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The hypolipidemic activity of chitosan nanopowder prepared by ultrafine milling

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ABSTRACT

The hypolipidemic activities of high and low molecular weights of chitosan nanopowders (HMW-chitosan-NP: 315 kDa; LMW-chitosan-NP: 51 kDa) prepared by ultrafine milling were evaluated in rats. The results showed that the hypolipidemic activity of chitosan nanopowder was better than ordinary chitosan, and LMW-chitosan-NP was superior to HMW-chitosan-NP in hypolipidimic activity. Compared with ordinary chitosan, chitosan nanopowder increased the fecal lipids and the activities of serum and liver lipoprotein lipase (LPL) and hepatic lipase (HL) of rats. Rats receiving LMW-chitosan-NP excreted less lipids in feces, but showed higher serum and liver LPL and HL activities compared with those fed HMW-chitosan-NP. These results suggested that compared with ordinary chitosan, the increased hypolipidemic activity of chitosan nanopowder might be attributed to its ability on increasing fecal lipid excretions and stimulating LPL and HL activities, and the better stimulation of LMW-chitosan-NP in activities of these lipases might help it to exceed HMW-chitosan-NP in hypolipidemic activity.

1. Introduction

Chitosan, a natural cationic polysaccharide, is obtained by the alkaline, partial deacetylation of chitin, which originates from shells of crustaceans. Due to the unique polycationic nature, chitosan has been proposed for various applications in food, pharmaceutical and chemical industries (Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004).

Growing evidences indicate that chitosan could reduce blood and liver triglyceride (TG) and total cholesterol (TC) levels of animals, exhibiting potent hypolipidemic activity (Gallaher, Munion, Hesslink, Wise, & Gallaher, 2000; Sugano, Watanabe, Kishi, Izume, & Ohtakara, 1988; Xia, Liu, Zhang, & Chen, 2011). Though some experiments have demonstrated the hypolipidemic effect of chitosan in humans, the positive results are generally related to long-term, high-dose supplementation (Baker, Tercius, Anglade, White, & Coleman, 2009; Jafer & Sampalis, 2007). For better application of chitosan in improving hyperlipidemia, it is of great significance to develop chitosan products of high hypolipidemic activity.

Nanosized chitosan has been extensively explored for pharmaceutical applications, as carriers for drug, gene and vaccine delivery (Muzzarelli, 2012; Qu, Lin, Zhang, Xue, & Zhang, 2013; Sajomsang et al., 2013; Subbiah et al., 2012). In recent years, the nanosized chitosan is found to have effective biological activities, such as antibacterial and antitumor activities (Ing, Zin, Sarwar, & Katas, 2012; Qi & Xu, 2006; Qi, Xu, Jiang, Hu, & Zou, 2004; Qi, Xu, & Chen, 2007). However, there are rare studies reporting the hypolipidemic activity of nanosized chitosan, which may be attributed to its preparation method. The main methods to prepare nanosized chitosan include ionotropic gelation, self-assembling and microemulsion method. These methods could only provide low concentration of products in liquid form, not suitable for hypolipidemic applications. In this study, the hypolipidemic activity of chitosan nanopowder prepared by ultrafine milling was evaluated in rats, and its hypolipidemic mechanism was investigated.

2. Materials and methods

2.1. Materials

High and low molecular weights of chitosan nanopowder (HMW-chitosan-NP: 315 kDa; LMW-chitosan-NP: 51 kDa) with particle size of 385.3 nm and 337.2 nm were prepared by ultrafine milling with a multidimensional swing high-energy nano-ball-mill

Abbreviations: AI, atherosclerosis index; BW, body weight; DD, deacetylation degree; FER, food efficiency ratio; HDL-cholersterol, high-density lipoprotein cholesterol; HF, high-fat group; HL, hepatic lipase; HMW-chitosan-NP, high molecular weight chitosan nanopowder; HMW-chitosan, high molecular weight chitosan; LDL-cholersterol, low-density lipoprotein cholesterol; LMW-chitosan, low molecular weight chitosan; LMW-chitosan-NP, low molecular weight chitosan nanopowder; LPL, lipoprotein lipase; NF, normal-fat group; TC, total cholesterol; TG, triglyceride.

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(W. Zhang, Zhang, Jiang, & Xia, et al., 2012). In animal experiment, two ordinary chitosan, namely high and low molecular weight chitosan (HWC-chitosan: 326 kDa; LMW-chitosan: 56 kDa) with particle size of 357.4 and 346.9 μm prepared by enzymatic method (Jin, Du, & Li, 2007) were used as controls of HMW-chitosan-NP and LMW-chitosan-NP, respectively. Triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-cholesterol), hepatic lipase (HL) and lipoprotein lipase (LPL) kits were purchased from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China). Other chemicals were of analytical grade.

2.2. Animals and diets

Male Sprague–Dawley rats (100 ± 10 g) were purchased from Shanghai Laboratory Animal Center (Shanghai, China). They were individually housed in metabolic cages in a controlled environment (20–22 °C; 50–60% relative humidity; 12-h light-dark cycle with lighting from 8:00 AM to 8:00 PM). All animal protocols were approved by the institutional animal care and use committee of Jiangnan University (Jiangsu, China).

Forty-eight rats were fed *ad libitum* with basic diet and water for seven days, and were then randomly divided into six groups (eight rats per group): normal-fat group (NF), fed a basic diet in which the composition conformed to AIN 76 (provided by Shanghai SLAC Laboratory Animal Co. Ltd., Shanghai, China); high-fat control group (HF), receiving high-fat diet containing 12% lard, 10% egg yolk powder, 1.5% cholesterol, 0.2% bile salt and 73.3% basic diet to 97% (in weight percent) plus 3% cellulose; two ordinary chitosan control groups (HMW-chitosan and LMW-chitosan) and two chitosan nanopowder groups (HMW-chitosan-NP and LMW-chitosan-NP), receiving 97% (in weight percent) high-fat diets plus 3% ordinary chitosan and chitosan nanopowder, respectively. The diets were prepared by blending powdered basic diet with other ingredients and test materials, and then pelleting.

2.3. Animal experimental design

During experimental period, rats were allowed free access to food and water. Body weight and food intake were recorded every day. At the end of six weeks, feces were collected for three days and lyophilized to determine the fat and cholesterol contents. All rats were given diethyl ether after fasting for 18 h. Blood was collected, and serum was obtained by centrifugation at 3000 g for 10 min. The liver were removed, rinsed in cold saline, patted between paper towels, and then weighed. The serum and liver samples were stored in a −20 °C freezer until used for further analysis.

2.4. The determinations of serum and liver lipids and lipase activities of rats

Serum TG, TC, HDL-cholesterol levels and LPL (EC3.1.1.34) activity were measured with commercial assay kits. The LDL-cholesterol content in serum was calculated by the Friedewald equation: LDL-cholesterol (mmol/L) = TC-HDL-choloseterol-TG/2.2 (Hellstrand et al., 2012). Liver lipid was extracted by the method of Folch, Lee, and Stanley (1957), and liver TG and TC levels were measured with commercial assay kits. Each liver (1 g) was homogenized with ice cold saline (9 ml) and centrifuged at 6000 g for 10 min. The supernatant was used to analyze the activities of liver HL (EC3.1.1.3) and LPL using commercial assay kits and expressed as units per gram protein.

2.5. The determinations of fat and cholesterol in feces

The lyophilized feces were milled and weighed. The fecal fat was determined gravimetrically by using the Soxhlet method with diethyl ester as the solvent (Deuchi, Kanauchi, Imasato, & Kobayashi, 1994). For fecal cholesterol determination, the fecal lipid was first extracted from 100 mg of lyophilized feces with 2 ml of chloroform-methanol (2:1 v/v) according to the method of Folch et al. (1957). The lower phase was dried under nitrogen gas. Fecal cholesterol was extracted using the method described by Zhao et al. (2006). In brief, the lipid extract was saponified in 2 ml of 1 mol/l KOH/methanol at 50 °C for 60 min. After cooling, 2 ml of distilled water was added, and the unsaponified cholesterol was extracted with 5 ml of n-hexane. The solvent was evaporated at 37 °C under nitrogen gas. The cholesterol content was determined with a commercial assay kit.

2.6. Statistical analysis

The test data was analyzed using DPS 7.05 software (Zhejiang University, Hangzhou, China). Values are expressed as mean ± SD ($n = 8$). Student-Newman–Kuels multiple range test were used to compare means at the 0.05 significance level.

3. Results and discussion

3.1. Effect of chitosan nanopowder on the growth of rats

The body weight (BW) gains and food intakes of rats are shown in Table 1. As previously reported, chitosan was able to reduce body weight gains of rats and mice fed high-fat diets (Sumiyoshi & Kimura, 2006; Zhang, Liu, Li, & Xia, 2008). In this study, similar effects were observed for chitosan nanopowders and the ordinary chitosan in rats. The BW gains of groups fed chitosan nanopowders were lower than the groups fed ordinary chitosan, and the LMW-chitosan-NP group showed increased BW gain compared with HMW-chitosan-NP group. As shown in Table 1, there was no significant difference in food intake among the four groups fed high-fat diets, and the groups showing low BW gains exhibited low food efficiency (FER), suggesting that the main reason for the difference in growth of rats in these groups was not food intake, but was the different nutrient efficiency caused by the added chitosan or chitosan nanopowders. Thus, these results indicated that at both high and low molecular weights, chitosan nanopowder was more effective than ordinary chitosan in inhibiting the increase of body weights of rats, and the inhibition ability of 51-kDa chitosan nanopowder was better than the 315-kDa nanopowder.

3.2. Effect of chitosan nanopowder on blood lipids of rats

Serum lipid concentrations of rats are shown in Table 2. After six weeks of diet, the serum TG, TC and LDL-cholesterol levels of HF group were much higher, and HDL-cholesterol level was lower than those of NF group, indicating that long time of high-fat diet resulted in abnormal change in blood lipid levels of rats. As compared to HF group, four groups fed ordinary chitosan and chitosan nanopowders (HMW-chitosan, LMW-chitosan, HMW-chitosan-NP, and LMW-chitosan-NP) registered significant decline in serum TG (by 22.4, 26.3, 33.6, and 42.8%, respectively), TC (by 46.8, 51.0, 53.9, and 58.5%, respectively) and LDL-cholesterol (by 62.9, 68.8, 73.2, and 79.0%, respectively), and an increase in HDL-C (by 24.2, 32.3, 43.5, and 50.0%, respectively). These results suggested that both ordinary chitosan and chitosan nanopowder could improve hyperlipidemia of rats fed high-fat diets, and the improvement effect of nanopowder were better. The data of serum lipids of rats also showed that the hypolipidemic activity of 51-kDa nanopowder was better than that of 315-kDa nanopowder. This result was in agreement with some previous literatures which reported that the hypolipidemic activity of chitosan or the related derivative of low molecular weight was better than that

Table 1
Body weight (BW) gain and food efficiency ratio (FER) of rats for six weeks of diets.

Group	Initial weight (g)	Final weight (g)	BW gain (g)	Food intake (g)	FER
NF	154.4 ± 5.0	473.3 ± 25.4	318.9 ± 18.3	29.8 ± 2.2	25.3 ± 1.7
HF	150.6 ± 4.6	533.5 ± 28.4	382.9 ± 26.4	29.7 ± 1.9	30.2 ± 2.3
HMW-chitosan	149.8 ± 8.2	516.7 ± 23.5	366.9 ± 24.3	29.7 ± 2.6	29.1 ± 1.7
HMW-chitosan-NP	153.5 ± 8.4	485.9 ± 24.3[a,b]	332.3 ± 21.6[a,b]	29.6 ± 2.8	26.5 ± 1.4[a,b]
LMW-chitosan	152.8 ± 6.2	502.5 ± 27.2[a]	349.7 ± 25.8[a]	30.9 ± 2.1	27.1 ± 1.9[a]
LMW-chitosan-NP	153.9 ± 5.7	469.1 ± 23.0[a,b]	315.2 ± 20.9[a,b]	30.6 ± 2.5	24.3 ± 1.5[a,b]

Abbreviations: BW: body weight; FER: food efficiency ratio = (BW gain × 100/food intake).
[a] There was significant difference between chitosan or chitosan nanopowder group and HF group ($p < 0.05$).
[b] There was significant difference between chitosan nanopowder group and the corresponding ordinary control group ($p < 0.05$).

Table 2
The serum lipids and atherosclerosis index (AI) of rats after six weeks of diets.

Group	TG (mmol/L)	TC (mmol/L)	HDL-cholesterol (mmol/L)	LDL-cholesterol (mmol/L)	AI
NF	0.91 ± 0.13	1.97 ± 0.12	0.84 ± 0.11	0.72 ± 0.14	1.35
HF	1.52 ± 0.14	5.16 ± 0.28	0.62 ± 0.06	3.85 ± 0.29	7.34
HMW-chitosan	1.18 ± 0.11[a]	2.74 ± 0.17[a]	0.77 ± 0.12[a]	1.43 ± 0.21[a]	2.55
HMW-chitosan-NP	1.01 ± 0.14[a,b]	2.38 ± 0.16[a,b]	0.89 ± 0.15[a]	1.03 ± 0.22[a,b]	1.67
LMW-chitosan	1.12 ± 0.15[a]	2.53 ± 0.21[a]	0.82 ± 0.14[a]	1.20 ± 0.26[a]	2.14
LMW-chitosan-NP	0.87 ± 0.15[a,b]	2.14 ± 0.16[a,b]	0.93 ± 0.17[a]	0.81 ± 0.17[a,b]	1.32

Abbreviations: TG: triglyceride; TC: total cholesterol; HDL-cholesterol: high-density lipoprotein cholesterol; LDL-cholesterol: low-density lipoprotein cholesterol; AI: atherosclerosis index = (TC-HDL-cholesterol)/HDL-cholesterol.
[a] There was significant difference between chitosan or chitosan nanopowder group and HF group ($p < 0.05$).
[b] There was significant difference between chitosan nanopowder group and the corresponding ordinary control group ($p < 0.05$).

of high molecular weight. LeHoux and Grondin (1993) found that high molecular weight chitosan (>750 kDa) were less effective as a hypocholesterolemic agent than a 70-kDa chitosan. Wu et al. (2012) found that the blood lipid-lowering ability of 70-kDa alkylsulfonated chitosan was better than a 200-kDa derivative.

Hyperlipidemia is a major cause of atherosclerosis, and the risk of atherosclerosis can be judged by the parameter of atherosclerosis index (AI) (Li, Chen, & Shen, 2011). As Table 2 shows, the AI of HF group was 7.34, much higher than that of NF group, which showed an AI value of 1.35. Compared with HF group, the ordinary chitosan and chitosan nanopowder groups showed great decrease in AI value. These results indicated that high-fat diet increased the atherosclerosis risk of rats, which could be decreased by both ordinary chitosan and chitosan nanopowder. After six weeks of diet, the AI values of HMW-chitosan, LMW-chitosan, HMW-chitosan-NP, and LMW-chitosan-NP groups were 2.55, 2.14, 1.67 and 1.32, respectively. It could be found from the comparison of AI values of ordinary chitosan and chitosan nanopowder groups that the nanopowder was more effective than ordinary chitosan in inhibiting atherosclerosis, and the inhibition ability of 51-kDa nanopowder was better in the two nanopowders, which was in consistent with their blood lipid-lowering effects as discussed above.

3.3. Effect of chitosan nanopowder on liver lipids of rats

The liver weight and liver lipids of rats are shown in Table 3, and the liver morphologies are shown in Fig. 1. As Table 3 shows, the liver weight, liver TG and TC levels of HF group were much higher than those of NF control. The liver of NF groups was dull red in color, but by contrast, the liver color of HF group became gray (Fig. 1). These results indicated that the feeding of high-fat diet resulted in high accumulation of lipids in liver of rats and led to severe fatty liver. Compared with HF group, decreased liver weight, liver TC and TG levels were observed for ordinary chitosan and chitosan nanopowder groups, and the decreased degrees in chitosan nanopowder groups were greater than the ordinary groups. The liver color of HMW-chitosan and LMW-chitosan groups was between red and gray, but HMW-chitosan-NP and LMW-chitosan-NP groups were close to red, which was similar to the NF control. These results indicated that chitosan nanopowder was more effective than ordinary chitosan in decreasing the accumulation of lipids in liver and preventing the development of fatty liver. Table 3 also shows that the liver weight, liver TC and TG of LMW-chitosan-NP group were lower than that of HMW-chitosan-NP group, indicating that 51-kDa nanopowder was more effective in reducing liver lipid accumulations. The data of liver parameters were in consistent with those of body weight gains and blood lipids, all proving the better hypolipidemic activities of chitosan nanopowder relative to ordinary chitosan and the superior hypolipidemic activity of 51-kDa nanopowder to 315-kDa nanopowder.

3.4. Effect of chitosan nanopowder on fecal lipid excretion of rats

The hypolipidemic activity of chitosan is related to its binding behavior of lipids in gastrointestinal tract, which decreases the lipid absorption of body by increasing their excretion in feces (Deuchi et al., 1994; Muzzarelli et al., 2006; Rodríguez & Albertengo, 2005; Sugano, Watanabe, Kishi, Izume, & Ohtakara, 1988; Xu, Huang, Qiu, Wu, & Hu, 2007). To analyze the hypolipidemic mechanism of chitosan nanopowder, the fecal lipids of rats were determined and the results were shown in Table 4. As Table 4 shows, the fecal fat and cholesterol contents of HF group were only 13.28% and 7.02 μmol/g, respectively. Compared with HF group, the fecal fat and cholesterol contents of rats were increased in HMW-chitosan

Table 3
The liver index, liver TC and TG of rats after six weeks of diets.

Group	Liver weight (%)	TG (μmol/g)	TC (μmol/g)
NF	2.92 ± 0.19	11.24 ± 1.80	8.88 ± 0.86
HF	4.38 ± 0.28	32.78 ± 3.34	13.49 ± 1.36
HMW-chitosan	3.82 ± 0.17[a]	18.82 ± 2.38[a]	12.14 ± 0.99[a]
HMW-chitosan-NP	3.32 ± 0.16[a,b]	15.39 ± 2.00[a,b]	10.06 ± 1.37[a,b]
LMW-chitosan	3.57 ± 0.26[a]	17.38 ± 2.44[a]	11.37 ± 1.17[a]
LMW-chitosan-NP	3.20 ± 0.22[a,b]	13.82 ± 2.15[a,b]	9.64 ± 1.30[a,b]

Abbreviations: TG: triglyceride; TC: total cholesterol.
[a] There was significant difference between chitosan or chitosan nanopowder group and HF group ($p < 0.05$).
[b] There was significant difference between chitosan nanopowder group and the corresponding ordinary control group ($p < 0.05$).

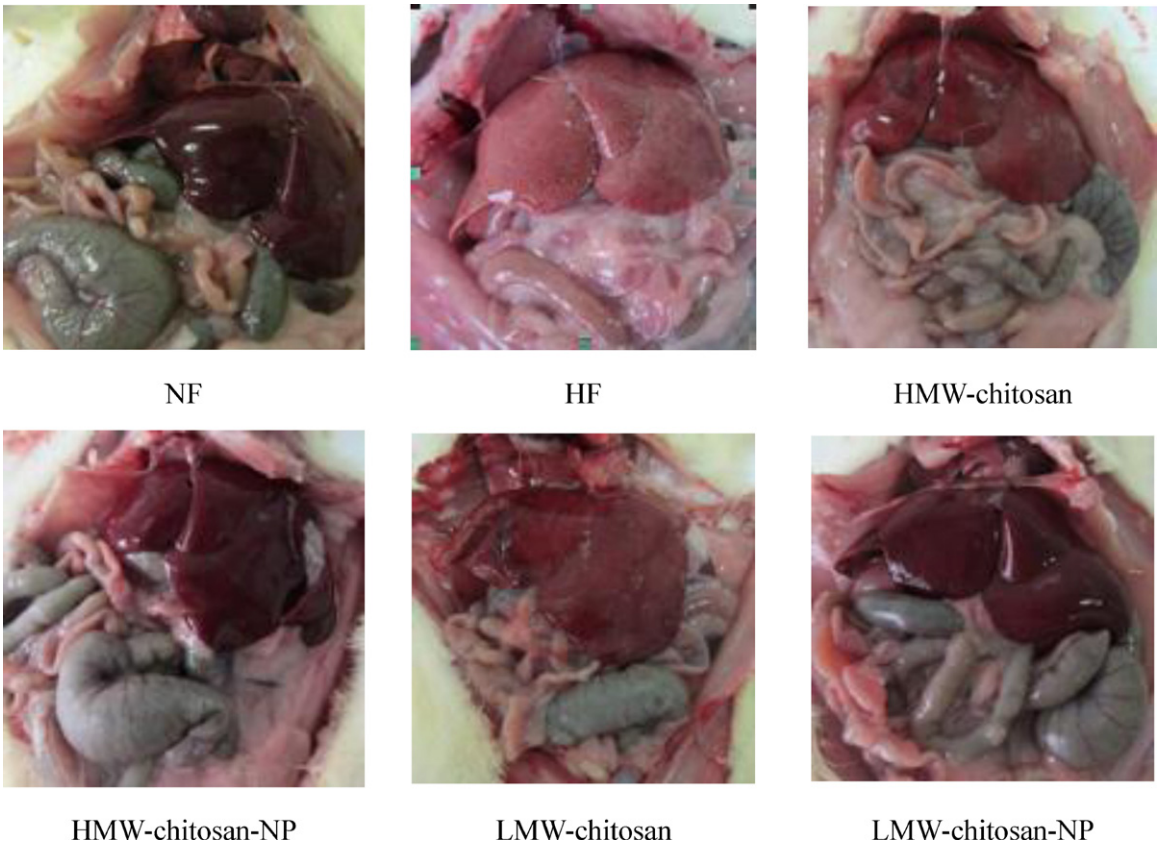

Fig. 1. The liver morphology of rats fed different diets.

(16.25% and 11.32 μmol/g, respectively), LMW-chitosan (14.78% and 9.37 μmol/g, respectively), HMW-chitosan-NP (20.59% and 14.48 μmol/g, respectively), and LMW-chitosan-NP groups (17.81% and 11.52 μmol/g, respectively). It can be found from the comparison of fecal lipids of rats in ordinary chitosan and chitosan nanopowder groups that decreasing the lipid absorption by increasing the lipid excretion in feces was one reason accounting for the increased hypolipidemic activity of chitosan nanopowder compared with ordinary chitosan. As compared to ordinary chitosan, the smaller particle size could provide chitosan nanopowder larger surface to interact with lipids, which might partly contribute to the increased excretion of lipids in feces. Besides, the decrease in particle size of chitosan might increase its dissolution rate in gastric juice, and in stomach, more molecular chains of chitosan nanopowder might participate in binding and trapping lipids, thus giving rise to the increased fecal lipid excretions of rats. The data of fecal lipids also shows that the fecal lipids of LMW-chitosan-NP group were lower than those of HMW-chitosan-NP group. However, the above results have shown that the hypolipidemic activity of LMW-chitosan-NP was better than HMW-chitosan-NP. These results suggested that some mechanism other than increasing fecal lipid excretion might work to help LMW-chitosan-NP to possess better hypolipidemic activity than HMW-chitosan-NP.

Table 4
The lipid content in feces of rats.

Group	Fat (%)	Cholesterol (μmol/g)
NF	4.94 ± 0.81	1.17 ± 0.08
HF	13.28 ± 0.94	7.02 ± 0.79
HMW-chitosan	16.25 ± 1.22[a]	11.32 ± 1.13[a]
HMW-chitosan-NP	20.59 ± 1.36[a,b]	14.48 ± 1.66[a,b]
LMW-chitosan	14.78 ± 1.46[a]	9.37 ± 1.32[a]
LMW-chitosan-NP	17.81 ± 1.92[a,b]	11.52 ± 1.40[a,b]

[a] There was significant difference between chitosan or chitosan nanopowder group and HF group ($p < 0.05$).
[b] There was significant difference between chitosan nanopowder group and the corresponding ordinary control group ($p < 0.05$).

3.5. Effect of chitosan nanopowder on activities of serum and liver lipases of rats

Lipoprotein lipase (LPL) and hepatic lipase (HL) are two key enzymes regulating lipid metabolism of animals (Connelly, 1999; Mead, Irvine, & Ramji, 2002). Some studies showed that some polysaccharides (including chitosan) could stimulate the activity of these two enzymes to exert hypolipidemic activity after the molecules are adsorbed by the body (Chen et al., 2008; Kwak, Kyung, Kim, Cho, & Rheem, 2010; Zhang et al., 2008; J. Zhang, Zhang, Mamadouba, & Xia, 2012). The activities of serum and liver LPL and HL of rats are shown in Table 5. As Table 5 shows, the two

Table 5
Serum and liver lipase activities of rats for six weeks of diets.

Group	Serum	Liver	
	LPL activity (U/g)	HL activity (U/g)	LPL activity (U/g)
NF	74.6 ± 5.3	101.2 ± 10.3	12.3 ± 1.5
HF	48.8 ± 3.5	54.8 ± 6.5	6.9 ± 0.8
HMW-chitosan	57.2 ± 4.0[a]	63.3 ± 5.9[a]	12.8 ± 1.3[a]
HMW-chitosan-NP	64.9 ± 4.5[a,b]	69.8 ± 5.2[a,b]	16.6 ± 1.0[a,b]
LMW-chitosan	61.7 ± 4.3[a]	71.3 ± 4.5[a]	13.5 ± 1.7[a]
LMW-chitosan-NP	66.8 ± 5.7[a,b]	82.8 ± 6.2[a,b]	17.8 ± 2.1[a,b]

Abbreviations: LPL: lipoprotein lipase; HL: hepatic lipase.
[a] There was significant difference between chitosan or chitosan nanopowder group and HF group ($p < 0.05$).
[b] There was significant difference between chitosan nanopowder group and the corresponding ordinary control group ($p < 0.05$).

nanopowder groups showed increased serum and liver LPL and HL activities compared with their corresponding ordinary chitosan control groups. This result suggested that the stimulating ability of nanopowder on activities of LPL and HL was better than that of ordinary chitosan. Therefore, except for increasing fecal excretion, the better stimulating ability of chitosan nanopowder on the activities of LPL and HL also contributed to the better hypolipidemic activity relative to ordinary chitosan. Table 5 also shows that the activities of serum and liver LPL and HL of LMW-chitosan-NP group were higher than those of HMW-chitosan-NP group. This result indicated that the better stimulating ability of LMW-chitosan-NP on LPL and HL activities might help it to exceed HMW-chitosan-NP in hypolipidemic activity.

4. Conclusion

This study showed that the chitosan nanopowder was more effective than ordinary chitosan in decreasing body weight and blood lipids and alleviating liver lipid accumulations of rats fed high-fat diet, exhibiting better hypolipidemic activity. The determinations of fecal lipids and serum and liver LPL and HL activities of rats revealed that compared with ordinary chitosan, the better hypolipidemic activity of chitosan nanopowder was attributed to its ability on increasing fecal lipid excretions and stimulating LPL and HL activities. The study also showed that the hypolipidemic activity of 51-kDa nanopowder was better than 315-kDa nanopowder. Compared with the rats fed 315-kDa nanopowder, the fecal lipid contents of rats receiving 51-kDa nanopowder was decreased, but these rats showed higher serum and liver LPL and HL activities, suggesting that the better improvement of 51-kDa nanopowder in LPL and HL activities might help it to exceed 315-kDa nanopowder in hypolipidemic activity.

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