Original Article

Mechanism study of chitosan on lipid metabolism in hyperlipidemic rats

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It has been reported that plasma and liver cholesterol concentrations decrease when animals are fed with chitosan, but the mechanism is unclear. Four wk old male SD (Sprague-Dawley) rats were fed a commercial rat diet (cholesterol-free diet, negative control, NC), cholesterol-enriched diet containing 5% of chitosan (CH) or cholesterol-enriched diet containing 5% of cellulose (CE) and 5% of lard for 12 weeks. We would investigated the effects of chitosan on plasma and liver cholesterol levels, liver weight, bile acids concentrations of fecal and hepatic LDL receptor mRNA expression. The results showed that chitosan could decrease levels of total cholesterol (TC), low density lipoprotein cholesterol (LDL-C) in plasma (p<0.05), and TC, total triglyceride (TG) in liver (p<0.05), and increase fecal bile acids excretion (p<0.05), but the levels of TG and HDL-C in plasma was unchanged (p>0.05). In addition, the result of RT-PCR test showed that saturated fat and cholesterol fed could significantly induce the reduction of LDL receptor mRNA levels, while chitosan could increase hepatic LDL receptor mRNA levels.
This study suggested that chitosan improve lipid metabolism by regulating TC and LDL-C by upregulating of hepatic LDL receptor mRNA expression, increasing the excretion of fecal bile acids.

Key Words: chitosan, cholesterol, bile acids, rat, receptor, mRNA

Introduction

Chitosan is the deacetylated of chitin, an aminopolysaccharide found in the exoskeletons and the fungal cell wall of various arthropods including insects, crabs and shrimp.[1] Although it is not derived from plants, it shares the same characteristics as dietary fiber, which is a indigestible polysaccharide by mammalian digestive enzymes. Several studies showed that chitosan might decrease the level of plasma cholesterol both in animal models[2-4] and humans.[5,6] Although chitosan has hypocholesterolemic effect; few studies have examined the mechanism by which this material exert this effect. Sugano *et al.*,[2] and Gallaher *et al.*,[7] reported chitosan increased fecal neutral sterol excretion and reduced liver cholesterol in rats, but Fukada *et al.*,[8] found no this effect.

The liver plays a central role in lipoprotein metabolism.[9] Besides the production of several apolipoproteins; the liver also produces enzymes and receptors involved in lipoprotein metabolism such as 3-hydroxy-3-methylglutaryl coenzyme-A (HMG CoA) reductase and the low density lipoprotein (LDL) receptor. HMG CoA reductase is the rate-limiting enzyme in endogenous sterol biosynthesis, this enzyme's activity in rats fed a chitosan-sterol diet was more elevated than in those fed a sterol diet but lower than in those fed normal diet, whereas HMG CoA reductase mRNA levels were normal.[10] The specific function of LDL receptor is to remove cholesterol-rich lipoprotein particles from the circulation,[11] which is a highly regulated pathway that has been shown to be down-regulated by dietary cholesterol in experimental animals[12] and in humans.[13] The principal tissue for clearance of LDL by LDL receptor is the liver. It has been demonstrated in experimental animals that as much as 75% of LDL catabolism occurs in the liver.[14] Michihiro *et al.*,[15,16] reported enokitake fiber, mushroom fiber and sugar beet fiber increased hepatic LDL receptor mRNA levels in rats, but there has no report of determining the effect of chitosan on hepatic LDL receptor expression.

The objective of the present study was to examine the effect of chitosan on plasma and liver cholesterol levels, liver weight and bile acid excretion using sterol diet for 12 wks in rats. Additionally, we determined whether this material would increase expression of the hepatic LDLR mRNA in rats.

Materials and methods

Materials

Chitosan, prepared from chitin deacetylated to 92%, was purchased from Xingcheng Biochemical Industry, Nantong. According to the supplier's statement, the molecular weight of chitosan preparation was approximately 120 kilodaltons (kDa) and it contained 7.5% nitrogen, 2.1% ash (as sulfate), and 5.2% moisture.

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Animals and diets

Thirty young male Sprague-Dawley rats (initial body weight 60~80g) were obtained from the Medical Experimental Animals Centre of Nantong University (Nantong, China). All rats were housed individually in suspended stainless steel wire cages in a room maintained at 22 to 24 ℃. There were 12 hr of daytime light between 6 AM and 6 PM and 12 hr of dark. The rats were fed a chow diet (Shuangshi Laboratory Animal Feed Science Co., Ltd, Suzhou) for 1 wk before switching to the experimental diets. They were provided with their respective diets and water on an ad libitum for 12 weeks. After appropriate periods of treatment, the animals were fasted for 15 hr from 6 PM to 9 AM before they were killed by decapitation. All protocols for animal experimentation and maintenance were approved by the Animal Ethics Committee in our university.

Rats were fed a AIN-93G [17] diet in NC group, whereas the rats in CH and CE groups were fed a modified AIN-93G diet containing 5 g/100 g lard, 1 g/100 g cholesterol, 0.25 g/100 g cholate and 5 g/100 g of test material i.e. chitosan or cellulose respectively. The composition of the modified AIN-93G diet was as follows (g/kg): casein, 200.0; cornstarch, 389.986; dextrinized cornstarch, 114; sucrose, 85.5; soybean oil, 50; AIN-93G mineral mix, 35; AIN-93G vitamin mix, 10; L-cystine, 3.0; lard 50; cholate, 2.5; cholesterol, 10; BHT, 0.014; and test materials, 50.

Experimental design

Rats were randomized into three groups of equal size (n = 10): commercial rat diet (cholesterol-free diet, negative control, NC) group, cholesterol-enriched diet containing 5% chitosan (CH) group and cholesterol-enriched diet containing 5% cellulose (CE) group. Body weight and food intake were recorded weekly and daily, respectively. A 3-d fecal collection was made in the last week. At the end of experimental period, the rats were anaesthetized, and blood was collected; the liver were removed and weighed, and a piece was immediately frozen in liquid nitrogen.

Analytical methods

Total cholesterol and triglyceride (TG) concentrations in the Plasma were determined using the commercial kits from Yilikang biological Technology Co. (Wenzhou, China). HDL and LDL in the plasma were separated by ultracentrifugation (194,000 × g for 3 hr at 10 °C), Total cholesterol of each fraction were measured by an enzymatic method with kit purchased from Yilikang biological Technology Co. (Wenzhou, China).

Lipids were extracted from livers by the method of Folch *et al.*,[18] Cholesterol and triglyceride were determined enzymatically (Sigma Diagnostics #352–100, St. Louis, MO) after solubilization in Triton X-100 in acetone. Bile acids were extracted from dried feces using organic solvents[19] and total bile acids measured enzymatically essentially as described by Sheltawy and Lowowsky.[20]

Preparation of RNA

Tissue samples (0.5-0.75 g wet weight) were extracted for total cellular RNA using TRI REAGENT (Sangon Biological Engineering & Tech and Service Co. Ltd., Shanghai). The A_{260}: A_{280} ratios were greater than 1.8, and the yield of RNA was about 1-1.5mg/g of tissue. The integrity and size distribution (quality) of RNA was examined by formaldehyde agarose gel electrophoresis.

RT-PCR

The mRNA expression for LDL-R was done in liver using RT-PCR kit from PROMEGA. 2 μg of total RNA template from different groups after treatment with DNase I (Ambion) was used in RT-PCR reaction. To the reaction mixture added 10 μl of 3X PROMEGA One Step RT-PCR buffer (2.5 mM $MgCl_2$ as final concentration), 2 μl of dNTP mix (10 mM of each dNTP), 5 μl of each forward and reverse gene specific primers (from 10 μM stock), 2 μl PROMEGA One Step RT-PCR Enzyme Mix, 1 μl RNase inhibitor (1 U/μl) and finally 25 μl of PCR grade RNase-free water (provided in the kit) to make total volume 50 μl. Mixed it gently by vortex and centrifuged it to collect all the components at the bottom of the PCR tubes. The PCR reaction was performed in the thermal cycle using following conditions: the RT reaction was performed at 40°C for 30 min, initial PCR activation was done at 94°C for 2 min, followed by 35 cycles of 94°C (denaturation) for 30 s, 58°C (annealing) for 30 s and 72°C (extension) for 1 min. Finally, incubated at 72°C for 10 min to extend any incomplete single strands.

Optimal oligonucleotide primer pairs for RT-PCR were selected with the aid of the software Gene Runner. The primer sequence (5' to 3') for rat LDL-R gene coding (+) strand was ACCGCCATGAGGTACGTAAG, noncoding (-) strand was GGGTCTGGACCCTTTCTCTC and for rat β-actin gene coding (+) strand was AGAGCTATG AGCTGCCTGAC, and the noncoding (-) strand was CTGCATCCTGTCAGCCTACG. The length of RT-PCR products for LDL-R and β-actin were 341 bp and 236 bp respectively. The PCR products were subjected to electrophoresis in a 2% agarose gel, and stained with ethidium bromide. The intensity of each band was quantified with NIH image software (free ware).

Statistical analyses

Data are presented as means ± SD and analysed using Statistica software (SAS Institute, Cary, NC). The significance of differences among treatment groups was determined by ANOVA with Student-Newman-Kuels multiple range test. Differences were considered significant at $p<0.05$.

Results

There were no significant differences in the body weight gain (NC, CE, and CH: 356 ± 34.9, 371 ± 37.4, and 361 ±35.2 g/12 weeks, respectively), food intake ((NC, CE, and CH: 24.3±5.5, 23.2±5.4, and 24.0±7.2 g/day, respectively). The relative liver weight in the CH group and the NC group were significantly lower than one in the CE groups ($p<0.05$). There was no difference of daily fecal dry weight between the CH group and the CE group ($p>0.05$), both of which were significantly greater than the NC group (Table1).

Changes in plasma lipid levels were presented in Table 2. At the end of 12th week, the plasma total cholesterol and LDL cholesterol level in CE group had increased to

Table 1. Body weight, food intake and relative liver weight in rats fed commercial rat diet, cholesterol-cellulose or cholesterol-chitosan for 12 wk[1]

Component	Diet group		
	NC	CH	CE
Initial body weight (g)	69.0±5.2	70.5±6.1	71.8±6.0
Body wt. Gain (g/12 wk)	356±34.9	361±35.2	371±37.4
Food Intake (g/d)	24.3±5.5	24.0±7.2	23.2±5.4
Liver weight (g/100 g body)	2.86±0.15[a]	3.10±0.43[a]	4.31±0.26[b]
Dry feces (g/d)	0.77±0.10[a]	2.06±0.29[b]	1.46±0.24[c]

[1]Values are means±SD, $n = 10$, Values within the same row bearing different superscripts are significantly different ($p < 0.05$).

Table 2. Plasma total cholesterol, LDL cholesterol, HDL cholesterol and triglyceride concentrations in rats fed commercial rat diet, cholesterol-cellulose or cholesterol-chitosan for 12 wk[1]

Component	Diet group		
	NC	CH	CE
Total cholesterol (mmol/L)	1.99±0.40[a]	2.09±0.26[a]	3.10±0.53[b]
LDL cholesterol (mmol/L)	0.38±0.09[a]	0.47±0.19[a]	0.70±0.24[b]
HDL cholesterol (mmol/L)	0.57±0.18	0.48±0.11	0.45±0.20
Triglyceride (mmol/L)	0.78±0.27	0.83±0.39	1.05±0.44

[1]Values are expressed as means ± SD, n =10. Means within the same row bearing different superscripts are significantly different ($p < 0.05$).

Table3. Total liver cholesterol, triglyceride concentrations and fecal bile acid excretion in rats fed commercial rat diet, cholesterol-cellulose or cholesterol-chitosan for 12 wk[1]

Component	Diet group		
	NC	CH	CE
liver cholesterol, *μmol/g liver*	6.20±1.26[a]	10.4±2.58b	17.2±3.06c
liver triglyceride, *μmol/g liver*	9.48±1.82[a]	12.8±2.11b	28.7±4.52c
Bile acid excretion, *μmol/d*	13.0± 3.67a	52.9±7.83b	16.8±4.82a

[1]Values are expressed as means ± SD, n =10. Means within the same row bearing different superscripts are significantly different ($p < 0.05$)

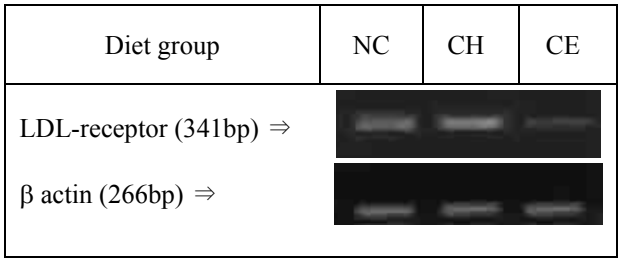

Figure 1. Hepatic LDL-R mRNA expression by RT-PCR in different groups: NC group; CH GROUP; CE group. β-actin was also amplified (house keeping gene).

62%, compared with NC group, whereas the CH group was lowered to the same level as the NC group. There were no significant differences in the HDL cholesterol and triglyceride concentrations in all groups.

Liver cholesterol and triglyceride concentrations were greater in the CE group ($p<0.05$), followed by the CH group ($p<0.05$), both of which were greater than the NC group (Table 3).

Daily fecal bile acid excretion in CH groups was two-fold greater than those of the NC and CE groups ($p<0.05$), there were no difference between the NC and CE groups ($p > 0.05$, Table 3).

RT-PCR products of expected size i.e. 341 bp and 236 bp were obtained for LDL-R and β-actin. Hepatic LDL receptor mRNA expression was greater in the CH group ($p<0.05$), and the NC group ($p<0.05$) than that in the CE group (Fig 1 & Fig 2).

Discussion

Several studies have shown the cholesterol-lowering effect of chitosan related to its dietary levels and particle size. On feeding a high cholesterol diet for 20 days, addition of 2 to 5% chitosan resulted in a significant reduction, by 25% to 30%, of plasma cholesterol without influencing food intake and growth. The concentration of liver cholesterol and triglyceride also decreased significantly.

Chitosan at the 10% level further reduced plasma cholesterol, but depressed growth.[2] The higher mol wt chitosan (>750 kDa) were found to be less effective as hypocholesterolemiant than 80-120 kDa chitosan.[21] In this study, dietary cholesterol and saturated fat increased plasma total and LDL cholesterol levels, and liver cholesterol and triglyceride concentrations, while chitosan moderated this cholesterol-induced increase (Table 2 & Table 3). There were no significant differences in the body weight gain and food intake in all groups (Table 1), and no adverse effects were evident at gross anatomical examination when experiments were conducted for up to 12 weeks but a bit of yellowish appearance of liver. It was suggested that chitosan at 5% of the diet was

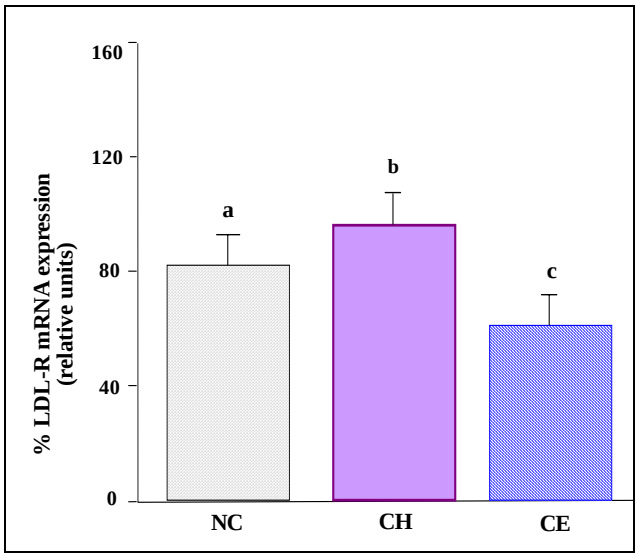

Figure 2. Hepatic LDL-R mRNA Contents in rats a commercial rat diet (cholesterol-free diet, negative control, NC), cholesterol-enriched diet containing 5% chitosan (CH) or cholesterol-enriched diet containing 5% cellulose (CE) for 12 weeks. Data is expressed as means±SD from 4 observations. Values that do not share a letter differ significantly, $p < 0.05$.

recommended deservingly for long-term feeding test in rat.

Although how chitosan reduced cholesterol was still uncertain, many studies indicated that increased bile acid excretion and/or decreased cholesterol absorption was responsible. [22] Chitosan acts as a weak anion exchange resin and exhibits a substantial viscosity in vitro. Either of these properties of chitosan could mediate its hypocholesterolemic effect. However, Sugano *et al.*,[21] found that chitosan preparations of different in vitro viscosities all demonstrated equivalent hypocholesterolemic effects, arguing against a role for viscosity. The anion exchange property of chitosan would seem to be favored as an explanation for its hypocholesterolemic properties. Gallaher *et al.*,[7] found that an equal mixture of chitosan and glucomannan, fed with 7.5% of the diet, reduced cholesterol absorption and increased bile acid excretion in rats fed with a cellulose-based diet. Sugano *et al.*,[2] noted an increase in cholesterol excretion in rats fed 5% chitosan, compared to cellulose treatment, and a change in the composition of the fecal sterols, resulted in excreting relatively more cholesterol and less coprostanol in rats treated with chitosan. In the study, we also found a strong trend to a decrease of bile acid excretion in rats fed 5% chitosan, compared to cellulose (Table 3). Increased bile acid excretion could reduce cholesterol concentrations because plasma or liver cholesterol would be utilized to maintain the bile acid pool.

Difference of LDL receptor mRNA abundance in some animals such as rabbits and guinea pigs could be correlated with differences in plasma total and LDL cholesterol concentrations.[23,24] We found lower levels of LDL receptor mRNA in rats fed high cholesterol concentrations, while chitosan increased hepatic LDL receptor mRNA expression compared to cellulose. The levels of hepatic LDL receptor mRNA in rats fed cholesterol-enriched and 5% chitosan even were greater than those rats fed cholesterol-free commercial rat diet (Fig 2). The results suggested that chitosan upregulate LDL mRNA receptor expression in rat liver with relation to improving liver function besides decreased cholesterol level. The rat liver weight in CH group was significantly lower than that of the CE group (Table 1), and there were smaller yellowish livers after treated with chitosan. These results indicate that the 5% chitosan treatment moderate cholesterol metabolism in rats, despite a greatly increased intake of cholesterol.

Our study indicated that chitosan lowered the plasma total cholesterol level by enhancement of the hepatic LDL receptor mRNA and chitosan might be potential to increase the excretion of fecal bile acids.

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