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Inhibitory Effect of TCCE on CCl_4-induced Overexpression of IL-6 in Acute Liver Injury

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Abstract *Terminalia catappa* L. leaves have been shown to protect against acute liver injury produced by some hepatotoxicants, but the active components and mechanisms are not clear. This study was designed to characterize the protective effects of the chloroform fraction of the ethanol extract of *T. catappa* leaves (TCCE) against carbon tetrachloride (CCl_4)-induced hepatotoxicity in mice, and analyze the changes in expression level of interleukin-6 (IL-6) in the process. It was found that TCCE pretreatment (10 or 30 mg/kg, ig) protected mice from CCl_4 toxicity, as evidenced by the reversed alterations in serum alanine aminotransferase (sALT) and serum aspartate aminotransferase (sAST) activities. Additionally liver tissues were subjected to RT-PCR, Western blot and immunohistochemistry to analyze changes in IL-6 expression. It was found that TCCE markedly suppressed the CCl_4-induced over-transcription of IL-6 gene. Consistent with the result, the expression of IL-6 protein was also blocked by TCCE in CCl_4-stimulated mice, especially in the area around central vein on liver tissue section. In conclusion, TCCE is effective in protecting mice from the hepatotoxicity produced by CCl_4, and the mechanisms underlying its protective effects may be related to the inhibition on the overexpression of IL-6 mainly around terminal hepatic vein.

Key words acute liver injury; TCCE; CCl_4; IL-6; expression

Terminalia catappa L. is a Combretaceous plant whose leaves are widely used as a folk medicine in Southeast Asia for the treatment of dermatosis and hepatitis [1]. More and more pharmacological studies have reported that the extract of *T. catappa* leaves and fruits have anticancer, antioxidant, anti-HIV reverse transcriptase, anti-inflammatory, antidiabetic effects and hepatoprotective activities [2–5], but the effective components and related mechanisms remain unknown.

Recently, data from our laboratory revealed that the chloroform fraction of the ethanol extract of *T. catappa* leaves (TCCE) has the strongest anti-inflammatory activity among all fractions from ethanol extract of *T. catappa* leaves [3]. Moreover, TCCE has been shown to protect against acute liver injury produced by some hepatotoxicants [6]. Except hepatotoxicants, hepatic necrosis could also be induced by cytokines released from inflammatory cells [7]. Interleukin-6 (IL-6), a 26 kD pleiotropic cytokine, has been reported to increase significantly in mRNA level after carbon tetrachloride (CCl_4) intoxication in rats and mediate the hepatic aspects of the acute phase response in patients [7–9].

The present investigation has been designed to study the effect of TCCE against CCl_4 induced hepatic damage in mice to elucidate a possible mechanism related to IL-6 expression underlying the hepatoprotective action of TCCE.

Materials and Methods

Animals

Male ICR mice weighing 18–22 g were obtained from Experimental Animal Center of Nanjing Medical University. All animals were fed with a standard laboratory diet and tap water ad libitum. They were maintained in a controlled environment at a temperature of 20–25 °C with a 12 h dark/light cycle, and acclimatized for at least 1 d prior to use.

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Chemicals

TCCE was provided by Institute of Materia Medica, Nanjing University School of medicine, with triterpenoid being the most abundant composition (23.8%, HPLC analysis) [3]. Dimethyl diphenyl bicarboxylate (DDB, a known hepatoprotective compound) was purchased from Beijing Union Pharmaceutical Factory (purity>99%). TCCE and DDB were prepared for intragastric administration by being suspended in saline, respectively. All other reagents were commercially available and were of analytic grade.

CCl_4 hepatotoxicity

TCCE (10 and 30 mg/kg) and DDB (200 mg/kg) were administrated intragastrically into mice once daily for 5 d consecutively, respectively (six mice in each group). Twelve hours after the final treatment, the mice were treated with CCl_4 (20 μl/kg, ip, dissolved in olive oil). Two re-ference groups were used: a group of non-intoxicated animals, which received respective vehicles (control group), and a group that received CCl_4 (CCl_4 group) following the treatment of saline (20 ml/kg, ig) for 5 d. After 24 h, mice were euthanized by decapitation and blood was collected using 1.5 ml Eppendorf tube. Serum was obtained after the blood was allowed to clot at room temperature for 30 min and centrifuged at 3000 rpm for 20 min. The whole liver was excised, weighed, and a small section (1 cm× 1 cm) was removed from the median lobe and fixed in 10% formalin in phosphate-buffered saline (PBS) to obtain the histological sections.

The remaining liver lobes intended for mRNA and protein analyses were frozen immediately in liquid nitrogen and stored at –82 °C before extraction.

Biochemical estimation

Serum alanine aminotransferase (sALT) and aspartate aminotransferase (sAST) levels, which were markers for hepatotoxicity, were determined with corresponding detection kit obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Evaluation of mRNA level by RT-PCR

Total RNA was extracted from frozen tissues by Tripure isolation reagent (Roche diagnostics corporation, Indianapolis, USA). The quality of the isolated RNA was verified by agarose gel electrophoresis. IL-6 and β-actin expression were evaluated by semiquantitative RT-PCR as previously described [6]. Briefly, 1 μg of total liver RNA was reversely transcribed using 0.5 μg oligo(dT) and incubated with AMV RT (Promega). The cDNA was amplified by PCR using specific oligonucleotide primers (Table 1).

PCR fragments were separated by gel electrophoresis and revealed by ethidium bromide staining. PCR amplification was verified to be exponential, and the amplification products were proportional to sample input.

Protein extraction and Western blot analysis

Frozen samples were homogenized in ice-cold PBS. Homogenates were centrifuged at 10,000 rpm for 10 min and the supernatants were collected and followed by protein concentration determination using the NJJC protein assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) with bovine serum albumin (BSA) as a control. For gel loading, the supernatants were heated for 4 min at 95 °C in a double volume of sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 1% bromophenol blue, and 25% glycerol). The samples (40 μg per lane) were separated by 12% SDS-PAGE and electro-transferred onto a polyvinylidene-difluoride (PVDF) membrane (Bio-Rad). The membrane was blocked with 5% skimmed milk for 2 h at room temperature, incubated overnight at 4 °C with primary antibodies directed against the IL-6 protein (R&D Systems, USA) in PBS+Tween 20 (PBST) at a dilution of 1:300, and β-actin (1:6000, Sigma) was used as a loading control. After the

Table 1 PCR primer sequences

Target gene	Sense primer (5′→3′)	Antisense primer (5′→3′)	Annealing temperature (°C)	Number of cycles
IL-6	CTGGTGACAACCACGGCC-TTCCCTA	ATGCTTAGGCATAACGCA-CTAGGTT	58	40
b	TGGAATCCTGTGGCATCC-ATGAAAC	TAAAACGCAGCTCAGTAA-CAGTCCG	58	30

membrane was washed for six times with 10 min each in PBST, it was incubated in the appropriate HRP-conjugated secondary antibody (diluted 1:400 in PBST) for 2 h. The blotted protein bands were stained with diaminobenzidine (DAB).

Immunohistochemical assessment

The IL-6 expression was also evaluated using the Elivision™ plus staining kit (Maixin_Bio, Fuzhou, China), according to the standard procedure described in the manufacturer's guides. In brief, slides were incubated with the primary anti-human IL-6 antibody (1:150, R&D Systems, USA) overnight at 4 °C. The secondary antibody was localized by incubating slides with the Elivision™ plus reagent (polymerized HRP-anti-Ms/Rb IgG) for 30 min at room temperature. Negative controls for immunohistochemical staining include slides that were not incubated with the primary or secondary antibody. Then DAB was used as the chromogen and the sections were counterstained with hematoxylin.

Statistical analysis

The $P<0.05$ level of probability was used as the criteria of significance. Comparison between different groups was made by one-way analysis of variance (ANOVA), followed by Duncan's new multiple range test.

Results

Protective effect of TCCE on CCl_4-induced liver injury in mice

Serum ALT and AST are hepatic enzymes that are released into the bloodstream when liver cells are damaged. In the present experiment, remarkable elevation in both sALT and sAST activities was observed as compared with the normal level in control group (Table 2). While 10 and 30 mg/kg TCCE significantly blocked the sALT and sAST elevation, especially the latter one reversed the sAST activity almost to normal level, and 200 mg/kg DDB also reduced the enzyme activities approximately to the same extent.

Inhibitory effect of TCCE on liver IL-6 mRNA expression in CCl_4-stimulated mice

The effect of TCCE on IL-6 mRNA expression was examined by RT-PCR. As shown in Fig. 1, the expression of IL-6 mRNA was not detected in the control group, but mice stimulated with CCl_4 (20 μl/kg) expressed high

Table 2 Effect of TCCE on the elevation of sALT and sAST activities in mice with liver injury induce by CCl_4

Group	Drug dose (mg/kg)	*n*	ALT (IU/L)	AST (IU/L)
Control	–	6	184 ± 38	363 ± 52
CCl_4	–	6	1454 ± 134*	1086 ± 180*
TCCE+CCl_4	10	6	781 ± 108*,#	672 ± 114*,#
TCCE+CCl_4	30	6	320 ± 95*,#	446 ± 55#
DDB+CCl_4	200	6	255 ± 40#	392 ± 53#

Data were represented in mean ± SD; *$P<0.01$ vs. control group; #$P<0.01$ vs. CCl_4 group.

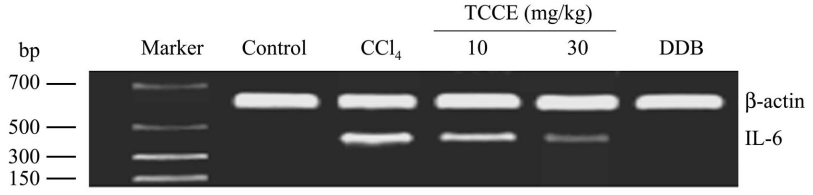

Fig. 1 Inhibitory effects of TCCE on IL-6 mRNA expression in CCl_4-stimulated mice

RT-PCR analysis of IL-6 at 24 h after CCl_4 administration in control mice, mice receiving CCl_4 alone and mice with pretreatment of TCCE (10 mg/kg and 30 mg/kg) or DDB (200 mg/kg). The particular procedure was performed as described in "Material and Methods" and PCR amplified products were analyzed by agarose gel electrophoresis. The house keeping gene, β-actin, was amplified as control. One of three representative experiments is shown.

levels of IL-6 mRNA. Furthermore, TCCE (10 mg/kg and 30 mg/kg) significantly suppressed the CCl_4-stimulated IL-6 mRNA expression, and so did DDB. In contrast, the level of β-actin mRNA remained the same under these conditions.

Inhibitory effect of TCCE on liver IL-6 protein level in CCl_4-stimulated mice

The inhibition of TCCE on IL-6 protein expression was further corroborated by Western blot results. Control animals showed only a weak signal for IL-6, and mice receiving CCl_4 alone showed a significant increase in IL-6 level. In contrast, in mice receiving TCCE or DDB, lower levels of IL-6 were found at 24 h after CCl_4 administration compared with CCl_4 alone (Fig. 2).

Effect of TCCE on the distribution of IL-6 by immunohistochemistry

We also studied the expression of IL-6 by immunohistochemical procedure. In normal liver, IL-6 showed no positive staining [Fig. 3(A)]. While 24 h after CCl_4 injection, there was an intensive immunostaining preferentially

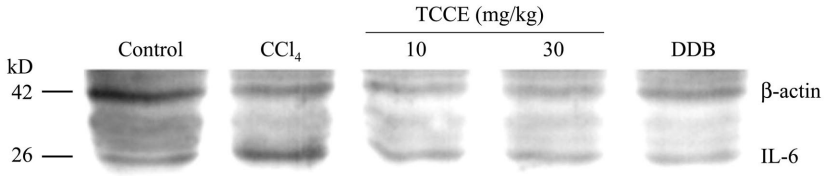

Fig. 2 Inhibitory effects of TCCE on IL-6 content in different groups of CCl_4-stimulated mice

Western blot analysis of IL-6 at 24 h after CCl_4 administration in control, CCl_4 alone, and TCCE (10 mg/kg and 30 mg/kg) or DDB (200 mg/kg) +CCl_4. The detailed experimental performances were described under "Material and Methods". One of three representative experiments is shown.

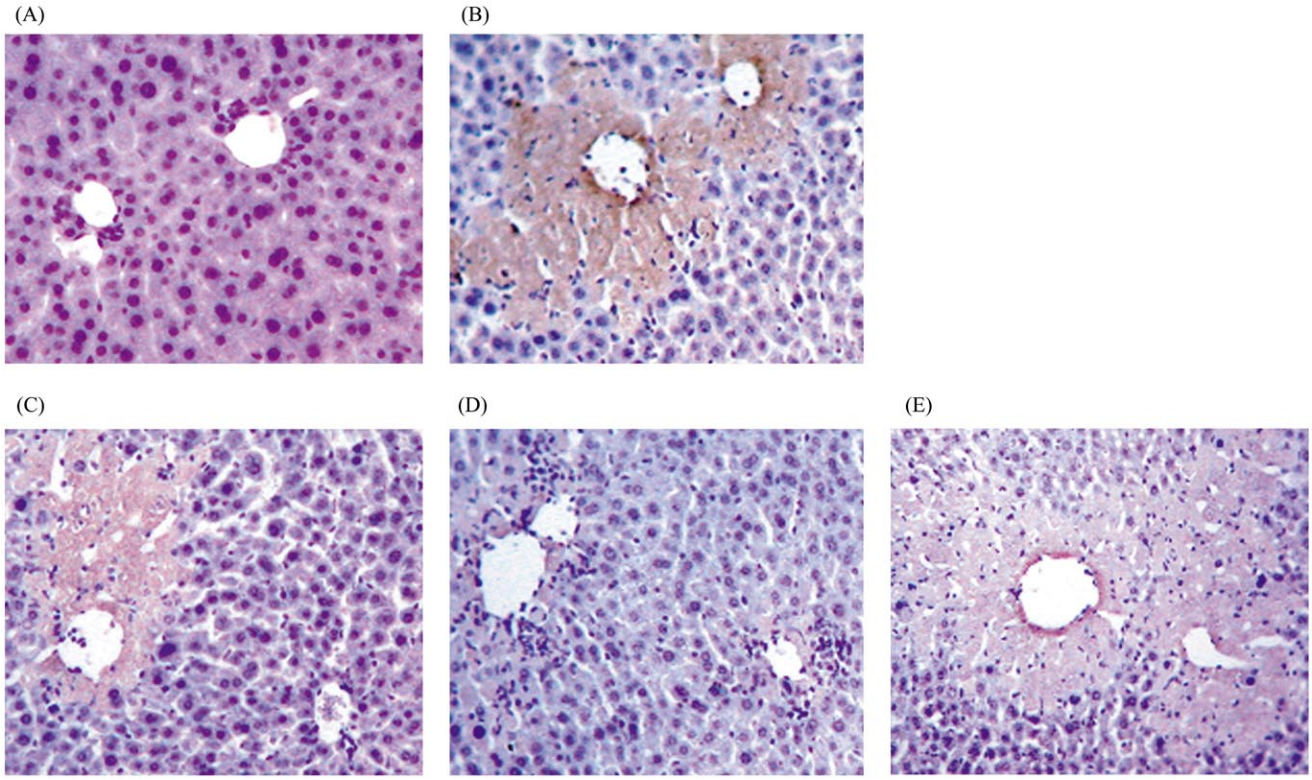

Fig. 3 Effect of TCCE on immunoreactivity in the CCl_4-injured liver

Immunohistochemical analysis results of IL-6 in different liver tissue at 24 h after CCl_4 administration. (A) Control. (B) CCl_4 alone. (C) 10 mg/kg TCCE and CCl_4. (D) 30 mg/kg TCCE and CCl_4. (E) 200 mg/kg DDB and CCl_4. Original magnification was ×100.

distributed around the terminal hepatic vein, showing hepatocytes plasma and membrane localization [Fig. 3(B)]. Following the administration of 10 mg/kg TCCE+CCl_4, a non-significant decrease in IL-6-positive cells can be observed [Fig. 3(C)] when compared with CCl_4 group, and the number of labeled hepatocytes markedly decreased when the mice were exposed to 30 mg/kg TCCE+CCl_4 [Fig. 3(D)]. However, a large number of positive cells were still detected in centrilobular region in the DDB+CCl_4 group [Fig. 3(E)].

Discussion

Liver injuries induced by CCl_4 are the best characterized system of xenobiotic-induced hepatotoxicity and commonly used models for the screening of anti-hepa-totoxic and/or hepatoprotective activities of drugs [10]. CCl_4 is metabolized into the trichloromethyl radical and other oxidant species, which can result in the disruption of structural and functional integrity in the liver. sALT and sAST are the most sensitive tests for diagnosis of liver diseases. The extend of hepatic damage is assessed by the increased serum level of cytoplasmic enzymes (AST and ALT) [11]. Present study revealed a significant increase in the activity of sALT and sAST within 24 h of the exposure of CCl_4, indicating a considerable hepatocellular injury. TCCE administrated orally at dose of 10 mg/kg and 30 mg/kg exhibited protection against CCl_4-indued damage in mice, suggesting its hepatoprotective action.

CCl_4-induced hepatic injury is initiated from the metabolism of CCl_4 to the trichloromethyl radical, which triggers lipid peroxidation and covalently binds to essential macromolecules. Secondary liver injury occurs from inflammatory processes [7] and it is well established that cytokines play an important role in the pathophysiology of liver diseases, which was further evidenced by the intensive immunostaining of IL-6 preferentially distributed around the terminal hepatic vein, which was the mainly damaged region in liver. It is interesting to find that the pretreatment of TCCE can dose-dependently inhibit both the acute liver injury and the overexpression of IL-6 in the specific area, which suggests that liver IL-6 level might be another criterion to screen new antihepatotoxic drugs.

On the other hand, IL-6 has diverse biological functions on different types of target cells. For example, it mediates the acute hepatic injury [7,8], acts as a mediator of lymphocyte function and cell differentiation [13], etc.. It has also been found that IL-6 exerts an important role in the process of liver regeneration induced by partial hepatoectomy in rats [14,15]. Cressman *et al.* [16] found that both impaired liver regeneration and liver necrosis occurred in IL-6 deficient mice, but the blunted DNA synthetic response in hepatocytes and non-parenchymal cells were different. So the inhibitory effect of TCCE on IL-6 gene expression awaits further studies in different experimental models in detail.

In summary, the data in this research indicate that TCCE has hepatoprotective activity and the mechanisms underlying its protective effects may be related to the inhibition on the overexpression of IL-6.

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