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Journal of Nutritional Biochemistry 17 (2006) 177–182

Journal of Nutritional Biochemistry

Effective protection of *Terminalia catappa* L. leaves from damage induced by carbon tetrachloride in liver mitochondria

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Received 31 May 2005; received in revised form 21 June 2005; accepted 21 June 2005

Abstract

The protective effects of chloroform extracts of *Terminalia catappa* L. leaves (TCCE) on carbon tetrachloride (CCl_4)-induced liver damage and the possible mechanisms involved in the protection were investigated in mice. We found that increases in the activity of serum aspartate aminotransferase and alanine aminotransferase and the level of liver lipid peroxidation (2.0-fold, 5.7-fold and 2.8-fold) induced by CCl_4 were significantly inhibited by oral pretreatment with 20, 50 or 100 mg/kg of TCCE. Morphological observation further confirmed the hepatoprotective effects of TCCE. In addition, the disruption of mitochondrial membrane potential (14.8%), intramitochondrial Ca^{2+} overload (2.1-fold) and suppression of mitochondrial Ca^{2+}–ATPase activity (42.0%) in the liver of CCl_4-insulted mice were effectively prevented by pretreatment with TCCE. It can be concluded that TCCE have protective activities against liver mitochondrial damage induced by CCl_4, which suggests a new mechanism of the hepatoprotective effects of TCCE.

Keywords: *Terminalia catappa* L.; Aspartate aminotransferase; Alanine aminotransferase; Lipid peroxidation; Mitochondrial membrane potential; Mitochondrial Ca^{2+} overload; Mitochondrial Ca^{2+}–ATPase activity

1. Introduction

Terminalia catappa L. is a tree from the Combretaceae family found in tropical and subtropical regions. The leaves, bark and fruit of the tree have been used in folk medicine for treatment of dermatitis and for antipyretic and homeostatic purposes. Recently, the fallen leaves of this plant have been used for preventing hepatoma and treating hepatitis in India, the Philippines and some other countries. Previous studies showed that the water extract of *T. catappa* L. leaves exert antioxidative, hepatoprotective and anti-inflammatory activities [1,2] and could prevent carcinogenesis [3]. But whereas the ethanol extract of *T. catappa* L. leaves contains more active components [2], the hepatoprotective effects of their chloroform soluble fraction [chloroform extracts of *T. catappa* L. leaves (TCCE)] and the possible mechanisms underlying the antihepatotoxicity have not been well investigated.

Evidence that cell death is involved in liver injury and liver disease have been accumulated. In fact, apoptosis and necrosis are crucial steps in the development of all kinds of liver injury, fibrosis, alcoholic liver disease and hepatitis [4,5]. It is also recognized that mitochondria play a key role in controlling cell death and that the function of mitochondria is not only to provide ATP by oxidative phosphorylation but also includes other roles such as the modulation of intracellular Ca^{2+} homeostasis, pH control and induction of

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CCl_4, carbon tetrachloride; DDB, dimethyl diphenyl bicarboxylate; TBA, thiobarbituric acid; TCCE, chloroform extracts of *Terminalia catappa* L. leaves.

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doi:10.1016/j.jnutbio.2005.06.008

apoptotic and excitotoxic cell death. Indeed, mitochondrial dysfunction contributes to a great number of human and animal diseases [6]. Changes such as the disruption of liver mitochondrial membrane potential, overload of hepatocellular Ca^{2+} and decrease in the activity of Ca^{2+}–ATPase located on the plasma membrane occur in the process of liver injury [7,8]. However, the effect of *T. catappa* L. leaves on liver mitochondria is unknown. Owing to our interest in the possible action of TCCE in preventing liver mitochondria injury induced by carbon tetrachloride (CCl_4), we aim to identify the possible mechanisms underlying their hepatoprotective effect.

2. Materials and methods

2.1. Plant material

Leaves of the *T. catappa* L. were collected in southern China in 1998 and identified by Mr. Gan Yao (Institute of Botany of Jiangsu Province, Chinese Academy of Sciences). A voucher specimen (No. 9808611) was deposited in the Institute of Materia Medica, School of Medicine, Nanjing University (Nanjing, P. R. China). TCCE were prepared as reported previously [2].

2.2. Chemicals

Fura-2/AM, rhodamine 123 (Rh123), succinate, rotenone and thiobarbituric acid (TBA) were purchased from Sigma (St. Louis, MO, USA). Aspartate aminotransferase (AST), alanine aminotransferase (ALT) and ATPase test kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, P. R. China). All other chemicals were of high purity from commercial sources.

2.3. Animals

Male ICR mice (Grade II, Certificate No. 97003, Experiment Animal Center of Southeastern University, Nanjing, P. R. China) weighing 20–25 g were used. All animals were fed a standard diet ad libitum and housed at a temperature of 20–25°C under a 12-h light/dark cycle throughout the experiment. The mice were randomly assigned. All animals received humane care and study protocols complied with the guidelines of the Nanjing University.

2.4. CCl_4-induced hepatotoxicity in mice

Mice were divided into six groups of six animals each. All mice except the normal ones received 0.15% of CCl_4 (in olive oil, 10 ml/kg ip). The normal and CCl_4 groups respectively received olive oil (10 ml/kg ip) and CCl_4 following 5 days of oral treatment with saline. Drug groups received CCl_4 following 5 days of oral treatment with 20, 50 or 100 mg/kg of TCCE or 200 mg/kg of dimethyl diphenyl bicarboxylate (DDB, as a positive reference) [9]. Mice were humanely killed 24 h after the treatment with CCl_4 and their blood was collected. Serum was separated by centrifugation, and serum AST and ALT activities were estimated spectrophotometrically using kits. After blood draining, liver sections were taken and fixed in 4% neutral-buffered formalin and prepared for examination under a photomicroscope. The remaining livers were removed and homogenized to analyze liver lipid peroxidation levels by measuring malondialdehyde formation using the TBA method [10]. Liver mitochondria were obtained to assess mitochondrial function.

2.5. Isolation of liver mitochondria

Mitochondria were prepared from the livers of mice according to the method of Aprille et al. [11]. In brief, mice livers were excised and homogenized in an isolation buffer containing 225 mM of mannitol, 75 mM of sucrose, 0.05 mM of EDTA and 10 mM of Tris–HCl (pH 7.4) at 4°C. The homogenate was centrifuged at $600\times g$ for 5 min to remove cell debris and the nuclear fraction. The resultant supernatant was centrifuged at $8800\times g$ for 10 min to sediment mitochondria. This pellet was washed twice with the same medium. Protein concentration was determined using Coomassie brilliant blue [12].

2.6. Measurement of mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi_m$) was evaluated according to Emaus et al. [13] from the uptake of the fluorescent dye Rh123, which accumulates electrophoretically into energized mitochondria in response to their negative inside membrane potential. Liver mitochondria isolated from livers of the mice from the normal, CCl_4, 20-mg/kg TCCE, 50-mg/kg TCCE, 100-mg/kg TCCE and 200-mg/kg DDB groups were prepared in the assay buffer (0.5 mg protein/ml) containing 225 mM of mannitol, 70 mM of sucrose and 5 mM of HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid), pH 7.2. $\Delta\Psi_m$ was assessed spectrophotometrically (Hitachi 850) with excitation at 505 nm and recording at 534 nm after the addition of 0.3 μM of Rh123 at 25°C. The membrane potential was calculated by the relationship $\Delta\Psi_m = -59 \log [Rh123]_{in}/[Rh123]_{out}$, assuming that the distribution of Rh123 between mitochondria and the medium follows the Nernst equation [14].

2.7. Measurement of mitochondrial free calcium

The intramitochondrial Ca^{2+} level was assayed by the change in fluorescent intensity (F) of the Ca^{2+} indicator dye fura-2. To load mitochondria with the fluorescent Ca^{2+} indicator fura-2, 0.5 mg protein/ml of mitochondria isolated from all groups' mice livers was incubated for 30 min at 30°C in a suspension medium containing 125 mM of sucrose, 65 mM of KCl, 5 mM of HEPES and 1 μM of fura-2/AM, pH 7.4, and then washed twice with the medium without the dye to eliminate free fura-2/AM. The final mitochondrial pellet was diluted in the suspension medium to obtain a protein concentration of 0.5 mg/ml. For every sample, the F of fura-2-loaded

Table 1
Effects of TCCE on serum AST and ALT and liver lipid peroxidation level

Group	AST (U/L)	ALT (U/L)	Lipid peroxidation (nmol malondialdehyde/mg/h)
Normal	401.3±35.2	220.0±51.3	3.83±0.45
CCl_4	813.8±117.0*	1252.3±173.2*	10.58±0.93*
CCl_4+TCCE (20 mg/kg)	777.5±96.8*	584.3±65.2*,**	6.31±0.94*,**
CCl_4+TCCE (50 mg/kg)	498.8±79.7**	339.0±52.0*,**	4.64±0.56*,**
CCl_4+TCCE (100 mg/kg)	387.5±40.6**	228.3±52.8**	4.02±0.55**
CCl_4+DDB (200 mg/kg)	411.3±45.8**	240.0±53.0**	4.78±0.76*,**

Values shown are the mean±S.D. of six determinations expressed as U/L. Drug group animals received various concentrations of TCCE at 24-h intervals before CCl_4 administration; 200 mg/kg of DDB was used as a positive reference.

* $P<.01$ versus the normal group.

** $P<.01$ versus the CCl_4 group.

mitochondria was recorded on a Hitachi 850 fluorescence spectrometer at an excitation wavelength of 340 nm and an emission wavelength of 510 nm. F_{max} was determined by adding 0.4% Triton X-100 and 1 mM of $CaCl_2$ to the mitochondrial suspension; F_{min} was measured by adding 10 mM of EGTA to the above system. The intramitochondrial Ca^{2+} content was calculated as follows: $K_d\ (F-F_{min})/(F_{max}-F)$ [15].

2.8. Analysis of mitochondrial Ca^{2+}–ATPase activity

Liver mitochondria were prepared in the assay buffer (0.5 mg protein/ml) containing 50 mM of Tris–HCl, 75 mM of KCl, 0.4 mM of EDTA and 6.0 mM of $MgCl_2$, pH 7.4. Ca^{2+}–ATPase activity was assayed by measuring phosphate release according to the protocol in the ATPase kit. One unit of the specific activity of the ATPase was defined as a micromole of inorganic phosphorus released from 1 mg of protein within 1 h (μmol Pi/mg protein/h).

2.9. Statistical analysis

Differences among all groups were analyzed by one-way analysis of variance followed by SNK q test; a P value of <.05 was accepted as statistically significant.

3. Results

3.1. Effects of TCCE on serum AST and ALT activities and liver lipid peroxidation level

Serum enzyme activity and liver lipid peroxidation level of mice in the prescriptions of TCCE are shown in Table 1. Serum AST and ALT activities increased remarkably (2.0-fold and 5.7-fold, respectively) after the injection of CCl_4. Also, the liver lipid peroxidation level in CCl_4-intoxicated mice was 2.8-fold of that in the normal mice. However, treatment with various concentrations of TCCE (20, 50 or 100 mg/kg) blocked the above changes significantly in a dose-dependent manner, and the elevations in serum AST and ALT activities and liver lipid peroxidation level were almost completely inhibited by 100 mg/kg of TCCE.

3.2. Histological observation

The histological changes associated with the hepatoprotective activity in three prescriptions of TCCE basically supported the estimation of the serum enzyme activities. The livers of CCl_4-intoxicated mice showed a massive fatty change, gross necrosis, broad infiltration of the lymphocytes and Kupffer cells around the central vein and loss of cellular boundary [Fig. 1(B)]. The histological pattern of

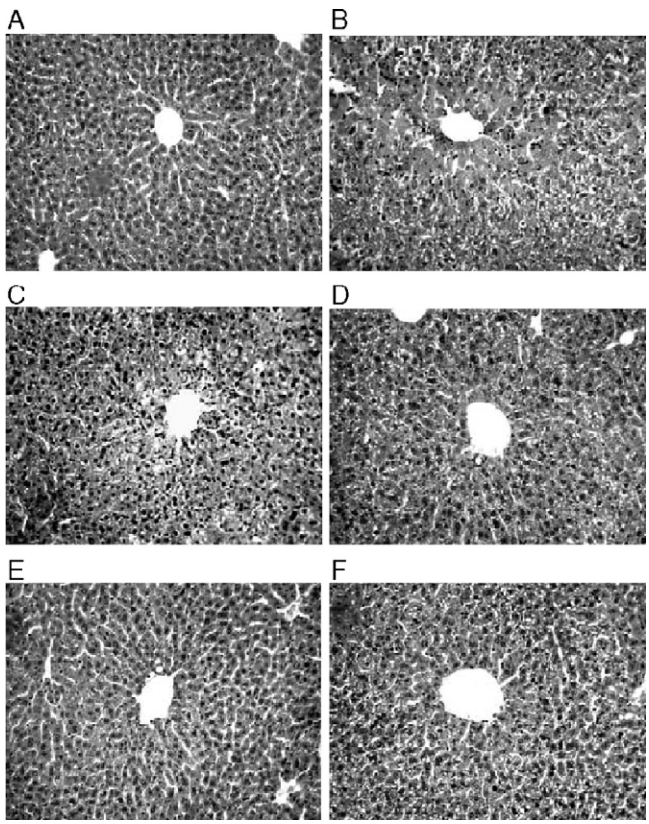

Fig. 1. Photomicrographs of liver sections taken from mice treated with CCl_4 with or without the pretreatment with TCCE. (A) Normal; (B) CCl_4; (C) CCl_4+TCCE (20 mg/kg); (D) CCl_4+TCCE (50 mg/kg); (E) CCl_4+TCCE (100 mg/kg); (F) CCl_4+DDB (200 mg/kg). (H&E stain, original magnification ×100).

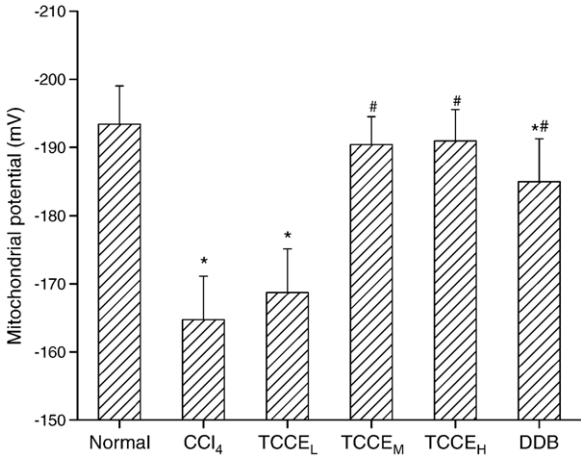

Fig. 2. Prevention of TCCE on mitochondrial membrane potential dissipation induced by CCl_4. Mice were treated with TCCE for 5 days before pretreatment with CCl_4. Liver mitochondria were then isolated and mitochondrial membrane potential was determined using Rh123. $TCCE_L$, $TCCE_M$ and $TCCE_H$ represent 20, 50 and 100 mg/kg of TCCE, respectively. DDB (200 mg/kg) was used as a positive reference. Values represent mean±S.D. ($n=6$). *$P<.01$ versus the normal group; $^{\#}P<.01$ versus the CCl_4 group.

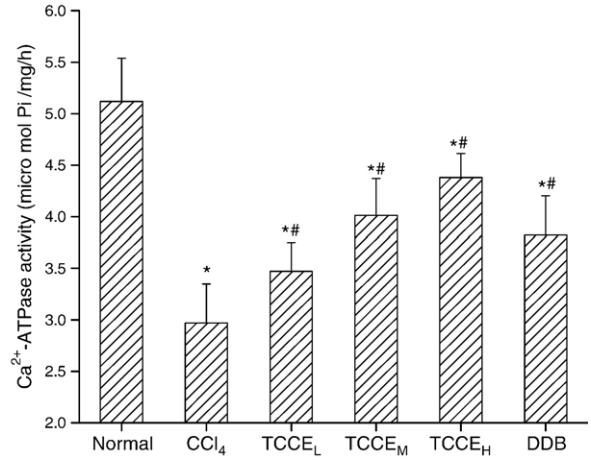

Fig. 4. Effect of TCCE on liver mitochondrial Ca^{2+}–ATPase activity in mice treated with CCl_4. Mice were treated with TCCE for 5 days before treatment with CCl_4. Liver mitochondria were then isolated and mitochondrial Ca^{2+}–ATPase activity was determined according the protocol in the kit. $TCCE_L$, $TCCE_M$ and $TCCE_H$ represent 20, 50 and 100 mg/kg of TCCE, respectively. DDB (200 mg/kg) was used as a positive reference. Values represent mean±S.D. ($n=6$). *$P<.01$ versus the normal group; $^{\#}P<.01$ versus the CCl_4 group.

the livers of the mice treated with TCCE only showed mild degrees of fatty change, necrosis and lymphocyte infiltration [Fig. 1(C)–(E)].

3.3. Effect of TCCE on mitochondrial membrane potential dissipation

Under the present experimental condition, the mitochondrial membrane potential of normal mice was −193.41±5.6 mV. This value dropped to −164.76±6.37 mV (14.8%, $P<.01$) when mice were intraperitoneally injected with CCl_4 (Fig. 2). TCCE preserved the mitochondrial membrane potential dissipated by CCl_4 in a dose-dependent manner. At a dose of 50 or 100 mg/kg of TCCE, the mitochondrial membrane potential was restored to that of normal mice. The dose of 20 mg/kg of TCCE seemed to increase the membrane potential as compared with the CCl_4 group, but this increase was not statistically significant.

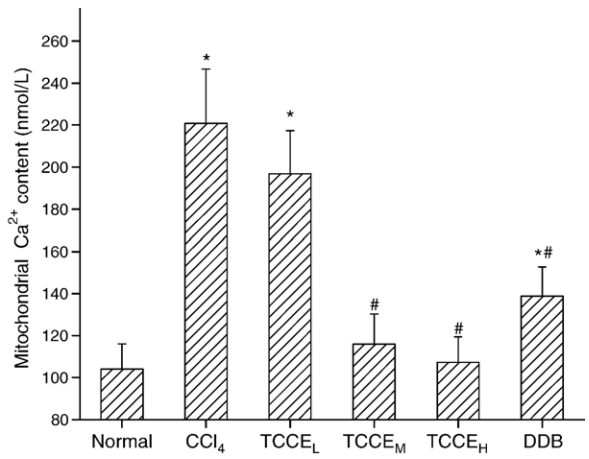

Fig. 3. Effect of TCCE on liver mitochondrial calcium content in mice treated with CCl_4. Mice were treated with TCCE for 5 days before pretreatment with CCl_4. Liver mitochondria were then isolated and mitochondrial free calcium content was determined using fura-2. $TCCE_L$, $TCCE_M$ and $TCCE_H$ represent 20, 50 and 100 mg/kg of TCCE, respectively. DDB (200 mg/kg) was used as a positive reference. Values represent mean±S.D. ($n=6$). *$P<.01$ versus the normal group; $^{\#}P<.01$ versus the CCl_4 group.

3.4. Effect of TCCE on mitochondrial calcium overload

Measurement of calcium content using the fluorescent probe fura-2 showed that intramitochondrial calcium concentration in CCl_4-intoxicated mice was much higher (2.1-fold, $P<.01$) than that in normal mice. However, the rise in calcium level induced by CCl_4 was effectively inhibited by pretreatment with 20, 50 or 100 mg/kg of TCCE, and the inhibitory rates in the 20-, 50- and 100-mg/kg TCCE groups reached 20.7%, 89.8% and 97.2%, respectively (Fig. 3).

3.5. Effect of TCCE on the decrease in mitochondrial Ca^{2+}–ATPase activity

The effect of TCCE on mitochondrial Ca^{2+}–ATPase activity is shown in Fig. 4. Mitochondrial Ca^{2+}–ATPase activity (3.59±0.47 μmol Pi/mg protein/h) in CCl_4-intoxicated mice was obviously lower than that in normal mice (5.12±0.32 μmol Pi/mg protein/h). However, the TCCE of various concentrations obviously blocked the decrease in mitochondrial Ca^{2+}–ATPase activity. The inhibitory rates of 20, 50 and 100 mg/kg of TCCE reached 23.3%, 48.4% and 65.6%, respectively.

4. Discussion

CCl_4-induced hepatic injury is often used as a model for hepatoprotective drug screening [16,17]. The results of the present study demonstrate that 20, 50 and 100 mg/kg of TCCE effectively protected mice against CCl_4-induced hepatotoxicity, providing evidence of the inhibitory effect of TCCE on increases in serum AST and ALT and liver lipid peroxidation levels. The histological changes observed in liver structures in mice further support this conclusion.

The level of ALT enzyme is an indicator of the degree of cell membrane damage whereas that of AST is an indicator of mitochondrial damage because mitochondria contain 80% of the enzyme [18]. We can speculate from our results that TCCE have a protective effect on both hepatocytes and their mitochondria.

It is now generally accepted that maintenance of mitochondrial membrane potential is necessary for mitochondria to carry out their function. In the present work, the effect of TCCE on liver mitochondrial membrane potential in CCl_4-intoxicated mice was assessed. It was found that treatment of mice with CCl_4 can damage liver mitochondria characterized by the disruption of mitochondrial membrane potential, which is in line with previous studies [7]. However, 20–100 mg/kg of TCCE effectively prevented the collapse of mitochondrial membrane potential, which indicates that TCCE give effective protection against mitochondria injury induced by CCl_4.

Evidence shows that the cell must maintain cytosolic Ca^{2+} at very low levels (0.1–0.2 μM) and that CCl_4 can result in hepatocellular Ca^{2+} overload [8], which can activate the mitochondrial Ca^{2+} uniporter in the mitochondrial inner membrane and induce a mitochondrial Ca^{2+} influx that eventually damages liver mitochondria [19]. Liver mitochondrial functions were further evaluated by measuring intramitochondrial Ca^{2+} concentrations in CCl_4-intoxicated mice with or without pretreatment with TCCE. The present results show that various concentrations of TCCE effectively suppressed the intramitochondrial Ca^{2+} overload induced by CCl_4, which suggests that TCCE could protect liver mitochondria against the toxicity of CCl_4 by preserving the mitochondrial calcium homeostasis.

It was believed that cells maintain a cytosolic Ca^{2+} homeostasis through the action of Ca^{2+}–ATPase located on the plasma membrane. This enzyme uses the energy of ATP to extrude cytoplasmic Ca^{2+} against a large concentration gradient into the extracellular space. However, more and more evidence suggest that mitochondria are particularly important in controlling cytoplasmic Ca^{2+} levels under pathological conditions. Ca^{2+}–ATPase located on the mitochondrial membrane can take up and retain large quantities of Ca^{2+} to buffer cytosolic Ca^{2+} levels and prevent damage to a cell [20]. Our studies show that TCCE in various concentrations obviously blocked the decrease in mitochondrial Ca^{2+}–ATPase activity. We therefore speculate that TCCE help maintain the mitochondrial calcium homeostasis through the protection of mitochondrial Ca^{2+}–ATPase. The results in the present study show that TCCE confer protection against liver mitochondria damage induced by CCl_4, which strongly suggests that TCCE have a potential clinical application in therapy for liver diseases.

Acknowledgments

This work was financially supported by the Natural Science Fund of Jiangsu Province of China (No. BK99051), the Analysis Fund of Nanjing University and Hong Kong Polytechnic University research grants. We would like to thank Mr. Yao Gan, an engineer of the Institute of Botany of Jiangsu Province (Chinese Academy of Sciences) for the identification of the plant *T. catappa* L. We also would like to thank Professor Zu Xuan Zhang (School of Medicine, Nanjing University) and Xian Chong Tao (Center of Modern Analysis, Nanjing University) for their support and assistance during this study.

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