Taohe Chengqi Tang ameliorates acute liver injury induced by carbon tetrachloride in rats

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Objective: To clarify the efficacy of Taohe Chengqi Tang (THCOT), a compound traditional Chinese herbal medicine, in protecting liver damage induced by carbon tetrachloride (CCL4) in rats.

Methods: Thirty male Wistar rats were divided into normal control group, untreated group, low-dose THCOT group (receiving 0.3 g/kg of THCOT), high-dose THCOT group (receiving 0.5 g/kg of THCOT), and positive control group (receiving silymarin 25 mg/kg). All testing substances were orally administered 1 hour before the intraperitoneal injection of CCl4 (1.5 mL/kg). Twenty-four hours after CCl4 injection, the rats were sacrificed to observe liver histopathological changes, and to evaluate activities of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT), and lipid peroxidation (LPO) and glutathione (GSH) levels in liver tissues.

Results: CCl4 injection elevated the serum AST and ALT activities, but THCOT significantly reversed this effect. The increase of hepatic LPO by CCl4 was markedly reduced by THCOT. Also, this herbal mixture increased hepatic GSH in the rats. In histopathology analysis, THCOT decreased the fatty accumulation, necrosis and lymphocyte infiltration. The in vitro study in rat brain showed that LPO induced by Fe3+/ascorbic acid was dose-dependently reduced by THCOT. According to the biochemical and morphological data, THCOT could protect the liver from CCl4-induced injuries.

Conclusion: THCOT seems helpful for protection of liver damage induced by chemicals depending on its anti-oxidant-like function, and THCOT is more effective than silymarin.

Keywords: traditional Chinese medicine; carbon tetrachloride; anti-oxidants; Taohe Chengqi Tang; rats

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桃核承气汤改善四氯化碳诱导的大鼠肝损伤

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目的：探讨桃核承气汤对四氯化碳诱导的大鼠肝损伤的保护作用。

方法：30 只大鼠分为正常对照组，模型组，大、小剂量桃核承气汤组和水飞蓟素组。桃核承气汤组灌胃桃核承气汤（0.3 g/kg 或 0.5 g/kg），水飞蓟素组灌胃水飞蓟素 25 mg/kg。灌胃后 1 h 腹腔注射四氯化碳 1.5 mL/kg。注射 24 h 后将大鼠处死，检测肝组织病理学改变，血液中天门冬氨酸氨基转移酶（aspartate amino transferase, AST）丙氨酸氨基转移酶（alanine aminotransferase, ALT）活性及肝脂质氧化程度。

结果：桃核承气汤不仅明显降低血液中 AST 及 ALT 活性，抑制四氯化碳引起的肝脏脂质过氧化，并且增加肝脏中谷胱甘肽含量；组织病理分析显示桃核承气汤具有抑制脂质堆积，肝细胞坏死及淋巴细胞浸润。

结论：桃核承气汤具有抗氧化作用，对肝损伤有保护作用，甚至功能优于水飞蓟素。

关键词：中药；四氯化碳；抗氧化剂；桃核承气汤；大鼠

Liver is the main organ for metabolism of exogenous chemical substance in human body. Free radical oxidative stress and lipid peroxidation (LPO) have been implicated in hepatic toxicity[1], but the agents effective to control liver disorders are still not applied in clinic. In traditional Chinese medicine, herbal mixture as prescription is employed to handle the hepatic disorders. Taohe Chengqi Tang (THCQT) is one of the herbal mixtures documented in Shang Han Lun and this prescription is composed of Semen Persicae (Taoren), Radix et Rhizoma Rhei Palmatii (Dahuang), Ramulus Cinnamomi (Guizhi), Natrii sulfas (Mangxiao) and Radix Glycerhrizae (Gancao). In clinic, THCQT has been documented to treat chronic hepatitis, amnorrhoea, diabetes mellitus, acute necrotic enteritis, and chronic cyclophosphamiditis[1]. However, the action mechanisms for this effectiveness of THCQT remained obscure.

Carbon tetrachloride (CCl₄) is activated by the cytochrome P450 to produce trichloromethyl radical (CCl₃), and with the combination of a molecule of oxygen to produce trichloromethylperoxyl radical (CCl₃O₂)[2,3]. These free radicals are believed to initiate the LPO of unsaturated fatty acid of membrane cells[3]. In addition, it has also been reported that it can decrease the activities of antioxidant enzymes, such as superoxide dismutase, catalase, glutathione reductase, microsomal cytochrome P450[4-6] and amounts of non-enzymatic antioxidants, such as glutathione (GSH), ascorbic acid[7,8]. Liver tissue necrosis is immediately induced by CCl₄, and it is also shown that the activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in serum obviously increased[8,9]. It is studied that some drugs have protection effect on liver injuries induced by carbon tetrachloride, such as cystamine[10], malotilate[11], cytochrome P450 inhibitor methoxsalen[12] and calcium blocker diliazem[13].

In an attempt to clarify the effects of THCQT on liver injury, we employed CCl₄ to induce liver injury in rats in this study. Also, we investigated the possible mechanism for this action of THCQT, especially the antioxidant-like action.

1. Materials and methods

1. 1 Chemicals Semen Persicae, Radix et
Rhizoma Rhei Palmatii, Ramulus Cinnamomi, Natrii sulfas and Radix Glycyrrhizae were purchased from Chuang Song Zong Pharmaceutical Co. Ltd., Kaohsiung, Taiwan. In the present study, we mixed the above herbs to boil for 30 minutes in 100 °C water bath and the extraction was carried out for twice. Then, the extracts were mixed and lyophilized to dry powder. CCl₄, sodium dodecyl sulfate, 2-thiobarbituric acid, malonaldehyde bis (dimethyl acetal), ethylenediaminetetraacetic acid disodium (EDTA), tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl), 5,5-dithio-bis(2-nitrobenzoic acid), reduced-form glutathione, ascorbic acid, and 2-thiobarbituric acid were purchased from Sigma Company. Also, olive oil and silymarin (Aldrich), n-butanol (Fluka), trichloroacetic acid and pyridine (Wako) were obtained from respective company.

1.2 Animals Male Wistar rats weighing (180±220) g were obtained from the National Laboratory Animal Breeding and Research Center, National Science Council, and fed with standard laboratory chow and reverse-osmosis water ad libitum. All animals were housed in an air-conditioned room with temperature of (22±3) °C, humidity of (55±5) % and half day of light. The use of animals was approved by the Institutional Animal Care and Use Committee.

1.3 Iron-dependent LPO Wistar rats were sacrificed under ether anesthesia and the brains were isolated after perfusion with cold saline. Brains were homogenized in Tris-HCl of pH 7.4 (1 : 10, W/V). A mixture containing 0.3 mL brain homogenate, 0.05 mL of 0.062 5 mmol/L ascorbic acid, 0.05 mL of 2.5 mmol/L FeCl₃, and 0.05 mL of THCQT at desired concentration (0.1, 0.3, 0.5, 1, 2 mg/mL) was incubated for 30 minutes at 37 °C. After incubation, LPO is characterized by the formation of malondialdehyde (MDA) from 2-thiobarbituric acid using spectrophotometric method (Beckman DU 650 spectrophotometer) as described by Okhawa et al[14]. The level of LPO was expressed as nmol of MDA per milligram of protein assayed by the method of Lowry et al[14].

1.4 CCl₄-induced acute hepatotoxicity Rats were divided into 5 groups to receive oral administration of testing substance. Low-dose THCQT group received 0.3 g/kg of THCQT, and high-dose THCQT group received THCQT at a dose of 0.5 g/kg, while the positive control group received silymarin (25 mg/kg in 1% sodium carboxymethyl cellulose, suspension solution). All testing substances were orally administered 1 hour before the intraperitoneal injection of CCl₄ (1.5 mL/kg in olive oil, 20%). Olive oil at same volume was injected intraperitoneally into the control group. Twenty-four hours after CCl₄ injection, the rats were sacrificed under ether anes-

1.5 Assessment of liver function Blood sample was centrifuged at 3 000 r/min (Kubota 8800 centrifuge, Japan) at 4 °C for 10 minutes to obtain the sera. Activities of AST and ALT in serum were tested spectrophotometrically by using Roche clinical test kits (Art. 0736414 and Art. 0736384).

1.6 Assay of LPO Following the method of Ohkawa et al[14], the frozen liver tissues (0.5 g) were homogenized in 0.1 mL of 1.15% KCl. Then, the homogenate was added 0.2 mL sodium dodecyl sulfate (8.1%), 1.5 mL acetic acid (20%) at pH 3.5 with NaOH. After addition of 1.5 mL 2-thiobarbituric acid (0.8%) to prepare 4 mL solution with distilled water, samples were incubated for 1 hour at 95 °C in water. Reaction was terminated by addition of 1 mL cool distilled water. Then, 5 mL n-butanol/pyridine solution (15 : 1, V/V) was used to extract the product MDA under shaking until well mixed and centrifuged at 4 000 r/min for 10 minutes. LPO was quantified by using the spectrophotometric method (Beckman DU650 spectrophotometer) as described by Okhawa et al[14]. The level of LPO in liver tissue was expressed as nmol of MDA per mg of protein that was measured by the method of Lowry et al[14] using bovine serum albumin as the standard.

1.7 GSH determinations The hepatic glutathione content was measured by the method of Ellman[16] as described by Sedlak et al[17]. The liver tissue homogenate (0.4 g) was prepared in 16 mL EDTA (0.02 mol/L) solution. Then, 5 mL of liver homogenate was added with 4 mL distilled water and 1 mL trichloroacetic acid (50%) to mix well and centrifuged at 3 000 × g for 15 minutes. The supernatant (2 mL) was added with 4 mL of 0.4 mol/L Tris-buffer solution (pH 8.9) and 0.1 mL of Ellman reagent (5,5-dithiobis(2-nitrobenzoid acid), 0.396% in methanol). Then, GSH concentration was measured at 412 nm by using calibration standards of authentic GSH. The concentration of GSH was expressed as μmol per gram of wet liver tissue.

1.8 Histopathological observation After draining blood, 0.5 cm³ of liver tissues were isolated from each lobe of the liver. The tissue was fixed in 10% neutral formalin for one to two weeks, dehydrated with graduating concentrations of ethanol solutions from 50% to 100% and embedded in paraffin, then cut into 4 to 5 μm thick sections, stained with hematoxylin-eosin and observed under a photomicroscope.

1.9 Statistical analysis Data were expressed as X ± s. Statistical software of SPSS 12.0 was used.
to analyze the data. One-way ANOVA was applied to statistical analysis of difference among groups and the Dunnett range post-hoc comparisons were used to compare difference between groups. $P < 0.05$ was considered statistically significant.

2 Results

2.1 LPO inhibition of THCQT in vitro Addition of Fe$^{3+}$ into rat brain homogenate to react at 37°C for 30 minutes, intense LPO was evidenced by the formation of MDA about (15.1 ± 0.4) nmol/mg brain protein and only (2.5 ± 0.3) nmol/mg brain protein in the THCQT (at dose 1 mg/mL) group. The maximal inhibitory effect on LPO of THCQT in rat brain homogenate was 90.3% with an IC$_{50}$-value of 0.5 mg/mL (Figure 1).

2.2 THCQT inhibited AST and ALT activities induced by CCl$_4$. In rats received peritoneal injection of CCl$_4$ (20%, 1.5 mL/kg) for 24 hours, the activities of serum AST and ALT were significantly higher than those in the normal control group. Like the effect of silymarin (25 mg/kg), oral administration of THCQT (0.5 g/kg) significantly reduced the activities of AST and ALT in rats receiving CCl$_4$ injection ($P < 0.05$) (Figure 2A and 2B).

Figure 1 Inhibitory effect of THCQT on FeCl$_3$-ascorbic acid-induced LPO in the homogenates of brain isolated from rats Values ($\bar{X} \pm S$) were obtained from each group of 5 animals.

![Figure 1](image1)

2.3 THCQT inhibited LPO induced by CCl$_4$ and elevated GSH level The hepatic LPO level was also increased 24 hours after CCl$_4$ injection. The increase of hepatic LPO was reduced by oral administration of THCQT (0.5 g/kg) or silymarin (25 mg/kg) (Figure 2C). The hepatic GSH level was increased 24 hours after CCl$_4$ injection to about 210% of the normal level. Oral administration of THCQT (0.5 g/kg) increased the GSH level markedly as showed in Figure 2D ($P < 0.05$).

![Figure 2](image2)

**Figure 2** Effects of THCQT and silymarin on activities of serum AST and ALT and levels of LPO and GSH in isolated liver tissues of CCl$_4$-injected rats

Activities of serum AST (A) and ALT (B) and levels of LPO (C) and GSH (D) in liver tissues were measured 24 hours after CCl$_4$ injection. THCQT or silymarin (25 mg/kg) was administered 1 hour before the injection. 1, Normal control group; 2, Untreated group; 3, 0.3 g/kg THCQT group; 4, 0.5 g/kg THCQT group; 5, Silymarin group. Values ($\bar{X} \pm S$) were obtained from each group of 6 animals. ** $P < 0.01$, vs normal control group; *** $P < 0.05$, vs untreated group.
2.4 Microscopic evaluation of THCQT protection effect on CCl4-injected rat liver  Histological examination showed the massive fatty formation, centrilobular necrosis, ballooning degeneration, infiltrating lymphocytes and loss of cellular boundary in the livers of CCl4 intoxicated rats (Figure 3B). The histological pattern of the livers in rats receiving THCQT (0.5 g/kg) or silymarin (25 mg/kg) became a normal lobular pattern with a mild degree of fatty accumulation, necrosis and lymphocyte infiltration (Figure 3C and 3D).

![Figure 3](image)

**Figure 3** Microscopic evaluation of the isolated livers of normal control rat or CCl4-injected rats (HE staining, ×200)
Microscopic evaluation of the effects of THCQT on CCl4-induced acute hepatotoxicity. (A) Normal control group; (B) Untreated group (CCl4 alone); Diffuse areas of necrosis, sinusoidal congestion, broad infiltration of lymphocytes and Kupffer cells around the central vein, fatty change, loss of cell boundaries and ballooning degeneration of liver damage are observed; (C) THCQT (0.5 g/kg) group; (D) Silymarin (25 g/kg) group.

3 Discussion

In the present study, we found that THCQT has the ability to protect liver damage induced by CCl4. In addition to the reduction of serum AST and ALT activities, histological observations and others provided the evidence to support the effectiveness of THCQT.

CCl4 is widely employed in the experimental cell or tissue injury model of free radical to induce acute liver injury[18]. Extracts from some herbs like *Coptidis rhizoma*[18], *Solanum nigrum* Linn[20], *Commiphora myrrha* (Arn) Engl bark[21], and *Phyllanthus amarus*[22] have also been investigated efficiently inhibiting CCl4-induced hepatotoxicity.

Superoxide anion (O$_2^-$) is one of the reactive oxygen species. In vivo, it is produced in phagocytosis, tissue ischemia, enzyme reaction, and the electron transportation of mitochondria or microsome[23]. Superoxide anion can be converted into H$_2$O$_2$ by the enzyme of superoxide dismutase. Superoxide anion and H$_2$O$_2$ can further convert into the more harmful hydroxyl radical (OH$^-$) after the reaction with ferrum ion[24, 25].

Free radical is harmful to protein, nucleic acid and unsaturated fatty acid of membrane in cell. Lipid peroxidation by hydroxyl radical with unsaturated fatty acid can be detected to indicate as the index of free radical injury[26]. LPO is easily induced by free radicals[8, 27] via the reaction of hydroxyl radical with unsaturated fatty acid. In this study, we employed the brain tissue to characterize the LPO inhibitory effect of THCQT.

In general, LPO is widely measured by using 2-thiobarbituric acid to form MDA[28]. Increase of MDA to indicate LPO level is an index used to identify the free radicals-induced injuries[21, 26]. Brain tissue has the enzymes such as monoamine oxidase to produce reactive oxygen species in spontaneous LPO reaction[17]. On the contrary, liver tissue has more antioxidant-like enzymes[22] to reduce the spontaneous LPO reaction. The antioxidant-like capacity in brain tissue was markedly lower than that in liver tissue. Thus, we employed brain tissue to assay and found THCQT produced a significant inhibitory effect on the spontaneous LPO reaction in brain tissue of rats. The antioxidant-like action of THCQT can be identified.

Iron-dependent LPO plays an important role in the reactive oxygen species-mediated tissue injuries[26, 29]. THCQT has an inhibitory effect on the iron-dependent LPO in brain homogenate that
seems similar to the protection effect on CCl₄-induced hepatotoxicity. Liver injury induced by CCl₄ is mainly due to the damage of free radicals and CCl₄-induced acute hepatotoxicity is widely used as an animal model for liver injury[4, 7, 10]. The proposed mechanism of the hepatotoxicity induced by CCl₄ is introduced as the metabolism activated by cytochrome P450 to produce CCl₃ and initiate the LPO of unsaturated fatty acid of membrane cell rapidly. Destruction of membrane cell resulted in cell necrosis following lesion development[4, 7, 30, 31]. After LPO of membrane, cell can cause the discharge of intracellular enzymes and electrolytes. Calcium ions will get into cells and accumulate to cause liver injury[32, 33].

Then, enzymes from hepatocytes released into the blood circulation during liver injury result in acute elevation of serum AST and ALT which have been used for evaluation of liver damage[45]. The scope for CCl₄-induced liver injury mainly diffused outward from the central vein. Histological changes included centrilobular necrosis, infiltrating lymphocytes and massive fatty changes. The present study observed that pre-treatment with THQCQ inhibited CCl₄-induced hepatotoxicity depending on the lowering of serum AST and ALT in addition to histological examination. This result is also observed in samples treated with silymarin as positive control.

The effect of CCl₄ on hepatic GSH or LPO level has also been studied. The involvement of endogenous GSH in the development of CCl₄-induced active liver injury and the response of GSH to hepatic lipid peroxidation during the development of injury in rats are still controversial. It has been documented that rats treated once with CCl₄ resulted a decrease of hepatic GSH concentration and an increase of LPO concentration during the progression of damage[45]. However, an increase of GSH concentration with a decrease of LPO concentration was observed in another similar study[34]. Di Simplicio et al[35] also observed the increase of hepatic GSH concentration during the progressed stage of liver injury in rats treated once with CCl₄. In mice treated once with CCl₄, hepatic GSH concentration was increased in spite of an increase in the hepatic LPO concentration during the progressed stage of liver injury[36]. Our results are consistent with that an increase of hepatic GSH concentration with an increase of LPO concentration during the progression of damage is observed in the liver of rats treated once with CCl₄. Administration of THQCQ significantly inhibited the increase of LPO level induced by CCl₄.

The present study showed that THQCQ has the ability to ameliorate acute hepatotoxicity induced by CCl₄. Scavenging of free radicals by this herbal mixture is contributed as one of the action mechanisms.

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REFERENCES

12. Labbe G, Descatoire V, Letteron P, Degott C, Tinel M, Larrey D, Carrion-Pavlov Y, Geneve J, Amouyal G, Pessayre D. The drug methoxsalen, a suicide substrate for cytochrome P450, decreases the metabolic activation, and prevents the hepatotoxicity, of carbon tetra-


