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Hepatoprotective effect of chitosan-caffeic acid conjugate against ethanol-treated mice

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ABSTRACT

The chitosan-caffeic acid (CCA) conjugate shows a hepatoprotective effect against oxidative stress-induced hepatic damage in cultured hepatocytes. The objective of this study is the verification of the hepatoprotective effect of the CCA *in vivo* against ethanol-induced liver injury in mice. The administration of ethanol resulted in the increase of the serum-aminotransferase activities (AST and ALT), triglycerides, total cholesterol, and lipid peroxidation. The CCA co-administration, however, significantly ($p < 0.05$) ameliorated these serum biomarkers. The antioxidant-enzyme activities in the liver tissue, including those of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), were significantly decreased by a chronic ethanol administration, whereas the hepatic lipid-peroxidation level was increased. Moreover, the chronic ethanol administration elevated the gene expression of pro-inflammatory cytokines such as tumor-necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in the liver tissue. The CCA co-administration, however, significantly ($p < 0.05$) increased the activities of the SOD, CAT, and GPx and caused the down-regulation of the TNF-α- and IL-6-gene expressions in the liver tissue. An histopathologic evaluation also supported the hepatoprotective effect of the CCA against ethanol-induced hepatotoxicity in the mice.

1. Introduction

The liver is an important organ that metabolizes endogenous and exogenous toxicants and is capable of metabolizing alcohol. The chronic consumption of alcohol causes a wide range of health problems, which lead to morbidity and mortality (Jung et al., 2016). The accumulating evidence has demonstrated that alcohol-induced liver damage may be associated with an increased oxidative damage due to the overproduction of reactive oxygen species (ROS) (Jung et al., 2016; Muller et al., 2013; Ozaras et al., 2003); therefore, agents that are capable of decreasing oxidative damage, represent promising therapeutic interventions for alcoholic liver damage. An array of studies have demonstrated that antioxidants are effective against alcohol-induced liver damage through a decreasing of ROS level (Je et al., 2013; Kasdallah-Grissa et al., 2007; Ozaras et al., 2003; Zhang et al., 2008).

Chitosan is a naturally occurring biopolymer composed of *N*-acetyl-D-glucosamine and D-glucosamine units and is obtained by the deacetylation of chitin. The biocompatibility, biodegradability, and less toxic nature of chitosan have led to the biomedical application, and it also exerted versatile bioactivities such as antioxidant, antimicrobial, anticancer, and anti-inflammatory effects (Lee and Je, 2013; Lee et al., 2014; Muzzarelli and Muzzarelli, 2005; Park et al., 2004). In addition, the chemical and/or enzymatic modification of chitosan for the enhancement of its chemical and physical properteis, such as solubility and bioactivities, is in development; for example, the conjugation of naturally bioactive molecules such as polyphenols onto chitosan leads to enhancements of the bioactivities and solubility in water (Aytekin et al., 2011; Lee and Je, 2013; Lee et al., 2014; Oh et al., 2016). Our previous work have demonstrated that the CCA exerted more-effective hepatoprotective effect against oxidative stress-induced hepatocyte damage *in vitro* compared to native chitosan (Ahn et al., 2016); however, *in vivo* evaluation of the CCA against oxidative stress-induced hepatotoxicity needs to be verified before the biomedical application can proceed. In the present study, the potentially beneficial effect of the

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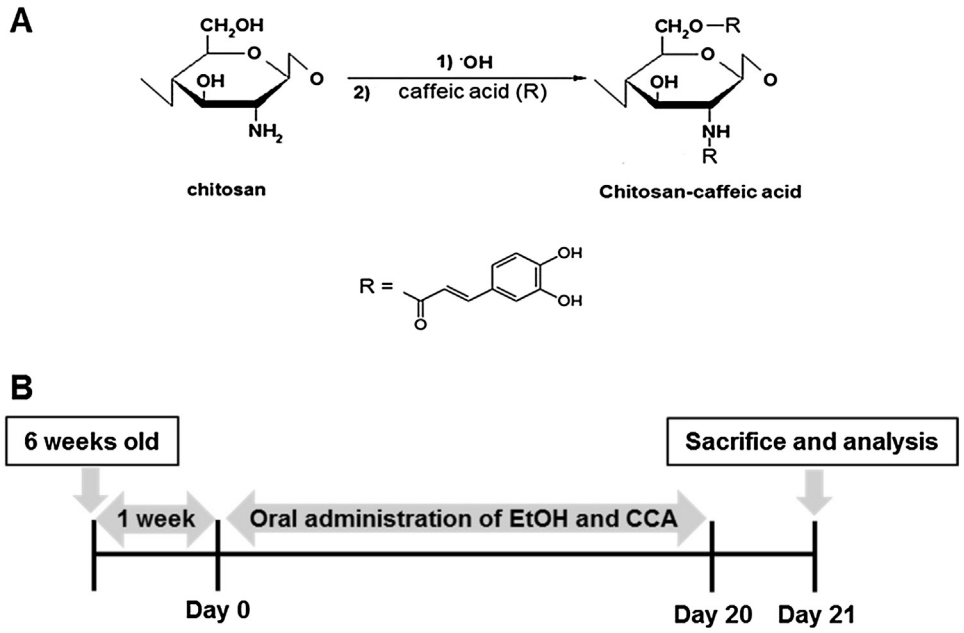

Fig. 1. Schematic diagram for (A) the preparation of the chitosan-caffeic acid conjugate (CCA) and (B) experimental design for hepatoprotective effect of CCA on ethanol-induced hepatotoxicity in mice.

CCA against the subacute alcohol-induced hepatic damage that is associated with liver injury and inflammation was investigated in Balb/c mice.

2. Material and methods

2.1. Preparation of the CCA

The chitosan was donated by Kitto Life Co. (Seoul, Korea). The average molecular weight and the degree of deacetylation are 310 kDa and 90%, respectively. The CCA conjugate was prepared using the optimal molar ratio of the chitosan residue (1 mol) and the caffeic acid (0.1 mol) according to the authors' previous method (Fig. 1) (Lee et al., 2014). The unreacted caffeic acid was removed by dialysis using a 1000 Da dialysis tube for 48 h, followed by lyophilization. The successful preparation of the CCA conjugate was confirmed using the ^{1}H NMR according to the previous result of Lee et al. (2014) and 7.81 mg/g of the caffeic acid was detected in the CCA using the Folin-Ciocalteau method. CCA is well soluble in distilled water up to 10 mg/mL.

2.2. Animals

The experimental methods were performed according to the National Institute of Health Guide for the Care and Use of Laboratory Animals, and they were approved by the institutional animal-care-and-use committee at Chonnam National University. The Balb/c mice (6 weeks old, 19 g to 21 g body weights, either sex) were obtained from Orient Bio Inc. (Seongnam, Korea). The animals were acclimated to temperature (22° C ± 2° C)- and humidity (55% ± 5%)-controlled rooms with a 12 h light-dark cycle for 1 week prior to use. The animals were fed tap water and a standard laboratory diet.

2.3. Experimental design

The Balb/c mice were randomly assigned to normal group (saline), ethanol (4 g ethanol/kg mice), CCA10 (4 g ethanol plus 10 mg CCA/kg mice), CCA20 (4 g ethanol plus 20 mg CCA/kg mice), and silymarin (4 g ethanol plus + 50 mg silymarin/kg mice). Mice were given free access to water and food. Ethanol was diluted in saline. CCA and silymarin (Sigma Chemical Co., St. Louis, MO, USA) were dissolved in 0.5% carboxymethyl cellulose (Sigma) and were orally administrated. During the experiment period, the body weights and survival rates were investigated every 2 days. After 20 days, the mice were anesthetized, and blood samples were collected into heparin tubes to determine the biochemical parameters (Fig. 1). The livers were frozen in liquid nitrogen and stored at −70 °C until the analysis. The livers were homogenized in five volumes of ice-cold homogenization buffer (250 mM sucrose, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA). The homogenates were centrifuged at 1000 × *g* for 10 min, and the supernatants were centrifuged at 12,000 × *g* for 30 min. The final supernatant-protein concentration was measured using the method of Bradford (1976) for which the bovine-serum albumin is used as the protein standard.

2.4. Serum biochemical analysis

The activities of the aspartate aminotransferase (AST) and alanineaminotransferase (ALT) in the plasma samples were determined using an enzymatic-analysis kit (Asan Pharmaceuticals, Hwasung, Korea) according to the Reitmen-Frankel method (Reitman and Frankel, 1957). The total cholesterol content in the serum was determined using a commercially available kit (Asan Pharmaceuticals, Hwasung, Korea) according to the method of Allain et al. (1974).

2.5. Determination of lipid peroxidation

The lipid-peroxidation concentrations in the serum and liver homogenates were determined by measuring the thiobarbituric-acid reactive substances (TBARS) based on the method of Ohkawa et al. (1979). Briefly, the serum or liver homogenates were mixed with thiobarbituric acid (TBA), followed by an incubation in boiling water for 30 min. After a centrifugation at 1000 × *g* for 10 min, the resulting colored upper layer was measured as 532 nm. The TBARS concentration was expressed as the nmol of malondialdehyde (MDA) per ml of serum or per mg of protein.

2.6. Determination of antioxidant enzyme activities in liver

The antioxidant-enzyme activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) in the liver homogenates were determined using a commercially available assay kit (Biovision, Milpitas, CA, U.S.A.) according to the manufacturer's instructions.

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