
The effect of \textit{trans}-ferulic acid and gamma-oryzanol on ethanol-induced liver injury in C57BL mouse

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\textbf{Abstract}

The effects of the oral administration of \textit{trans}-ferulic acid and gamma-oryzanol (mixture of steryl ferulates) with ethanol (5.0 g per kg) for 30 days to C57BL mice on ethanol-induced liver injury were investigated. Preventions of ethanol-induced liver injury by \textit{trans}-ferulic acid and gamma-oryzanol were reflected by markedly decreased serum activities of plasma aspartate aminotransferase, alanine aminotransferase and significant decreases in hepatic lipid hydroperoxide and TBARS levels. Furthermore, the \textit{trans}-ferulic acid- and gamma-oryzanol-treated mice recovered ethanol-induced decrease in hepatic glutathione level together with enhancing superoxide dismutase activity. These results demonstrate that both \textit{trans}-ferulic acid and gamma-oryzanol exert a protective action on liver injury induced by chronic ethanol ingestion.

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\textbf{Keywords:} \textit{Trans}-ferulic acid; Gamma-oryzanol; Protective action on liver injury; Ethanol and oxidative stress

\section*{Introduction}

Reactive oxygen species (ROS) are involved in variety of diseases and also rise in the situation of toxic compound uptake such as ethanol (\textit{Koch et al.}, 2004). ROS are scavenged by the antioxidant defense system including enzymes such as superoxide dismutase (SOD) and antioxidants such as glutathione (GSH). The production of free radicals or ROS in the tissue exceeds the ability of the antioxidant system to eliminate the oxidative stress, causing some physiological disorders (Halliwell, 1997). Free radical production and alcoholic liver diseases are often associated with the development of immune reaction toward liver, involving the formation of hydroxyethyl free radicals generated during ethanol oxidation by CYP2E1 (\textit{Reinke et al.}, 1994). Ethanol consumption causes liver injury by inducing hepatotoxicity, oxidative stress (\textit{Albano et al.}, 1987; \textit{Zima et al.}, 2001) and decrease in antioxidant levels (\textit{Bondy and Dyer}, 1994).

A therapeutic strategy aimed at reducing hepatotoxicity through ethanol-induced oxidative stress has become highly sought-after, because liver is one of the most important organs that metabolize some chemical compounds for energy acquirements or detoxification. Curcumin is a phenolic phytochemical responsible for the yellow color of turmeric and important for minimizing pathological and toxic effects associated with ethanol-induced oxidative stress (\textit{Naik et al.}, 2004). Hydroxy cinnamic acid derivatives such as 4-hydroxy-3-methoxycinnamic acid (\textit{trans}-ferulic acid) and its steryl
esters (gamma-oryzanol) also have similar phenolic structure. Resources of trans-ferulic acid and gamma-oryzanol are potentially enormous because of their ubiquitous distribution in primary plant cell walls and crop bran (Tanaka, 1971; Tanaka and Koto, 1975; Xu and Godber, 1999). Trans-ferulic acid and gamma-oryzanol also have some physiological activities such as inhibition of tumor promotion (Yasukawa et al., 1998), reduction of serum cholesterol levels (Guardiola et al., 1996) and antioxidant properties in several oil models (Xu et al., 2001; Nystrom et al., 2005). The objective of this study was to analyze the effects of the oral administration of trans-ferulic acid and gamma-oryzanol on ethanol-induced liver injury in mice.

Materials and methods

Materials

Trans-ferulic acid, 2-thiobarbituric acid and 2,6-di-tert-butyl p-cresol (butyl hydroxytoluene, BHT) were purchased from Tokyo Kasei Kogyo Co., Ltd (Tokyo, Japan). Gamma-oryzanol was purchased from Oryza oil & Fat chemical Co., LTD (Aichi, Japan), which contained cycloartenyl ferulate (29.5%), 24-methylene cycloartanyl ferulate (24.1%), campesteryl ferulate (19.2%), \( \beta \)-sitosteryl ferulate (14.7%) and other components of 604 and 616 in molecular weight (12.5%). Averaged molecular weight was 600.2. The chemical formulae of trans-ferulic acid and main components in gamma-oryzanol are shown in Fig. 1. Dimethyl sulfoxide, cytochrome C, superoxide reductase, xanthin oxidase, xanthine, glutathione, \( \alpha \)-phthaldialdehyde (OPT) and 2,4 dinitro phenylhydrazine (DNPH) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Phosphate buffer saline (PBS) powder was purchased from Nissui Pharmaceutical Co., LTD (Tokyo, Japan). Triphenylphosphine (TPP) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Japan (Tokyo, Japan). 3-PeDPP was prepared from 3-bromoperylene and TPP and identified as previously described (Chotimarkorn et al., 2005a). The purified 3-perylene diphenylphosphine (3-PeDPP) was kept as the solid under \( \text{N}_2 \) gas in the dark at \(-20^\circ\text{C}\) until use.

Animals

Male c57BL/6J wild-type mice (8 weeks of age, 19–23 g) were obtained from Clea Japan, Inc (Tokyo, Japan). The mice were kept (\( n = 5 \) each case) in room with 50% relative humidity and a 12/12 h light/dark cycle at 20–22 °C. Experimental mice (\( n = 90 \)) were fed with water and the commercial diet type MF from Oriental Yeast Co., Ltd (Tokyo, Japan) for 1 week until there were 9 weeks old. Ninety mice were randomly divided into six groups as follows: group I, negative control mice (\( n = 15 \)), were given daily dose of distilled water (5.0 g per kg) orally with an orogastric tube for 30 days and group II, positive control mice (\( n = 15 \)), were given daily dose of ethanol (5.0 g per kg) orally with an orogastric tube for 30 days. Group III (\( n = 15 \)) were administered daily dose of trans-ferulic acid (0.025 mmol) with ethanol (5.0 g per kg) for 30 days. Group IV (\( n = 15 \)) were given daily dose of gamma-oryzanol (0.025 mmol) with ethanol (5.0 g per kg) for 30 days. Group V (\( n = 15 \)) were given daily dose of trans-ferulic acid (0.025 mmol) for 30 days. Group VI (\( n = 15 \)) were given daily dose of gamma-oryzanol (0.025 mmol) for 30 days. After sacrificing mice with diethyl ether vapor, the main artery was immediately perfused with
PBS containing heparin to remove red blood cells and clots from the livers, and the livers were excised out. Each liver was stored at −85°C for the following chemical and biochemical measurements. All experiments with animals were approved by the Animal Care Committee of Tokyo University of Marine Science and Technology and performed in accordance with the guidelines of the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Liver homogenate preparation

The livers were homogenized in cold PBS (1:10 w/v) at 4°C for 10 min by Ace homogenizer model AM-3 (Nihonseiki Kaisha, Tokyo, Japan). Cell debris were removed by centrifugation at 1500g for 10 min with a Kubota model 5700 (Tokyo, Japan) at 4°C. The resulting supernatants were subsequently used for the following analyses.

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) assay

AST and ALT assay were measured according to the method of Reitman and Frankel (1957) by using DNPH as coloring reagent.

Superoxide dismutase (SOD) activity assay

SOD activity was measured according to the method of McCord and Fridovic (1969). One unit of SOD was defined as the enzyme, which was required to inhibit 50% of the reduction rate for cytochrome c per mg of protein.

Determination of GSH

GSH in liver tissue was measured according to the method of Hissin and Hilf (1976) using OPT. The reaction is based on formation of the fluorescent product between OPT and GSH. The fluorescence intensity of the fluorescent product was determined with an Olympus IX70 inverted microscope (Olympus, Tokyo, Japan) as objective lens and an Olympus UMNBV2 cube filter unit (420–440 nm for excitation, 475 nm for emission). Image data were digitally acquired with an Olympus model C-3040 Camedia digital camera equipped with a NY-2000S eyepiece-mount adapter (MeCan Imaging Inc., Saitama, Japan) according to the manufacture’s instructions. The acquired data were converted into 8-bit gray scale digital data and gray scale pixel densities were calculated with an ImageJ software (National Institutes of Health, Bethesda, MD). The resulting mean pixel densities were used as the fluorescence intensities at arbitrary unit (a.u.).

Statistical analysis

Data were analyzed by one way analysis of variance (ANOVA) followed by Duncan’s multiple range test (DMRT) using a commercially available statistic software package (SPSS for windows, V. 13.0, Chicago, USA). Results were presented as mean ± S.D. p-Value <0.01 were regarded as statistically significant.

Results

Administration of daily dose of ethanol (5.0 g/kg orally) for 30 days to c57BL mice caused significant
increase in serum enzymes such as AST and ALT (235.4 ± 42.1 and 122.4 ± 28.5 IU/L), compared to control (57.8 ± 25.6 and 42.3 ± 27.4 IU/L), trans-ferulic acid (53.4 ± 23.4 and 47.3 ± 22.1 IU/L), gamma-oryzanol (60.3 ± 21.1 and 49.5 ± 18.9), co-administration of trans-ferulic acid (80.3 ± 25.2 and 53.4 ± 22.4 IU/L) and gamma-oryzanol (92.5 ± 33.4 and 59.8 ± 18.9 IU/L) with ethanol for 30 days (Fig. 2).

Co-administration of trans-ferulic acid or gamma-oryzanol to c57BL mice with ethanol for 30 days showed a potent inhibition of ethanol stimulated lipid peroxidation and oxidative stress in liver. 3-PeDPP was also addressed to examine directly the lipid hydroperoxide production in liver tissue of ethanol treated c57BL mice. Markedly strong increases in 3-PeDPP fluorescence were found in liver tissues of daily dose of ethanol (Fig. 3A), which reflected high levels of lipid hydroperoxide. The lower fluorescence intensities of 3-PeDPO liver in control treatment, trans-ferulic acid, gamma-oryzanol, co-administration of trans-ferulic acid, gamma-oryzanol with ethanol for 30 days were observed, which illustrated low levels of lipid hydroperoxide in the tissues (Fig. 3B–F). 3-PeDPO fluorescence were 36.9 ± 15.8, 40.1 ± 12.1, 42.4 ± 11.1, 118.1 ± 35.4, 75.2 ± 38.7 and 83.6 ± 25.3 for control treatment, trans-ferulic acid, gamma-oryzanol, ethanol, ethanol plus trans-ferulic acid and ethanol plus gamma-oryzanol, respectively (Fig. 3G).

TBARS levels were significantly lower in liver, 0.48 ± 0.28 nmol/g sample for the control group, 0.54 ± 0.18 nmol/g sample for trans-ferulic acid, 0.50 ± 0.17 nmol/g sample for gamma-oryzanol, 0.83 ± 0.22 nmol/g sample for the ethanol plus trans-ferulic acid group and 0.92 ± 0.45 nmol/g sample for the ethanol plus gamma-oryzanol group, than those of ethanol-treated group, 1.85 ± 0.34 nmol/g sample (Fig. 4).

Administration of trans-ferulic acid (14.1 ± 2.8 nmol/mg protein) and gamma-oryzanol (14.3 ± 1.8 nmol/mg protein) maintained GSH levels of control treatment (14.2 ± 2.2 nmol/mg protein), co-administrations of trans-ferulic acid and gamma-oryzanol plus ethanol (13.6 ± 3.1 and 14.4 ± 3.6 nmol/mg protein, respectively) were significantly higher than those of daily dose of ethanol (5.5 ± 0.4 nmol/mg protein) (Fig. 5). SOD activities of co-administrations of trans-ferulic acid and gamma-oryzanol plus ethanol were 125.3 ± 27.4 and 98.8 ± 22.6 unit per mg protein, respectively, compared to 53.5 ± 12.5 unit per mg protein for ethanol treated group. The SOD activities of control, trans-ferulic acid and gamma-oryzanol groups showed levels similar to one another, 92 ± 25.8, 94.5 ± 17.9 and 90.7 ± 17.0 unit per mg protein, respectively (Fig. 6).

Discussion

Oxidative stress is the result of an increase in intracellular pro-oxidant species such as hydrogen peroxide (H2O2), hydroxyl radical (OH-) and superoxide anion radical (O2•−). High intracellular concentrations of these molecules lead to promotion of lipid peroxidation in tissue. Indeed, ethanol-induced liver damage is thought to be due to oxidative stress that results from ethanol consumption (Cahill et al., 2002). The mechanisms underlaying alcohol-induced hepatotoxicity are complex and have many factors. Several studies have suggested that several oxygen-generating radicals, which remove GSH from tissue (Dupont et al., 2000; Masalkar and Abhang, 2005), inhibit glutathione synthesis (Videla et al., 1991; Oh et al., 1997), and impair the antioxidant defense system (Nordmann et al., 1992), and might play a role in causing alcoholic liver disease, as 95% of ingested ethanol is metabolized in liver (Lieber, 1997).

The therapeutic strategy aimed at reducing hepatotoxicity of ethanol-induced oxidative stress has become highly sought-after, because liver is one of the most important organs which metabolize some chemical compounds for energy acquirement or detoxification. In particular, the use of antioxidants such as vitamin E, glutathione prodrugs and curcumin is an important preventive method of minimizing pathological and toxic effects associated with oxidative stress (Kunchandy and Rao, 1990; Lee et al., 1995; Meister, 1998). Ferulic acid and its sterol ferulate are found ubiquitously in plant cell
walls and crop bran (Wallace and Fry, 1994; Colquhoun et al., 1994) and have antioxidant activities (Xu and Godber, 1999; Xu et al., 2001; Nystrom et al., 2005; Bourne and Rice-Evans, 1997; Hiramitsu and Armstrong, 1991; Kanski et al., 2002). This paper is the first report that ferulic acid and its steryl ferulate are useful for preventing the liver injury by ethanol ingestion.

The abnormal higher level of serum AST and ALT observed in ethanol treated mice in this study (Fig. 2) is the consequence of daily dose of ethanol. Trans-ferulic acid and gamma-oryzanol gave a high hepatoprotective effect of reducing the increased AST and ALT activities by ethanol. The observed significant decreases in the activities of these enzymes suggest that trans-ferulic acid and gamma-oryzanol preserve the liver injury by the toxic effects of daily dose of ethanol such as curcumin.

The hepatic lipid peroxidation was associated with chronic ethanol administration, as an indicator of oxidative stress. The present results showed a significant ($p < 0.01$) decrease in LOOH levels in the hepatic tissue of trans-ferulic acid- or gamma-oryzanol-treated mice with oxidative stress induced by a daily dose of ethanol as shown in Fig. 3B and C. The 3-PeDPP is a non-fluorescent compound, but when it is oxidized to 3-PeDPPO, the resultant oxidation products exhibit dramatically increased fluorescence intensity, with the excitation (440 nm) and emission (470 nm) wavelengths (Chotimarkorn et al., 2005a, b, 2006). In agreement with this finding, co-administration of trans-ferulic acid and
gamma-oryzanol showed significantly decreased levels of TBARS (p<0.01), compared to only ethanol administration (Fig. 3). These observations demonstrate the antioxidant effects of \textit{trans}-ferulic acid and gamma-oryzanol against ethanol-induced oxidative stress. These results were consistent with several previous reports showing that \textit{trans}-ferulic acid decreased lipid peroxidation with an antioxidation in bulk and emulsion of methyl linoleate (Nystrom et al., 2005), synaptosomal and neuronal cell culture (Kanski et al., 2002), and low density lipoprotein (Bourne and Rice-Evans, 1997). \textit{Trans}-ferulic acid can readily form a resonance-stabilized pheroxyl radical, which accounts for its potent antioxidant activity (Kanski et al., 2002). On the other hand, the gamma-oryzanol was a mixture of at least 10 types of phytosterol ferulate (Xu and Godber, 1999), but antioxidative mechanisms of gamma-oryzanol remain unclear. Kikuzaki et al. (2002) reported that cycloartenyl and 24-methylene cycloartanyl ferulates showed no activity in the liposomal system and suggested that their bulky structures might hinder their contact with lipid bilayers in liposomes. However, it has been found that gamma-oryzanol exhibits antioxidant properties in many types of \textit{in vitro} model systems, such as cholesterol oxidation accelerated by 2,2’-azobis 2-methylpropionamide (Xu et al., 2001), porcine retinal homogenate oxidation accelerated by ferric ion (Hiramitsu and Armstrong, 1991), pyrogallol autoxidation (Kim and Godber, 2001) and pharmaceutical oils (Juliano et al., 2005). Based on these previous reports, it seems reasonable to consider that gamma-oryzanol would also function as an antioxidant against ethanol-induced liver injury.

The tissue antioxidant status under ethanol administration as a model of hepatotoxic and oxidative damage \textit{in vivo} was investigated in this study, since impairments of enzymatic in antioxidative defense systems have been...
shown in alcoholics (Nordmann et al., 1992). The significant increase in hepatic GSH level was observed in co-administration of trans-ferulic acid or gamma-oryzanol with ethanol, compared to the daily dose of ethanol (Fig. 4). GSH plays an important role of the non-enzymatic ROS scavenging in the tissues, maintaining adequate hepatocyte viability, and preventing liver injury (Artell et al., 2003). As shown in Fig. 5, significantly increasing hepatic SOD activities in trans-ferulic acid and gamma-oryzanol groups suggest that these compounds may improve an antioxidative response of defense system. The similar increase in SOD activity has been observed in our recent study for effects of gamma-oryzanol on macrophage cell line RAW 264.7, though cell types were different from each other (Nagasaka et al., 2007). Mechanisms for the activation or induction of SOD are now investigated and will be described elsewhere.

In conclusion, the present study demonstrates that oral administrations of trans-ferulic acid and gamma-oryzanol with potentially huge resources on earth exert a protective action on liver injury induced by chronic ethanol ingestion.

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References


