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Full Paper

Hypolipidaemic and antioxidant activities of chito-oligosaccharides in hyperlipidaemic rats induced by high-fat diet

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Abstract: Hyperlipidaemia (HLP), characterised by an abnormally high level of serum lipids, can increase the risk of developing many diseases in the human body. HLP is prevalent all over the world and is attracting increasing attention. However, pharmacological drugs or therapies for effectively treating hyperlipidaemia are not yet available. Recent efforts have been made to seek natural anti-hyperlipidaemic compounds with fewer side effects. Chito-oligosaccharides (COS), the partially hydrolysed products of chitosan, have attracted increasing interest due to their hypolipidaemic and antioxidant effects. In this study the effects of COS on serum lipid levels, hepatic indicators and antioxidant activity were investigated in rats after 6 weeks of intragastric administration. COS can dramatically decrease total serum cholesterol, triglyceride, low-density lipoprotein cholesterol, alanine aminotransferase, aspartate aminotransferase, malondialdehyde and liver coefficient. COS can elevate levels of high-density lipoprotein cholesterol, the activity of lipoprotein lipase, hepatic lipase, superoxide dismutase and glutathione peroxidase. The results of this study reveal the antihyperlipidaemic and antioxidation activities of COS in hyperlipidaemic rats, suggesting its potential use as a natural hypolipidaemic drug therapy.

Keywords: chito-oligosaccharides, antihyperlipidaemic activity, antioxidant activity

INTRODUCTION

Hyperlipidaemia (HLP), also known as dyslipidaemia, increases risk of developing hypertension and coronary disease. It has a close relationship with hyperglycaemia, hypertension, coronary disease and aging [1-3]. Nowadays, hyperlipidaemia has become a severe public health problem. Currently, drug therapy is the primary treatment, although there are many studies

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demonstrating that consuming antilipaemic agents for a long period of time would cause some side effects such as diarrhoea, muscle aches or pains, nausea, indigestion and weakness [4-6]. Finding natural hypolipidaemic agents with few or no side effects would attract great attention.

Chitosan, composed of β-(1→4)-linked N-acetyl-glucosamine (GlcNAc unit) and deacetylated glucosamine ($GlcNH_2$ unit), is a derivative of chitin – a major component of the cell walls of fungi and the exoskeletons of crustaceans and invertebrates [7]. It is reported that chitosan has hypolipidaemic activity [8-10]; however, chitosan is insoluble in water and cannot be well absorbed, which is the major restricting factor preventing its application in medicine. It has been reported that the absorption of chitosan is significantly influenced by its molecular weight (MW), the absorption increasing with decreasing MW [11]. Recently, Zhang et al. [10] also found that the hypolipidaemic activity of low-MW chitosan was better than that of high-MW chitosan.

Chito-oligosaccharides (COS), which have a low molecular weight and good water solubility and absorption, are the partially hydrolysed product of chitosan. COS exhibit numerous biological functions such as anti-inflammatory [12], antibacterial [13, 14], antitumour [15, 16], antifungal [17], antioxidant [18, 19], bone strengthening [20], wound healing [21] and enzyme inhibiting activities [22]. In recent years, COS have attracted considerable interest due to their hypolipidaemic activity. Singh et al. [23] observed that COS have significant hypolipidaemic activity in both alloxan induced and normal mice. Xia et al. [24] also found that COS prepared from *Clanis bileinata* could be a suitable alternative source of hypolipidaemic agent for humans. However, due to the difficulties of preparation and separation of COS to a single polymerisation degree (DP), the COS used was almost a mixture, with a DP distribution of two to eight – a range too wide to determine which DP was mainly responsible for the hypolipidaemic activity. To better understand the exact DP or DP range of COS exerting hypolipidaemic activity, and the hypolipidaemic mechanisms of COS, a method of obtaining COS with a narrower distribution of DP must be investigated. In this study the preparation of COS with a narrow distribution of DP was developed and the hypolipidaemic and antioxidant effects of COS in rats investigated.

MATERIALS AND METHODS

Materials

Chitosan was purchased from Golden-Shell Pharmaceutical Co. (Taizhou, China). Triacylglycerol (TG), total cholesterol (TC), low- and high-density lipoprotein cholesterol (LDL-C and HDL-C), aspartate aminotransferase (AST), alanine aminotransferase (ALT), hepatic lipase (HL), lipoprotein lipase (LPL), superoxide dismutase (SOD), malonaldehyde (MDA), glutathione peroxidase (GSH-Px) and all other assay kits used in this study were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Chitosanase, with 174 $U \cdot ml^{-1}$ activity, was obtained from Ocean University of China (Qingdao, China). COS standard samples including GlcN to $(GlcN)_6$ were purchased from Long Line Biotechnology Co. (Zhaoqing, China). Simvastatin was obtained from Sinopharm Group Rongsheng Pharmaceutical Co. (Wuzhi, China). All other reagents were of analytical grade.

Preparation of COS

Three grams of chitosan was dissolved in 1.0% (v/v) aqueous acetic acid (100 mL) and the pH of the solution adjusted to 5.6 with 1 M NaOH. The solution was placed in a water bath at 50ºC and then chitosanase (1.0 mL) was added to initiate the hydrolysis reaction. After 4 hr, the reaction

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was terminated by heating the mixture solution to 95ºC for 15 min. After rapid cooling to room temperature and evaporation to one-third of the original volume, the solution was lyophilised [19].

Characterisation of COS

The composition and content of the oligosaccharide in the hydrolysed chitosan was analysed by a Waters 1525 HPLC equipped with an evaporative light scattering detector. The column used was a Shodex NH_2P-50 4E column (4.6 mm×250 mm). Mobile phases A (water) and B (acetonitrile) were used with the following gradient: 75% B (0 - 5 min.), 75% ~ 65% B (5 - 40 min.), 65% B (40 - 50 min.), 65% ~ 75% B (50 - 60 min.). The detector temperature was set at 60ºC; the flow rate and the column temperature were maintained at 0.6 mL·min.$^{-1}$ and 35ºC respectively, and the injection volume was set as 20 μL.

An external standard method was used to determine the content of the oligosaccharides in the COS sample. Standards of COS (DP 1 to 6) in water at concentrations of 0.5, 1.0, 2.0, 4.0, 6.0 and 8.0 mg·mL^{-1} respectively were used.

Animals and Diets

Male Sprague-Dawley rats (180 ± 20 g) were purchased from Shanghai Laboratory Animal Center (Shanghai, China). The rats were individually housed in metabolic cages in a controlled environment (23 ± 1°C, 50-60% relative humidity, 12 hr light-dark cycle with lighting from 8:00 a.m. to 8:00 p.m.). All animal protocols were approved by the institutional animal care and use committee of Huaqiao University (Xiamen, China) [HDSPF/SQ-20170013].

After acclimation for one week, the rats were randomly divided into six groups (ten rats per group) as follows: 1) normal control group (NC), fed with a commercial diet provided by Shanghai Laboratory Animal Centre; 2) model control group (MC), receiving a high-fat diet containing 10.0% (w/w) lard, 10.0% (w/w) egg, 0.2% sodium cholate and 1.0% cholesterol in the commercial diet (100% w/w); 3) positive control group (PC), treated with the high-fat diet while orally given simvastatin (7.0 mg·kg^{-1}) once a day for six weeks; 4-6) groups with high-, middle- and low-dose COS (H-COS, M-COS and L-COS), treated with the high-fat diet while receiving 375.0, 187.5 and 93.75 mg·kg^{-1} COS respectively once daily for six weeks.

Experimental Design

The rats were allowed free access to food and water during the experiment and their body weight and food intake were recorded every day. At the end of the six weeks, all the rats were given diethyl ether after fasting for 18 hr. The blood of each rat was collected from the tail vein, and the serum obtained by centrifugation at 3000 rpm for 10 min. The liver was removed, rinsed in cold saline, patted between paper towels and then weighed. A portion of each liver was excised and fixed in a 10% formalin solution. The plasma and liver samples were stored at −20°C in a freezer until further analysis.

Serum Lipid, Liver Enzyme and Lipase

Serum TG, TC, LDL-C, HDL-C levels and AST and ALT activities were measured according to the procotol of the commercial assay kits. Each liver (1.0 g) was homogenised with ice-cold saline (9.0 mL) and then centrifuged at 6000 rpm for 10 min. The supernatant was used for determining the activities of HL and LPL, expressed as units per gram protein, according to the procotol of the commercial HL and LPL assay kits. The measurements were conducted in triplicate.

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Determination of Serum SOD, MDA and GSH-Px Contents

Serum SOD, MDA and GSH-Px contents were analysed according to the procotol of the commercially available assay kits. The measurements were conducted in triplicate.

Histopathologic Examination

Formalin-fixed liver samples were sectioned and mounted on a slide. They were then stained with hematoxylin and eosin and examined under a light microscope.

Statistical Analysis

All data were presented as means ± SD. Analysis of variance (ANOVA) was used to compare the means of the two groups in the rat study. Statistical significance at 95% and 99% probability levels were set at $p < 0.05$ and $p < 0.01$ respectively.

RESULTS AND DISCUSSION

Characterisation of COS

The oligosaccharides detected in the COS sample are GlcN, $(GlcN)_2$, $(GlcN)_3$, $(GlcN)_4$ and $(GlcN)_5$ with their contents being 1.24%, 12.83%, 40.42%, 33.04% and 12.46% respectively (Figure 1). Among them, $(GlcN)_3$ and $(GlcN)_4$ together account for more than 70% of the total content.

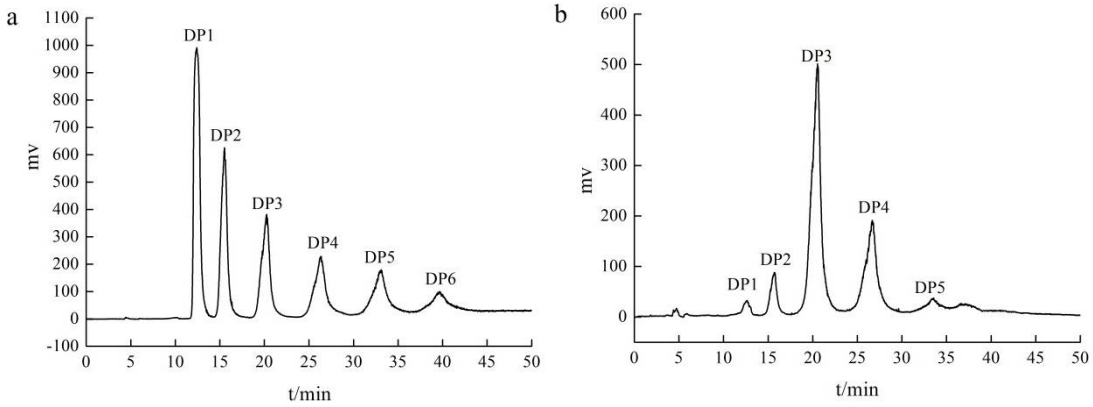

Figure 1. HPLC chromatogram: (a) COS standard (DP of 1 to 6); (b) COS prepared sample

Serum Lipids

Serum lipid concentrations of the rats are listed in Table 1. After six weeks of high-fat diet treatment, the serum TC, TG and LDL-C levels in the MC group remarkably increase ($p < 0.05$) while the HDL-C level decreases ($p < 0.05$) relative to the NC group, suggesting that a long-term high-fat diet can lead to abnormal changes in the blood lipid levels of rats. As shown in Table 1, changes in the levels of blood lipids are observed in the COS treated groups compared to the MC group. TC, TG and LDL-C of L-COS, M-COS and H-COS groups are remarkably lower than those of the MC group ($p < 0.05$), while HDL-C is significantly higher than that of MC ($p < 0.05$). Similar effects are observed in the PC group ($p < 0.05$).

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Table 1. Effects of COS on serum lipid levels in rats

Group	TC (mmol·L^{-1})	TG(mmol·L^{-1})	HDL-C (mmol·L^{-1})	LDL-C(mmol·L^{-1})
NC	5.22 ± 1.27	2.57 ± 0.74	2.80 ± 0.36	0.65 ± 0.10
MC	8.68 ± 1.52$^{\Delta\Delta}$	4.06 ± 0.99$^{\Delta\Delta}$	1.63 ± 0.51$^{\Delta\Delta}$	1.81 ± 0.58$^{\Delta\Delta}$
H-COS	5.71 ± 1.38**	2.82 ± 0.52**	2.47 ± 0.63**	1.21 ± 0.33*
M-COS	6.34 ± 1.30**	3.14 ± 0.37*	2.17 ± 0.45*	1.26 ± 0.48*
L-COS	6.60 ± 1.09*	3.24 ± 0.46*	2.00 ± 0.70	1.33 ± 0.35*
PC	5.93 ± 1.04**	2.95 ± 0.47**	2.33 ± 0.61*	1.28 ± 0.29*

Note: Values are expressed as means ± SD (n = 10); $^{*}p < 0.05$; $^{**}p < 0.01$ compared with MC group; $^{\Delta\Delta}p < 0.01$ compared with NC group.

Lipase Activity and Liver Coefficient

The HL and LPL activities and liver coefficient of the rats are summarised in Table 2. Compared with the NC group, HL and LPL activities of the MC group drastically decrease ($p < 0.05$) while the liver coefficient increases ($p < 0.05$). Feeding a high-fat diet while giving COS (middle and high doses) and simvastatin can significantly slow down the downward trend of HL and LPL activity ($p < 0.05$) and reduce the liver coefficient ($p < 0.05$).

Table 2. LPL activity, HL activity and liver coefficient in rats

Group	LPL（U·mg protein^{-1}）	HL（U·mg protein^{-1}）	Liver coefficient$^{\#}$
NC	1.21 ± 0.27	1.23 ± 0.13	2.39 ± 0.20
MC	0.59 ± 0.03$^{\Delta\Delta}$	0.66 ± 0.02$^{\Delta\Delta}$	5.13 ± 0.65$^{\Delta\Delta}$
H-COS	1.01 ± 0.09**	1.16 ± 0.18**	3.98 ± 0.79**
M-COS	0.89 ± 0.13*	1.04 ± 0.10**	4.23 ± 0.81*
L-COS	0.71 ± 0.03	0.84 ± 0.20	4.43 ± 0.59*
PC	1.02 ± 0.05**	1.22 ± 0.11**	4.04 ± 0.61**

Note: Values are expressed as means ± SD (n = 10); $^{*}p < 0.05$; $^{**}p < 0.01$ compared with MC group; $^{\Delta\Delta}p < 0.01$ compared with NC group; $^{\#}$ ratio of weight of liver (g) to animal weight (g).

The regulation of blood lipid metabolism is mainly directed by lipoprotein metabolism enzymes, including LPL, HL, lecithin cholesterol acyltransferase and cholesteryl ester transfer protein. Among these enzymes, LPL is particularly critical for the development of hyperlipidaemia [25]. LPL can promote TG hydrolysis into chylomicron and very-low-density lipoproteins, which are important sources for the synthesis of HDL-C. The increased LPL can be hydrolysed to yield large amounts of very-low-density lipoproteins and chylomicron in the blood, resulting in an increased HDL-C [26, 27]. As shown in Table 2, COS treatment can obviously increase HL and LPL activities while reducing the liver coefficient in rats with hyperlipidaemia. These results suggest that COS can improve the metabolism of triglycerides in the liver by increasing the activity of total lipase, thus reducing the risk of fatty liver.

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Liver Index

As shown in Table 3, after six weeks of the diet, serum ALT and AST levels of the MC group are much higher than those in the NC group, indicating that a long-term high-fat diet causes damage to liver cells. These effects are significantly attenuated in the COS-treated groups, as M-COS and H-COS can remarkably improve ALT and AST levels ($p < 0.05$). The results indicate that COS treatment is not toxic to the liver and can reduce the injury caused by administration of a high-fat diet. COS supplementation apparently has a certain protective effect against liver injury caused by a high-fat diet.

Table 3. Liver index in rats

Group	ALT（$IU \cdot L^{-1}$）	AST（$IU \cdot L^{-1}$）
NC	10.71 ± 2.02	10.96 ± 3.02
MC	19.68 ± 2.91$^{\Delta\Delta}$	16.54 ± 3.63$^{\Delta\Delta}$
H-COS	12.25 ± 3.42**	11.45 ± 3.74**
M-COS	13.60 ± 3.13**	12.87 ± 3.83*
L-COS	17.18 ± 2.77	14.17 ± 3.63
PC	12.73 ± 2.09**	11.37 ± 2.90**

Note: Values are expressed as means ± SD (n = 10); $^{*}p < 0.05$; $^{**}p < 0.01$ compared with MC group; $^{\Delta\Delta}p < 0.01$ compared with NC group.

Serum MDA, SOD and GSH-Px

Serum MDA, SOD and GSH-Px activities are listed in Table 4. Compared with the NC group, the antioxidant activities of the MC group decrease ($p < 0.05$) while the content of MDA increases ($p < 0.05$). After treatment with COS for six weeks, the antioxidant activities of the COS (L-COS, M-COS and H-COS) groups and the PC group improve. Specifically, the SOD and GSH-Px activities dramatically increase ($p < 0.05$) while the MDA content is obviously reduced ($p < 0.05$). Administration of high-fat diets could lead to an increase of oxidative stress which can result in many degenerative diseases such as cardiovascular and cerebrovascular diseases, hyperlipidaemia, hypertension and diabetes [28]. As a positive free radical scavenging system, SOD and GSH-Px can prevent the body from being damaged by free radicals, whereas MDA content is an indicator of response to the lipid peroxidation levels of the body. Therefore, the activities of SOD and GPx were assayed as an indicator of serum or liver antioxidant capacities. COS has also been reported to exhibit a high antioxidant activity [29-31].

Liver Appearance and Histopathologic Examination

At the end of the experiment the livers were removed for histopathologic assay. As shown in Figure 2, the liver of the NC group is dull red in colour, its edge surface smooth and sharp and its texture soft. In contrast, the liver from the MC group became pale, rough edged with tissue swelling and less elastic. These results indicate that high-fat diet treatment induces high accumulation of lipids in the liver of rats. The colours of the livers in L-COS and M-COS groups are between red and pale, but those in H-COS and PC groups are almost red, which is similar to the NC group.

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Table 4. Antioxidant activities in rats

Group	SOD（U·mL^{-1}）	MDA（nmol·mL^{-1}）	GSH-Px（U·L^{-1}）
NC	105.50 ± 3.23	2.68 ± 0.35	3706.52 ± 118.31
MC	79.70 ± 6.64$^{\Delta\Delta}$	7.85 ± 0.50$^{\Delta\Delta}$	2677.40 ± 165.17$^{\Delta\Delta}$
H-COS	97.04 ± 3.60**	3.08 ± 0.49**	3674.16 ± 165.76**
M-COS	93.91 ± 4.96**	4.46 ± 0.67**	3006.74 ± 283.82**
L-COS	85.56 ± 8.09	6.11 ± 0.53	2855.73 ± 154.49*
PC	98.74 ± 5.33**	3.39 ± 0.39**	3443.60 ± 191.13**

Note: Values are expressed as means ± SD (n = 10); $^{*}p < 0.05$; $^{**}p < 0.01$ compared with MC group; $^{\Delta\Delta}p < 0.01$ compared with NC group.

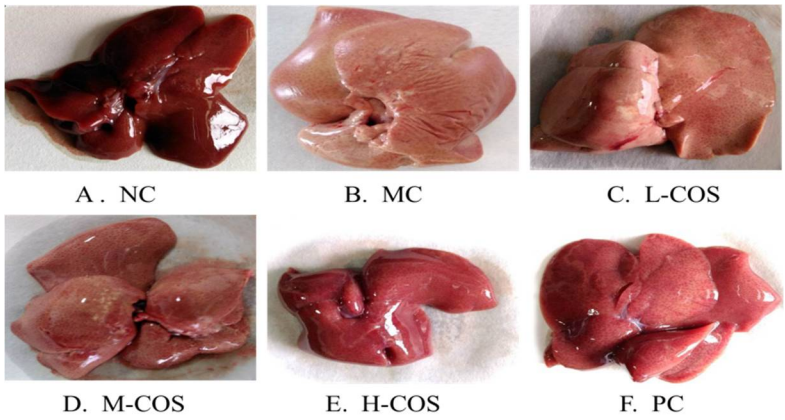

Figure 2. Liver morphology of rats fed different diets

Histopathologic examination of hepatocytes (Figure 3) reveals that the liver in the NC group is complete and clear and liver cells around the central veins are radial. By contrast, high-fat diet treatment induces a significant accumulation of hepatic lipid droplets in the MC group, with liver cell nuclear extrusion to the side and abnormal central vein. The lipid droplets in the COS treatment groups significantly decrease in a dose-dependent manner.

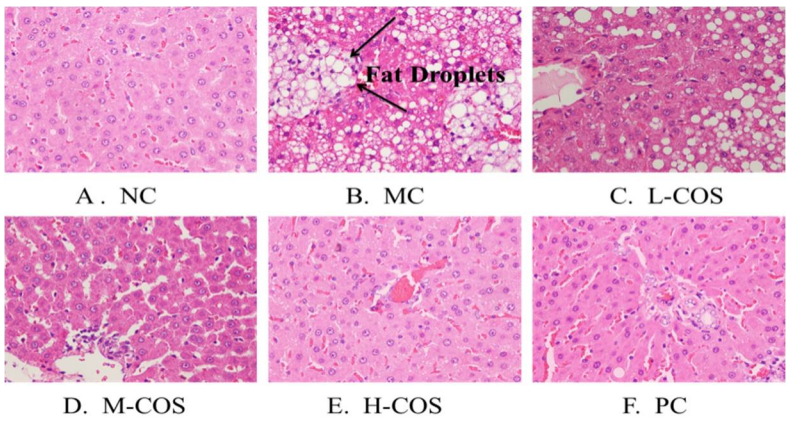

Figure 3. Histologic examination of liver tissues with a light microscope at ×400

CONCLUSIONS

COS with a narrow DP of 1 to 5 was prepared and its hypolipidaemic activity was investigated in this study. Results indicate that COS with a narrow DP efficiently decreases serum TC, TG, LDL-C, ALT, AST, MDA and the liver coefficient, but increases the content of HDL-C as well as the activities of LPL, HL, SOD and GSH-Px.

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