Hepatoprotective Effect of Chitosan Oligosaccharides on CCl₄-induced Liver Injury in Rats

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Abstract
Various toxic materials cause hepatocellular injury and finally hepatic fibrosis or cirrhosis. It is well known that development of hepatic fibrosis is mediated by hepatic stellate cells (HSCs) and transforming growth factor-betas (TGF-βs) signalling mechanism. The aim of this study is to demonstrate hepatoprotective effect of chitosan oligosaccharides (COS) using carbon tetrachloride (CCl₄) intoxicated rat model. For this study, three groups of animals were prepared. Group A was given corn oil only, but group B and C were given CCl₄ once a week for 4 weeks. And group C was additively given COS daily for 4 weeks. After then, serological, histological, and western blot analyses were performed. COS treatment prevented increment of liver weight and elevation of serum ALT level. It also reduced hepatocellular damages and prevented development of hepatic fibrosis and collagen deposit. The immunohistochemistry and western blot analysis showed that COS treatment decreased activation of HSCs and expression of type I TGF-β receptor. These results suggest that COS effectively protects liver from CCl₄-induced hepatocellular injuries and hepatic fibrosis.

Keywords: chitosan oligosaccharides (COS), carbon tetrachloride (CCl₄), liver, fibrosis, hepatoprotection

INTRODUCTION
Progressive hepatic fibrosis is a main cause of the hepatic failure in chronic viral or toxic hepatitis. It is reported that hepatic fibrosis results from various causes such as infection of hepatitis B or C virus, administration of toxins or drugs, inadequate hemodynamics and autoimmune diseases. Factors that play the key roles in the process of hepatic fibrosis are activation of the hepatic stellate cells (HSCs) and increased secretion of transforming growth factor beta 1 (TGF-β1) (1-3).

Among various hepatotoxic chemicals, CCl₄ is commonly used in many studies to induce acute toxic hepatitis or chronic toxic hepatitis accompanying with hepatic fibrosis (4-10). Mechanisms of CCl₄-induced hepatotoxicity are various (11-12). Most important mechanism is that CCl₄ produces reactive radicals such as trichloromethyl and trichloromethyl peroxyl radicals and reactive aldehydes such as trans-4-hydroxy-2-nonenal (HNE). These radicals induce dysfunctions of various cellular components, inhibition of lipoprotein secretion and chain reaction of lipid peroxidation. Moreover, CCl₄ activates various cytokines such as tumor necrosis factor alpha (TNF-α), nitric oxide (NO) and TGFs to direct toward cellular destruction or tissue fibrosis. Fortunately, various antioxidants such as vitamin E, ascorbic acid, reduced glutathione, butylated hydroxy anisole and sulphhydryl compounds protect against lipid peroxidation (13). These antioxidants enhance the antioxidative defensive system against the reactive radicals which can induce tissue injuries.

Chitosan is also considered as one of the antioxidative materials. It is a glucosamine polymer obtained by deacetylation of chitin, which is found in the crustacean exoskeleton. Chitosan has various biological functions and high level of safety. Thus chitin and chitosan are widely used in many studies for various purposes such as wound dressings, gene carrier molecules, anticancer, hypcholesteremic, immunostimulating, anticoagulating and antimicrobial agents (14).

There are some reports about the effects of the chitosan and COS on liver functions. Low molecular weight chitosan (70 kDa) increases 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) activity and HMG-CoA RNA transcription and decreases cholesterol from plasma and liver without any adverse effects (15). Low molecular weight chitosan (9 kDa) scavenges oxidative radicals more effectively than high molecular weight chitosan (760 kDa) (16). Chitosan also has antioxidative effects on CCl₄-induced liver injury (17). Pretreatment with COS brings out hepatoprotective effects on acute CCl₄ intoxication (18). In addition, COS (1 ~ 3 kDa and 3 ~ 5 kDa) protects the liver from the 2, 3, 7, 8-tetrachlorodibenzo- p-dioxin-induced oxidative stress by increasing the activity of glutathione S-transferase and glutathione peroxidase and the hepatic content of glutathione (19).

However, there is no report about the hepatoprotective and
antifibrogenetic effects of COS on prolonged and repetitive toxic liver injury. For this reason, we aimed to investigate effects of COS on repetitive CCl4-induced liver injury and hepatic fibrosis. Alterations of the serological markers, histological findings and molecular factors such as the expression of TGF-βs and their receptors were evaluated to explain the hepatoprotective and antifibrogenetic effects of COS.

MATERIALS AND METHODS

Preparation of COS

COS was provided by professor Jung Woo Kim of Seonam University and it was prepared from low molecular weight chitosan showing more than 98% of deacetylation and lower than 10 cps viscosity (20). COS was prepared by enzymatic method and it was composed of monomer (7.8%), dimer (0.8%), trimer (13.1%), tetramer (18.5%), pentamer (21.3%), hexamer (30.3%), and more than heptamer (8.2%) by HPLC analysis.

Preparation of Animals

Eight-week-old 15 male Sprague-Dawley rats (Dea Han Biolink, Co., Ltd., Daejeon, Korea) were housed under controlled temperature (23 ± 2°C), humidity (55 ± 5%), and dark-light cycle (12-12 hours). Animals were allowed tap water and standard food ad libitum. For this study, rats were randomly subdivided into three groups; normal control group (group A), negative control group (group B), and experimental group (group C). Group B and C were given CCl4 (Sigma, USA, 1 mg/kg, in corn oil 1:1, v/v, 10:00 p.m.) once a week for 4 weeks by oral gavage. But group A were given only same volume of corn oil (Sigma, USA). At 12 hours after the CCl4 administration, group C was daily administered COS (100 mg/kg, 10:00 a.m.) once a week for 4 weeks. All kinds of animal treatments, including CCl4 and COS administration, were carried out at the same time of a day. One day after the last treatment of COS, animals were sacrificed under ether anesthesia. All experimental procedures were carried out in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals.

Serum enzyme analysis

Whole blood sample was collected by puncture of the right ventricle, and then serum was separated. All samples were stored at −80°C for serum enzyme analysis. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and albumin were measured by serum analyser (CIBA Corning Diagnostics Corp., USA).

Histology

The whole liver was rapidly excised after perfusion of 4°C normal saline, and then weight of each liver was measured. Acquired liver tissues were fixed in the 4°C neutral buffered formalin for 24 hours and embedded in paraffin. Tissue blocks were cut at 5 µm and tissue slices were attached on the slide glass coated with 3-aminopropyl triethoxysilane (Sigma, USA). Hematoxylin-eosin stain and Masson's trichrome stain were done to compare the histological features and the deposition of the extracellular matrix.

Immunohistochemistry

Tissue slices were deparaffined and hydrated with xylene and serial ethanol. To block the endogenous peroxidase, 3% H2O2 (in 60% methanol) was applied on the tissue slice for 15 minutes. To avoid the nonspecific antigen-antibody reaction, tissue slices were treated with the protein blocking agent (ThermoShandon, USA) for 1 hour, and then they were incubated overnight at 4°C with diluted primary antibodies. Biotinylated secondary antibody (ThermoShandon, USA) and streptavidin peroxidase complex (ThermoShandon, USA) were attached for 1 hour and 30 minutes, respectively, at room temperature. Immunolabeling was visualized with the 0.2% diaminobenzidine solution (Sigma, USA). Between each process, tissue slides were rinsed with phosphate buffered saline (pH 7.4) three times for ten minutes. After immunolabeling process, tissue slices were briefly stained with hematoxylin and sealed with cover glass. Primary antibodies used in immunohistochemistry were anti-TGF-β1, anti-TGF-β2, anti-TGF-β3, anti-type I TGF-β receptor, anti-type II TGF-β receptor, anti-caspase 3 (SantaCruz Biotechnology, USA), anti-alpha smooth muscle actin (Sigma-Aldrich, USA), and anti-collagen type I (Chemicon, USA).

Western Blot Analysis

Fifty milligram of unfixed fresh liver tissue of each animal was homogenized and then, crude protein was extracted with 1 ml of modified RIPA buffer (50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 mM Na3VO4, 1 mM NaF, 1× protease inhibitor cocktail) during one hour at 4°C on the rocking platform. After centrifugation for 30 minutes at 4°C and 20,000 x g, the supernatant was separated and stored at −80°C.

Protein concentration of each supernatant was measured by the Bradford method (21). Protein samples (30 µg) were loaded on 10% sodium dodecyl sulfate polyacrylamide gel and electrophoresed. Separated proteins were transferred to the polyvinylidene fluoride (PVDF) membrane (Millipore, USA) with the Semi-Dry Transfer Cell (Bio-Rad Laboratories, Inc., USA). Membranes were treated with 5% nonfat dry milk in Tris buffered saline containing 0.1% Tween-20 (TBS-T, pH 7.6) for 1 hour to inhibit nonspecific antigen-antibody reactions. Primary antibody diluted in TBS-T at optimal concentration was attached to the membranes overnight at 4°C. The horseradish