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Hypocholesterolaemic effects of different chitosan samples *in vitro* and *in vivo*

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Abstract

This study determined the hypocholesterolaemic effects of chitosan preparations with different physicochemical properties. Chitosans with smaller particle size had better cholesterol-binding capacities. Chitosans exhibited potent hypocholesterolaemic effect in rats; those with higher degree of deacetylation and molecular weight seemed to reduce plasma triglyceride, total cholesterol and low-density-lipoprotein cholesterol levels and elevate the high-density-lipoprotein cholesterol level more effectively, although not all differences were significant. We concluded that the hypocholesterolaemic mechanism of chitosan was by adsorption, electrostatic force and entrapment.
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Keywords: Chitosan; Hypocholesterolaemic; Particle size; Degree of deacetylation; Molecular weight

1. Introduction

Chitosan, a polyglucosamine derived from chitin, is a cellulose-like polymer located mainly in the exoskeletons of arthropods, such as crabs, shrimps, lobsters and insects (Razdan & Pettersson, 1994). It can be defined both chemically and physiologically as a dietary fibre since it is a polysaccharide, which cannot be digested by digestive enzymes of humans (Razdan & Pettersson, 1996). It has exhibited a potent hypocholesterolaemic activity in rats (Ikeda et al., 1993; Simunek & Bartonova, 2005; Sugano et al., 1980) and humans (Guerciolini, Radu-Radulescu, Boldrin, Dallas, & Moore, 2001; Maezaki et al., 1993). However, its hypocholesterolaemic mechanism is still unclear.

Under different reaction conditions, chitosans with different physicochemical properties can be prepared. Degree of deacetylation and molecular weight of chitosan are two important characteristics which greatly affect its chemical and physiological five properties. It is well known that chitosan is the only abundant cationic polymer having an amino group in its chemical structure (Maezaki et al., 1993; Muzzarelli, 1996), and degree of deacetylation (DD) is a characteristic greatly different from those of other dietary fibres. Higher DD means that there are more free amino groups in the chitosan molecule and more positive charge in chitosan solution. It was reported that the hypocholesterolaemic activity of chitosan was better when DD was high (90% deacetylated), which might be due to the electrostatic force between chitosan and anion substances, such as fatty acid and bile acid (Deuchi, Kanauchi, Imasato, & Kobayashi, 1995; Vahouny, Satchithanandam, Cassidy, Lightfoot, & Furda, 1983). However, our previous study showed that binding capacities of chitosans with bile acids and triglycerides had no correlation with DD *in vitro* (Zhou, Xia, Zhang, & Yu, 2006).

Molecular weight is also related to the hypocholesterolaemic effect of chitosan, and the mechanism might be similar to that of certain dietary fibres, such as guar gum and

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pectin (Vahouny et al., 1983). The molecular weight of chitosan is proportional to its viscosity and either of these two parameters can represent the size of the molecule. The entrapment caused by a viscous polysaccharide, which would reduce the absorption of fat and cholesterol in the diet, was thought of as the hypocholesterolaemic mechanism of dietary fibres (Kanauchi, Deuchi, Imasato, Shizukuishi, & Kobayashi, 1995). However, the relationship between the molecular weight of chitosan and its hypocholesterolaemic effect is still under discussion. Sugano, Watanabe, Kishi, Izume, and Ohtakara (1988) showed that the hypocholesterolaemic action of chitosans in rats was independent of their molecular weight within the tested viscosity range (30–1620 cps). Another report found that high molecular weight chitosans (>750 kDa) were less effective as hypocholesterolaemic agents than a 70 kDa preparation (LeHoux & Grondin, 1993). In broiler chickens, feeding a low viscosity chitosan diet reduced plasma triacylglycerol and total cholesterol concentration, and a medium- and high-viscosity chitosan-containing diets reduced plasma total cholesterol and elevated HDL-cholesterol concentration, compared with control-fed animals (Razdan & Pettersson, 1996). The effect of chitosan on the apparent fat digestibility in rats was greater as its viscosity increased (Deuchi et al., 1995).

In the present study, the cholesterol-binding capacities of chitosans with different physicochemical properties were tested *in vitro*. The effects of chitosans with different degrees of deacetylation and molecular weight on plasma lipid concentrations in rats were compared, in order to elucidate the influence of the physicochemical properties of chitosans, especially DD and molecular weight, on their hypocholesterolaemic effects, and to propose a mechanism.

2. Materials and methods

2.1. Materials

The chitosan samples used in this study were prepared from sea crab shell chitin by alkali fusion in our laboratory (Xia & Wang, 1992). Sodium taurocholate and cholesterol were purchased from Sigma–Aldrich (St. Louis, MO). Triglyceride, total cholesterol, high-density-lipoprotein (HDL) cholesterol and low-density-lipoprotein (LDL) cholesterol kits were purchased from Zhejiang Dongou Bioengineering Co. Ltd., China.

2.2. Preparation of chitosans and determination of their properties

2.2.1. Preparation of chitosans with different degree of deacetylation

Briefly, 100 g of dried chitin was added to a 50% (w/v) NaOH solution in a flask. The reaction mixture was stirred for 1, 2, 6, or 9 h in a 95 °C water bath, to obtain chitosan samples with 64%, 73%, 81% and 90% degree of deacetylation, respectively. After cooling, the reaction mixtures were filtered. The solid was collected, washed with deionised water to neutral pH, and dried at 60 °C under vacuum.

2.2.2. Preparation of chitosans with different molecular weights

Chitosan sample was dissolved in 2% acetic acid to obtain a reaction mixture with a final concentration of 2 g chitosan/100 g solution. The reaction solution was heated with stirring for 2, 4 or 8 h. The reaction mixture was neutralised with 1 mM NaOH solution. Absolute ethanol was added to the neutralised solution until the final ethanol concentration reached 70 ml/l, to completely precipitate the chitosan. The chitosans with different molecular weights, but with the same degree of deacetylation, were collected by filtration, washed with deionised water, dried at 60 °C under vacuum and powdered.

2.2.3. Determination of the degree of deacetylation

The degree of deacetylation (DD) was determined, according to a colloid titration method (Cho, No, & Meyers, 1998). Briefly, dried chitosan was dissolved in 0.2 M CH_3COOH/0.1 M CH_3COONa solution to a final concentration of 0.02% (w/v), and 0.0025 M polyvinyl sulfate potassium salt (PVSK) and 0.1% (w/v) toluidine blue (TB) were added, as titrant and indicator, respectively. The DD was calculated as follows:

$$NH_2(\%) = \frac{N \times (V_1 - V_2) \times M}{5 \times C} \times 100 \quad (1)$$

where N is the concentration of PVSK (M); M is equivalent to 161.15 which is the molecular weight of a glucosamine residue; C, the concentration of chitosan (g/ml); V_1 the volume of PVSK (ml) when titrating the chitosan; and V_2 is the volume of PVSK when titrating the deionised water, which is a control (ml). Then,

$$DD(\%) = \frac{NH_2\%}{9.94\%} \times 100 \quad (2)$$

where 9.94% is the theoretical NH_2 percentage of chitosan.

2.2.4. Determination of molecular weight

The molecular weight of chitosan was determined using an Ubbelohde viscometer at 30 °C (Zhang & Neau, 2001). The intrinsic viscosity $[\eta]$ of chitosan samples was measured in a 0.2 M CH_3COOH/0.1 M CH_3COONa solution. The viscosity average molecular weight (M_v) was then calculated using the Mark–Houwink equation: $[\eta] = k(M_v)^{\alpha}$, in which the constants k and α depended on the DD, as follows (Wang, Bo, Li, & Qin, 1991):

$$k = 1.64 \times 10^{-30} \times DD^{14}\ cm^3/g \quad (3)$$

$$\alpha = -1.02 \times 10^{-2} \times DD + 1.82 \quad (4)$$

2.2.5. Determination of particle size

Particle size of chitosan was determined by a two-sieve method with standard sieves, according to the method in Chinese Pharmacopoeia, with some modifications. Chitosan

was sieved through two sieves with known diameter. The sieves were shaken for at least 15 min and the chitosan passing through the first sieve was collected. The sizes of sieve mesh for powdered chitosans were 125, 150 and 180 μm, and those for flaked chitosans were 2800, 3350 and 5600 μm.

2.3. Cholesterol-binding capacities of chitosans in vitro (Experiment I)

The cholesterol-binding capacity of chitosan *in vitro* was measured by the method of Nagaoka et al. (2001) with some modifications. Cholesterol micellar solution (1 ml) containing 10 mM sodium taurocholate, 0.4 mM cholesterol, 1 mM oleic acid, 132 mM NaCl, and 15 mM sodium phosphate buffer (pH 7.4), was prepared by sonication. Sixty milligrams of chitosan samples with different properties was added to 5 ml of micellar solution. Cellulose was used as a positive control and the micellar solution without chitosan was used as a substrate blank, to calculate the recovery. Each sample was prepared in triplicate. Then the mixtures were incubated for 2 h in a 37 °C shaker bath. Mixtures were transferred to centrifuge tubes and centrifuged at 16000 rpm for 20 min at 37 °C. The supernatant was collected for the determination of cholesterol enzymatically. The amount of binding was calculated as the amount of cholesterol in the supernatant of the substrate blank subtracted from the amount in the supernatant of the samples. The binding capacity of chitosan was expressed as milligram of bound cholesterol per gram of chitosan.

2.4. Hypocholesterolaemic effects of chitosan in rats (Experiments II and III)

2.4.1. Animals and diets

Male Sprague–Dawley rats weighing 100 ± 10 g (Experiment II) and 120 ± 10 g (Experiment III) were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences. They were individually housed in metabolic cages in a controlled environment (22 ± 1 °C, 50–60% relative humidity, 12 h light-dark cycle with lighting from 8:00 a.m. to 8:00 p.m.). All animal protocols were approved by our animal care and use committee.

In Experiment II, the rats were fed *ad libitum* with a commercial diet and water for 5 days, and were then randomly assigned to five groups (8–9 rats per group): normal-fat group (NF), receiving normal-fat diet (commercial diet, the compositions conform to GB14924.3); high-fat control group (HF), receiving high-fat diet containing 10% lard, 15% egg yolk powder, 1% cholesterol and commercial diet to 97% (in weight percent) plus 3% cellulose; and three chitosan groups (D73, D81 and D90), receiving 97% high-fat diet plus 3% chitosans (73%, 81% and 90% deacetylated, respectively). The diets were prepared by blending the powered commercial diet with the ingredients and test materials and forming bars.

In Experiment III, rats were assigned to four groups which were NF, HF, LW and HW groups. The addition of chitosan was at 5% (w/w), and two chitosan groups were studied, corresponding to the chitosans with low and high molecular weight. Other conditions were the same as Experiment II.

2.4.2. Experimental design

In Experiment II, water and diets were available *ad libitum*, body weight and food intake were recorded daily during the experimental period. Rats were derived of food overnight after being fed test materials for 3 weeks, and blood was withdrawn from the tail vein to measure the plasma lipid level. After 8 weeks of treatment, rats were deprived of food for about 18 h and blood was withdrawn.

In Experiment III, rats were deprived of the food overnight after the acclimation and blood was drawn from the tail vein to measure the initial plasma lipid level. During the experimental period, water and diets were available *ad libitum*, body weight and food intake were recorded daily, and blood was withdrawn every 2 weeks from the tail vein for determining plasma lipids. At the end of 6 weeks, rats were deprived of food for about 18 h and blood was collected.

2.4.3. Lipid analysis

Plasma samples were extracted from blood after centrifugation at 3000 rpm for 10 min. They were kept at −20 °C, prior to analysis of lipid parameters. Plasma triglyceride (TG), total cholesterol (TC), LDL cholesterol (LDL-C) and HDL cholesterol (HDL-C) concentrations were measured with commercial assay kits.

2.4.4. Statistical analysis

All data are expressed as means ± SE. Differences between the groups were determined by one-way analysis of variance, using a statistical analysis software program (SAS Institute, Cary, NC, USA). Differences among means were inspected using Student–Newman–Kuels multiple range test and results were considered significant if the value of p was <0.05.

3. Results

3.1. Cholesterol-binding capacity of chitosans in vitro (Experiment I)

Properties of chitosans prepared for testing cholesterol-binding capacity were listed in Table 1. There were nine chitosan samples to carry out this study (numbered 1–9). Chitosans with a wide range of degree of deacetylation (64%, 73%, 81% and 90%, respectively) were prepared and the molecular weight varied from 284 kDa to 2750 kDa. The particle sizes of major chitosan samples were 150 μm (100 mesh) and 180 μm (80 mesh). These chitosans were called powdered chitosans. The other two samples (samples 1 and 4) had significantly large particle size (5 mm and 3 mm, approximately), which were called flake chitosans.

Table 1
Physicochemical properties of chitosan samples

	DD (%)	M_v (kDa)	[η] (ml/g)	Particle size (μm)
Experiment I, II				
1	64	2750	1038	5600
2 (D73)	73	528	284	150
3	73	791	438	180
4	73	1510	889	3350
5	81	284	226	150
6 (D81)	81	544	431	180
7	81	988	780	180
8	90	354	380	150
9 (D90)	90	499	518	150
Experiment III				
LW	73	584	318	150
HW	73	936	609	150

DD, Degree of Deacetylation; M_v, viscosity average molecular weight; [η], intrinsic viscosity; samples numbered 1–9 were tested in Experiment I; D73, D81 and D90 represented the chitosans which were 73%, 81% and 90% deacetylated, studied in Experiment II; LW and HW represented the chitosans with lower and higher molecular weight studied in Experiment III.

Cholesterol-binding capacities of chitosans with different physicochemical properties are shown in Fig. 1. It showed that the physicochemical properties of chitosan had no conclusive effect on their cholesterol-binding capacities. Chitosan samples 2–4 which had the same degree of deacetylation (73%), showed that binding capacity was enhanced decreasing molecular weight. However, the result was reversed when DD was 90%, and no trend was observed when DD was 81%. Meanwhile, chitosan samples with similar molecular weight (samples 2, 6 and 9) did not show a trend between DD and cholesterol-binding capacity. However, we found that cholesterol-binding capacity changed greatly with particle size. Flake chitosans (samples 1 and 4) showed less binding capacity than powder chitosans and they bound even less cholesterol than cellulose powder. Moreover, when the DD and particle size were same, binding capacity of chitosan increased with molecular weight (6 and 7, 8 and 9).

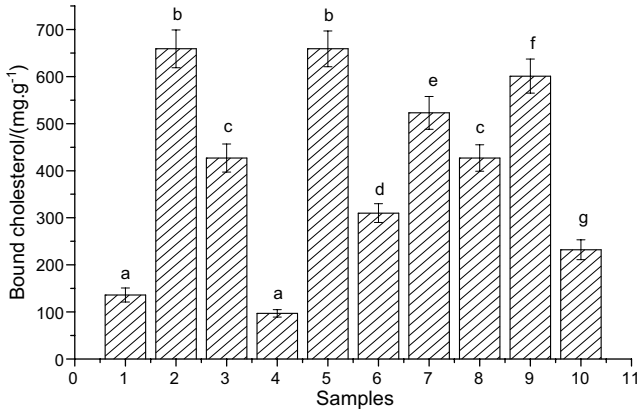

Fig. 1. Cholesterol-binding capacities of nine chitosan samples and cellulose *in vitro*. Samples 1–9 are chitosans (listed in Table 1) and 10 is cellulose. Values are expressed as means ± SE of three determinations. Values with different letters are significantly different at $p < 0.05$.

3.2. Hypocholesterolaemic effects of chitosans with different degree of deacetylation in rats (Experiment II)

In rats fed different diets, body weight gain during the experimental period is shown in Fig. 2. At the beginning, body weight increased steadily. After 5 weeks, rats in the NF and three chitosan groups grew slowly while those in the HF group grew as before. As Table 2 shows, in rats fed chitosan, weight gain was lower than that in rats fed normal-fat and high-fat diets, but food intake was more, although no differences were significant. Food efficiency in the five groups had the same trend as body weight gain. Rats fed chitosan with the highest degree of deacetylation had lowest body weight gain and food efficiency while food intake among the three chitosan groups was similar.

Plasma lipid concentrations of rats in the middle and at the end of the experiment are shown in Table 3. In the third week, triglyceride (TG) concentration in the NF group was significantly lower than in the other four groups. In the

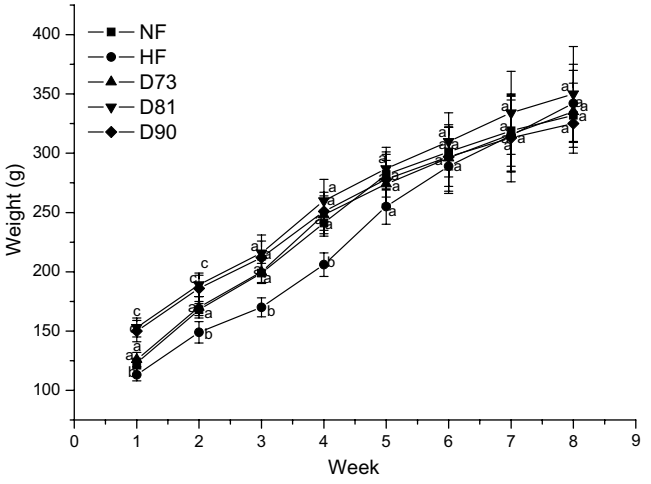

Fig. 2. Body weight gain in rats fed normal fat (NF), high fat + cellulose (HF), high fat + 73% deacetylated chitosan (D73), high-fat + 81% deacetylated chitosan (D81) or high fat + 90% deacetylated chitosan (D90) diets for 8 weeks (Experiment II). Values are expressed as means ± SE ($n = 8$ or 9). Values with different letters at the same time are significantly different at $p < 0.05$.

Table 2
Food intake, body weight gain and food efficiency ratio in rats fed normal fat (NF), high fat + cellulose (HF), high fat + 73% deacetylated chitosan (D73), high fat + 81% deacetylated chitosan (D81), or high fat + 90% deacetylated chitosan (D90) diets for 8 weeks (Experiment II)

	Body weight (BW) gain (g)	Food intake (g)	Food efficiency ratio (g BW gain × 100/g food intake)
NF	210 ± 35^{a}	20.25 ± 1.98^{a}	21.2 ± 1.36^{a}
HF	229 ± 47^{a}	20.23 ± 3.56^{a}	23.1 ± 2.02^{a}
D73	208 ± 32^{a}	22.59 ± 5.21^{a}	$18.8 \pm 1.96^{a,b}$
D81	198 ± 29^{a}	23.19 ± 4.36^{a}	17.4 ± 1.87^{b}
D90	175 ± 28^{a}	23.09 ± 4.02^{a}	$15.5 \pm 1.01^{b,c}$

Values are expressed as means ± SE ($n = 8$ or 9). Values with different letters within the same line are significantly different at $p < 0.05$.

Table 3
Plasma lipid concentration in rats fed normal fat (NF), high fat + cellulose (HF), high fat + 73% deacetylated chitosan (D73), high fat + 81% deacetylated chitosan (D81), or high fat + 90% deacetylated chitosan (D90) diets after 3 and 8 weeks (Experiment II)

	NF	HF	D73	D81	D90
TC (mM)					
3 weeks	1.76 ± 0.19^{a}	3.82 ± 0.47^{b}	3.16 ± 0.35^{c}	3.05 ± 0.32^{c}	2.91 ± 0.36^{c}
8 weeks	1.29 ± 0.24^{a}	4.11 ± 0.45^{b}	2.73 ± 0.21^{c}	2.63 ± 0.19^{c}	2.16 ± 0.15^{d}
TG (mM)					
3 weeks	0.89 ± 0.12^{a}	1.58 ± 0.21^{b}	1.33 ± 0.25^{b}	1.38 ± 0.19^{b}	1.25 ± 0.29^{b}
8 weeks	0.70 ± 0.16^{a}	1.65 ± 0.14^{b}	0.78 ± 0.12^{a}	0.77 ± 0.06^{a}	0.66 ± 0.08^{a}
HDL-C (mM)					
3 weeks	0.55 ± 0.10^{a}	0.42 ± 0.06^{a}	0.50 ± 0.09^{a}	0.53 ± 0.11^{a}	0.65 ± 0.12^{a}
8 weeks	0.45 ± 0.17^{a}	0.39 ± 0.11^{a}	0.51 ± 0.10^{a}	0.56 ± 0.16^{a}	0.77 ± 0.09^{b}
LDL-C (mM)					
3 weeks	0.80 ± 0.15^{a}	2.68 ± 0.25^{b}	2.05 ± 0.23^{c}	1.89 ± 0.19^{c}	1.69 ± 0.29^{c}
8 weeks	0.88 ± 0.21^{a}	2.97 ± 0.46^{b}	1.85 ± 0.21^{c}	1.72 ± 0.38^{c}	1.09 ± 0.44^{a}

Values are expressed as means ± SE ($n = 8$ or 9). Values with different letters within the same row are significantly different at $p < 0.05$.

final week, TG in the chitosan groups lowered significantly and was near to that in the NF group. The D90 group had the lowest TG concentration. Total cholesterol (TC) concentration in the chitosan groups was significantly lower than in the HF group and higher than in the NF group in the third and final week. High-density-lipoprotein cholesterol (HDL-C) concentrations in the five groups showed no significant differences, except for the D90 group, which had significantly higher HDL-C level in the final week. Low-density-lipoprotein cholesterol (LDL-C) level had the similar trend as total cholesterol, with the D90 group exhibiting the best hypocholesterolaemic effect.

3.3. Hypocholesteroalaemic effects of chitosan with different molecular weight in rats (Experiment III)

Rats fed chitosan had less body weight gain than those fed chitosan-free diets, while the food intake was similar (Table 4). Food efficiency was lower in the chitosan group, especially in the HW group. As shown in Fig. 3, receiving a high-fat diet significantly increased the body weight gain of rats. As time went by, chitosan effectively retarded body weight gain in rats and the highest molecular weight chitosan exhibited the best effect.

Table 4
Food intake, body weight gain and food efficiency ratio in rats fed normal fat (NF), high fat + cellulose (HF), high fat + low molecular weight chitosan (LW), or high fat + high molecular weight chitosan (HW) diets for 6 weeks (Experiment III)

	Body weight (BW) gain (g)	Food intake (g)	Food efficiency ratio (g BW gain × 100/g food intake)
NF	216 ± 15^{a}	20.87 ± 3.65^{a}	24.9 ± 2.11^{a}
HF	233 ± 30^{b}	22.65 ± 2.76^{a}	24.5 ± 2.36^{a}
LW	187 ± 15^{a}	19.00 ± 5.12^{a}	$23.4 \pm 1.69^{a,b}$
HW	152 ± 11^{a}	20.86 ± 5.25^{a}	17.4 ± 1.47^{c}

Values are expressed as means ± SE ($n = 8$ or 9). Values with different letters within the same line are significantly different at $p < 0.05$.

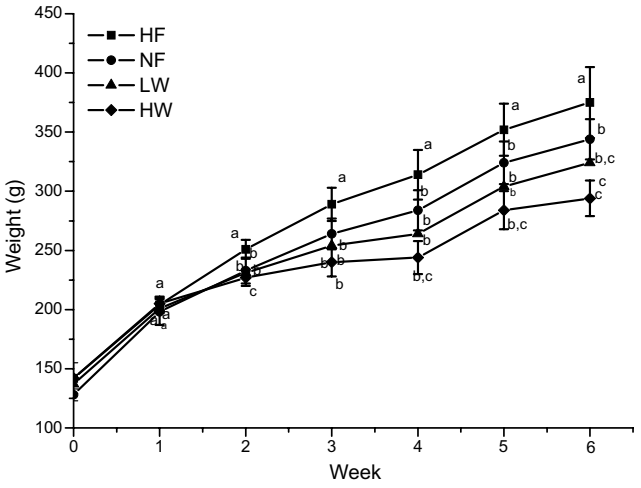

Fig. 3. Body weight gain in rats fed normal fat (NF), high fat + cellulose (HF), high fat + low molecular weight chitosan (LW) or high fat + high molecular weight chitosan (HW) diets for 6 weeks (Experiment III). Values are expressed as means ± SE ($n = 8$ or 9). Values with different letters at the same time are significantly different at $p < 0.05$.

The time course of the effects of chitosan with different molecular weight on plasma lipid levels is shown in Table 5. Total cholesterol level increased significantly after 2 weeks in groups fed high-fat diets and chitosan reduced this increase. Subsequently, chitosan further lowered the cholesterol level and that with higher molecular weight seemed to exhibit the better hypocholesterolaemic effect. Chitosan also showed a potent hypolipidaemic effect, especially with increasing of molecular weight. HDL-C level did not differ among the five groups and only increased a little in rats fed chitosan. Rats in the chitosan groups had significant lower LDL-C levels than those in the HF group. Although the results had no significant difference, chitosan with higher molecular weight showed a better hypocholesterolaemic effect than low molecular weight chitosan.

Table 5
Plasma lipid concentration in rats fed normal fat (NF), high fat + cellulose (HF), high fat + low molecular weight chitosan (LW), or high fat + high molecular weight chitosan (HW) diets for 6 weeks (Experiment III)

	NF	HF	LW	HW
TC (mM)				
0 weeks	2.23 ± 0.40^{a}	2.20 ± 0.30^{a}	2.10 ± 0.35^{a}	1.99 ± 0.16^{a}
2 weeks	2.02 ± 0.30^{a}	4.03 ± 0.60^{b}	3.13 ± 0.32^{c}	3.02 ± 0.38^{c}
4 weeks	2.31 ± 0.45^{a}	4.76 ± 0.34^{b}	3.01 ± 0.54^{a}	2.79 ± 0.66^{a}
6 weeks	1.96 ± 0.32^{a}	4.93 ± 0.52^{b}	2.99 ± 0.61^{a}	2.58 ± 0.57^{a}
TG (mM)				
0 weeks	1.31 ± 0.33^{a}	1.14 ± 0.27^{a}	1.24 ± 0.30^{a}	1.37 ± 0.33^{a}
2 weeks	1.10 ± 0.15^{a}	1.21 ± 0.14^{a}	1.19 ± 0.19^{a}	1.25 ± 0.22^{a}
4 weeks	1.09 ± 0.28^{a}	$1.38 \pm 0.29^{a,b}$	0.94 ± 0.14^{a}	0.80 ± 0.11^{a}
6 weeks	0.85 ± 0.20^{a}	1.45 ± 0.21^{b}	0.75 ± 0.15^{a}	$0.51 \pm 0.12^{a,c}$
HDL-C (mM)				
0 weeks	0.86 ± 0.14^{a}	0.83 ± 0.18^{a}	0.72 ± 0.13^{a}	0.69 ± 0.18^{a}
2 weeks	0.80 ± 0.18^{a}	0.78 ± 0.16^{a}	0.77 ± 0.13^{a}	0.74 ± 0.12^{a}
4 weeks	0.81 ± 0.13^{a}	0.60 ± 0.16^{a}	0.80 ± 0.17^{a}	0.79 ± 0.10^{a}
6 weeks	0.82 ± 0.20^{a}	0.51 ± 0.11^{b}	$0.83 \pm 0.20^{a,b}$	0.90 ± 0.22^{a}
LDL-C (mM)				
0 weeks	0.77 ± 0.14^{a}	0.85 ± 0.21^{a}	0.92 ± 0.14^{a}	0.68 ± 0.10^{a}
2 weeks	0.72 ± 0.21^{a}	2.70 ± 0.26^{b}	2.02 ± 0.31^{c}	1.71 ± 0.35^{c}
4 weeks	1.10 ± 0.21^{a}	3.73 ± 0.30^{b}	1.87 ± 0.21^{c}	1.65 ± 0.20^{c}
6 weeks	0.75 ± 0.14^{a}	3.76 ± 0.47^{b}	1.82 ± 0.20^{c}	1.45 ± 0.15^{c}

Values are expressed as means ± SE ($n = 8$ or 9). Values with different letters within the same row are significantly different at $p < 0.05$.

4. Discussion

In this study, hypocholesterolaemic effects of chitosans with different physicochemical properties were tested *in vitro* and *in vivo*. The results showed that particle size, degree of deacetylation and molecular weight all influenced the hypocholesterolaemic effect of chitosan.

This is the first report of the binding capacity of chitosan in cholesterol micellar solution. Powder chitosans exhibited better cholesterol-binding capacity than cellulose. However, chitosan in the flake form bound less cholesterol than cellulose. This is consistent with the report that chitosan with finer particle size could effectively lower the plasma and liver lipid level in rats (Sugano et al., 1980). In addition, the powder form of chitosan exhibited a greater rate of adsorption of oil, compared to the flake type (Ahmad, Sumathi, & Hameed, 2005). Generally, when the weight is fixed, the smaller the particle size is, the bigger is the total surface area. Powdered chitosan has looser pore structure, compared to flake chitosan, which may facilitate adsorption. This suggests that the interaction between chitosan and cholesterol in this experiment is adsorption, and the pore structure of chitosan contributes to its hypocholesterolaemic effect. However, chitosan can be dissolved in the acidic conditions of the stomach, so this adsorption action may weaken in the body.

The degree of deacetylation is a property of chitosans which greatly determines their physiological properties (Deuchi et al., 1995). We investigated the hypocholesterolaemic effect of chitosan with different degree of deacetylation in rats and found that the effect tended to strengthen as the DD of chitosan increased. Rats fed diets containing the highest deacetylated chitosan significantly lowered plasma cholesterol and LDL-C, and increased HDL-C level.

Food efficiency ratio decreased with increasing DD. Deuchi et al. (1995) observed a similar result. This may be due to the positive charge in the chitosan molecule. Chitosan can interact by electrostatic force with fatty acid and bile acid, which have negative charge, in the digestive tract. Chitosan which is highly deacetylated has more free amino groups and hence more positive charge, so the electrostatic force strengthens. Reduction of fatty acid and bile acid will lead to less absorption of fat from the diets (Gallaher, Munion, Hesslink, Wise, & Gallaher, 2000; Sugano et al., 1988), and the reduction of endogenetic cholesterol because of the interruption of enterohepatic bile acid circulation (Razdan & Pettersson, 1996), will influence cholesterol metabolism.

Molecular weight is another important parameter of chitosan, which corresponds to the viscosity. Chitosan is soluble in the acidic conditions of the stomach and forms a gel when the molecular weight is high. When fat and chitosan in the diets are eaten together, the viscous chitosan will entrap the fat droplet in the stomach. In the small intestine, which is at neutral pH, chitosan forms a precipitate and prevents the digestion of fat. This has been proved *in vitro* (Zhou et al., 2006).

In our present study, chitosan with higher molecular weight limited the body weight gain of adult rats significantly, reduced the food efficiency ratio and lowered plasma lipid, compared to the HF group in Experiment III. Furthermore, in Experiment III, when the DD and particle size were comparable, chitosan with higher molecular weight also exhibited better cholesterol-binding capacity *in vitro*. These results verified the effect of viscosity on hypocholesterolaemic activity. However, the effect of molecular weight on the lipid-lowering activity of chitosan seemed was not obvious. These results indicated that the viscosity in the upper gastrointestinal tract was not the major factor influencing the hypocholesterolaemic effect of chitosan. Above a certain viscosity, the effect of molecular weight increases is small. A glucosamine oligomer was not effective (Sugano et al., 1988), which confirmed that the viscosity of chitosan was required for its hypocholesterolaemic effect.

So, we can conclude that the physicochemical properties of chitosans will affect their hypocholesterolaemic activities. When the particle is finer, and degree of deacetylation and molecular weight is relatively high, the effect is better. Electrostatic action, adsorption and entrapment are the probable hypocholesterolaemic mechanisms of chitosan.

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