



Tetrandrine attenuates lipopolysaccharide-induced fulminant hepatic failure in D-galactosamine-sensitized mice

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ABSTRACT

Fulminant hepatic failure (FHF) remains an extremely poor prognosis and high mortality; better treatments are urgently needed. Tetrandrine (TET), a traditional anti-inflammatory drug, has been reported to exhibit hepatoprotective activities in several liver injury models. We now investigated the effects and underlying mechanisms of TET on lipopolysaccharide (LPS) and D-galactosamine (D-GalN)-induced FHF in mice. TET (50, 100, and 200 mg/kg) was given intraperitoneally 1 h before LPS/D-GalN injection in mice. The mortality and liver injury was evaluated subsequently. The results showed that administering TET to mice reduced mortality and improved liver injury induced by LPS/D-GalN in a dose-dependent manner. In addition, TET dose-dependently inhibited LPS/D-GalN-induced NF- κ B activation, serum and hepatic tissues tumor necrosis factor- α (TNF- α) production, caspase-3 activation and hepatocellular apoptosis, myeloperoxidase (MPO) activity, intercellular adhesion molecule-1 (ICAM-1) and endothelial cell adhesion molecule-1 (ECAM-1) expression. Our experimental data indicated that TET might alleviate the FHF induced by LPS/D-GalN through inhibiting NF- κ B activation to reduce TNF- α production.

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1. Introduction

Fulminant hepatic failure (FHF), induced by bacteria, viral hepatitis, alcohol and other hepatotoxic drugs, is a severe clinical syndrome which results in massive hepatocytes necrosis [1]. Except liver transplantation, there are still lack of available effective therapies, with a high mortality (80–90%) observed in the patients [2,3]. Lipopolysaccharide (LPS) and D-galactosamine (D-GalN)-induced acute liver injury in mice is a promising model similar to FHF in clinic. In this model, pro-inflammatory mediators including interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor- α (TNF- α) and adhesion molecules play important roles [4]. Among these factors, TNF- α , which induces hepatocyte apoptosis in the early stages and neutrophil transmigration in the later stages, acts as a pivotal mediator in the progression of acute liver injury [5]. Thereby, interfering with pro-inflammatory cytokines especially TNF- α is likely to be therapeutically effective in patients with FHF [6]. In addition, concerning the regulation of TNF- α , NF- κ B resides in

an essential position. It is normally located in an inactive state in cytoplasm, then enters the nucleus and initiates transcription of TNF- α genes when activated by LPS [7].

Tetrandrine (TET) (Fig. 1), a bis-benzylisoquinoline alkaloid, is isolated from the root of the Han-Fang-Ji (Chinese herb *Radix Stephania tetrandra* S. Moore), which is traditionally used for patients with arthritis, arrhythmia, hypertension, inflammation and silicosis [8,9]. It is reported that tetrandrine has cytoprotective and cytotoxic, immunosuppressive, free radical scavenging, anti-proliferative and anti-inflammatory activities [8,10,11]. Furthermore, recent studies have shown that tetrandrine has hepatoprotective effect on various liver injury models [8,12,13].

Based on these effects of TET, in this study, we used LPS/D-GalN-induced fulminant hepatic failure model to examine the effect of TET on liver injury and then intend to clarify its mechanisms.

2. Materials and methods

2.1. Animals

Balb/c mice (6–8 weeks old; weight range, 20–22 g) were obtained from the Laboratory Animal Center of the Chongqing

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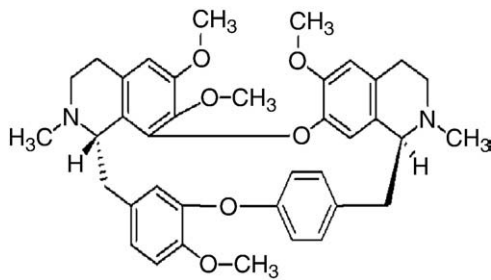


Fig. 1. Chemical structure of tetrandrine.

Medical University (Chongqing, PR China). All mice received human care according to the guidelines of the Local Institutes of Health guide for the care and use of laboratory animals. They were maintained under controlled conditions (22 °C, 55% humidity and 12 h day/night rhythm) and fed standard laboratory chow.

2.2. Materials

TET (C₃₈H₄₂O₆N₂, MW:622.8, purity >98%) determined by HPLC as previously described [14] was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). LPS (*Escherichia coli*, 0111:B4), D-GalN were obtained from Sigma (St. Louis, MO, USA). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) detection kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Caspase-3 colorimetric assay kit was purchased from Beyotime institute of biotechnology (Nanjing, China). Mouse tumor necrosis factor- α (TNF- α) enzyme-linked immunosorbent assay (ELISA) kit was purchased from Bender MedSystems (Vienna, Austria). Intercellular adhesion molecule-1 (ICAM-1), endothelial cell adhesion molecule-1 (ECAM-1) and β -actin antibodies were products of Abcam (Cambridge, UK). TransAM™ NF- κ B transcription factor assay kit was purchased from Active Motif (Carlsbad, USA). Other chemicals were obtained from the local market.

2.3. Experimental protocols

Mice were injected intraperitoneally (i.p.) with TET (50, 100, and 200 mg/kg, respectively) 1 h prior to challenge experimentation. Mice were challenged i.p. with LPS (50 μ g/kg) and D-GalN (700 mg/kg). In addition, the doses of TET (50, 100, and 200 mg/kg) were chosen because TET alone did not induce liver injury as determined by evaluating lethality, liver enzymes, cytokines, and liver histology (data not shown). Mice were sacrificed by decapitation at different time points; blood samples and liver tissues were collected for further determinations.

2.4. Survival rate and histopathological analysis

The survival rate of mice was monitored for 48 h after LPS/D-GalN injection. Liver specimens for histopathological analysis were obtained at 6 h after LPS/D-GalN administration. Liver tissues from individual mouse were fixed in 10% neutral-buffered formalin and stained with hematoxylin–eosin to analyze morphology.

2.5. Analysis of liver enzymes

Hepatocyte damage was assessed 6 h after LPS/D-GalN administration by measuring serum enzyme activities of ALT and AST using corresponding detection kits according to the manufacturer's instructions.

2.6. Hoechst 33342-staining

To analyze the typical morphological signs of apoptosis, paraffin-embedded sections were deparaffinized and stained with Hoechst 33342, a fluorescent dye that has been widely used for analysis of nuclear morphology, in an aqueous dilution of 1:10,000 for 5 to 10 min. Hoechst 33342-stained tissue was examined with a fluorescence microscope and photographed. For quantification of apoptosis, 100 nuclei were randomly viewed, and apoptosis was counted.

2.7. Caspase-3 protease activities

Caspase-3 protease activities in the liver tissue were measured using a caspase-3 colorimetric assay kit, according to the manufacturer's instructions. Briefly, after homogenization of whole liver tissue in cell lysis buffer, homogenates were centrifuged for 1 min at 10,000 g, and the supernatant (100 μ g protein) was incubated with Ac-DEVD-pNA substrate for caspase-3, and reaction buffer for 90 min at 37 °C. Absorbance was measured at 405 nm as caspase protease activity.

2.8. Measurement of TNF- α levels

Mouse serum and hepatic samples were assayed for murine TNF- α by enzyme-linked immunosorbent assay as described by the manufacturer. Previous studies have shown that administration of LPS/D-GalN significantly increased TNF- α levels, which peaked at 1.5 h post-injection [15–17]. Therefore, serum and hepatic tissue levels of TNF- α were measured at 1.5 h after the LPS/D-GalN challenge.

2.9. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from hepatic samples using Trizol reagent according to the manufacturer's protocol. First-strand complementary DNA (cDNA) was synthesized. The cDNA samples were then incubated at 90 °C for 7 min to stop the reaction. For amplification of TNF- α cDNA, the sequences of primers were 5'-GGC AGG TCT ACT TTG GAG TCA TTG C-3' (sense) and 5'-ACA TTC GAG GCT CCA GTGAAT TCG G-3' (antisense). The primers used for amplification of β -actin cDNA as an internal standard were 5'-TGG AAT CCT GTG GCA TCC ATG AAA C-3' (sense) and 5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3' (antisense). The PCR products of TNF- α and β -actin were 300 and 349 base pairs (bp) in length, respectively. cDNA was amplified in 25 μ l reaction system. PCR reactions were initiated at 94 °C for 5 min, followed by 34 cycles of amplification (denaturation at 94 °C for 1 min, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s) with a final primer extension at 72 °C for 7 min. The PCR products were separated on 2% agarose gel and stained with ethidium bromide. The intensity of each TNF- α mRNA band was quantified by the Kodak molecular imaging system (Kodak Gel Logic 1500, Kodak MI software, USA) and normalized to values for β -actin.

2.10. Measurement of MPO activity

Frozen liver tissues were thawed and homogenized in phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide. The enzyme activity was determined spectrophotometrically using a MPO detection kit according to the manufacturer's instructions. MPO activity was assessed according to the absorbance measured at 450 nm and normalized by the total protein concentration of the same sample.

2.11. Western blotting

Total proteins from frozen hepatic samples were prepared according to the method described by the protein extract kit (Pierce

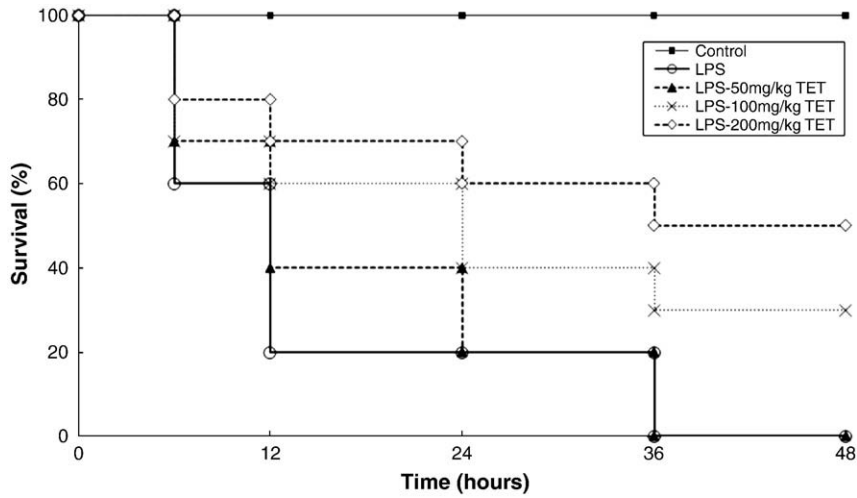


Fig. 2. Effect of TET on survival rate in mice ($n = 10$). Mice were pretreated intraperitoneally (i.p.) with TET (50, 100, and 200 mg/kg) 1 h prior to LPS/D-GalN injection. Survival of mice was monitored for 48 h.

Biotechnology, Rockford, USA). Protein concentrations were determined by BCA protein assay kit. Protein extracts were fractionated on 12% polyacrylamide-sodium dodecyl sulfate (SDS) gel and then transferred to nitrocellulose membrane. The membrane was blocked

with 5% (w/v) fat-free milk in Tris-buffered saline (TBS) containing 0.05% tween-20, followed by incubation with a rabbit primary polyclonal antibody at 4 °C overnight. Then the membrane was treated with horseradish peroxidase-conjugated goat anti-rabbit

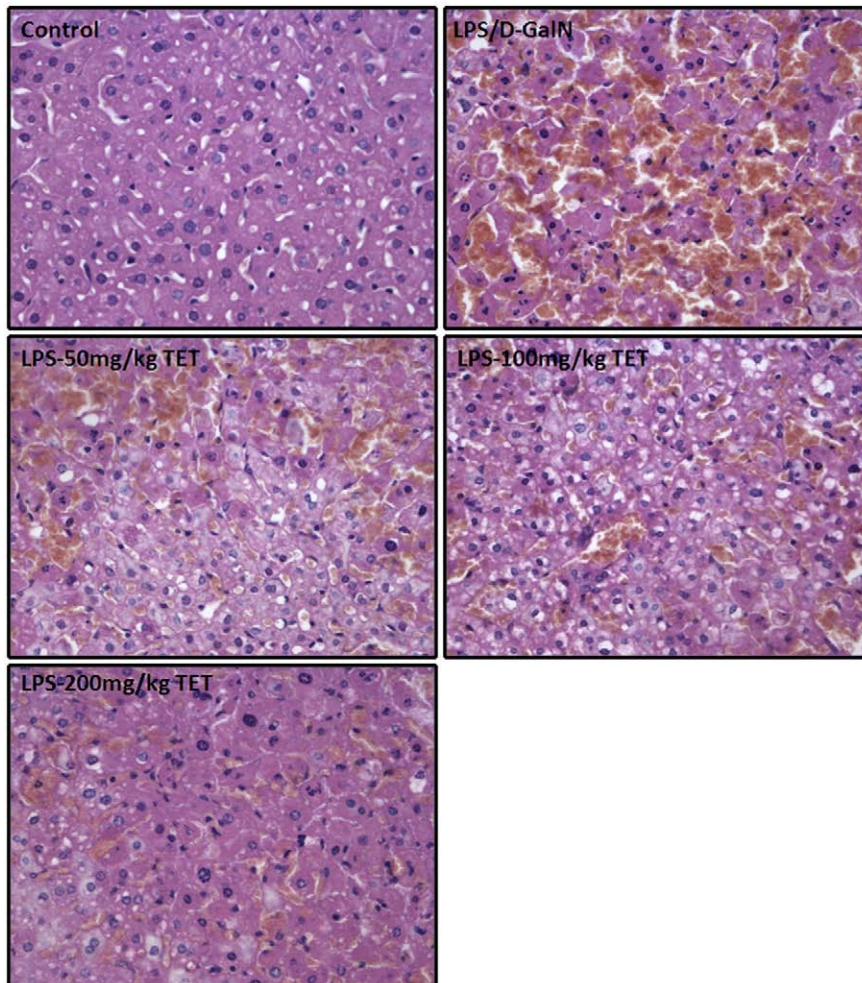


Fig. 3. Hematoxylin and eosin-stained livers from the treated mice ($n = 6$). Mice were pretreated i.p. with vehicle (PBS) or TET (50, 100, and 200 mg/kg) 1 h before LPS/D-GalN injection. Liver specimens were obtained at 6 h after administration of LPS/D-GalN. Original magnification, 400 \times .

secondary antibody (1:10,000). Antibody binding was visualized with an ECL chemiluminescence system and short exposure of the membrane to X-ray films (Kodak, Japan).

2.12. NF- κ B p65 transcription factor assay

Nuclear protein extracts were obtained from liver tissues using a Nuclear Extract Kit (Active Motif, USA) according to the manufacturer's instructions. The p65 DNA binding activity was assessed by the TransAM™ NF- κ B Kit (Active Motif, USA) according to the manufacturer's instructions.

2.13. Statistical analysis

Results were analyzed using Student's test or by ANOVA where appropriate. All data in this study were expressed as mean \pm standard (S.D.). *P* values less than or equal to 0.05 was considered significant.

3. Results

3.1. Effects of TET on LPS/d-GalN-induced lethality and liver injury

All mice survived in control group. After LPS/d-GalN administration, death of mice occurred at 6 h and the lethality reached to 100% at 48 h in LPS/d-GalN-treated group. Pretreatment with TET significantly decreased the lethality in a dose-dependent manner (Fig. 2).

To investigate effect of TET on liver injury, HE staining of the liver was performed. In control group, it showed no pathological abnormality. The morphology of liver parenchyma was good with no congestion and inflammation in the sinusoids. However, in LPS/d-

GalN group, it showed severe pathological abnormality including hepatocytes necrosis, congestion, destruction of hepatic architecture, massive immigration of inflammatory cells in the sinusoids. However, in TET (50, 100, and 200 mg/kg)-pretreated groups, the area and extent of necrosis was attenuated and the infiltration of inflammatory cells was reduced dose-dependently (Fig. 3).

Further, as shown in Fig. 4, at 6 h after administration of LPS/d-GalN, serum ALT and AST levels were significantly increased in mice, which are quantitative markers of the extent of hepatocellular damage. However, in TET pretreated groups, serum ALT and AST were attenuated in a dose-dependent manner.

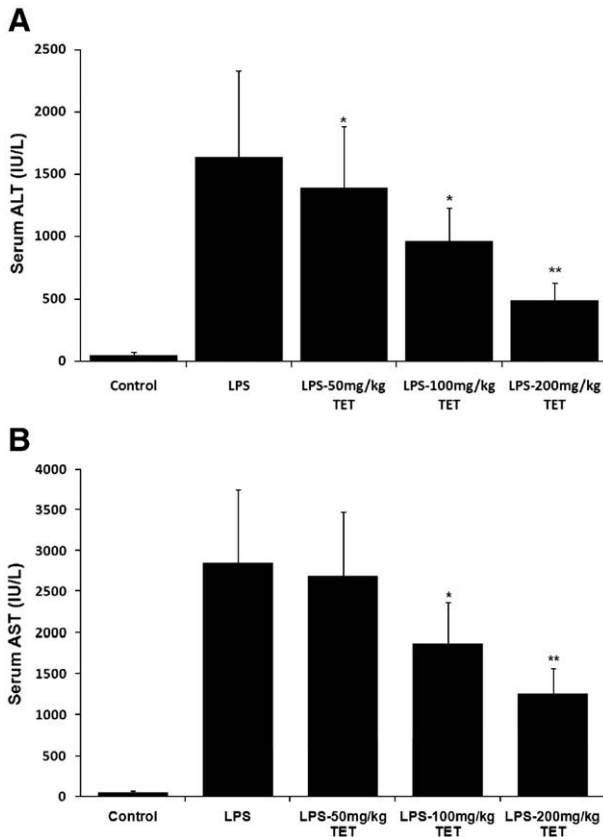


Fig. 4. Effect of TET on the serum ALT (A) and AST (B) concentration in mice. Mice were pretreated i.p. with TET (50, 100, and 200 mg/kg) 1 h before the injection of LPS/d-GalN. Serum samples were collected 6 h after co-injection, and serum aminotransferases activity was determined. Each value is mean \pm S.D. (*n* = 6). **P* < 0.05, ***P* < 0.01 versus LPS/d-GalN group.

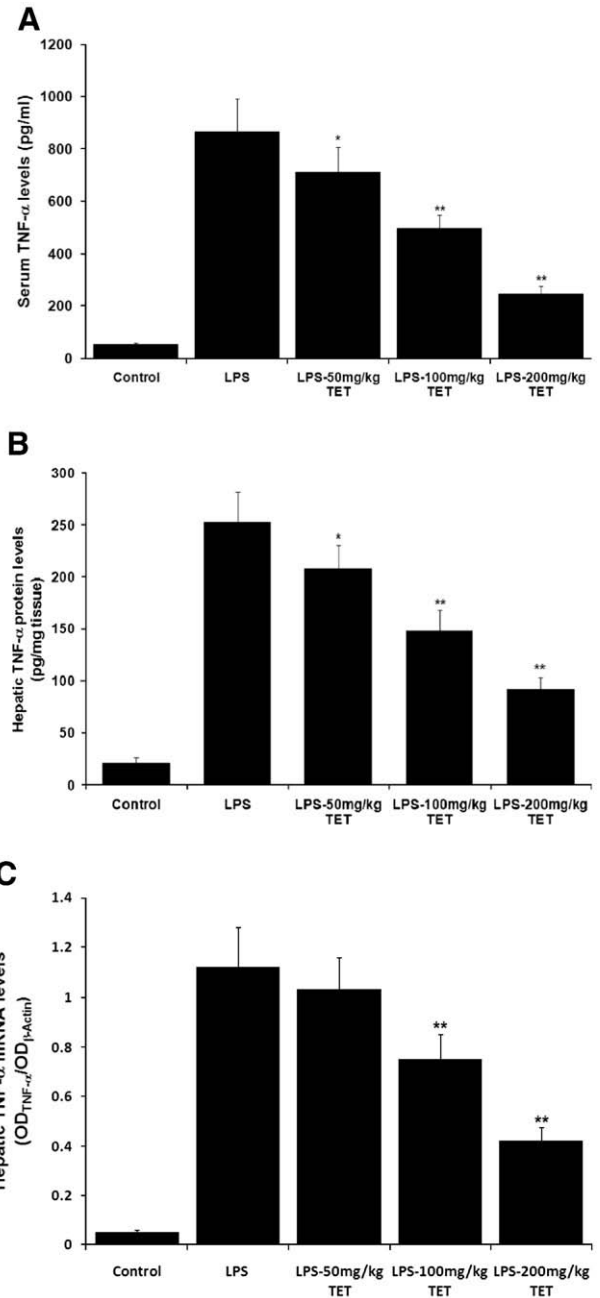


Fig. 5. Effect of TET on the changes of serum TNF- α levels (A), hepatic TNF- α protein levels (B) and mRNA levels (C) in mice. Mice were pretreated i.p. with TET (50, 100, and 200 mg/kg) 1 h before the injection of LPS/d-GalN. Blood and liver samples were obtained at 1.5 h after LPS/d-GalN injection. Each value is mean \pm S.D. (*n* = 6). **P* < 0.05, ***P* < 0.01 versus LPS/d-GalN group.

3.2. Effects of TET on LPS/D-GalN-induced TNF- α production

The production of serum TNF- α and hepatic TNF- α after LPS/D-GalN injection was significantly increased as compared with the control group. However, this elevation of TNF- α levels was suppressed by pretreatment with TET in a dose-dependent manner. A similar observation was made regarding the TNF- α mRNA level in liver tissue (Fig. 5).

3.3. Effects of TET on LPS/D-GalN-induced hepatocellular apoptosis

Hoechst 33342 staining was used to detect apoptotic hepatocytes. Injection of LPS/D-GalN induced high numbers of apoptotic cells,

whereas pretreatment with TET showed dose-dependently less hepatocellular apoptosis, indicating less severe cellular damage in the TET-treated mice (Fig. 6A). The caspase-3 activity in liver tissue 6 h after the LPS/D-GalN injection was markedly higher than the control group but was attenuated by pretreatment with TET in a dose-dependent manner (Fig. 6B).

3.4. Effects of TET on LPS/D-GalN-induced hepatic inflammation

To determine whether TET interfered with LPS/D-GalN-induced inflammatory responses, the levels of ICAM-1, ECAM-1 and MPO were examined. They were significantly elevated after LPS/D-GalN injection.

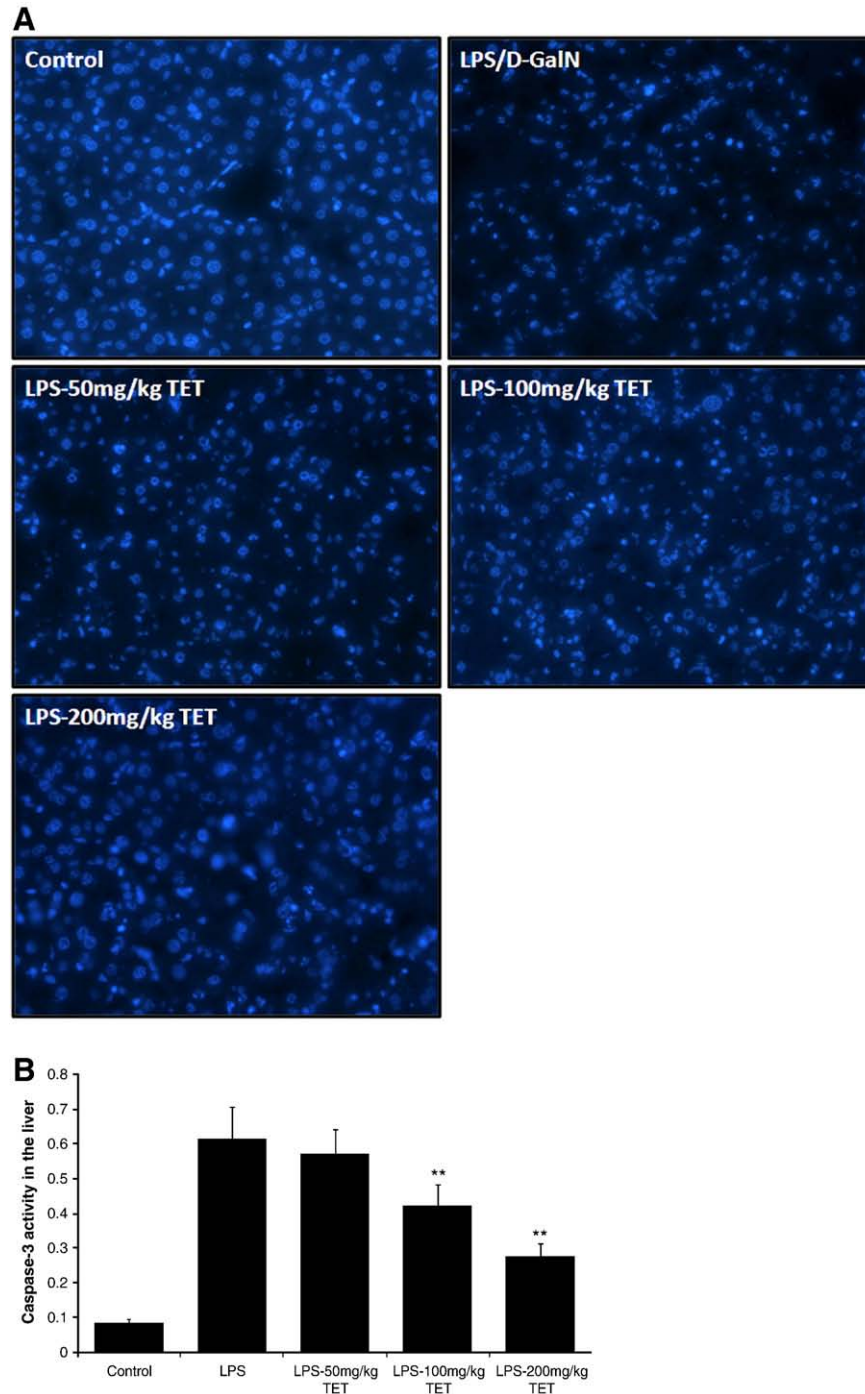


Fig. 6. Effect of TET on LPS/D-GalN-induced hepatocellular apoptosis in mice. (A) Hoechst 33342 staining. Original magnification, 400 \times . (B) the activity of caspase-3. Mice were pretreated i.p. with TET (50, 100, and 200 mg/kg) 1 h before LPS/D-GalN injection. Liver samples were obtained at 6 h after LPS/D-GalN injection. Each value is mean \pm S.D. ($n = 6$). ** $P < 0.01$ versus LPS/D-GalN group.

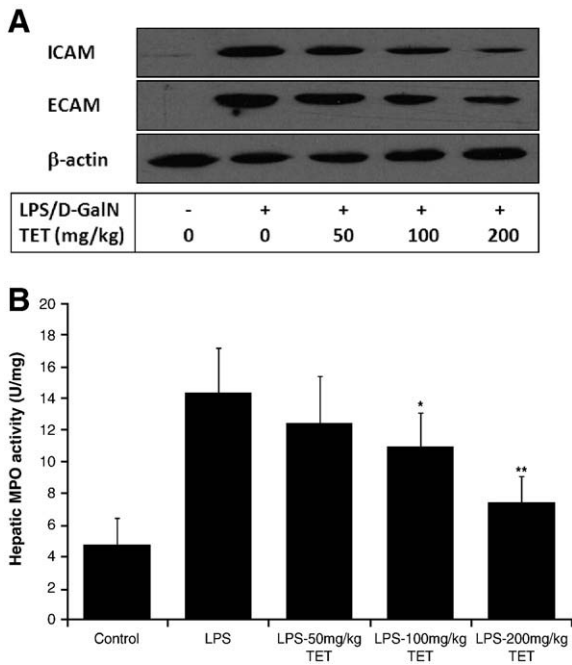


Fig. 7. Effect of TET on the changes of ICAM, ECAM protein expression (A) and MPO activity (B) in liver of mice. Mice were pretreated i.p. with TET (50, 100, and 200 mg/kg) 1 h before administration of LPS/D-GalN. Liver samples were obtained at 6 h after LPS/D-GalN injection. Each value is mean \pm S.D. ($n = 6$). * $P < 0.05$, ** $P < 0.01$ versus LPS/D-GalN group.

Pretreatment with TET (50, 100, and 200 mg/kg) reduced the levels of ICAM-1, ECAM-1 and MPO activity in a dose-dependent manner (Fig. 7).

3.5. Effects of TET on LPS/D-GalN-induced the activity of NF- κ B

The p65 is a main subunit of NF- κ B. Thereby, nuclear NF- κ B activity was assayed by using a NF- κ B p65 transcription factor assay method. As shown in Fig. 8, in LPS/D-GalN group, NF- κ B activity markedly increased compared with the control group. However, TET pretreatment dramatically inhibited LPS/D-GalN-induced NF- κ B activity in a dose-dependent manner.

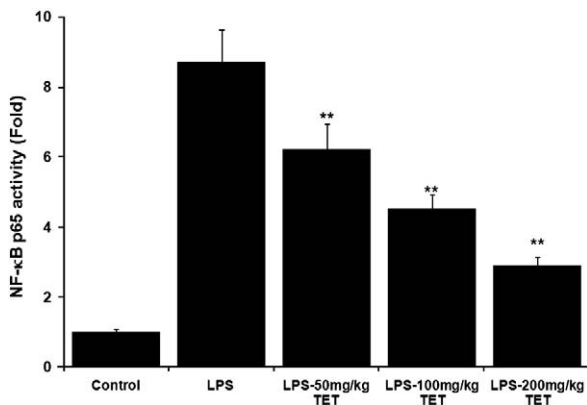


Fig. 8. Effect of TET on LPS/D-GalN-induced NF- κ B activation in mice. The NF- κ B p65 activity in the nuclear was measured by ELISA to show NF- κ B activation. Mice were pretreated i.p. with TET (50, 100, and 200 mg/kg) 1 h before administration of LPS/D-GalN. Liver samples were obtained at 1 h after LPS/D-GalN injection for determining p65. Each value is mean \pm S.D. ($n = 6$). ** $P < 0.01$ versus LPS/D-GalN group.

4. Discussion

LPS/D-GalN-induced liver injury has been widely used to elucidate the mechanisms of clinical liver dysfunction and evaluate the efficiency of hepatoprotective agents [17]. Upon stimulated by LPS, Kupffer cells secrete various pro-inflammatory cytokines, including IL-1, IL-6 and TNF- α [18]. D-GalN, a specific hepatotoxic agent, is often used to enhance the sensitivity to inflammatory injury to hepatocytes [19]. Co-injection of LPS and D-GalN into mice produces acute liver injury with severe hepatic congestion, resulting in rapid death [18,19]. In our study, mice with LPS/D-GalN injected have a high lethality and severe pathological injury including hepatocellular apoptosis and necrosis. With TET pretreated, the lethality significantly decreased in a dose-dependent manner, accompanied with palliative hepatic pathological injury and serum ALT and AST reduced. These results indicated that TET alleviated LPS/D-GalN-induced liver injury, consistent with previous researches that TET protects mice from concanavalin A-induced hepatitis [20,21].

TNF- α plays a crucial role in LPS/D-GalN-induced acute liver injury [22], which has been confirmed by researches with TNF- α knockout or TNF-receptor p55 knockout mice [23,24]. Moreover, anti-TNF- α antibody could decrease the lethality, while inhibition of TNF- α is in parallel with attenuated hepatocellular necrosis and apoptosis [2,19,25]. In terms of apoptosis, the overproduction of TNF- α activates caspase-3, a original family of cysteine proteases, to trigger it [26]. It has been showed that TET significantly suppressed LPS-induced transcription of proinflammatory cytokines including IL-4, IL-8 and TNF- α in human monocytic cells [27]. In our study, TET significantly decreased serum TNF- α expression as well as the mRNA and protein level of TNF- α in liver tissue. Meanwhile, depletion of apoptotic cells and suppression of caspase-3 activity were observed in TET-treated groups, suggesting TET might relieve liver injury through inhibiting hepatocellular apoptosis. However, a previous study in rat primary hepatocytes has yielded contradictory results, which TET-induced hepatocytes apoptosis through caspase-3 activation, and this apoptosis process might be involved in TET-increased the generation of reactive oxygen species (ROS) [28]. We speculate that the differential effect of TET on hepatocytes apoptosis is dependent on differential molecular mechanisms. In rat primary hepatocyte experiment, TET directly induced static hepatocyte to generate ROS, which activated caspase-dependent mitochondrial hepatocyte apoptosis pathway. But in our present experiment, TET mainly inhibited LPS-activated macrophages to reduce TNF- α production. The reduced TNF- α attenuated hepatocellular apoptosis; on the other hand, inhibited neutrophils transmigration, which might reduce neutrophils-generated ROS. In fact, previous reports by others have shown that TET inhibited the accumulation of ROS induced by PMA, ischaemia-reperfusion or leukotriene B4 in neutrophils [29–31].

Furthermore, TNF- α induces neutrophil transmigration in the later stage of the liver injury, causing massive hepatocytes necrosis [32,33]. The overproduction of TNF- α stimulated endothelial cells to express various adhesion molecules [34]. Of these adhesion molecules, endothelial cell adhesion molecule-1 (ECAM-1) and intercellular adhesion molecule-1 (ICAM-1) are pivotal in regulating the interaction between neutrophils and endothelial cells, as well as neutrophil recruitment in the inflammatory response [35,36]. Inhibiting ECAM-1 expression reduces leukocyte adhesion and transmigration across the endothelium [34,37]. The present study showed the level of ICAM-1 and ECAM-1 in TET-treated groups had an evident decline, compared to LPS/D-GalN group. In according with these findings, myeloperoxidase (MPO), which is mainly excreted by neutrophils, decreased in mice pretreated with tetrandrine before LPS/D-GalN administration [38]. The results indicated that TET might down-regulated the inflammatory response to protect against LPS/D-GalN-induced acute liver injury.

It is well known that NF- κ B is an important transcriptional factor involved in the expression of TNF- α [39,40]. In unstimulated cells, NF- κ B is sequestered in an inactive form in the cytoplasm bound to inhibitory I κ B proteins. Stimulation leads to the rapid phosphorylation, ubiquitinylation, and ultimately proteolytic degradation of I κ B, which frees NF- κ B to translocate to the nucleus and activate the transcription of TNF- α genes [41]. Additionally, It is demonstrated that TNF- α is reduced in mice deficient in the activation of NF- κ B [42]. TET has been found to intervene with NF- κ B activation and nuclear translocation in vitro in several cell lines [21,42]. Therefore, we investigated whether NF- κ B activity in hepatocytes was affected by TET. In our study, pretreatment with TET dose-dependently inhibited LPS-induced NF- κ B activity. These observations revealed that the transcriptional inhibition of TNF- α by TET was probably due to inhibit NF- κ B activity.

To conclude, we have demonstrated that TET has potent protection against LPS/D-GalN-induced FHF. It decreased the high lethality, alleviated hepatic pathological injury, inhibited hepatocytes apoptosis and reduced hepatic inflammatory responses including MPO and adhesion molecules. Such protective effects were probably carried out through suppression of NF- κ B activation, which inhibited TNF- α production. Therefore, it is expected that TET might contribute to delaying FHF progression and prolonging life in patients with liver damage.

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