Sasa veitchii extract suppresses carbon tetrachloride-induced hepato- and nephrotoxicity in mice

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Abstract

Objective The aim of this study was to investigate the therapeutic effects of a Sasa veitchii leaf extract (SE) on carbon tetrachloride (CCl₄)-induced hepato- and nephrotoxicity.

Methods Seven-week-old male ddY mice were orally administered SE or saline for seven days. Twenty-four hours after the last SE or saline administration, the mice were intraperitoneally injected with 3 g/kg CCl₄ or olive oil. The mice from each group were euthanized and bled for plasma analysis 24 h after the CCl₄/olive oil injection.

Results We found that pretreatment with SE completely abolished the CCl₄-induced mortality in the mice after 24 h. The mice pretreated with SE exhibited significantly decreased levels of functional markers, and reduced histological damage in both the liver and the kidney. Furthermore, we found that the SE pretreatment decreased lipid peroxidation and calcium levels in the liver. Although SE could not induce the free radical-scavenging metallothioneins, the plasma biological antioxidant power was significantly increased in the mice pretreated with SE.

Conclusion Our findings demonstrate that prophylactic treatment with SE protects mice from CCl₄-induced lethal toxicity by decreasing oxidative stress in the liver and kidney, presumably by increasing biological antioxidant power.

Keywords Carbon tetrachloride · Sasa veitchii · Antioxidant · Liver · Kidney

Introduction

Reactive oxygen species (ROS) are various forms of activated oxygen. A disproportionately large amount of ROS and the absence of their scavenging systems in cells lead to oxidative stress and increase the risk of several human diseases, including hepatic injury, carcinogenesis, and inflammation [1]. The liver plays a central role in the maintenance of systemic lipid homeostasis and is especially susceptible to ROS-induced damage. Carbon tetrachloride (CCl₄) is widely used to develop experimental animal models of liver failure (caused by free radical production) that mimic human hepatic toxicity. Although the main target organ of CCl₄ is the liver, toxic effects of CCl₄ are also observed in other organs, including the kidneys, testis, and brain [2–5], and the nephrotoxic effect of CCl₄ is also associated with free radical production [2].

To prevent the damage caused by ROS, living organisms have developed an antioxidant system, which includes non-enzymatic antioxidants and enzymes, such as catalase, superoxide dismutase, and peroxidase [6]. In addition to these natural antioxidants, other synthetic or natural ROS scavengers may reduce the incidence of free radical-mediated diseases. The use of antioxidants in the prevention and cure of various diseases is intensifying, and there is considerable interest in the study of antioxidant activities of molecules, such as polyphenols and carotenoids [6–8]. Antioxidants appear to act against disease processes by increasing the levels of endogenous antioxidant enzymes and decreasing lipid peroxidation [9, 10].
Bamboo grasses are common perennial plants belonging to several species of the genus *Sasa* in the *Gramineae* family. They are widely distributed in Asian countries, and their roots and leaves have been used in food and medicine. *Sasa veitchii* has been utilized in Asia for a long time. In Japan, its leaves have been used to wrap sushi sheets to protect against bacterial spoilage. Furthermore, previous studies have demonstrated that an *S. veitchii* extract (SE) shows antitumor [11], antiulcer [12], antiviral [13], anti-inflammatory [14], and anti-allergic [15] activities. More recently, SE has been found to contain a large number of bioactive molecules, such as polyphenols and flavones [16, 17]. In addition, it has been found that SE has antioxidant properties and protects against lipid peroxidation [18–20]. However, no study has investigated the protective activity of SE against CCl₄-induced hepatotoxicity.

Therefore, in this study, we examined the protective effects of SE on the liver and kidney damages induced by CCl₄. In our investigation, we found that SE pretreatment attenuated the CCl₄-induced acute liver and kidney injury and showed that its protective effect might be due to the inhibition of oxidative stress.

**Materials and methods**

**Animal treatment**

Seven-week-old male ddy mice were purchased from Japan SLC (Shizuoka, Japan). They were housed under the standard conditions of controlled temperature (24 ± 1 °C), humidity (55 ± 5 %), and light (12:12-h light/dark cycle) and were provided food and water ad libitum. All experiments were approved by the Institutional Animal Care and Experiment Committee of Kinjo Gakuin University (No. 110).

**Preparation of SE**

*Sasa veitchii* leaves were obtained from Sunchlon Co., Ltd. (Nagano, Japan) and used to prepare an SE sample according to the procedure, as shown in Fig. 1. Fresh leaves of *S. veitchii* were cut into small pieces, and magnesium in chlorophyll of *S. veitchii* was replaced by copper by soaking the leaves in CuSO₄-containing boiled water. This substitution prevents the decomposition of chlorophyll. Then, cell walls were hydrolyzed by boiling in an aqueous 15 % (w/v) NaOH solution for 80 min. HCl was then added to the obtained solution, which contained hydrolyzed cell walls and cytoplasm components, and a precipitate was formed. The precipitate was collected by centrifugation and dissolovd by adding 20 % (w/v) NaOH to pH 7 (about 50 mL of 20 % NaOH per 1 kg of paste).

Finally, this solution was diluted with distilled water to adjust the concentration of Cu-chlorophyll to 0.25 % (w/v). The diluted solution (SE) contained other components, including minerals, carbohydrates, fat, protein, and silicic acid, as shown in Table 1. One milliliter of SE was made from 2.82 g of *S. veitchii* leaves according to the company data.

**Preliminary confirmation of mortality in animal experiments**

Mice were divided into two groups of 10 mice each and were pretreated for seven days with 0.2 mL of SE or saline as a
control, which were administered once per day by oral gavage. Then, all mice received 3 g/kg CCl₄ in an olive oil emulsion by intraperitoneal (i.p.) injection at 5 mL/kg body weight. The mice were monitored for mortality for 24 h following the CCl₄ injection. Following the experiment, any surviving mice were euthanized with pentobarbital.

**Evaluation of SE prophylaxis**

Mice were divided into four groups of six mice each and were pretreated for seven days with 0.2 mL of SE or saline as a control, which were administered once per day by oral gavage. Then, the mice received 3 g/kg CCl₄ in an olive oil emulsion or olive oil alone by i.p. injection at 5 mL/kg body weight. Twenty-four hours following the injection, the mice were euthanized and bled to obtain plasma. The resulting plasma samples were stored at −80 °C. The liver and kidney were harvested and stored at −80 °C or fixed in 15 % neutral buffered formalin (pH 7.2).

**Plasma biochemical analysis**

Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured using the Transaminase CII kit (Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturer’s instructions, as previously described [21, 22]. Concentrations of plasma creatinine and blood urea nitrogen (BUN) were measured using a creatinine liquid reagents assay (Diazyme, Poway, CA, USA) and a BUN test (Wako Pure Chemical Industries), respectively, according to the manufacturers’ instructions, as previously described [23, 24]. For relative quantification, calibration curves were prepared using standard solutions.

**Histopathological findings**

For histological analysis, a portion of the left liver lobe and left kidney from each animal were perfused with 15 % phosphate-buffered neutral formalin (pH 7.2), dehydrated, and embedded in paraffin. Embedded tissues were sectioned at 4 μm and stained with hematoxylin and eosin (H&E). Histopathological features were observed using a light microscope.

**Measurement of malondialdehyde levels in the liver and kidney**

Total malondialdehyde (MDA) levels in the liver and kidney were examined using a colorimetric thiobarbituric acid reactive substance microplate assay kit (FR40, Oxford Biochemical Research, Oxford, MI, USA) according to the manufacturer’s protocol, as previously described [23, 24].

**Measurement of hepatic Ca concentration**

Liver specimens (0.2–0.3 g each) were digested in 0.5 mL of concentrated nitric acid (60 %; Kanto Chemical, Tokyo, Japan) in glass test tubes. The digested samples were incubated at 80 °C for 1 h, and then, the temperature was gradually increased (10 °C/h) until it reached 130 °C. The samples were maintained at 130 °C until they became transparent and were then diluted to a 5-mL final volume with distilled water. The Ca concentration was determined by atomic absorbance using a Z-2300 spectrophotometer (Hitachi, Tokyo, Japan).

**Determination of metallothionein (MT) levels in the liver and kidney**

Hepatic metallothionein (MT) protein levels were determined by the Cd-saturation/hemolysate (Cd–hem) method. Liver tissue was homogenized with five volumes of 0.25 M sucrose. The homogenates were centrifuged at 18,000×g for 20 min at 4 °C to separate postmitochondrial supernatants, and suitable aliquots were used for the MT assay by the Cd–hem method, as described previously [25, 26].

**Determination of biological antioxidant power (BAP) in plasma**

The BAP was assessed in plasma using a free radical analytical system (FRAS4; Wismerll Co., Ltd, Parma, Italy). A redox potential, including glutathione peroxidase and superoxide dismutase, was measured using the FRAS4 BAP test. Briefly, plasma samples (5 μL) were dissolved in a colored solution containing ferric ions and a chromogenic sulfur-derived compound and incubated for 5 min. Following the incubation, the degree of discoloration was found to be directly proportional to the ability of the plasma to reduce ferric ions. The amount of the reduced ferric ions was determined using a photometer to assess the intensity of discoloration, and the BAP results were expressed in micromoles of reduced Fe/L.

**Statistical analysis**

Results of the acute CCl₄ toxicity were analyzed by the $X^2$ test. Statistical significance of the differences between two groups was estimated using a two-tailed Student’s $t$ test. Multiple comparisons were made by one-way analysis of variance with post hoc Tukey–Kramer’s test. All statistical analyses were performed using the SPSS 19.0 software (Chicago, IL, USA). Values of $P < 0.05$ were considered statistically significant.
Table 2 Effect of pretreatment with SE on acute CCl₄-induced lethal toxicity

|        | Live | Dead |
|--------|------|------|
| CCl₄   | 6    | 4    |
| SE + CCl₄ | 10** | 0    |

**P < 0.01 versus CCl₄-treated group

Results

Effect of SE on CCl₄-induced lethal toxicity

To test if SE counteracts the acute CCl₄ toxicity, we calculated the mortality rate at 24 h after CCl₄ i.p. injection in mice. As shown in Table 2, the CCl₄-induced mortality occurred in 40 % (4/10) of the mice pretreated with the saline control. However, pretreatment with SE for 1 week completely abolished the CCl₄-induced acute lethal toxicity at 24 h after the injection (0/10). These data suggest that SE has the potential to prevent the CCl₄-induced lethal toxicity.

Effect of SE on CCl₄-induced acute toxicity as assessed by functional markers and morphology

Next, we examined whether SE affected the CCl₄-induced acute toxicity by assessing hepatic and renal functional markers. As expected, administration of CCl₄ severely increased the plasma concentrations of ALT (Fig. 2a) and AST (Fig. 2b) and mildly increased the concentrations of creatinine (Fig. 2c) and BUN (Fig. 2d) compared to those in the control group. Pretreatment with SE suppressed the CCl₄-induced increases in these hepatic and renal functional markers. There were no significant differences between the control and SE groups.

In parallel with the measurement of functional markers, we conducted histopathological studies. H&E-stained liver sections from the control and SE groups showed a normal cell morphology and well-preserved cytoplasm, in addition to a clear, plump nucleus (Fig. 3a, b). However, we observed extensive cell death, particularly around the central vein, in the mice treated with CCl₄ (Fig. 3c).

Fig. 2 Effect of pretreatment with SE on hepatic and renal function. Mice received SE or saline (control) once daily for seven days by oral gavage. Twenty-four hours following the final SE or saline dose, the mice received 3 g/kg CCl₄ i.p. Plasma levels of hepatic and renal markers were determined 24 h after the CCl₄ administration. a, b, c, and d represent ALT, AST, creatinine, and BUN data, respectively. The data are the mean ± standard deviation (SD) for four-to-six mice per group. **P < 0.01 versus the control group and ##P < 0.01 versus the CCl₄-treated group.
Pretreatment with SE ameliorated some, but not all, liver cell death (Fig. 3d).

Histologic examination of the glomeruli and tubules in the kidney did not reveal any abnormalities in the control and SE groups (Fig. 3e, f). Exposure to CCl_4 induced swelling, degeneration, and the appearance of a protein columna, a morphology that indicates the penetration of protein into renal proximal tubules (Fig. 3g). However, exposure to CCl_4 had no effect on the Henle’s loop, distal tubules, collecting ducts, and medullae, suggesting that CCl_4 triggered mild renal toxicity. In contrast, pretreatment with SE protected the CCl_4-treated mice from CCl_4-induced renal toxicity and restored almost normal kidney histology and the normal glomerular architecture (Fig. 3h). These data suggest that SE has the potential to prevent the CCl_4-induced hepato- and nephrotoxicity.

**Effect of SE on CCl_4-induced acute toxicity as assessed by MDA levels and Ca levels**

To further investigate the SE protective activity against CCl_4 intoxication, we measured the oxidative stress in tissues of the target organs. In the liver, the CCl_4 treatment significantly increased the MDA levels ($P < 0.01$), and these effects were ameliorated by the pretreatment with SE (Fig. 4a). In the kidney, exposure to CCl_4 also led to increases in the MDA levels ($P < 0.05$), and although SE pretreatment resulted in a decreasing trend, no statistically significant change was observed ($P = 0.095$, Fig. 4b). Our previous findings have suggested that the CCl_4-induced toxicity is milder in the kidney than in the liver [25, 27], which might explain why the SE-mediated protection of the kidney was undetectable in our present study.

In addition, we found that the hepatic Ca levels in the mice pretreated with SE decreased compared to those in the control CCl_4-treated mice (Fig. 5). In the kidney, no significant changes were observed among the groups (data not shown).

**Determination of SE’s properties as assessed by MT levels and plasma BAP**

To further investigate the SE activity against CCl_4 intoxication, we tested MT levels and found no significant differences in MT levels in both the liver and the kidney among the groups (Fig. 6).

To test if SE protects against the CCl_4-induced toxicity by inducing antioxidant responses, we determined plasma BAP levels in the mice treated with SE or control saline. Treatment with SE increased the BAP levels compared to those in the saline controls (Fig. 7).
Our present study demonstrated that pretreatment with SE largely prevented the acute toxicity induced by CCl₄, as assessed by mortality, blood functional markers, and histological changes in both the liver and the kidney. These results indicated that SE counteracted, through its antioxidant properties, the free radical-associated cell injury induced by metabolites of CCl₄.

CCl₄-induced toxicity is a stepwise process [9, 27]. The first step involves metabolic activation of CCl₄ by the cytochrome P450 isozyme CYP2E1. CCl₄ is then converted to trichloromethyl and/or trichloromethyl peroxyl free radicals, which react with sulfhydryl groups, such as glutathione, or are scavenged by antioxidant enzymes. In the second step, overproduction of these free radicals increases oxidative stress, which is associated with alterations in Ca homeostasis and the initiation of signal transduction responses. The third step is the depletion of ATP and an increase in cellular levels of Ca. Through these steps, cell death is observed as the fourth step. In our present study, extensive liver cell death (fourth step) around the central vein was observed in CCl₄-injected mice, and pretreatment with SE attenuated the liver cell death to some extent. In contrast to the liver injury, kidney injury was milder, with the area of injury limited to proximal tubules, suggesting that the main target organ of CCl₄ is the liver. In addition to the morphological changes, hepatic calcium levels decreased after pretreatment with SE. There has been evidence suggesting that upregulation of the cytosolic Ca²⁺ concentration is a terminal event in the progression of toxic liver injury to cell death [28]. These data suggest that SE counteracted the CCl₄-induced toxicity from earlier stages (at least the third step).

Numerous studies have reported that antioxidants can prevent hepatic damage and nephropathy by counteracting free radicals and preventing lipid peroxidation [3, 29–31]. As a marker of lipid peroxidation, we measured the MDA levels. MDA is an end product of lipid peroxidation, which can react with sulfhydryl groups [32] and potentially crosslink proteins, reducing or abolishing the protein function [33]. Pretreatment with SE significantly prevented lipid peroxidation in the liver and kept it at almost the control level. This effect might be explained by the capacity of SE or an SE-induced gene product to increase the antioxidant activity.
MT is a low-molecular-weight protein with a high cysteine content, and it has been proposed to play a role in protecting against oxidative stress [34, 35]. For example, MT has been shown to be a scavenger of hydroxyl radicals in vitro, and cells with high levels of MT are resistant to radiation [36, 37]. MT production is induced in many organs and not only by various metals, such as Zn, Cd, and Cu, but also by non-metallic compounds [25, 38, 39]. Several investigations have reported that pretreatment with Zn or low doses of Cd (known inducers of MT) resulted in reduced lethality after administration of high doses of Cd [40, 41]. Moreover, Klaassen and Liu [42] have indicated that 14C-labeled CCl₄ or a CCl₄ metabolite binds to Zn-induced MT and protects against the CCl₄-induced hepatotoxicity. We have previously reported that Zn-induced MT (about 30 times higher than in the control) prevented the CCl₄-induced lethal toxicity in mice [23]. In contrast, MT-knockout mice were more susceptible than wild-type mice were to CCl₄-induced hepatotoxicity [43]. These data suggest that MT is involved in an adaptive mechanism to decrease the toxicity of CCl₄. Since Okada et al. have reported that SE has the potential to show radical-scavenging activity [20], we hypothesized that the prevention of CCl₄-induced lethal toxicity by SE is due to the induction of MT. This hypothesis is also supported by the fact that SE contains Zn and Cu (Table 1). However, we found no significant differences in the MT levels in the liver and kidney (Fig. 6). These results suggest that the protection provided by SE against the CCl₄-induced toxicity is not mediated by MT.

Most studies, including ours, used extracts obtained from S. veitchii leaves. The leaf extract consists of multiple components; however, the bioactive or pharmacologically active substance has not been identified. One possible bioactive molecule candidate is chlorophyll, which has documented antioxidant activity [44] and contains in abundance. Furthermore, Serpeloni et al. [45] have demonstrated that the cisplatin-induced oxidative stress is suppressed in mice treated with chlorophyll (0.5 mg/kg) for 13 days by oral gavage. Although the duration of SE administration was shorter in our study, the amount of chlorophyll in our SE was much greater than 0.5 mg per day. Therefore, chlorophyll in SE could contribute to the suppression of CCl₄-induced oxidative stress. Recently, many new flavonoids and polyphenols have been discovered in S. veitchii using advanced analysis techniques [17, 46, 47]. Since these compounds have antioxidant properties, it is possible that a flavonoid or polyphenol is the bioactive molecule. In addition, other antioxidant molecules derived from plants have been found [7, 8]. Further analysis of S. veitchii components will lead to the identification of the antioxidant substance.

In conclusion, our study demonstrates that S. veitchii leaf extract increases the BAP of plasma and protects
against CCl₄-induced mortality, in addition to significantly suppressing the CCl₄-induced hepatic and renal toxicity in mice. Further investigations will elucidate how SE components protect against CCl₄-induced lethal toxicity to allow SE to be used in the future for human health protection and disease prevention.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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