Effect of Chitosan Oligosaccharide on 2,3,7,8-Tetrachlorodibenzo-p-dioxin-Induced Oxidative Stress in Mice

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The abilities of two types of chitosan oligosaccharides, chitosan oligosaccharide I (1-kDa< MW<3-kDa) and chitosan oligosaccharide II (3-kDa< MW<5-kDa), to prevent 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced oxidative stress were examined in ICR mice. Chitosan oligosaccharide I had no effect on TCDD-induced alterations in lipid peroxidation and glutathione S-transferase activity or liver weight change. However, mice treated with chitosan oligosaccharide II were protected from TCDD-induced lipid peroxidation, inhibition of glutathione peroxidase and glutathione S-transferase activities, and losses in body and liver weights. These results suggest that chitosan oligosaccharide might be a potential agent for combating TCDD-induced pathogenesis.

Key words chitosan oligosaccharide; glutathione peroxidase; glutathione S-transferase; lipid peroxidation; 2,3,7,8-tetrachlorodibenzo-p-dioxin

Polyhalogenated aromatic hydrocarbons (PAHs) are highly persistent environmental contaminants that pose a potential risk to human health. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is the prototypical representative of these widely dispersed pollutants and one of the most potent toxins and tumor promoters known to man. TCDD exposure in experimental animals results in an array of tissue and species-specific responses, including the following: dermal toxicity, immunotoxicity, hepatotoxicity, carcinogenicity, teratogenicity, and neurobehavioral, endocrine, and metabolic alterations.1–4) Recent studies have demonstrated that oxidative stress occurs in various tissues of TCDD-treated animals and is considered an important mechanism in the toxicity of TCDD.2,3) Oxidative stress following TCDD exposure in laboratory animals has been demonstrated to increase the production of reactive oxygen species, lipid peroxidation, DNA and membrane damage, and possible enzyme inhibition.2–4) Chitooligosaccharides are known to be biologically active compounds.5) Chitohexaose and its N-peracetylated derivative were shown to inhibit the growth of murine cancer cells.6) N-Acetylichitohexose protected against Candida albicans infection and increased the activities of macrophages, T lymphocytes, and natural killer cells in tumor-bearing mice.5) The current authors previously demonstrated the effects of chitosan oligosaccharides on enzymes for cancer chemoprevention8) and various chemical mutagens.9–9) In this study, the effects of two types of chitosan oligosaccharides, chitosan oligosaccharide I (1-kDa< MW<3-kDa) and chitosan oligosaccharide II (3-kDa< MW<5-kDa), on the oxidative stress resulting from exposure to TCDD were investigated in mice.

MATERIALS AND METHODS

Materials Water soluble chitosan oligosaccharides were prepared from 1% chitosan by an ultrafiltration membrane reactor system (Millipore Minitan system; molecular weight cut-off 5000, 3000, and 1000 membrane) which recycles a chitosanase (derived from Bacillus pumilus BN-262).

Animals and Treatments Five-week-old male ICR mice were purchased from the Dae-Han Laboratory Animal Research Center (Eumsung, Korea). The animals were housed ten per cage. After a one-week acclimation period, chitosan oligosaccharide I, chitosan oligosaccharide II, or the vehicle alone were administered for 14 consecutive days (intragastric application at doses of 500 mg/kg). A single dose of 25 μg TCDD/kg was administered by oral gavage with corn oil as the vehicle on day 8. At the end of the application regimen, the mice were killed by cervical dislocation. The livers were perfused with a cold 0.15 m KCl buffer (pH 7.0) and homogenized in 0.25 m sucrose. Microsomes and the cytosol fractions were prepared from liver homogenates by differential centrifugation.

Determination of Lipid Peroxidation Micromolar malondialdehyde (MDA) production was used as an index of lipid peroxidation. Microsomes (300 μg/ml) were incubated at 37 °C for 60 min with 0.4 mM FeSO4 and 0.2 mM ascorbic acid in 0.1 m Tris–HCl buffer solution (pH 7.5). The reaction was stopped by trichloroacetic acid (TCA) and 2-thiobarbituric acid (TBA) in succession, and the solution was then heated at 100 °C for 15 min. After centrifugation of precipitated protein, the color reaction of the MDA–TBA complex optical density was detected at 530 nm using a Gilford spectrophotometer (Gilford Instrument Laboratories, Ltd., Oberlin, OH, U.S.A.).

Glutathione Peroxidase (GSH-Px) Activity The GSH-Px activity of cytosolic preparations of livers was determined using the coupled assay developed by Paglia and Lawrence,10,11) with hydrogen peroxide as the substrate. The reaction was started by the addition of 2.2 mM hydrogen peroxide as the substrate. The change in absorbance at 340 nm was measured for 1 min, and the activity was expressed as the μmol of NADPH oxidized/min/mg protein.

Glutathione S-Transferase (GST) Activity The GST
activity of cytosolic preparations of livers was measured using a modification of the procedure developed by Habig et al.\textsuperscript{12} with 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate. The GST activity was expressed as the slope/min/mg protein.

**Total Reduced GSH** The GSH content of liver homogenates was assayed by an enzymatic recycling procedure in which it is sequentially oxidized by 5,5'-dithiobis-(2-nitrobenzoic acid) and reduced by a NADPH-generating system (glucose-6-phosphate/glucose-6-phosphate dehydrogenase). The extent of 2-nitro-5-thiobenzic acid formation was monitored at 405 nm. The GSH content was calculated in comparison with a standard GSH curve. The GSH levels were expressed as nmol/mg protein.

**Protein** The protein content was determined by a bicinchoninic acid protein assay kit (Sigma, St. Louis, MO, U.S.A.) with bovine serum albumin as the standard.

**Data Analysis** The data were analyzed for statistical significance using Student’s t-test. p Values less than 0.05 were considered to be significant.

**RESULTS AND DISCUSSION**

**Lipid Peroxidation** The results in Table 1 indicate that TCDD treatment produced a 23.3% increase in microsomal MDA content as compared to the control. Chitosan oligosaccharide I administration to the mice did not affect microsomal MDA production compared with the control group. Treatment of mice with both chitosan oligosaccharide I and TCDD resulted in no change in microsomal MDA production relative to animals treated with TCDD only. However, the administration of chitosan oligosaccharide II to mice resulted in a 20.3% decrease in microsomal MDA production relative to the control animals. Mice treated with chitosan oligosaccharide II and TCDD exhibited a 24.9% decrease in microsomal MDA production relative to animals treated with TCDD alone.

**GSH-Px** TCDD administration inhibited GSH-Px activity. Chitosan oligosaccharide I administration produced a 2.1-fold increase in GSH-Px activity, relative to the control group. Treatment of mice with both TCDD and chitosan oligosaccharide I resulted in an increase in GSH-Px activity that was significantly different than that produced by TCDD alone. Chitosan oligosaccharide II treatment of the animals resulted in a 1.8-fold increase in the activity of this enzyme compared to the control group and a 2.4-fold increase in the TCDD-treated animals (Fig. 1).

GSH-Px plays an important role in the removal of peroxides from the cell, and the accumulation of H$_2$O$_2$ results in lipid peroxidation. The increased lipid peroxidation by TCDD may involve the inhibition of GSH-Px activity. In TCDD-treated animals, a good correlation exists between GSH-Px activity and survival. Good inverse correlations exist between mixed-function oxidase activity and survival.\textsuperscript{13} The inhibition of GSH-Px activity and the induction of mixed-function oxidase activity may both be part of the pleiotropic response that occurs following binding of the ligand to the TCDD receptor and the interaction of this complex with a dioxin-responsive regulatory element on DNA.

**GST** TCDD administration inhibited GST activity. Chitosan oligosaccharide I treatment of the animals had no effect on hepatic GST activity and did not prevent the inhibition of this enzyme activity by TCDD. However, chitosan oligosaccharide II treatment of mice resulted in a 2.0-fold increase in hepatic GST activity and a 5.4-fold increase in enzyme activity in TCDD-treated animals (Fig. 2).

**GSH** Following exposure to TCDD, the total GSH content was increased. Chitosan oligosaccharide I and chitosan

![Figure 1](image1.png)

**Figure 1.** Effects of Chitosan Oligosaccharide I (COS I) and Chitosan Oligosaccharide II (COS II) on TCDD-Induced GSH-Px Activity

1: Control; 2: TCDD; 3: COS I; 4: TCDD + COS I; 5: COS II; 6: TCDD + COS II.

Data shown are mean values with bars indicating the S.D. of the mean. a) Represents a statistically significant difference from group 1 (control) and b) represents a statistically significant difference from group 2 (TCDD) (p<0.05).

![Figure 2](image2.png)

**Figure 2.** Effects of Chitosan Oligosaccharide I (COS I) and Chitosan Oligosaccharide II (COS II) on TCDD-Induced GST Activity

1: Control; 2: TCDD; 3: COS I; 4: TCDD + COS I; 5: COS II; 6: TCDD + COS II.

Data shown are mean values with bars indicating the S.D. of the mean. a) Represents a statistically significant difference from group 1 (control) and b) represents a statistically significant difference from group 2 (TCDD) (p<0.05).

<table>
<thead>
<tr>
<th>Group</th>
<th>Samples</th>
<th>Concentration (mg/kg)</th>
<th>Malondialdehyde formation (Optical density at 530 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>0.871 ± 0.062</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>TCDD</td>
<td>1.074 ± 0.093</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>COS I</td>
<td>0.967 ± 0.114</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>TCDD + COS I</td>
<td>1.079 ± 0.067</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>COS II</td>
<td>0.694 ± 0.098</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>TCDD + COS II</td>
<td>0.807 ± 0.079</td>
<td></td>
</tr>
</tbody>
</table>

COS I, COS II, or the vehicle alone were administered for 14 consecutive days. A single dose of 25 µg TCDD/kg was administered on day 8. Each value is the mean ± S.D. of 10 mice. a) Represents a statistically significant difference from group 1 (control) and b) represents a statistically significant difference from group 2 (TCDD) (p<0.05).
The mechanism by which TCDD causes sustained oxidative stress is unclear at present. The importance of cytochrome P450 1A1 and cytochrome P450 1A2 in the TCDD-mediated oxidative stress response may be related to the production of hydrogen peroxide by the peroxidase activity during cytochrome P450 metabolic cycling. Cytochrome P450s sequentially transfer two electrons to molecular oxygen from NADPH. In the course of electron transfer, some of the activated oxygen can be released as a superoxide and/or H₂O₂. The cytochrome P450s have clearly been shown to contribute significantly to the total cellular production of reactive oxygen in rat liver, even in the absence of enzyme induction. Park et al. indicated that an increase in aromatic hydrocarbon (Ah) receptors or cytochrome P450 1A1 could be associated with the TCDD-mediated oxidative stress response. Cytochrome P450 1A1 induction may result in an increased production of reactive oxygen species, perhaps due to futile cycling of the P450 in its effort to unsuccessfully metabolize TCDD.

A knowledge of the types of reactive oxygen species associated with TCDD-induced oxidative stress is essential to understanding the mechanisms involved in the tissue-damaging effects of TCDD. Stohs et al. suggest that hydrogen peroxide and superoxide anion, as well as a hydroxyl radical, may be involved in TCDD-induced oxidative stress. If some of the toxic processes induced by TCDD are mediated by free radicals and/or reactive oxygen species, the administration of antioxidants such as butylated hydroxyanisole (BHA) should provide protection against TCDD. When rats were pretreated with BHA for 3 days prior to treatment with a lethal dose (2 × LD₅₀) of TCDD, followed by the daily administration of BHA, the animals did not die. Protection from an acute lethal dose of TCDD by BHA has been confirmed by other investigators.

In this study, chitosan oligosaccharide I had no effect on TCDD-induced lipid peroxidation, glutathione S-transferase activity, or loss of liver weight. However, chitosan oligosaccharide II provided protection against TCDD-induced lipid peroxidation (microsomal MDA production), inhibition of GSH-Px and GST activities, and losses of body and liver weights. Previously, we found that chitosan oligosaccharide II exhibited significant activities of superoxide and hydroxyl radical scavenging in vitro. Therefore, the differences in the abilities of chitosan oligosaccharide I and chitosan oligosaccharide II to protect against TCDD-induced oxidative stress may depend on the fact that chitosan oligosaccharide II is an antioxidant and free radical scavenger via a one electron process, while chitosan oligosaccharide I is not. These results suggest that chitosan oligosaccharide may be a promising source for the research and development of potential drugs for combating TCDD-induced pathogenesis.

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**REFERENCES**