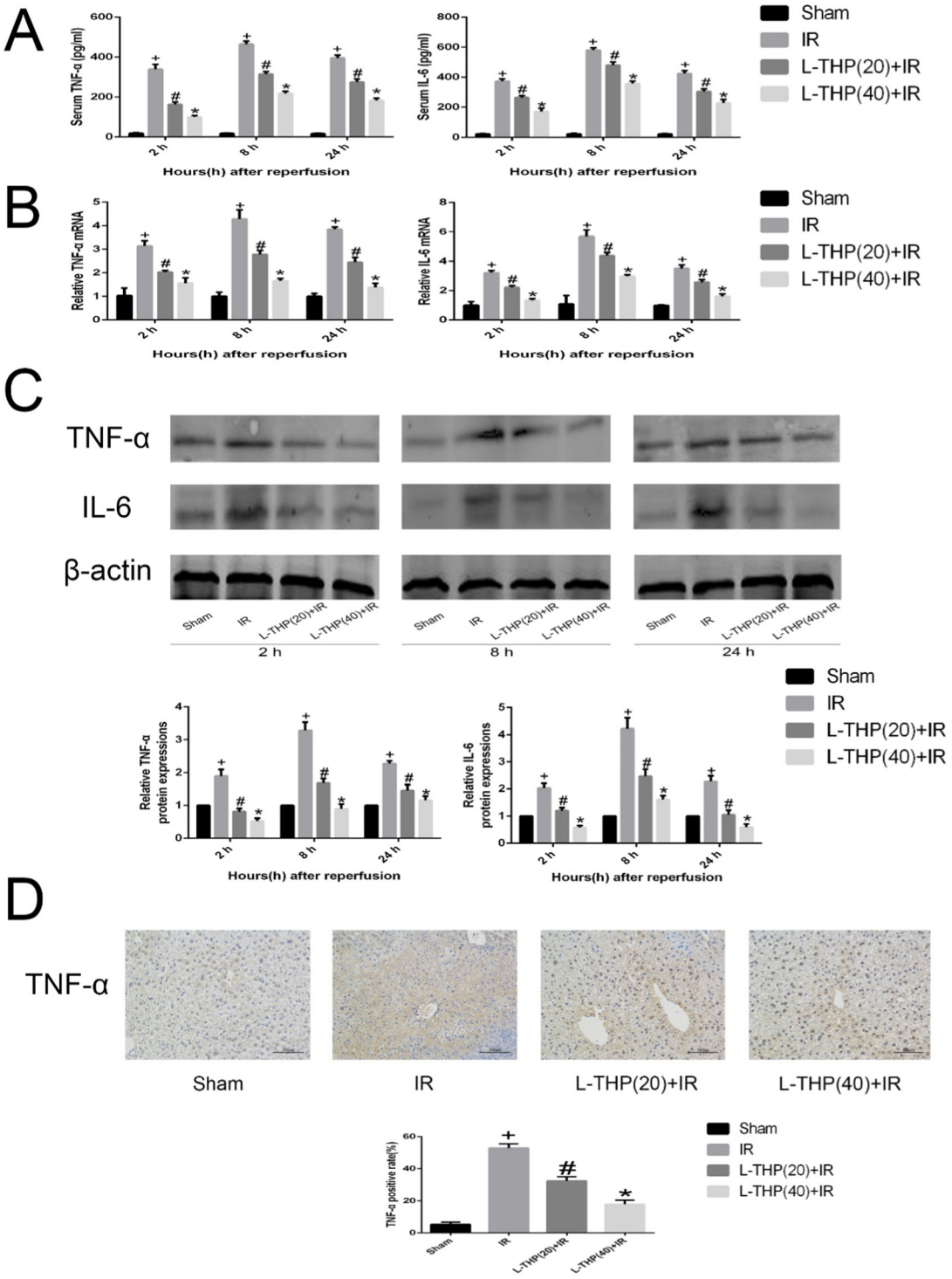

Fig. 3. L-THP pretreatment inhibited the released of TNF-a and IL-6. (A) The serum TNF-α and IL-6 levels were detected by ELISA and were reduced by L-THP pretreatment at three time points. Data were expressed as mean ± SD. ($n = 6$, $^{+}P < 0.05$ for Sham versus IR, $^{\#}P < 0.05$ for L-THP(20) + IR versus IR, and $^{*}P < 0.05$ for L-THP(20) + IR versus L-THP(40) + IR). (B) The mRNA expression of TNF-α and IL-6 in each group was detected by qRT-PCR. Data were expressed as mean ± SD. ($n = 6$, $^{+}P < 0.05$ for Sham versus IR, $^{\#}P < 0.05$ for L-THP(20) + IR versus IR, and $^{*}P < 0.05$ for L-THP(20) + IR versus L-THP(40) + IR). (C) The protein levels of TNF-α and IL-6 were determined by western blotting at three time points. Data were expressed as mean ± SD. ($n = 3$, $^{+}P < 0.05$ for Sham versus IR, $^{\#}P < 0.05$ for L-THP(20) + IR versus IR, and $^{*}P < 0.05$ for L-THP(20) + IR versus L-THP(40) + IR). (D) Representative immunohistochemical staining (×200) showing the expression of TNF-α at 8 h. ($n = 6$, $^{+}P < 0.05$ for Sham versus IR, $^{\#}P < 0.05$ for L-THP(20) + IR versus IR, and $^{*}P < 0.05$ for L-THP(20) + IR versus L-THP(40) + IR).

In addition to apoptosis, autophagy is another important process during organ ischemia and reperfusion damage, including liver. Beclin-1 and LC3 belong to biomarkers of cell autophagy. The Bcl-2-Beclin-1 complex is a significant mediator that regulates cell autophagy [47]. While Bcl-2 is inactivated, free Beclin-1 enhances cell autophagy. Transformation from LC3 I-to-LC3 II is crucial for the formation of autophagosomes [48]. The results of our research showed that L-THP pretreatment downregulates the expression of Beclin-1 and LC3. Based

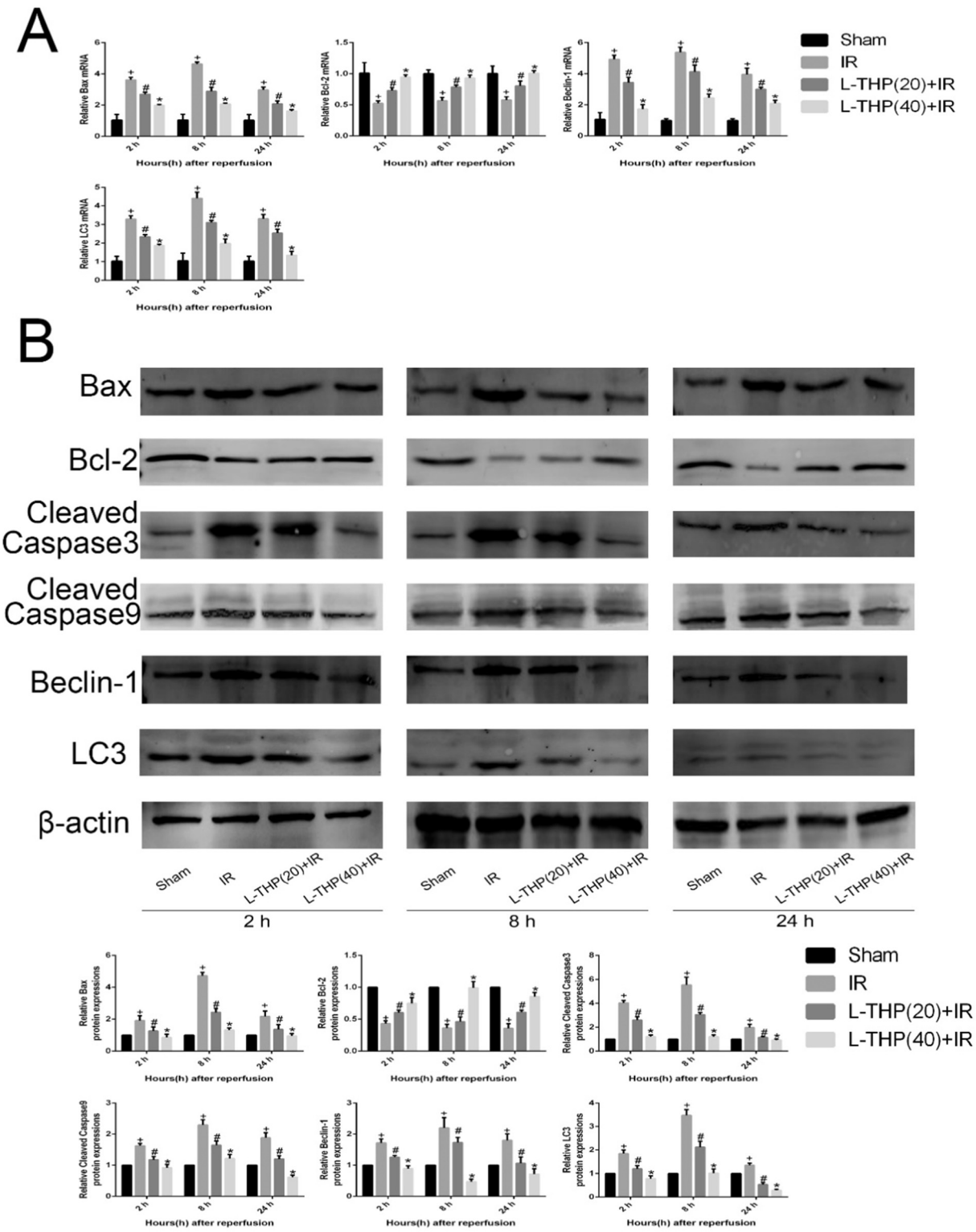

Fig. 4. L-THP pretreatment alleviated apoptosis and autophagy in hepatic IR. (A) The mRNA expression of Bax, Bcl-2, Beclin-1 and LC3 was determined by qRT-PCR. Data were expressed as the mean ± SD. ($n = 6$, $^{+}P < 0.05$ for Sham versus IR, $^{\#}P < 0.05$ for L-THP(20) + IR versus IR, and $^{*}P < 0.05$ for L-THP(20) + IR versus L-THP(40) + IR). (B) The protein levels of Bax, Bcl-2, Caspase 3, Caspase 9, Beclin-1, and LC3 was determined by western blotting. The gray values were calculated and data were expressed as the mean ± SD. ($n = 3$, $^{+}P < 0.05$ for Sham versus IR, $^{\#}P < 0.05$ for L-THP(20) + IR versus IR, and $^{*}P < 0.05$ for L-THP(20) + IR versus L-THP(40) + IR). (C) The expression of Bax, Bcl-2, Beclin-1, LC3 and caspase3 in the liver tissue at 8 h was detected by immunohistochemistry. The positive areas and total area were analyzed by Image-Pro Plus software 6.0. ($n = 6$, $^{+}P < 0.05$ for Sham versus IR, $^{\#}P < 0.05$ for L-THP(20) + IR versus IR, and $^{*}P < 0.05$ for L-THP(20) + IR versus L-THP(40) + IR). (D) TUNEL staining showed apoptotic hepatocytes in four groups at 8 h. The percentage of TUNEL-positive cells was analyzed by Image-Pro Plus software 6.0. ($n = 6$, $^{+}P < 0.05$ for Sham versus IR, $^{\#}P < 0.05$ for L-THP(20) + IR versus IR, and $^{*}P < 0.05$ for L-THP(20) + IR versus L-THP(40) + IR).

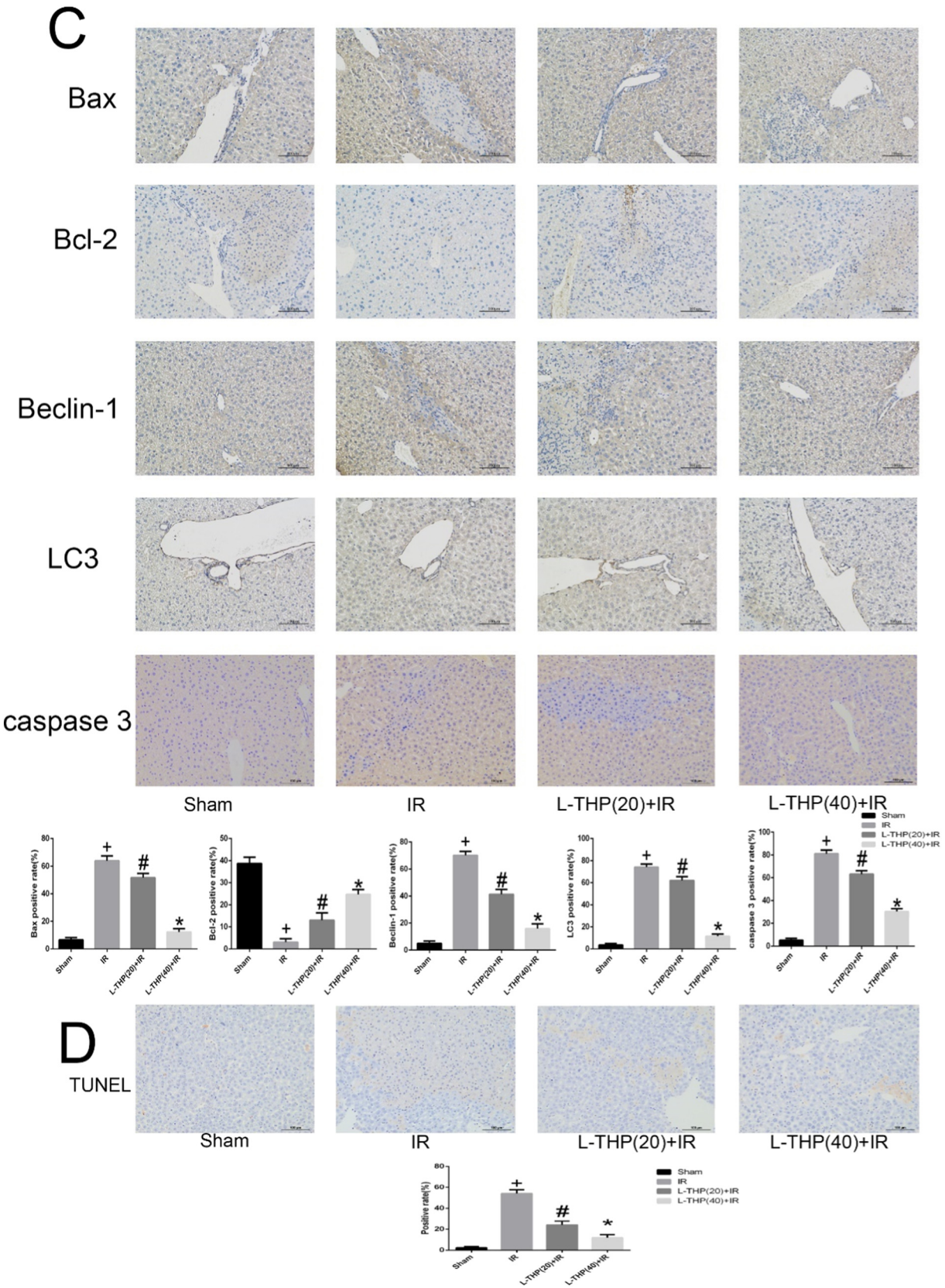

Fig. 4. (*continued*)

on the above results, we concluded that L-THP pretreatment alleviated IR injury by downregulating hepatocyte apoptosis and autophagy. Taken together, we found that L-THP alleviated the release of proinflammatory factors induced by hepatic IR, and alleviated liver cell apoptosis and autophagy via preventing the ERK/NF-κB signaling pathway activation; however, there were several limitations in our study and further investigation to verify the safety of L-THP for clinical use is warranted.

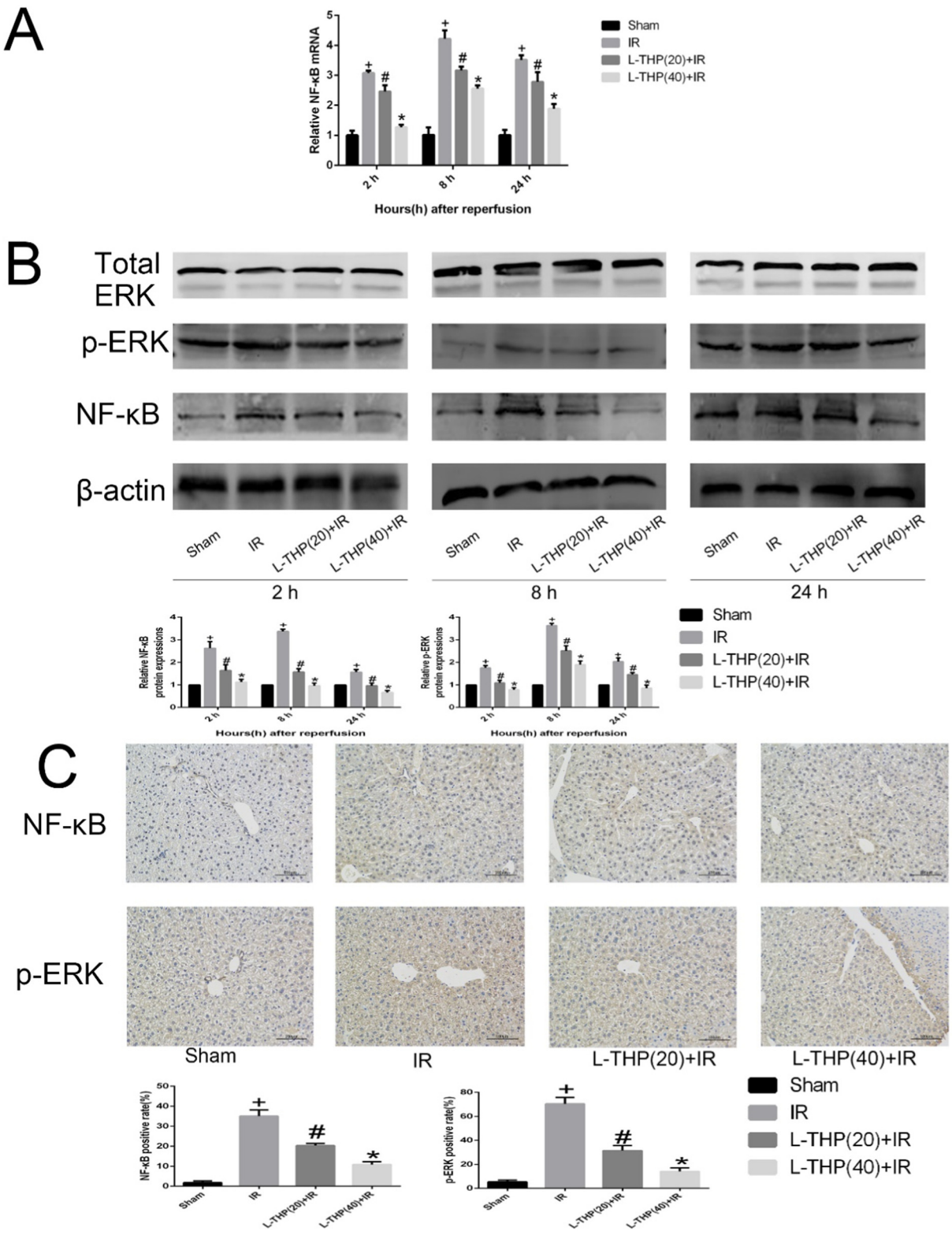

Fig. 5. L-THP pretreatment downregulated ERK/NF-κB pathway in hepatic IR mice. (A) The mRNA expression of NF-κB was detected by qRT-PCR. Data were expressed as the mean ± SD. ($n = 6$, $^{+}P < 0.05$ for Sham versus IR, $^{\#}P < 0.05$ for L-THP(20) + IR versus IR, and $^{*}P < 0.05$ for L-THP(20) + IR versus L-THP (40) + IR). (B) The protein levels of ERK, p-ERK and NF-κB were determined by western blotting. Data were expressed as the mean ± SD. ($n = 3$, $^{+}P < 0.05$ for Sham versus IR, $^{\#}P < 0.05$ for L-THP(20) + IR versus IR, and $^{*}P < 0.05$ for L-THP(20) + IR versus L-THP(40) + IR). (C) Representative immunohistochemical staining (×200) showing the expression of p-ERK and NF-κB at 8 h. ($n = 6$, $^{+}P < 0.05$ for Sham versus IR, $^{\#}P < 0.05$ for L-THP(20) + IR versus IR, and $^{*}P < 0.05$ for L-THP(20) + IR versus L-THP(40) + IR).

5. Conclusion

Our study showed that L-THP effectively alleviated hepatic IR injury in mice. L-THP alleviated inflammatory reactions in the liver and hepatocyte apoptosis and autophagy. And this protective effect is partly based on the inhibition of the TNF-α-mediated ERK/NF-κB pathway.

Conflict of interests

The authors report no conflicts of interest in the present study.

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Author contributions

Li weiwu, Tong Liu, and Jiao Feng conducted the experiments. Sainan Li analyzed the data. Yuqing Mao, Xiaoming Fan, and Chuanyong Guo provided the reagents and materials.

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