Tao-He-Cheng-Qi-Tang Ameliorated the Acute Liver Injury Induced by Carbon Tetrachloride (CCl_4) in Rats

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Running title: Effect of Tao-He-Cheng-Qi-Tang on Anti-Oxidation and Acute Liver Injury

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Abstract

In Chinese traditional medicine, Tao-He-Cheng-Qi-Tang (THCQT) has widely been used to treat the patients with hepatitis or chronic pyelonephritis. However, this action mechanism of THCQT is still unknown. The present study is thus designed to clarify the efficacy of THCQT to protect acute hepatitis induced by carbon tetrachloride (CCl_4) in rats. Actually, we observed that THCQT can protect the liver from CCl_4-induced injuries due to the morphological and biochemical data. Moreover, the increase of hepatic lipid peroxidation (LPO) by CCl_4 was markedly reduced by THCQT. Also, this herbal mixture increased hepatic glutathione (GSH) in the rat. The in vitro study in rat brain, LPO induced by Fe(3+)/ascorbic acid was dose-dependently reduced by THCQT. In conclusion, we suggest that THCQT seems helpful for protection of liver damage, induced by chemical depending on the antioxidant-like activity, and THCQT even showed more significantly effect than the silymarin.

Key words：Chinese Traditional Medicine, Carbon Tetrachloride, Hepatoprotective, Antioxidant

Introduction

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 the hepatotoxicity,[1] but the agents effective to control liver disorders are still not applied in clinic. In Chinese traditional medicine, herbal mixture as prescription is employed to handle the hepatic disorders. Tao-He-Cheng-Qi-Tang (THCQT) is one of the herbal mixtures documented in Shang-Han-Lun and this prescription is composed of *Prunus persica (L.) Batsch, Rheum palmatum L., Cinnamomum cassia Presl, Natrii sulfas., Glycyrrhiza uralensis Fisch*. In clinic, THCQT has been documented to treat chronic hepatitis, amenorrhea, diabetes mellitus, acute necrotic enteritis, and chronic pyelonephritis.[2] However, the action mechanisms for this effectiveness of THCQT remained obscure.

Carbon tetrachloride is activated by the cytochrome P450 to produce trichloromethyl radical (CCl_3), and with the combination of a molecule of oxygen to produce trichloromethylperoxyl radical (CCl_3O_2).[3, 4] These free radical are believed to initiate the lipid peroxidation of unsaturated fatty acid of membrane cell.[3] In addition, it has also been reported that it can decrease the activities of antioxidant enzymes, such as superoxide dismutase(SOD), catalase, glutathione reductase(GSSG-R), microsomal cytochrome P450[5, 6] and amounts of non-enzymatic antioxidants, such as

glutathione(GSH), ascorbic acid.[6] Liver tissue necrosis is immediately induced by CCl_4,[7] it is also shown that the level of Glutamate oxaloacetate transaminase(GOT), Glutamate pyruvate transaminase(GPT) in serum has obviously increased.[7, 8] It is studied that some drugs have protection effect on liver injuries induced by carbon tetrachloride, such as cystamine,[9] malotilate,[10] cytochrome P450 inhibitor methoxsalen[11] and calcium blocker diltiazem.[12]

In an attempt to clarify the effect of THCQT on liver injury, we employed carbon tetrachloride (CCl_4) to induce liver injury as an animal model in this study. Also, we investigated the possible mechanism for this action of THCQT, especially the antioxidant-like action.

Materials and Methods

Chemicals

Composition of THCQT includes *Prunus persica (L.) Batsch, Rheum palmatum L., Cinnamomum cassia Presl., Natrii sulfas.,Glycyrrhiza uralensis Fisch* 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 ^{o}C water bath and the extraction was carried out for twice. Then, the extract was mixed and lyophilized to dry powder. Carbon tetrachloride, sodium dodecyl sulfate, 2-thiobarbituric acid, malonaldehyde bis[dimethyl acetal] (MDA), 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 each company.

Animals

Male Wistar rats weighing 180~220g were obtained from the National Laboratory Animal Breeding and Research Center, National Science Council, to feed with standard laboratory chow and RO water ad libitum. All animals were housed in an air-conditioned room with 22 ± 3 ^{o}C, humidity 55 ± 5% and half day of light. The

use of animals was approved by the Institutional Animal Care and Use Committee.

Iron-dependent lipid peroxidation

Wistar rats were sacrificed under ether anesthesia and the brains were isolated after perfusion with cold saline. Brains were homogenized in Tris-HCl PH7.4 (1:10, w/v). A mixture containing 0.3ml brain homogenate, 0.0625mmol/l ascorbic acid 0.05ml, 2.5mmol/l $FeCl_2$ 0.05ml and 0.05ml of THCQT at desired concentration was incubated for 30 minutes at 37^{o}C. After incubation, lipid peroxidation (LPO) is characterized by the formation of malondialdehyde (MDA) from 2-thiobarbituric acid using spectrophotometric method (Beckman DU 650 spectrophotometer) as described[13] The level of lipid peroxidation was expressed as nmol of MDA per milligram of protein assayed by the method of Lowry *et al.*[14]

Carbon tetrachloride-induced acute hepatotoxicity

Rats were divided into 6 groups to receive oral administration of testing substance. THCQT group Ⅰ received 0.3g/kg of THCQT and THCQT group Ⅱ with THCQT 0.5g/kg, to compare the positive control group receiving silymarin (25mg/kg in 1% Sodium carboxymethyl cellulose, suspension solution). All testing substances were orally administered 1 hour before the intraperitoneal injection of CCl_4 (1.5ml/kg in olive oil, 20%). Olive oil at same volume was injected intraperitoneally into the control group. After 24 hours of CCl_4 injection, the rats were sacrificed under ether

anesthesia, blood was withdrawn from the carotid artery and serum was obtained to assay the activities of glutamate-oxalate-transaminase (GOT) and glutamate-pyruvate-transaminase (GPT). The livers were isolated for biopsy and kept at -80 ^{o}C until the determination of LPO or glutathione (GSH).

Assessment of liver function

Blood sample was centrifuged at 3000 rpm (Kubota 8800 centrifuge, Japan) at 4^{o}C for 10 minutes to obtain the sera. Assay of GOT and GPT in serum was performed spectrophotometrically using Roche clinical test kits (Art.0736414 and Art.0736384).

Assay of lipid peroxidation (LPO)

Following the method of Ohkawa *et al.*,[13] the frozen liver tissues (0.5g) were homogenized in 0.1ml of 1.15% KCl. Then, the homogenate was added 0.2ml sodium dodecyl sulfate (8.1%), 1.5ml acetic acid (20%) at pH 3.5 with NaOH. Addition of 1.5ml 2-thiobarbituric acid (0.8%) to prepare 4ml solution with distilled water, samples were incubated for 1 hour at 95^{o}C in water. Reaction was terminated by addition of 1ml cool distilled water. Then, 5ml n-butanol/pyrimidine solution (15:1, v/v) was used to extract the product MDA under shaking until well mixed and centrifuged at 4000 rpm for 10 minutes. LPO was quantified using the spectrophotometric method (Beckman DU650 spectrophotometer) as described by

Ohkawa *et al.*[13] The level of liver tissue LPO was expressed as the 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.

Glutathione (GSH) determinations

The hepatic glutathione content was measured by the method of Ellman[15] as described by Sedlak and Lindsay.[16] The liver tissue homogenate (0.4g) was prepared in 16ml EDTA (0.02mol/l) solution. Then, 5ml of liver homogenate was added with 4ml distilled water and 1ml trichloroacetic acid (50%) to mix well and centrifuged at 3000 g for 15 minutes. The supernatant (2ml) was added with 4ml of 0.4mol/l tris-buffer solution (pH8.9) and 0.1ml of Ellman reagent (5,5'-dithiobis (2-nitro benzoid acid, 0.396 % in methanol)). Then, GSH concentration was measured at 412nm using calibration standards of authentic GSH. The concentration of GSH was expressed as μmol per gram of wet liver tissue.

Histopathological observation

After draining blood, 0.5cm^3 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 concentration of ethanol solutions from 50~100% and embedded in paraffin, then cut into 4~5μm thick sections, stained with hematoxylin-eosin and observed under a photomicroscope.

Statistical analysis

Data are expressed as the mean ± SD for the number (n) of animals in the group as indicated. Repeated measures analysis of variance (ANOVA) was used to analyze the changes in plasma glucose and other parameters. The Dunnett range post-hoc comparisons were used to determine the source of significant differences where appropriate. $P < 0.05$ was considered statistically significant.

Results

In rats received peritoneal injection of CCl_4 (20%; 1.5ml/kg) for 24 hours, the values of serum GOT and GPT were significantly higher than those in the vehicle-treated control group. Like the effect of silymarin (25mg/kg), oral administration of THCQT (0.5g/kg) significantly reduced the values of GOT and GPT in rats received CCl_4 injection (Fig. 1A and Fig. 1B).

The hepatic LPO levels were also increased 24 hours after CCl_4 injection. The increase of hepatic LPO was reduced by oral administration of THCQT (0.5g/kg) or silymarin (25mg/kg) (Fig. 2A). The hepatic glutathione level was increased 24 hours after CCl_4 injection to about 210% of the normal level. Oral administration of THCQT (0.5g/kg) increased the glutathione level markedly as showed in Fig. 2B.

Histological examination showed the massive fatty formation, centrilobular necrosis, ballooning degeneration, infiltrating lymphocytes and loss of cellular boundary in the livers of CCl_4 intoxicated rats (Fig. 3B). The histological pattern of the livers in rats received THCQT (0.5g/kg) or silymarin (25mg/kg) became a normal lobular pattern with a mild degree of fatty accumulation, necrosis and lymphocyte infiltration (Fig. 3C and Fig. 3D).

Addition of Fe^{+2} into rat brain homogenate to react at 37^oC for 30 minutes, intense lipid peroxidation (LPO) was evidenced by the formation of MDA

(malonaldehyde bis [dimethyl acetal]) about 15.1 ± 0.4nmol/mg brain protein and only 2.5 ± 0.3nmol/mg brain protein. The maximal inhibitory effect on LPO of THCQT in rat brain homogenate was 90.3% with an IC_{50}-value of 0.5mg/ml (Fig.4).

Discussion

In the present study, we found that THCQT has the ability to protect liver damage induced by CCl_4. In addition to the reduction of serum GOT and GPT, histological observations and others provided the evidence to support the effectiveness of THCQT.

The experimental cell/tissue injury model of free radical is widely employed CCl_4 to induce the acute liver injury.[17] Basically, CCl_4 is metabolized by the cytochrome P450 to become trichloromethyl radical (CCl_3) that can combine a molecule of oxygen to form the trichloromethylperoxyl radical (CCl_3O_2). The formed free radical is believed to initiate the lipid peroxidation of unsaturated fatty acid in cell membrane.[3, 4] Also, it can decrease the activities of antioxidant enzymes including SOD, catalase, glutathione reductase (GSSG-R), and microsomal cytochrome P450[5, 6] as well as the amounts of non-enzymatic antioxidants, such as glutathione (zGSH), ascorbic acid.[6, 18] Liver tissue necrosis is easily induced by CCl_4[7] to result in an increase of glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) in serum. Drugs showing the protection of liver injuries induced by CCl_4 are documented as cysteamine,[9] malotilate,[10] methoxsalen[11] and diltiazem.[12]

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.[19] Superoxide anion can be inverted into H_2O_2 by the enzyme of superoxide dismutase (SOD). Superoxide anion and H_2O_2 can further convert into the more harmful hydroxyl radical (OH^-) after the reaction with ferrum ion. [20, 21]

Free radical is harmful to protein, nucleid 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.[22] Determination of reactive oxygen species is recently employed the electron spin resonance and chemiluminescence[23] although these methods are so expensive. Assay of hydroxyl radical was also estimated by the deoxyribose method.[24] Lipid peroxidation (LPO) is easily induced by free radicals[9, 21] 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, lipid peroxidation is widely measured using 2-thiobarbituric acid to form MDA.[13] Increase of MDA to indicate LPO level is an index used to identify the free radicals induced injuries.[21, 22] 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[25] to reduce the spontaneous LPO reaction. The antioxidant-like capacity in brain tissue was markedly lower than

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, 27] THCQT has an inhibitory effect on the iron-dependent LPO in brain homogenate that seems similar to the protection effect on CCl_4 induced hepatotoxicity. Liver injury induced by CCl_4 is mainly due to the damage of free radicals and CCl_4-induced acute hepatotoxicity is widely used as an animal model for liver injury.[1, 4, 28] The proposed mechanism of the hepatotoxicity induced by CCl_4 is introduced as the metabolism activated by cytochrome P450 to produce trichloromethyl radical (CCl_3) and initiate the lipid peroxidation of unsaturated fatty acid of membrane cell rapidly. Destruction of membrane cell resulted in cell necrosis following lesion development.[4, 7, 28, 29] After LPO of membrane, cell can cause the discharge of intracellular enzymes and electrolytes. Calcium ions will get into cells and accumulated to cause liver injury.[30, 31] Then, enzymes from hepatocytes released into the blood circulation during liver injury to result in the acute elevation of serum GOT and GPT that has been used for evaluation of liver damage.[8] The scope for CCl_4 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 THCQT inhibited CCl_4-induced hepatotoxicity depending on the lowering of serum GOT and GPT in addition to histological examination. This result is also observed in samples treated with silymarin as positive control.

The effect of CCl_4 on hepatic GSH or LPO level has also been studied. The involvement of endogenous GSH in the development of CCl_4-induced active liver injury and the response of GSH to hepatic lipid peroxidation during the development of injury in rats is still controversial. It has been documented that rats treated once with CCl_4 resulted a decrease of hepatic GSH concentration and an increase of LPO concentration during the progression of damage.[6] However, an increase of GSH concentration with a decrease of LPO concentration was observed in another similar study.[32] Di Simplicio and Mannervik[33] also observed the increase of hapatic GSH concentration during the progressed stage of liver injury in rats treated once with CCl_4. In mice treated once with CCl_4, hepatic GSH concentration was increased in spite of an increase in the hepatic LPO concentration during the progressed stage of liver injury.[34] 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 CCl_4. Administration of THCQT significantly inhibited the increase of LPO level induced by CCl_4.

In conclusion, the present study showed that THCQT has the ability to ameliorate acute hepatotoxicity induced by CCl_4. Scavenging of free radicals by this herbal mixture is contributed as one of the action mechanisms.

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Figure Legends

Fig. 1. Effects of THCQT and silymarin on the activities of GOT (A) and GPT (B) in serum of CCl_4-injected rats. Activities of serum GOT and serum GPT were measured 24 hours after CCl_4 injection. THCQT or silymarin (25mg/kg) was administered 1 hour before the injection. Values (mean ± SD) were obtained from each group of 6 animals. * $P < 0.05$ compared with CCl_4 group. # # $P < 0.01$ compared with the normal control group. CON: control; Sily: silymarin; GOT: glutamate oxaloacetate transaminase; GPT: Glutamic Pyruvic Transaminase.

Fig. 2. Effects of THCQT and silymarin on the levels of LPO (A) and GSH (B) in the isolated livers of CCl_4-injected rats. The hepatic levels of LPO and GSH were measured 24 hours after CCl_4 injection. THCQT or silymarin (25mg/kg) was administered 1 hour before the injection. Values (mean ± SD) were obtained from each group of 6 animals. * $P < 0.05$ compared with CCl_4 group. # $P < 0.05$ compared with the normal control group. CON: control; Sily: silymarin; LPO: lipid peroxidation; GSH: glutathione.

Fig. 3. Microscopic evaluation of the isolated livers of normal control rat (A) or CCl_4-injected rats (B). CCl_4-injected rats received THCQT (0.5g/kg) or silymarin

(25mg/kg) were shown in the panel C and panel D, respectively.

Fig. 4. The inhibitory effect of THCQT on $FeCl_2$-ascorbic acid induced LPO in the homogenates of brain isolated from rats. Values (mean ± SD) were obtained from each group of 5 animals. LPO: lipid peroxidation.