Protective Effect of a Lignan-Containing Flaxseed Extract against CCl₄-Induced Hepatic Injury

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ABSTRACT. Carbon tetrachloride (CCl₄)-induced hepatotoxicity is a commonly used model for investigating lipid peroxidation-related tissue injury. The involvement of free radical-mediated reactions in the development of CCl₄-induced hepatic injury has been implicated in various in vitro and in vivo studies [7]. Using electron spin resonance measurements, the formation of trichloromethyl and carbon dioxide anion radicals from CCl₄ metabolism has been demonstrated in perfused liver and in vivo [5]. These trichloromethyl and carbon dioxide anion radicals react with macromolecules and initiate lipid peroxidative reactions, resulting in the disruption of cellular and subcellular structures and subsequent generalized tissue damage [11].

As some antioxidants may scavenge free radicals and inhibit lipid peroxidation, treatment with several antioxidants such as vitamin E and thiopalmatic acid has been shown to protect against CCl₄-induced hepatic damage [2–4, 13]. In addition to chemically synthesized antioxidants, dietary antioxidants also protect against CCl₄-induced lipid peroxidation [28]. Recently lignans have attracted attention as effective antioxidants in the context of the relationship between diet and degenerative diseases such as cancer and cardiovascular diseases [10, 26, 31]. In addition to soybean [1], schisandra fruits [9] and whole grains [29], flaxseed is also known as a rich source of plant lignans [15]. Flaxseed contains relatively high amounts of secoisolariciresinol diglucoside (SDG) [17], which has protective effects against hypercholesterolemic arteriosclerosis, diabetes, and metastasis of cancer [12, 21, 20]. The antioxidative effect of flaxseed lignans on CCl₄-induced liver injury has not, however, been reported. In this study, we show how flaxseed extract also provides protection for the liver against CCl₄-induced necrosis.

KEY WORDS: carbon tetrachloride-induced liver injury, comet assay, flaxseed extract, glutathione, lignan.

MATERIALS AND METHODS

Chemicals and flaxseed extract: Reduced and oxidized glutathione (GSH and GSSG, respectively), N-ethylmaleimide (NEM), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), glutathione reductase and 2- and 4-vinylpyridine were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.) and stored at –20°C. Seed-oil (amani-oil) from flaxseed was purchased from Kanto-kagaku (Tokyo, Japan). Two hundred-ml of the oil was mixed with 1,000-ml of 80% methanol.

Animal treatment and histological procedure: Inbred strain of WKH/Hkm (WKH) rats were cared for according to the principles of the ‘Guide for the Care and Use of Laboratory Animals’ prepared by Rakuno Gakuen University. WKH rats (200–250 g) were maintained on a 12-hr light/dark cycle at 20°C and allowed food and water ad libitum. Animals were randomly assigned into four groups each of five animals. In the pretreatment group, animals were treated intragastrically with flaxseed extract at a daily dose of 1.6 g/kg body weight for three days. Control ani-
mals were given an equal volume of corn oil as the vehicle for the flaxseed extract. Twenty-four hours after the final dose, animals were administered with an intraperitoneal bolus of CCl₄ (2 g/kg). Control animals were given phosphate buffered saline (PBS) only. Twenty-four hours after injection of CCl₄, rats were ether-anaesthetized, sacrificed and autopsied. Hepatic tissue samples were excised after circulation with ice-cold PBS, rinsed with ice-cold PBS and used for measurement of glutathione content. DNA strand breaks or prepared for histological observations. For histological observation, the hepatic samples were fixed in 10% formalin for three days. Three-micrometer thick paraffin sections prepared by a routine procedure were stained with hematoxylin and eosin.

Measurement of glutathiones: The concentrations of GSH and GSSG were determined according to the method described by Tietze [30]. Briefly, rat liver homogenates were suspended in trichloroacetic acid were centrifuged at 17,000 × g for 15 min at 2°C. To assay for total glutathione, 700 μl of solution I (0.3 mM nicotinamide adenine dinucleotide phosphate (NADPH), 100 μl of solution II (6 mM DTNB) and glutathione sample were mixed in a final volume of 1.0 ml. Optical density at 412 nm was monitored and glutathione content was calculated. The GSSG content of tissue extracts was determined following preliminary reaction of the GSH contained therein with excess NEM. Following incubation with NEM (final concentration 0.02 M) for 40–60 min at 25°C, the solution was extracted at least 10 times with ether to ensure complete removal of the unreacted sulphydryl reagent, which is an inhibitor of glutathione reductase.

Measurement of DNA strand-breaks (comet assay): The comet assay of isolated hepatocytes was performed basically according to the method of Singh et al. [25] under alkaline conditions with slight modification. Briefly, about 200 mg liver tissue was mixed with 3 ml of hypotonic buffer (75 mM NaCl, 24 mM EDTA, pH 7.5), homogenized with Potter homogenizer, centrifuged at 700 rpm for 5 min, suspended in the hypotonic buffer, centrifuged and resuspended in the hypotonic buffer. The isolated liver cells were embedded in 1% low melting-point agarose (Life Technologies, Co., Ltd., Tokyo, Japan) and deposited on top of a 1% agarose base layer (Nakarai Techs Co., Ltd., Osaka, Japan) on a fully frosted slides (Matsunami Glass Indust. Ltd., Tokyo, Japan). After solidification of the agarose containing approximately 10⁶ of the cells, 1% agarose was deposited on the second layer. After solidification of the top layer of agarose the slides were placed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl, 1% Na-sarcosinate, 10% dimethyl sulfoxide and 1% Triton X-100, pH 10.0) for one hour at 4°C in a dark room. After lysis, cell membrane and cytosol were lysed and isolated nucleus were remained in the agarose. The slides were incubated in an electrophoretic buffer (0.3 M NaOH, 1 mM EDTA) for 30 min. Electrophoresis was carried out at 25 V and approximately 400 mA for 25 min at room temperature. The slides were neutralized in 0.4 M Tris-HCl solution (pH 7.5) for 20 min, stained with propidium iodide, and then photographed under a fluorescent microscope (Olympus Optical Co., Ltd., Tokyo, Japan). DNA strand breaks measured by this assay are expressed as the “tail momentum” which is the product of the fraction of DNA that has exited the nucleus multiplied by the distance migrated. An application program to calculate tail momentum was developed by Dr. Furusawa of the National Institute of Radiological Sciences using Interactive Data Language (IDL version 5.4, Adam Net Co., Tokyo) with the analytical protocol (the comet analysis program is provided free by Dr. Furusawa) [24].

RESULTS

In the rats without pretreatment of flaxseed extract, liver cell cords were disrupted, hepatocytes were changed into severe vacuolated degeneration and nucleus of some hepatocytes were less stained or disappeared, which indicated necrosis of hepatocytes, was induced 24 hr after injection of CCl₄ (Fig. 1C). Infiltration of several immune cells was observed. Number of necrotic cells was 276 in 1000 hepatocytes. In contrast, the extent of vacuolated degeneration of hepatocytes was lower in flaxseed-extract-pretreated CCl₄-injected rats than that in the CCl₄-injected rats without pretreatment (Fig. 1D). Infiltrated of immune cells were fewer in pretreated rats than that in the rats without pretreatment. Number of necrotic cells was 64 in 1000 hepatocytes. Frequency of necrotic cells was significantly (P<0.001 by χ² test) decreased from that in CCl₄-injected rat without pretreatment. Flaxseed extract per se showed almost no influence on the hepatic tissue (Fig. 1B).

Depletion of hepatic GSH has been shown to be associated with enhanced toxicity of chemicals including CCl₄. In our experiments, levels of hepatic GSH significantly decreased after intraperitoneal injection of CCl₄ (P<0.05). The CCl₄ treatment caused a 46.5% decrease in average hepatic GSH level (Fig. 2). On the other hand, the hepatic GSH level in flaxseed-extract-pretreated CCl₄-injected rats was not significantly different from that in untreated rats but significantly higher than that in CCl₄-treated rats without pretreatment (Fig. 1D). These data shows that flaxseed extract suppressed CCl₄-induced decrease of GSH. Similarly, GSSG content was decreased in the CCl₄-treated rats than that of control rats. When rats were pretreated with flaxseed extract and injected with CCl₄, GSSG level was not different from that in the control rats but significantly higher than that in the CCl₄-treated rats without pretreatment. As a consequence, average total glutathione contents decreased after CCl₄-treatment and this decrease was not observed in flaxseed extract-pretreated and CCl₄-injected rats.

Predicted from histological observations, hepatic cells were injured after CCl₄-injection. Both cell death and CCl₄-induced DNA breaks cause greater elongation of comets, which results in an increase of tail momentum in single-cell-gel-electrophoresis (Fig. 3). In this experiment, tail momentum was almost three times increased from that in control rats after injecting CCl₄ (P<0.001). This increase in
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Tail momentum was much smaller in lignan-fraction-treated rats. In the rats injected with flaxseed extract but not with CCl₄-injection, there was not shown significant difference of tail momentum. These data suggested that the pretreatment with flaxseed extract reduced frequency of DNA strand breaks induced by CCl₄ in the liver cells.

Fig. 1. Histological alterations of liver in CCl₄-injected and control WKHA rats. Liver tissue was fixed and stained with hematoxylin-eosin 24 hr after injection of CCl₄ injected into the intrapentoneal cavity of rats (C). Arrowhead shows necrotic cells, of which nucleus disappeared. Flaxseed extract was intragastrically injected for 3 days into two groups of rats (B and D). CCl₄ was injected in a group of rats after the preliminary injection of flaxseed extract (D). Untreated control was shown in A. Bars represent 50 µm.

Fig. 2. Effect of flaxseed extract and CCl₄ treatment on hepatic glutathione levels. Rats were orally injected with flaxseed extract or corn oil for 3 days and then intrapentoneally injected with CCl₄ or PBS. Open and closed bars represents content of reduced and oxidized glutathiones respectively (nmol/mg tissue weight). Each bar represents the mean ± standard deviation, n=3 (CCl₄-injected rats) or 4 (rats without CCl₄-injection).

Fig. 3. Effect of flaxseed extract and CCl₄ treatment on tail momentum of rat liver cells in comet images. Rats were orally injected flaxseed extract or corn oil for 3 days and then intrapentoneally injected with CCl₄ or with PBS. Each bar represents the mean ± standard deviation, n=3 (CCl₄-injected rats) or 4 (rats without CCl₄-injection).

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DISCUSSION

Flaxseed is one of the richest sources of plant lignans [15]. In the present study, we showed that pretreatment with flaxseed extract reduced the extent of CCl₄-induced liver-necrosis (Fig. 1) and DNA strand breaks (Fig. 3) of hepatic cells in comparison with untreated controls. These data suggest that flaxseed extract provides protection against CCl₄-induced injury of the liver cells. Reportedly, SDG, which is most abundant lignan in flaxseed, scavenges hydroxyl radicals in vitro [8, 19]. Flaxseed also contains isoflavonoids which are known to reduce H₂O₂-induced DNA strand breaks in human colon cells [18]. It has been reported that extract of Schisandra fruits, which also includes antioxidative lignans, also provides protection against CCl₄-induced hepatic injury [32]. Lignans from Saururus chinensis [26] and Machilus thunbergii [31] also protect CCl₄-injured primary cultures of rat hepatocytes. Lignins included in flaxseed appear to modulate oxidative damage through its antioxidative effects in the liver.

Comet assay has previously been used for the detection of cellular damage induced by toxic chemicals [23, 27]. This method can be used for small numbers of cells with relatively high sensitivity and provides quantitative index for DNA damages [6, 14, 16]. In the present study, we also detected a statistically significant difference between flaxseed-extract-pretreated and untreated CCl₄-injected rats from the small amount of liver tissue (Fig. 3). Since comet assay can be used for both peripheral blood cells and small biopsy samples, time-course of protective effects of flaxseed may be observed. Experiments for time-course study and long term-effects of flaxseed lins are now in progress.

Flaxseed extract may also protect in vivo the antioxidative system consisting of glutathiones (Fig. 2). Depletion of GSH provides evidence for the destruction of the protection system against lipid peroxidation [10]. Lignans have been shown to protect this defense system against oxidative damages through activation of GST and glutathione peroxidase [9, 31]. It has been reported that GSH in hepatic cells is depleted twenty-four hours following oral administration of CCl₄ and that this depletion is associated with a decrease in the hepatic activities of antioxidant enzymes [10]. This generalized impairment in the hepatic antioxidant defense mechanism was indication of hepatocellular damage. In this study, flaxseed extract protected against depletion of average levels of GSH. At the same time, the total amount of glutathiones may increased from that of control rats. Thus, flaxseed extract not only protect the oxidation of GSH, but also affect intracellular glutathione content. Mechanism of flaxseed extract for protection against GH depletion should be studied further.

As a preliminary analysis, the lignan composition of flaxseed extracts was estimated within 0.5% by HPLC analysis. Electron capture peaks were matching to five lignans, namely, SDG, secoisolariciresinol, lariciresinol, pinosylvin and matairesinol (data not shown). As we injected 1.6 g/kg/day of flaxseed extract, the amount of lignans given amounted to 8 mg/kg/day. This amount is comparable with those reported in experiments about protective effects of SDG [20–22]. In the amount of 15 to 22 mg/kg/day, SDG reduced serum cholesterol in rabbits [20], protect rats against streptozotocin-induced diabetes [22] and delayed the development of type II diabetes in Zucker rat [21] through its radical scavenging and antioxidative effects. The broad agreement of the injected amount of lignans between flaxseed extract in the present study and the studies using SDG provide a suggestion that liver-necrosis was suppressed by radical scavenging and antioxidative effect of SDG.

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REFERENCES


