

Kampo formula “Hochu-ekki-to” suppressed carbon tetrachloride-induced hepatotoxicity in mice

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Abstract

Objective The aim of this study was to investigate whether pretreatment with the Japanese herbal medicine “Hochu-ekki-to” (TJ-41) has an ameliorative effect on carbon tetrachloride (CCl₄)-induced hepatotoxicity through anorexia prevention.

Methods Twenty-four hours before CCl₄ injection, TJ-41 or saline solution was intraperitoneally administered. Furthermore, 24 h after TJ-41 injection, mice were intraperitoneally administered 1.6 g/kg CCl₄ or olive oil. Moreover, 24 h after CCl₄/olive oil injection, mice from each group were euthanized and bled for plasma analysis.

Results Mice injected with CCl₄ exhibited severe anorexia. Moreover, CCl₄ increased the plasma levels of hepatic injury markers (i.e., alanine aminotransferase and aspartate aminotransferase) as well as lipid peroxidation and hepatic Ca levels. Pretreatment with TJ-41 recovered the CCl₄-induced anorexia and plasma levels of the hepatic injury markers. Moreover, CCl₄-induced lipid peroxidation and hepatic Ca levels decreased upon TJ-41 pretreatment. In addition, hepatic metallothionein levels in the TJ-41 + CCl₄-treated group were decreased by >50 % compared with the levels in the TJ-41-treated group, implying that metallothionein was consumed by CCl₄-induced radicals.

Conclusion Our results suggest that TJ-41 attenuates CCl₄-induced hepatotoxicity, presumably by the induction of metallothionein, which in turn scavenges radicals induced by CCl₄ exposure.

Keywords Carbon-tetrachloride · Liver · Hochu-ekki-to · Oxidative stress

Introduction

The liver is one of the most important organs in the human body and has multiple functions such as detoxification, protein synthesis, and production of chemicals necessary for digestion. In addition, the liver is one of the most easily damaged organs owing to its sensitivity to chemical toxic agents [1]. Acute liver injury is usually referred to as the rapid development of hepatocellular dysfunction and has a poor prognosis. It frequently results from drug administration, viral infection, and toxic or hepatic ischemic-reperfusion injury [2].

Carbon tetrachloride (CCl₄) belongs to a class of chemicals widely used as grain fumigants, anthelmintics, and intermediates of the synthesis of chlorofluorocarbons called haloalkanes [3]. CCl₄ is one of the most extensively studied hepatotoxicants because its exposure mimics human hepatotoxicity. CCl₄-induced hepatotoxicity is a multifactorial process [4–6]: the first step involves the metabolic activation of CCl₄ by cytochrome P450 2E1 (CYP2E1), resulting in the formation of trichloromethyl and trichloromethyl peroxy radicals; second step involves radical binding, wherein the free radicals react with the sulfhydryl groups of antioxidant enzymes (e.g., glutathione and proteins' thiols); third step is the overexpression of these free radicals, leading to several deleterious effects

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such as enhanced membrane lipid peroxidation and covalent macromolecule binding; and fourth step is ATP depletion and intracellular Ca elevation. Through these steps, necrosis is induced; this induction is termed as the fifth step. Multiple compounds have been reported to show protective effects against CCl₄-induced hepatotoxicity, and associated protective mechanisms have been proposed to act on each of the five steps [7–10].

The Japanese herbal medicine “Hochu-ekki-to” (TJ-41) is a Kampo medicine comprising ten medicinal herbs. It has long been used in Japan for the treatment of cancers, rheumatoid arthritis, and atopic dermatitis [11, 12]. In addition, TJ-41 has traditionally been used against anorexia [13]. Because CCl₄ is an anorexic agent, we hypothesized that TJ-41 suppresses CCl₄-induced toxicity by preventing anorexia.

Therefore, in this study, we investigated whether pre-treatment with TJ-41 was sufficient to attenuate CCl₄-induced acute hepatotoxicity.

Materials and methods

Animal treatment

Six-week-old male ddY mice were purchased from Japan SLC (Shizuoka, Japan). Following arrival at our facility, the mice were maintained under standard conditions of controlled temperature (24 ± 1 °C), humidity (55 ± 5 %), and light (12:12-h light/dark cycles) with free access to water and food. The experiments were performed using seven-week-old animals. Following the protocol of the animal experiment, all surviving mice were killed using pentobarbital. All experiments were approved by the Institutional Animal Care and Experiment Committee of the Kinjo Gakuin University (No. 129).

Experimental protocol

The mice were divided into four groups, and the members of each group were kept in the same cage. Twenty-four hours before the CCl₄ injection, animals of Group-2 (TJ-41 group) and Group-4 (TJ-41 + CCl₄ group) were intraperitoneally (i.p.) administered 10 % TJ-41 solution (Tsumura, Tokyo, Japan) at 10 mL/kg (1 g/kg). Furthermore, animals in Group-1 (control group) and Group-3 (CCl₄ group) were i.p. administered equivalent volumes of the saline vehicle. Moreover, 24 h after the TJ-41 or saline injection, the members of both Group-3 and Group-4 were i.p. administered 1.6 g/kg (at 10 mL/kg) of CCl₄. This CCl₄ dose was in accordance with previous studies [8, 14–16]. Animals in Group-1 and Group-2 were i.p. injected with equivalent volumes of olive oil. In addition,

24 h after the CCl₄/olive oil injection, mice from each group were euthanized and bled for plasma analysis. The food intake after CCl₄ or olive oil administration was calculated per day. The resulting plasma samples were stored at –80 °C before conducting alanine aminotransferase (ALT) and aspartate aminotransferase (AST) assays. The liver was harvested from each animal and separate samples from each liver were stored at –80 °C [pending thiobarbituric acid reactive substances (TBARS) assay].

Measurement of ALT and AST

Plasma ALT and AST activities were measured using the Wako Transaminase CII Test (Wako Chemical, Osaka, Japan) in accordance with the manufacturer’s instructions and as previously described [17].

Measurement of hepatic Ca and Zn concentrations

Liver specimens (0.2–0.3 g each) were digested in 0.5 mL of concentrated nitric acid in glass test tubes. The digested samples were held at 80 °C for 1 h, with a subsequent gradual increase in temperature (10 °C/h) until the samples reached 130 °C. The samples were held at 130 °C until the specimens became transparent and were then diluted to a final volume of 5 mL with distilled water and used for the determination of Ca and Zn concentrations by atomic absorbance spectroscopy using a Z-2300 spectrophotometer (Hitachi, Tokyo, Japan).

Measurement of malondialdehyde levels in liver

Total malondialdehyde (MDA) levels in the liver were examined with a colorimetric TBARS microplate assay kit (FR40; Oxford Biochemical Research, Oxford, MI, USA) in accordance with the manufacturer’s protocol and as previously described [18].

Determination of metallothionein (MT) levels

We i.p. administered 1 g/kg TJ-41 at 10 mL/kg. The control group was i.p. administered an equivalent volume of saline. Twenty-four hours after the TJ-41 or saline injection, the liver was harvested from each animal. Hepatic MT protein levels were determined by the Cd saturation–hemolysate method (Cd-hem method), as previously described [17].

Statistical analysis

All data from the control and treatment groups were obtained from the same number of replicated experiments. All experiments were independently performed at least

twice. The results were analyzed using one-way analysis of variance with post hoc Tukey–Kramer test. All statistical analyses were performed using SPSS 19.0 software (Chicago, IL, USA). *p* values of <0.05 were considered statistically significant.

Results and discussion

First, we measured food intake and found that CCl₄ decreased food intake by more than 75 % in mice compared with the intake in control mice (Fig. 1). This result is consistent with previously reported data [7]. In comparison with CCl₄-injected groups, TJ-41 + CCl₄ groups partially increased food intake (from 1.18 g to 2.52 g). In addition, the saline- and TJ-41-injected groups showed approximately the same level of food intake (5.45 and 5.30 g,

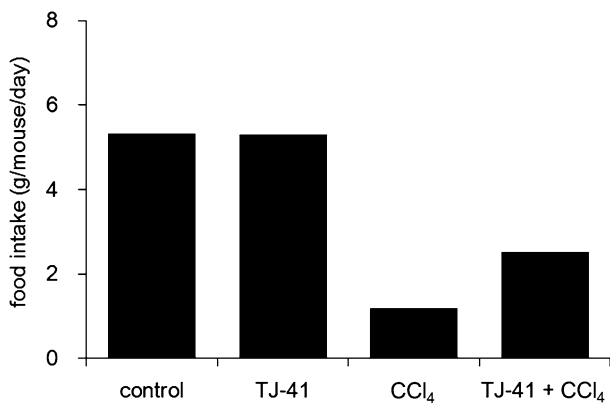


Fig. 1 Effect of pretreatment with TJ-41 on acute CCl₄ toxicity as measured by food intake. Mice were injected i.p. with 10 % TJ-41 solution. 24 h after pretreatment, mice were injected i.p. with 1.6 g/kg CCl₄. Amount of food intake during 24 h was determined after i.p. injection with CCl₄. Food intake was calculated total food intake/number of mouse/per day

respectively). Several reports have indicated a correlation between anorexia and hepatic injury [19, 20]. In addition, it has been reported that anorexia is a sign of hepatic injury. These data suggests that TJ-41 improves CCl₄-induced anorexia presumably by preventing liver injury.

Together with the food intake measurements, we analyzed the plasma ALT and AST activities (Fig. 2). These are known markers of liver injury and dysfunction. The control and TJ-41 groups showed normal levels of ALT (Fig. 2a) and AST (Fig. 2b) activities. Pretreatment with CCl₄ increased the ALT and AST plasma concentrations, whereas pretreatment with TJ-41 suppressed ALT and AST activities by 54 and 34 %, respectively.

To further investigate the protective effect of TJ-41 against CCl₄, we calculated the hepatic Ca content (Fig. 3) because exposure to CCl₄ is known to elevate hepatic Ca levels [21]. In this study, pretreatment with CCl₄ increased hepatic Ca levels, whereas pretreatment with TJ-41 decreased the levels. These findings suggest that TJ-41 inhibits CCl₄-induced hepatotoxicity.

Several studies have suggested that one possible molecular mechanism involved in CCl₄ hepatotoxicity is disruption of the delicate oxidant/antioxidant balance, which can lead to liver injury via oxidative damage [4, 22]. Moreover, CCl₄ is a prototypical lipid peroxidant that induces early lipid peroxidation in the liver [8]. As a marker of lipid peroxidation, MDA concentration was measured in all animal groups. Pretreatment with CCl₄ significantly increased MDA levels, whereas pretreatment with TJ-41 abolished CCl₄-induced MDA upregulation (Fig. 4), suggesting that TJ-41 itself and/or TJ-41-induced gene products have an antioxidant effect. One potential mechanism underlying this is the induction of MT. MT has antioxidant properties against reactive oxygen species (ROS) via scavenging of free radicals [23]; MT is estimated to exhibit approximately 300-fold higher scavenging activity than that exhibited by glutathione (GSH). MT

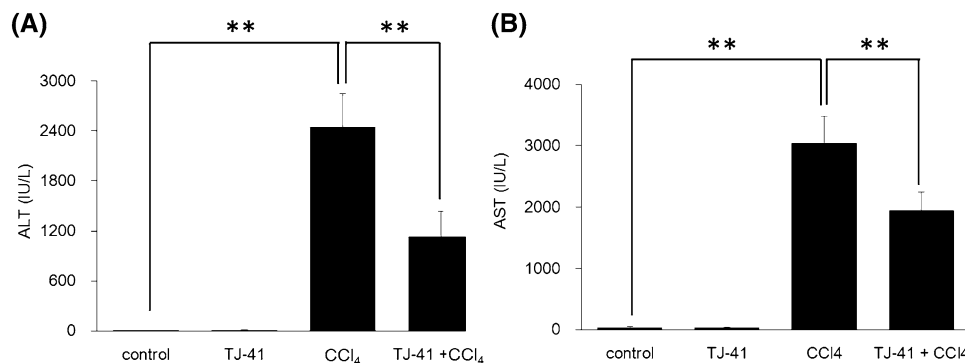


Fig. 2 Effect of pretreatment with TJ-41 on ALT and AST activities. Mice were injected i.p. with 10 % TJ-41 solution. 24 h after pretreatment, mice were injected i.p. with 1.6 g/kg CCl₄. ALT (A) and AST (B) levels in liver was determined 24 h after i.p.

injection with CCl₄. Data are presented as mean ± SD of five or six mice. Double asterisk significant difference between compared values (***p* < 0.01)

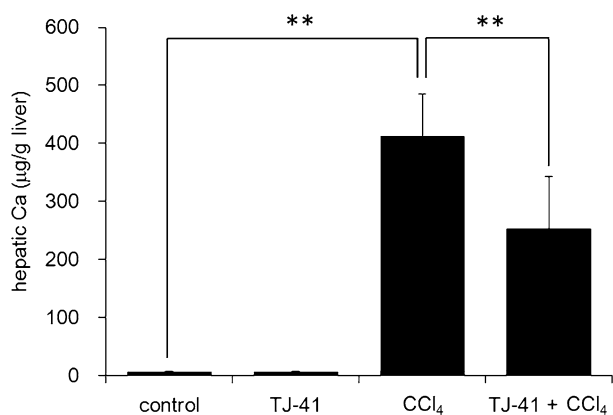


Fig. 3 Effect of pretreatment with TJ-41 on hepatic Ca concentration in acute CCl₄-induced toxicity. Mice were injected i.p. with 10 % TJ-41 solution. 24 h after pretreatment, mice were injected i.p. with 1.6 g/kg CCl₄. Ca concentration in liver was determined 24 h after i.p. injection with CCl₄. Data are presented as mean ± SD of five or six mice. *Double asterisk* significant difference between compared values (** $p < 0.01$)

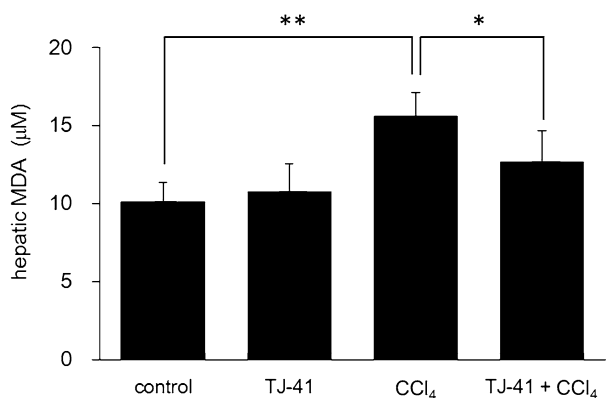


Fig. 4 Effect of pretreatment with TJ-41 against acute CCl₄ toxicity, as measured by MDA levels. Mice were injected i.p. with 10 % TJ-41 solution. 24 h after pretreatment, mice were injected i.p. with 1.6 g/kg CCl₄. MDA levels in liver was determined 24 h after i.p. injection with CCl₄. Data are presented as mean ± SD of five or six mice. *Single asterisk* and *double asterisk* significant difference between compared values (* $p < 0.05$ and ** $p < 0.01$, respectively)

expression is upregulated in many organs, not only by various metals such as Zn and Cd but also by non-metallic compounds [24, 25]. MT has the potential for application against hepatic injury. For example, acetaminophen (APAP)-induced hepatotoxicity is prevented by Zn-induced MT or using MT knockout (KO) mice [26, 27]. In addition, not only APAP but also other hepatic injury chemicals such as Cd and bromobenzene reportedly show hepatoprotective effects by pretreatment with Zn or using MT-KO mice [28–30]. We also reported that Zn-induced MT expression has a protective effect against CCl₄-induced hepato- and nephrotoxicity [17, 18]. Our current study demonstrates that i.p. injection of TJ-41 increased hepatic

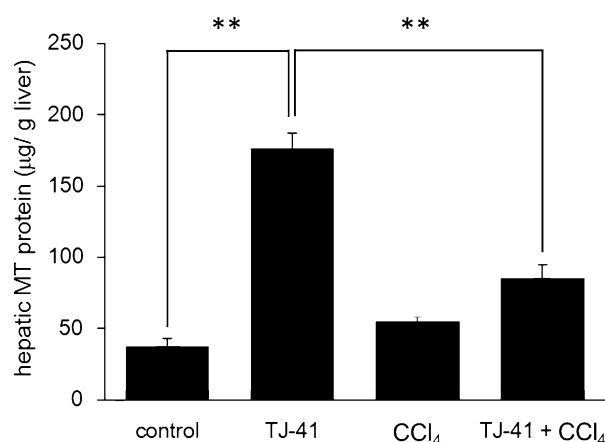


Fig. 5 Effect of pretreatment with TJ-41 against acute CCl₄ toxicity, as measured by hepatic MT levels. Mice were injected i.p. with 10 % TJ-41 solution. 24 h after pretreatment, mice were injected i.p. with 1.6 g/kg CCl₄. MT levels in liver was determined 24 h after i.p. injection with CCl₄. Data are presented as mean ± SD of five or six mice. *Double asterisk* significant difference between compared values (** $p < 0.01$)

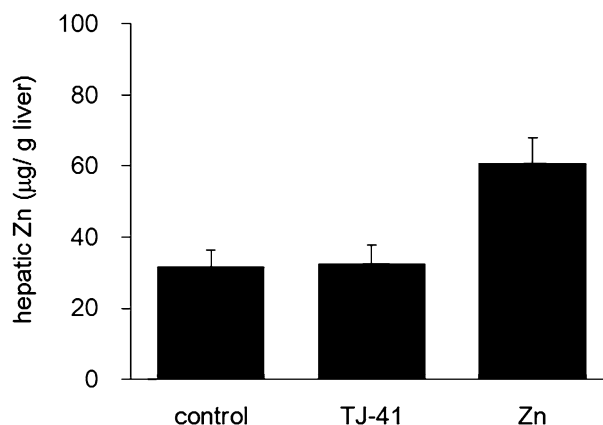


Fig. 6 Effect of pretreatment with TJ-41 on Zn levels in mouse liver. Mice were injected i.p. with 10 % TJ-41 solution or subcutaneous with 50 mg/kg ZnSO₄ (as Zn) (Nacalai Tesque, Kyoto, Japan). 24 h after pretreatment, the liver samples were collected followed by determining the Zn contents. Data are presented as mean ± SD of five or six mice

MT levels by >175 µg/g liver (Fig. 5). In addition, we showed that the TJ-41-induced increase of hepatic MT levels was attenuated by >50 % following CCl₄ injection, suggesting that MT is consumed by CCl₄-derived radicals. The Cd-hem assay, used to determine MT in our present study, is based on the quantification of metal ions (Cd) bound to MT molecules. Because oxidized MT does not bind to Cd and is not detected in this assay, we speculate that CCl₄-derived free radicals preferentially attacked MT, resulting in a loss of Cd-binding activity. Moreover, Itoh et al. reported that saponins such as Echnoside A and sakuraso-saponin induced MT (200 µg/g liver) and had protective effects against CCl₄-induced hepatotoxicity

[31]. Because the MT induction level in this study was the same as that in the study by Itoh et al., their data support our hypothesis.

In this study, the main compound that induced MT remains elusive. One candidate compound is Zn, which is the most potent inducer of MT. However, hepatic Zn is not increased by pretreatment with TJ-41 (Fig. 6). Taken together, MT induction does not explain the association of Zn with TJ-41. Another possibility is that the compound is a saponin derivative because TJ-41 contains derivatives such as saikosaponin b2. This may support our hypothesis because Itoh et al. reported the Echnoside A and sakurasaponin induced MT [31]. Other possibility is an additive effect since TJ-41 comprises ten different medicinal herbs that have possibility of unknown compound which might induce MT. Further investigation is thus needed to elucidate the active component of TJ-41.

In conclusion, we have demonstrated that pretreatment with TJ-41 suppresses CCl₄-induced acute hepatotoxicity; we hypothesize that the hepatoprotective effect of TJ-41 is attributable to its antioxidant role, presumably by the induction of MT. To the best of our knowledge, this is the first evidence suggesting that TJ-41 protects against CCl₄-induced acute hepatotoxicity. Although further investigation is needed to clarify the active component of TJ-41, these findings are expected to improve self-medication or therapeutic products against free radical-induced organ injury and disease.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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