Preventive effect of a galactoglucomannan (GGM) from *Dendrobium huoshanense* on selenium-induced liver injury and fibrosis in rats

Li-Hua Pan, Jun Lu, Jian-Ping Luo*, Xue-Qiang Zha, Jun-Hui Wang

School of Biotechnology and Food Engineering, Hefei University of Technology, Hefei 230009, People’s Republic of China

**Abstract**

This study was carried out to investigate the preventive effects of galactoglucomannan (GGM), a homogeneous polysaccharide from *Dendrobium huoshanense*, on liver injury and fibrosis induced by sodium selenite. Sprague–Dawley rats injected subcutaneously with sodium selenite at the dosage of 3.28 mg kg⁻¹ b. wt. were set as the model groups. Rats treated with sodium selenite at the dosage of 3.28 mg kg⁻¹ b. wt. and GGM at 50–200 mg kg⁻¹ b. wt. were set as the prevention groups. Biochemical and histological analysis showed that GGM significantly ameliorated selenium-induced liver injury and fibrosis in rats. Oral administration of GGM effectively attenuated the toxicity of selenium to liver tissue, which was judged both by the decreased activities of serum hepatic enzymes, including alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH), and by liver histopathological examination. Meanwhile, GGM also reduced the levels of H₂O₂ and malondialdehyde (MDA), elevated the levels of GSH, restored the fluidity of hepatic plasma membrane, and retained the activities of endogenous enzymes including superoxide dismutase (SOD), catalase (CAT) and glutathione S-transferase (GST). The prevention of selenium-induced liver injury and fibrosis by GGM was further supported by the reduced expression of transforming growth factor-β1 (TGF-β1) and type I collagen. These results suggested that GGM may be developed into a novel antioxidant agent for the prevention of liver injury and fibrosis.

*Corresponding author. Fax: +86 551 2901539.*

**Keywords:** *Dendrobium huoshanense* Galactoglucomannan Liver injury Hepatic fibrosis Hepatoprotection Selenium

1. Introduction

Chronic liver injuries can be caused by various factors including viral infection, alcohol abuse, fat accumulation, parasitic infection, cholestasis and autoimmune diseases (Bataller and Brenner, 2005). These chronic liver injuries may lead to hepatic fibrosis development eventually, which is characterized by excessive production and deposition of extracellular matrix (ECM) consisting of collagens, proteoglycans, fibronectins and hyaluronic acid (Trappoliere et al., 2009). A large body of evidence has suggested that oxidative stress plays an important role in the fibrogenesis both in animal models of liver fibrosis in rats and in patients with chronic liver diseases (Kim et al., 2009; Povero et al., 2010).

Selenium is an essential micronutrient for animal and human nutrition (Navarro-Alarcon and Cabrera-Vique, 2008). Although supply with appropriate selenium is beneficial to the normal biological functions of the body, excessive selenium intake can cause toxicosis (Rayman et al., 2008). It has been shown that selenium at high concentrations can trigger oxidative stress in a wide variety of tissues (Manikandan et al., 2010), suggesting that selenium-induced oxidative stress primarily involving the generation of free radicals such as superoxide anion (O₂⁻) and H₂O₂ is one of the main reasons leading to selenium toxicosis (Manikandan et al., 2010; Spallholz et al., 2004). Liver is the major site for detoxification. In liver, inorganic selenium is converted into organic forms that are less toxic than inorganic forms (Rayman et al., 2008). If the intake of selenium is beyond the capacity of liver detoxification, liver injury will occur and even hepatic fibrosis will arise (Zhang et al., 2008; Manikandan et al., 2010).

*Dendrobium huoshanense* C. Z. Tang et S. J. Cheng (Orchidaceae) is a famous herb used in the traditional Chinese medicine (TCM) for centuries. Its stems are customarily used as materials to make herbal tea or a functional beverage for the protection of eye, liver and stomach (Luo et al., 2008). Crude polysaccharides from hot-water extracts of *D. huoshanense* have showed to possess antioxidative function in streptozotocin-induced diabetic cataractogenesis of rat lens by the inhibition of nitric oxide generation (Luo et al., 2008). Furthermore, the main active fraction has been isolated from crude polysaccharides, and this active fraction has been proved to be a galactoglucomannan (GGM) by the identification of chemical structures using GC–MS and NMR techniques (Zha et al., 2007). In this study, our aim is to assess the potential role of...
GGM in preventing liver injury and fibrosis induced by excessive selenium.

2. Materials and methods

2.1. Chemicals

Sodium selenite, 4,6-diamidino-2-phenylindole (DAPI) and 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Commercial kits for alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), hydrogen peroxide (H$_2$O$_2$), malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) and glutathione S-transferase (GST) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China).

2.2. Preparation of polysaccharide GGM

Isolation and purification of crude polysaccharides from *D. huoshanense* were described previously (Zha et al., 2007). In short, the water-soluble polysaccharides were extracted from the fresh stems and fractionated by DEAE-Cellulose anion-exchange chromatography. Fraction eluted with double-distilled water was collected and purified by gel filtration chromatography of Sephacryl S-200 to give one homogenous fraction (designated as GGM). GGM is a galactoglucomannan consisting of glucose, mannose and galactose in molar ratio 31:10:8 and its molecular weight was 2.2 × 10$^4$ Da. No absorption at 280 nm and a negative response to the method of Lowry et al. (1951) confirmed that GGM does not contain proteins. The repeating unit of GGM has been reported by Zha et al. (2007).

2.3. Animals and treatments

Sprague–Dawley (SD) rats weighing 105 ± 5 g were supplied by Experimental Animal Center of Anhui Medical University. Rats were left undisturbedly to acclimatize for 1 week prior to the experiments. All rats were free access to normal rat diet and water, and were housed in a controlled environment with 22 ± 1 °C and 50% relative humidity under a 12-h dark/light cycle throughout the experiment period. Sodium selenite was dissolved in 0.9% NaCl solution (pH 8.6). To initiate liver injury, the rats were subcutaneously injected twice with sodium selenite at a dose of 3.28 mg kg$^{-1}$ body weight on the first day and on the third day into three groups including normal control (NC), selenite control (SC), vitamin C control (VC), low dose of GGM set, 60 rats were divided into six groups including normal control group and 200 mg kg$^{-1}$ body weight GGM for HP group, each day 50% relative humidity under a 12-h dark/light cycle throughout the experiment period. Sodium selenite was dissolved in 0.9% NaCl solution (pH 8.6). To initiate liver injury, the rats were subcutaneously injected twice with sodium selenite at a dose of 3.28 mg kg$^{-1}$ body weight on the first day and on the third day into three groups including NC, SC and HP groups and were treated as the first set. After the fifteenth day of the experiments, they were not administered with GGM and maintained until the 50th day.

At the end of each set of experiments, the rats were euthanized. Peripheral blood was collected from ophthalmic veins and centrifuged (3000 × g, 10 min) to obtain blood serum. Livers and spleens were quickly excised and rinsed with ice-cold normal saline. After these tissues were removed excess moisture, they were weighed and packed in centrifuge tubes. The collected tissues and blood samples were stored at −80 °C before further analysis. All protocols involving animal treatments complied with the national guidelines for the care and use of laboratory animals (Ministry of Health, P.R. China, 1998).

2.4. Biochemical parameters

The activities of serum ALT, AST and LDH were determined spectrophotometrically using commercial kits according to the manufacture’s instructions. Liver tissue samples were homogenized with ice-cold 0.9% NaCl solution (pH 8.6) and centrifuged at 4000 g (4 °C) for 10 min. The resulting supernatants were used for the determination of H$_2$O$_2$, MDA and GSH, SOD, CAT, GST using commercial kits according to the manufacture’s instructions. Protein contents were determined using the method of Bradford (1976).

2.5. Membrane fluidity

According to the method of Hashimoto et al. (1999), liver tissues were homogenized with ice-cold 0.1 M sucrose buffer solution (pH 7.4) containing 10 mM Tris–HCl and then centrifuged at 1000 × g (4 °C) for 10 min. The resulting supernatants were centrifuged at 9000 × g (4 °C) for 10 min and further centrifuged at 100,000 × g (4 °C) for 30 min. The obtained plasma membrane-rich pellets were resuspended in sucrose buffer solution (pH 7.4) containing 10 mM Tris–HCl and incubated with the fluorescence probe 1,6-diphenyl-1,3,5-hexatriene (DPH) at room temperature for 10 min. The fluidity of hepatic plasma membrane was determined by measuring the fluorescence polarization of DPH. Fluorescence polarization (P) was measured by exciting the samples at an excitation wavelength of 358 nm and an emission wavelength of 430 nm on an Amino–Bowman AB2 SLM spectrofluorimeter (Thermospec-tronic, Madison, WI, USA).

2.6. Histopathological observation

Liver tissues were fixed in 10% buffered neutral formalin for 10 h, and then embedded in molten paraffin. Tissue sections of 3 μm thickness were deparaffinized and stained with hematoxylin and eosin (HE) for histological examinations. The histological observations of liver sections were made under light microscope.

2.7. Immunofluorescence staining

Thin sections (3 μm) of liver tissues were deparaffinized and blocked with 10% goat serum. Then they were incubated with primary antibodies rabbit anti-collagen I in 1: 100 dilution, mouse anti-collagen III in 1: 100 dilution or rabbit anti-transforming growth factor–β1 (TGF-β1) in 1:200 dilution overnight at 4 °C. After washed thrice with PBS, the sections were stained with goat anti-rabbit FITC-conjugated secondary antibody in 1:200 dilution and sheep anti-mouse Cys-conjugated secondary antibody in 1:500 dilution. The nuclei were counterstained with DAPI. The sections were viewed using a FV1000 laser scanning confocal microscope (Olympus, Tokyo, Japan).

2.8. Statistical analysis

The data were expressed as mean ± standard deviation. The differences between groups were analyzed using one-way analysis of
Effects of galactoglucmannan from Dendrobium huoshanense on body weight, liver index and spleen index in selenite-induced liver injury on the 15th day and fibrosis on the 50th day in rats.

Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Body weight (g)</th>
<th>Liver index (%)</th>
<th>Spleen index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>10</td>
<td>18.9 ± 1.2</td>
<td>3.904 ± 0.1072</td>
<td>0.3383 ± 0.0191</td>
</tr>
<tr>
<td>SC</td>
<td>5</td>
<td>20.4 ± 7.7</td>
<td>3.706 ± 0.0956</td>
<td>0.4234 ± 0.0184</td>
</tr>
<tr>
<td>LP</td>
<td>6</td>
<td>105 ± 9.7</td>
<td>3.6473 ± 0.1173</td>
<td>0.3996 ± 0.0276</td>
</tr>
<tr>
<td>MP</td>
<td>8</td>
<td>121 ± 12.1</td>
<td>3.772 ± 0.1171</td>
<td>0.3953 ± 0.0286</td>
</tr>
<tr>
<td>HP</td>
<td>9</td>
<td>125 ± 12.2</td>
<td>3.8323 ± 0.1911</td>
<td>0.3965 ± 0.0455</td>
</tr>
<tr>
<td>VC</td>
<td>7</td>
<td>170 ± 10.7</td>
<td>3.7871 ± 0.1988</td>
<td>0.3890 ± 0.0297</td>
</tr>
</tbody>
</table>

Day 50

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Body weight (g)</th>
<th>Liver index (%)</th>
<th>Spleen index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>10</td>
<td>247 ± 2.4</td>
<td>3.7786 ± 0.1062</td>
<td>0.3522 ± 0.0332</td>
</tr>
<tr>
<td>SC</td>
<td>5</td>
<td>186 ± 18.1</td>
<td>2.6344 ± 0.1755</td>
<td>0.4634 ± 0.0143</td>
</tr>
<tr>
<td>HP</td>
<td>8</td>
<td>220 ± 18.4</td>
<td>3.4621 ± 0.3499</td>
<td>0.3431 ± 0.0383</td>
</tr>
</tbody>
</table>

Note: Various groups are normal control group (NC), selenite-induced control group (SC), vitamin C control group (VC), selenite-induced + polysaccharide at low dose group (LP), selenite-induced + polysaccharide at medium dose group (MP) and selenite-induced + polysaccharide at high dose group (HP).

p < 0.05 indicate significant difference between the NC and the other groups.

p < 0.01 indicate significant difference between the NC and the other groups.

p < 0.001 indicate significant difference between the NC and the other groups.

p < 0.0001 indicate significant difference between the NC and the other groups.

3. Results

3.1. Body weight and weights of liver and spleen

The excessive ingest of selenite caused a significant decrease in the body weight of rats as compared with the control rats. A significant decrease in body weight of rats occurred between SC group and polysaccharide-treated groups (Table 1). After 15 days of treatment, the body weight of rats from SC group was 28.3% lower than that of rats from NC group and 20.8% lower than that of rats from HP group, respectively. The body weights of rats from MP group and HP group were close to that of rats from NC group and there were no significant difference between them. As a positive control, the body weight of rats from VC group was significantly lower than that of rats from NC. After 50 days of treatment, the body weight of rats from SC group was 24.7% lower than that of rats from NC group and 20.8% lower than that of rats from HP group, respectively. No significant difference occurred between body weights of rats from NC group and HP group after 50 days of treatment. As far as the liver and spleen growth were concerned, liver index (the ratio of liver weight to body weight) and spleen index (the ratio of spleen weight to body weight) were also shown in Table 1. Compared with NC group, the liver indices were decreased significantly and the spleen indices were increased significantly in SC group both on the 15th day and on 50th day of the experiments. However, there was no difference in liver index and spleen index between HP group and NC group.

3.2. Serum biochemical parameters

ALT, AST and LDH are the important hepatic metabolic enzymes. When liver is damaged, these enzymes will be released from liver into serum. Thus, the activities of these enzymes in serum can reflect the degree of liver injury (Zhang et al., 2005). The experiments showed that selenite markedly increased the activities of serum ALT, AST and LDH from rats of SC group as compared with the NC group. But GGM effectively reduced the activities of these enzymes. As shown in Fig. 1, the activities of ALT, AST and LDH in serum from polysaccharide-treated groups were decreased with increased dose of GGM and the dose-dependent influence was strongly significant (p < 0.05). At the dose of 200 mg kg⁻¹ day⁻¹, GGM effectively inhibited the selenite-increased activities of ALT, AST and LDH, and no notable difference in activities of AST and LDH was observed between the NC group and HP group (p < 0.01). The activities of ALT, AST and LDH in serum from VC group were near to those from LP group. It was noted that in the serum from SC group, AST activity determined on 50th day was significantly lower than that on 15th.

3.3. Hepatic biochemical parameters

Hepatic biochemical parameters concerning H₂O₂, MDA, GSH and membrane fluidity are showed in Table 2. Compared with the NC group, selenite led to significant increases in H₂O₂ and MDA levels in liver from rats of SC group both on the 15th and on 50th day of feeding. In contrast, selenite-induced H₂O₂ and MDA production in liver was inhibited by GGM. In HP group that was treated with GGM at 200 mg kg⁻¹ day⁻¹, H₂O₂ and MDA levels in liver were decreased by 39.5% and 34.6%, respectively, after feeding of 15 consecutive days. Meanwhile, selenite caused a markedly decrease in GSH level in rat liver. This decrease could be reversed by GGM. When GGM dose reached 200 mg kg⁻¹ day⁻¹, GSH level in rat liver from HP group was near to that of NC group. Damage of selenite to plasma membrane of liver was indicated by membrane fluidity by measuring the fluorescence polarization of DPH. The experimental results showed that selenite resulted in a significant decrease in...
the fluidity of hepatocyte plasma membranes, while GGM restored the fluidity of hepatocyte plasma membranes to normal level. Since selenite-induced liver injury is contributed to oxidative stress triggered by excessive selenite (Wang et al., 2007), the activities of antioxidative enzymes including SOD, CAT, and GST in liver are measured. As shown in Table 2, selenite treatment caused significant decrease in the activities of liver CAT both on 15th day and on 50th day of feeding as compared to NC group. At the same duration, the activities of GST in liver of rats from SC group were increased markedly. When GGM was given to rats with selenite treatment, the activity of CAT rose and the activity of GST dropped. Although the activity of SOD in liver was decreased in SC group and increased in the groups treated with GGM, no statistical differences were observed between different groups. The positive control VC group also exhibited the capacity of restoring these hepatic biochemical parameters, but H2O2 level and fluorescence polarization were higher than those in NC group.

3.4. Histopathological changes in liver

Liver histopathological examination with hematoxylin and eosin (H&E) showed normal hepatic architecture in liver sections from rats of NC group (Fig. 2A). However, liver sections taken from rats of SC group exhibited large areas of liver necrosis after 15 days of treatment (Fig. 2B) and large number of fibrous tissues and pseudolobules after 50 days of treatment (Fig. 2D). In contrast, GGM treatment markedly alleviated these histopathological changes. Only punctate necrosis and sinusoidal damage were occasionally found in liver sections from rats of HP group after 15 days.
Fig. 3. Immunohistochemical staining of TGF-β1 and collagens (I and III) in liver sections. Liver sections taken from rats of normal control group (A), selenite-induced control group (B), selenite-induced + polysaccharide at high dose group (C) on the 4th day were stained with antibodies specific for TGF-β1 (green). Liver sections taken from rats of normal control group (D), selenite-induced control group (E), selenite-induced + polysaccharide at high dose group (F) on the 50th day were stained with antibodies specific collagen I (green) and collagen III (red). The pictures display representative photomicrographs of each group at an original magnification of 40×.

3.5. Immunohistochemical expression of TGF-β1 and collagens

The expression of TGF-β1, which has been suggested to be the principal mediator of liver fibrosis, was detected by immunohistochemical staining. On the fourth day of treatment, few expression of TGF-β1 was noticed in liver sections of rats from NC group (Fig. 3A), but the expression of TGF-β1 was observed notably in liver sections of rats from SC group (Fig. 3B). On the contrary, treatment with GGM obviously suppressed the expression of TGF-β1 induced by selenite in comparison with SC group (Fig. 3C). Immunohistochemical staining using polyclonal antibodies specific to collagens I and III was used to reveal collagen accumulation in liver tissue of rats. Although there is few expression of collagens in liver sections of rats from NC group after 50 days of treatment (Fig. 3D), strong expression of collagen I (green) and weak expression of collagen III (red) were showed in the areas of liver pseudolobule in SC group (Fig. 3E). In the GGM treatment group, collagen expression was markedly inhibited (Fig. 3F), only weak accumulation was found in central vein and liver cell gaps.

4. Discussion

Many studies have indicated that the activities of ALT, AST and LDH in serum increased both in a dose dependent manner and in a time dependent manner after selenium treatment (Wang et al., 2007; Zhang et al., 2008). Thus, these hepatic enzymes in serum have been used as the biochemical markers for evaluating liver injury and fibrosis. Our examination showed that the activities of ALT, AST and LDH in serum of rats from SC group were much higher than those from NC group. These increased enzyme activities could be decreased by the oral administration of galactoglucomannan (GGM), a homogeneous polysaccharide from D. huoshanense. Similar results were found in the prevention of CCl4-induced rat liver fibrosis by the polysaccharides from Ganoderma lucidum (Park et al., 1997). In particular, ALT activity may show a dynamic change during of liver injury and fibrosis development and its activity is relatively high in the early stage of liver injury and low in the end stage, which is the so-called “bilirubin-transaminase separation” phenomenon (Li et al., 2007). This phenomenon of high ALT level in the early stage and low ALT level in end stage of liver injury was also observed in our experiments, which has been contributed to the change of biosynthetic function during the progression of liver injury (Li et al., 2007; Chen et al., 2008). In the early stage, the injured hepatic cells release ALT into blood because of the increase of membrane permeability, leading to a sharp increase in ALT activity in serum. However, in cases of liver fibrosis development in the end stage or serious hepatic damage, hepatic cells lose biosynthetic function, leading to a decrease in ALT synthesis and ALT secretion into blood (Li et al., 2007).

It has been known that selenium toxicity comes from increased thiol oxidation and superoxide generation (Stewart et al., 1999). When excessive selenium is ingested, it will be reduced by GSH to form GS–SeH and GS–Se–SG, which are ultimately converted into CH3Se− in liver. CH3Se− may react with oxygen to produce reactive oxygen species (ROS), which result in oxidative stress (Ganther, 1968; Ganther, 1971; Kice et al., 1980). It has been reported that selenium-induced liver injury was caused mainly by oxidative stress in liver (Spallholz, 1994). In this study, the significant increase in H2O2 and MDA levels and the significant decrease in GSH level were found in liver tissues of rats treated with selenite, suggesting that a severe oxidative stress occurred in liver tissues. Studies have showed that the plasma membrane is easily attacked by excessive oxidative stress, leading to the increase of membrane permeability and the release of ALT into blood.
by oxidative stress and the change of plasma membrane fluidity has been used as the important index to evaluate the injured state of cells (Gala et al., 1999; Sergent et al., 2005; Shimanoouchia et al., 2009). As shown in our study, the decrease in the fluidity of hepatocyte plasma membrane in rats treated with selenite was consistent with the increase in oxidative stress, indicating that the oxidative stress caused liver cell injury. The decrease in oxidative stress and increase in the fluidity of hepatocyte plasma membrane in rats treated with selenite and GGM suggested that this polysaccharide from D. huoshanense possesses the preventive effect on rat liver injury and fibrosis induced by selenite. Further, our results indicated that the treatment with GGM might impede the consumption of SOD and CAT, and for that reason the concentration of these enzymes remains higher activities than selenium treatment group. In our preliminary acute toxicity test, GGM at 1000 mg kg⁻¹ body weight was proved to be innocuous to rats (Data not shown). Thus, all these findings indicate that GGM exerts a preventive effect on selenite-induced liver injury and fibrosis in rats possibly through its antioxidative function.

The activation and proliferation of HSCs is the key to liver fibrogenesis because the activated HSCs express large amount of ECM including Type I and Type III collagens (Shi et al., 2009). It has been found that the activation and proliferation of HSCs is triggered by various cytokines and TGF-β1 appears to be a critical mediator during liver injury and fibrosis development (Fu et al., 2008). TGF-β1 stimulates the synthesis of ECM proteins and inhibits their degradation (Bataller and Brenner, 2005). Our results on immunohistochemical staining of TGF-β1 and collagens showed that GGM may decrease the enhanced expression of TGF-β1 and collagens by selenite in liver tissues of rats, indicating that the inhibition of TGF-β1 by GGM might play an important role in the down-regulation of collagen accumulation in rat liver.

In conclusion, our experimental results confirmed that GGM may protect against liver injury and fibrosis induced by excessive selenium intake, which can be deduced not only from biochemical parameters of serum and liver tissues, but also from histological observation of liver tissues. This protective effect of GGM might be contributed to its potential of scavenging selenium-induced oxidative stress, which resulted in the inhibition of TGF-β1 and the decrease of collagen synthesis in liver. Our research suggested that GGM may be developed into a novel antifibrotic agent for prevention of the liver injury diseases although further work remains to be done.

Acknowledgements

This research was financially supported by the National Natural Science Foundation of China (20872024 and 21006019) and the Key Project for Science and Technology Research from Ministry of Education of China (109091).

References

