Functional comparison of high and low molecular weight chitosan on lipid metabolism and signals in high-fat diet-fed rats

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Abstract

The present study examined and compared the effects of high- and low-molecular weight (MW) chitosan, a nutraceutical, on intestinal and liver lipid metabolism in rats fed with high-fat diet. Both high- and low-MW chitosan decreased liver weight, elongated small intestine, improved the dysregulation of blood lipids and liver fat accumulation, and increased fecal lipid excretion in high-fat diet-fed rats. Supplementation of both high- and low-MW chitosan significantly inhibited the decreased phosphorylated AMP-activated protein kinase (AMPK)α and peroxisome proliferator-activated receptor (PPAR)α protein expressions and the increased lipogenesis/cholesterogenesis-associated protein expressions (sterol regulatory element binding protein (SREBP)1c, SREBP2, and PPARγ) and the decreased apolipoprotein (Apo)E and microsomal triglyceride transfer protein (MTTP) protein expressions in the livers of high-fat diet-fed rats. Both high and low-MW chitosan supplementation could also suppress the increased MTTP protein expression and the decreased angiopoietin-like protein (Angptl)4 protein expression in the intestines of high-fat diet-fed rats. Comparison between high and low-MW chitosan, high-MW chitosan has a higher efficiency than low-MW chitosan on the inhibition of intestinal lipid absorption and the increase of hepatic fatty acid oxidation, which can improve liver lipid biosynthesis and accumulation.

Keywords: high and low molecular weight chitosan; lipid metabolism; liver lipid accumulation
1. Introduction

The Western pattern diet, which generally characterized by high calories, high protein, high fat, and high salt, accompanied with low physical activity level tends to become obese and increase the risk of having the metabolic syndrome [1]. Obesity is a risk factor for the occurrence of chronic diseases, such as diabetes, cardiovascular disease, and cancer. There are 39% of women and 39% of men aged ≥ 18 overweight and 18% of children and adolescents aged 5-19 overweight or obese in 2016 [2]. The insulin resistance and dyslipidemia induced by obesity and diabetes are the risk factors for the occurrence of nonalcoholic fatty liver disease (NAFLD) [3,4]. How to prevent and improve obesity and diabetes has become an important issue to reduce the prevalence of NAFLD.

Supplementation of chitosan has been reported to be capable of regulating the metabolism of carbohydrates and lipids. Chitosan could reduce the activity of intestinal disaccharides, increase the excretion of lipids, improve the insulin resistance, and prevent hepatic lipid accumulation in diabetic animal models [5-8]. Zeng et al. (2008) have shown that molecular weight (MW) and water solubility are the important factors to influence the absorption and distribution of chitosan in mice by oral administration; the decreased MW and increased water solubility can enhance the absorption of chitosan molecules [9]. Chiu et al. (2017) have also found that low-MW chitosan produces greater effects than chitosan oligosaccharide to improve the abnormal lipid metabolism and intestinal disaccharidase activity in high-fat diet-fed rats [5]. Kondo et al. (2000) have shown that low-MW chitosan can prevent the progression of low-dose streptozotocin (STZ)-induced slowly progressive non-insulin-dependent diabetes mellitus [10]. Yao et al. (2008) found that high-MW chitosan possessed more potential in decreasing hyperglycemia and hypercholesterolemia in STZ-induced diabetic rats than that of low-MW chitosan [11]. Both high- and low-MW
chitosan have been suggested to be capable of decreasing liver gluconeogenesis and increasing muscle glucose uptake to alleviate hyperglycemia in a type-1 diabetic animal model [7]. The effects of both high- and low-MW chitosan on intestinal and liver lipid metabolism still need to be clarified. The present study examined and compared the effects of high- and low-MW chitosan on intestinal and liver lipid metabolism in rats fed with high-fat diet. This study focuses on the ability of comparatively high- and low-MW chitosan to inhibit the intestinal lipid metabolism and explore the difference from the effects of high- and low-WM chitosan on fatty liver in high-fat diet fed rats.

2. Results

2.1. Effects of high- and low-MW chitosan on body weight, organ weight, and plasma biochemical indices in HF diet-fed rats

After eight weeks of feeding different diets, the changes in body weight and organ weight were shown in Table 1. The final body weight in the HF diet-fed group was significantly higher than the NC group, which could not be reversed by both high- and low-MW chitosan supplementations. Both high- and low-MW chitosan supplementations significantly inhibited the increased liver weight in HF diet-fed rats (Table 1). There was no significant difference in relative adipose tissue weight between the HF diet-fed group and the HF diet-fed + high- or low-MW chitosan group, although high-MW chitosan could reduce the weight of peripheral adipose tissue weight compared to the normal control group (Table 1). Both high- and low-MW chitosan supplementations significantly prolonged the length of the small intestine compared to the normal control group (Table 1).
Table 1. The changes of body weight, liver and adipose tissue weight, and small intestine length in rats fed with different experimental diets for 8 weeks.

<table>
<thead>
<tr>
<th></th>
<th>NC</th>
<th>HF</th>
<th>HC</th>
<th>LC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>539.9±22.5*</td>
<td>578±47.3</td>
<td>518.3±34.1*</td>
<td>543.4±18.9*</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>14.5±1.5*</td>
<td>33.3±4.9</td>
<td>20.0±1.9*</td>
<td>23.0±3.2*,#</td>
</tr>
<tr>
<td>Relative liver weight (g/100g BW)</td>
<td>2.7±0.2*</td>
<td>5.9±0.7</td>
<td>3.9±0.4*</td>
<td>4.2±0.5*</td>
</tr>
<tr>
<td>Adipose tissue weight (g)</td>
<td>28.7±6.3</td>
<td>23.1±5.2</td>
<td>18.0±5.6</td>
<td>20.7±3.1</td>
</tr>
<tr>
<td>Relative adipose weight (g/100g BW)</td>
<td>5.2±1.0*</td>
<td>4.1±0.8</td>
<td>3.5±1.0</td>
<td>3.8±0.5</td>
</tr>
<tr>
<td>Perirenal adipose weight (g)</td>
<td>16.7±3.6</td>
<td>14.0±3.4</td>
<td>10.7±2.7*</td>
<td>12.1±2.2</td>
</tr>
<tr>
<td>Epididymal adipose weight (g)</td>
<td>12.0±3.0*</td>
<td>9.1±2.0</td>
<td>7.3±3.3</td>
<td>8.6±1.5</td>
</tr>
<tr>
<td>Small intestine length (cm)</td>
<td>116.3±2.6</td>
<td>116.0±5.2</td>
<td>132.6±11.4*</td>
<td>129.1±10.3*</td>
</tr>
<tr>
<td>Relative small intestine length (cm/100g BW)</td>
<td>21.4±1.2</td>
<td>20.7±1.6</td>
<td>25.6±1.4*</td>
<td>23.5±1.9*,#</td>
</tr>
</tbody>
</table>

Data are presented as mean ± S.D. for each group (n=7-8).

*p<0.05, versus HF group. #p<0.05, versus HC group.

NC: normal control + 5% cellulose; HF: High-fat diet + 5% cellulose; HC: High-fat diet + 5% High molecular weight chitosan; LC: High-fat diet + 5% Low molecular weight chitosan.

Changes in the levels of blood lipids and TNF-α are shown in Table 2. The blood levels of TC, LDL-C+VLDL-C, VLDL-C, LDL-C, TC/HDL-C, and TNF-α were significantly increased in the HF diet group, which could be significantly reversed by both high- and low-MW chitosan supplementations. Unexpectedly, the blood TG level was decreased in the HF diet group compared to NC group (Table 2). Both high- and low-MW chitosan supplementations significantly inhibited the HF diet group-decreased blood TG level, but it was no statistically significant difference compared to NC group (Table 2). Moreover, the blood levels of liver function markers AST and ALT were significantly increased in the HF diet group, which could be significantly reversed by both high- and low-MW chitosan.
supplementation (Table 2).

**Table 2.** The changes of plasma lipids, TNF-α, and liver functional markers levels in rats fed with different experimental diets for 8 weeks.

<table>
<thead>
<tr>
<th></th>
<th>NC</th>
<th>HF</th>
<th>HC</th>
<th>LC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total cholesterol (mg/dL)</strong></td>
<td>63.1±9.3*</td>
<td>87.4±11.</td>
<td>56.2±7.9*</td>
<td>52.3±17.0*</td>
</tr>
<tr>
<td><strong>HDL-C (mg/dL)</strong></td>
<td>44.7±5.8*</td>
<td>9.1±7.9</td>
<td>26.7±2.7</td>
<td>25.5±4.1</td>
</tr>
<tr>
<td><strong>LDL-C + VLDL-C (mg/dL)</strong></td>
<td>18.4±7.2*</td>
<td>58.3±12.0</td>
<td>29.5±10.2*</td>
<td>26.7±18.7*</td>
</tr>
<tr>
<td><strong>VLDL-C (mg/dL)</strong></td>
<td>14.9±8.1*</td>
<td>28.8±8.2</td>
<td>16.0±6.4*</td>
<td>14.0±12.7*</td>
</tr>
<tr>
<td><strong>LDL-C (mg/dL)</strong></td>
<td>3.5±1.6*</td>
<td>29.5±11.4</td>
<td>13.6±7.7*</td>
<td>12.7±10.5*</td>
</tr>
<tr>
<td><strong>TC/HDL-C (mg/dl)</strong></td>
<td>1.4±0.2*</td>
<td>3.2±0.9</td>
<td>2.1±0.5*</td>
<td>2.1±0.9*</td>
</tr>
<tr>
<td><strong>HDL-C/LDL-C + VLDL-C ratio</strong></td>
<td>2.8±1.2*</td>
<td>0.5±0.2</td>
<td>1.0±0.4*</td>
<td>2.2±2.7*</td>
</tr>
<tr>
<td><strong>Triglyceride (mg/dL)</strong></td>
<td>96.2±43.3*</td>
<td>34.5±5.1</td>
<td>44.1±8.8*</td>
<td>45.8±13.0*</td>
</tr>
<tr>
<td><strong>TNF-α (pg/dL)</strong></td>
<td>10.9±2.3*</td>
<td>36.8±13.7</td>
<td>17.1±3.6*</td>
<td>22.8±7.3*</td>
</tr>
<tr>
<td><strong>ALT (U/L)</strong></td>
<td>15.7±3.5*</td>
<td>72.2±32.1</td>
<td>25.5±15.9*</td>
<td>35.1±25.0*</td>
</tr>
<tr>
<td><strong>AST (U/L)</strong></td>
<td>42.1±16.6*</td>
<td>79.1±42.8</td>
<td>39.4±22.2*</td>
<td>59.7±31.2</td>
</tr>
</tbody>
</table>

Data are presented as mean ± S.D. for each group (n=7-8).

* p<0.05, versus HF group.

NC: normal control + 5% cellulose; HF: High-fat diet + 5% cellulose; HC: High-fat diet + 5% High molecular weight chitosan; LC: High-fat diet + 5% Low molecular weight chitosan.

ALT=alanine aminotransferase; AST=aspartate aminotransferase

2.2. **Effects of high- and low-MW chitosan on the lipid metabolism in adipose and liver tissues and feces of HF diet-fed rats**

As shown in Figures 1A and 1B, high-MW, but not low-MW, chitosan supplementation significantly decreased the TG level and LPL activity in the perirenal adipose tissues compared to the HF diet group, although there were no significant changes in the HF diet group compared to the NC group. High-MW, but not low-MW, chitosan supplementation could also significantly increase the lipolysis rate compared to the HF diet group (Figure 1C).
Figure 1. Effects of high- or low-MW chitosan on TG (A), lipoprotein lipase (LPL) (B), and lipolysis rate (C) in perirenal adipose tissues of HF diet-fed rats. The rats were fed with different experimental diets for 8 weeks. Data are presented as mean ± SD (n = 8). NC, normal control + 5% cellulose; HF, high fat diet + 5% cellulose; HC, high fat diet + 5% high-MW chitosan; LC, high fat diet + 5% low-MW chitosan. *p < 0.05, versus HF group. #p < 0.05, versus HC group.

We next investigated the effects of chitosan on lipid-related profiles in the livers of HF diet-fed rats. As shown in Table 3, rats had severe TC and TG accumulation in the livers of HF diet-fed rats, which could be significantly reversed by both high- and low-MW chitosan supplementations. Moreover, the histological examination showed severe hepatic vacuolization in HF diet-fed rats, which could be significantly reversed by both high- and low-MW chitosan supplementations (Figure 2).

Table 3. The changes of hepatic cholesterol and triglyceride levels in rats fed with different experimental diets for 8 weeks.

<table>
<thead>
<tr>
<th></th>
<th>NC</th>
<th>HF</th>
<th>HC</th>
<th>LC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mg/g liver)</td>
<td>2.7±1.0*</td>
<td>152.6±13.7</td>
<td>52.6±28.4*</td>
<td>65.4±33.2*</td>
</tr>
<tr>
<td>(g/liver)</td>
<td>0.04±0.02*</td>
<td>5.1±0.8</td>
<td>1.1±0.6*</td>
<td>1.6±0.9*</td>
</tr>
<tr>
<td>Triglyceride</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mg/g liver)</td>
<td>12.1±5.0*</td>
<td>96.0±22.2</td>
<td>49.2±20.1*</td>
<td>58.7±29.0*</td>
</tr>
<tr>
<td>(g/liver)</td>
<td>0.2±0.1*</td>
<td>3.1±0.5</td>
<td>1.0±0.4*</td>
<td>1.4±0.8*</td>
</tr>
</tbody>
</table>

Data are presented as mean ± S.D. for each group (n=7-8).
*p<0.05, versus HF group.
NC: normal control + 5% cellulose; HF: High-fat diet + 5% cellulose; HC: High-fat diet + 5% high-MW chitosan; LC: High-fat diet + 5% low-MW chitosan.
molecular weight chitosan; LC: High-fat diet + 5% Low molecular weight chitosan.

**Figure 2.** Effect of high- or low-MW chitosan on hepatic morphology in HF diet-fed rats. The livers were isolated from rats fed with different experimental diets for 8 weeks. Tissue sections were stained with hematoxylin and eosin. NC, normal control + 5% cellulose; HF, high fat diet + 5% cellulose; HC, high fat diet + 5% high-MW chitosan; LC, high fat diet + 5% low-MW chitosan.

The fecal lipid-related profiles were also tested and shown in Table 4. Both high and low-MW chitosan supplementations significantly increased the fecal weights and fecal TC and TG levels compared to the HF diet group.

**Table 4.** The changes of fecal weight, total cholesterol and triglyceride concentration in rats fed with different experimental diets for 8 weeks.

<table>
<thead>
<tr>
<th>Diet</th>
<th>NC</th>
<th>HF</th>
<th>HC</th>
<th>LC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feces wet weight (g/day)</td>
<td>2.9±0.2</td>
<td>2.8±0.2</td>
<td>3.1±0.5*</td>
<td>3.6±0.5*</td>
</tr>
<tr>
<td>Feces dry weight (g/day)</td>
<td>2.2±0.2</td>
<td>2.0±0.2</td>
<td>2.4±0.5*</td>
<td>2.9±0.5*</td>
</tr>
<tr>
<td>Total cholesterol (mg/g feces)</td>
<td>2.4±0.4*</td>
<td>12.2±1.5</td>
<td>24.2±4.8*</td>
<td>23.2±4.7*</td>
</tr>
<tr>
<td>(mg/day)</td>
<td>5.4±1.1*</td>
<td>24.3±3.4</td>
<td>59.4±20*</td>
<td>66.5±17.3*</td>
</tr>
<tr>
<td>Triglyceride (mg/g feces)</td>
<td>0.4±0.2*</td>
<td>0.7±0.2</td>
<td>1.8±0.4*</td>
<td>2.0±0.3*</td>
</tr>
<tr>
<td>(mg/day)</td>
<td>0.9±0.4*</td>
<td>1.4±0.5</td>
<td>4.5±1.5*</td>
<td>5.7±1.4*</td>
</tr>
</tbody>
</table>

Data are presented as mean ± S.D. for each group (n=7-8).
* p<0.05, versus HF group.
NC: normal control + 5% cellulose; HF: High-fat diet + 5% cellulose; HC: High-fat diet + 5% High molecular weight chitosan; LC: High-fat diet + 5% Low molecular weight chitosan.

2.3. **Effects of high- and low-MW chitosan on lipometabolic signals in the liver, blood, and intestine of HF diet-fed rats**

To evaluate the mechanisms of preventive effects of high- and low-MW chitosan on HF diet-altered lipid homeostasis, we investigated the expressions of lipometabolic mediators...
(AMPKα, PPARα, PPARγ, SREBP1c, and SREBP2) [12] and lipid transport-related proteins (ApoE, MTTP, and Angptl4) [13-15]. As shown in Figure 3, rats fed HF diet significantly inhibited the protein expressions of phosphorylated AMPKα (decreased pAMPKα/AMPKα ratio) and PPARα in the livers, which could be significantly reversed by both high- and low-MW chitosan supplementations. The effects of high-MW chitosan on AMPK phosphorylation and PPARα protein expression were significantly higher than low-MW chitosan (Figure 3). Moreover, both high- and low-MW chitosan supplementations significantly inhibited the increased protein expressions of PPARγ, SREBP1c, and SREBP2 in the livers of HF diet-fed rats (Figure 4). The effects of chitosan on the expressions of MTTP, ApoE, and Angptl4 proteins were shown in Figure 5. HF diet feeding significantly decreased the protein expressions of liver ApoE and MTTP, plasma Angptl4, and intestinal Angptl4, and increased the protein expression of intestinal MTTP, which could be significantly reversed by both high- and low-MW chitosan supplementations. The effects of high-MW chitosan on MTTP, ApoE, and Angptl4 protein expressions were significantly better than low-MW chitosan (Figure 5).

**Figure 3.** Effect of high- or low-MW chitosan on hepatic AMPKα and PPARα protein expressions in HF diet-fed rats. The rats were fed with different experimental diets for 8 weeks. Protein expressions of phosphorylated AMPKα/AMPKα (A) and PPARα (B) were determined by Western blotting. Densitometric analysis for protein levels corrected to AMPKα or β-actin (internal control) was shown. Data are presented as mean ± SD (n = 6). NC, normal control + 5% cellulose; HF, high fat diet + 5% cellulose; HC, high fat diet + 5% high-MW chitosan; LC, high fat diet + 5% low-MW chitosan. *p < 0.05, versus HF group. #p < 0.05, versus HC group.
Figure 4. Effect of high- or low-MW chitosan on hepatic SREBP2, SREBP1c and PPARγ protein expressions in HF diet-fed rats. The rats were fed with different experimental diets for 8 weeks. Protein expressions of SREBP2 (A), SREBP1c (B), and PPARγ (C) were determined by Western blotting. Densitometric analysis for protein levels corrected to β-actin (internal control) was shown. Data are presented as mean ± SD (n = 6). NC, normal control + 5% cellulose; HF, high fat diet + 5% cellulose; HC, high fat diet + 5% high-MW chitosan; LC, high fat diet + 5% low-MW chitosan. *p < 0.05, versus HF group. #p < 0.05, versus HC group.

Figure 5. Effect of high- or low-MW chitosan on MTTP, ApoE, and Angptl4 protein expressions in the liver, blood, or intestine of HF diet-fed rats. The rats were fed with different experimental diets for 8 weeks. Protein expressions of hepatic ApoE and MTTP (A), blood Angptl4 (B), and intestinal MTTP and Angptl4 (C) were determined by Western blotting. Densitometric analysis for protein levels corrected to β-actin (internal control) was shown. Data are presented as mean ± SD (n = 6). NC, normal control + 5% cellulose; HF, high fat diet + 5% cellulose; HC, high fat diet + 5% high-MW chitosan; LC, high fat diet + 5% low-MW chitosan. *p < 0.05, versus HF group. #p < 0.05, versus HC group.
3. Discussion

Absorption of chitosan in the intestine is mainly affected by the molecular weight and water solubility of chitosan. Low-MW chitosan is more easily absorbed in the intestine than high-MW chitosan [9]. The absorbed chitosan can be rapidly distributed from circulation to liver and kidney. The intestinal absorption and tissue distribution may be the factors to affect the healthy activities of chitosan. Sugano et al. (1980) have been shown that chitosan (200 kDa) supplementation possesses the potential in lowering blood cholesterol levels in HF diet-fed rats for 20 days [16]. High-MW chitosan (830 kDa) has been found to suppress lipid accumulation in liver and adipose tissues in a diabetic rat model for 10 weeks [6]. Gades and Stern (2003) have shown that chitosan complex (Absorbitol®) supplementation for 4 days can increase the fecal fat excretion in men [17]. Sugano et al. (1988) have further indicated that the effects of lowering blood cholesterol by chitosan are independent of their MW [18]. Nevertheless, Zhang et al. (2012) have indicated that the potential for hypolipidemic activity by low-MW chitosan (39.8 kDa) is higher than that of high-MW chitosan (712.6 kDa) in HF diet-fed rats for 8 weeks; but the increase in fecal fat and cholesterol excretion by high-MW chitosan is better than that of low-MW chitosan [19]. Chitosan oligosaccharides (≤ 1 kDa and ≤ 3 kDa) have also been found to possess better lipid-lowering effects than higher MW chitosan in HF diet-fed rats for 6 weeks [20]. However, Chiu et al. (2017) have recently reported that low-MW chitosan (80 kDa) supplementation provides a better improvement than chitosan oligosaccharide (0.719 kDa) on lipid metabolism in HF diet-fed rats for 10 weeks [5]. Yao et al. (2008) have also found that the effects of high MW chitosan (1000 kDa) on lipid-lowering and fecal fat excretion-increasing are greater than that of low-MW chitosan (14 kDa) in a diabetic rat model for 4 weeks [11]. In the present study, we also demonstrated
that high-MW chitosan (740 kDa) had a higher efficiency than low-MW chitosan (91 kDa) on the inhibition of intestinal lipid absorption and the increase of hepatic fatty acid oxidation in HF diet-fed rats for 8 weeks; but there was no significant difference between high- and low-MW chitosan on the increase in fecal TC and TC excretion. For comparison of the effects of high- and low-MW chitosan supplementations, the differences in the intestinal absorption efficiency of chitosan and their adsorption capacity of dietary lipids may result in their different outcome on the regulation of blood and liver lipid metabolism.

LPL in adipose tissue plays an important role in lipid metabolism. It can hydrolyze the core triglyceride of chylomicrons and VLDL to free fatty acid and monoglycerides. Fatty acid is uptaken and re-esterified and stored in adipose tissue or used as an energy source in muscle. Gaidhu et al., (2010) have shown that the LPL activity is increased in epididymal and visceral adipose tissues, and the lipolysis rate is decreased in subcutaneous and visceral adipose tissues in HF diet (60% kcal from fat)-fed rats [21]. In the present study, we found that high-MW, but not low-MW, chitosan supplementation significantly decreased the TG level and LPL activity and increased the lipolysis rate in the perirenal adipose tissues compared to the HF diet group. These effects of high-MW chitosan resulted in the enhancement of lipolysis and the decrease of TG storage in the adipose tissues.

MTTP, an endoplasmic reticulum (ER)-resident chaperone, can assemble chylomicrons in the enterocytes. The increase in intestinal MTTP expression can be observed in high-cholesterol and HF diet-fed inositol-requiring enzyme 1 (IRE1β)-deficient mice, leading to hyperlipidemia and fatty liver [22]. Angptl4 is known as an endogenous inhibitor of LPL. Angptl4-deficient mice has been found to increase intestinal pancreatic lipase (PL) activity and lipid accumulation and reduce lipid excretion in the feces, leading to increased weight gain and fat mass [23]. Therefore, MTTP and Angptl4 play an important role in intestinal lipid digestion and absorption. In the present study, the increased MTTP and decreased
Angptl4 protein expressions were observed in the intestines of HF diet-fed rats. Feeding both high- and low-MW chitosan could significantly inhibit the effects of HF diet on MTTP and Angptl4, leading to suppress dietary TG hydrolysis and absorption and increase fecal lipid excretion. High-MW chitosan was more effective than low-MW chitosan in inhibiting MTTP protein expression, indicating that high-MW chitosan may have higher potential in reducing dietary lipid absorption and increasing lipid excretion than low-MW chitosan in the intestine. However, there was no significant difference on the hypolipidemic effect and the increased fecal lipid excretion in HF diet-fed rats between high- and low-MW chitosan supplementations, suggesting chitosan may possess multiple molecular targets on intestinal lipid metabolism.

AMPK activation plays a key role in the regulation and maintenance of cell homeostasis [24]. Increasing AMPK activation has been shown to improve NAFLD through the inhibition of de novo lipogenesis, the increase of hepatic fatty acid oxidation, and the enhancement of mitochondrial function and integrity in adipose tissue [25]. When AMPK is activated, the adipogenic transcription factors (eg. SREBPs and PPARγ) are downregulated to deactivate transcriptional activity, which impairs lipid synthesis in the liver and improves hepatic lipid metabolism. SREBP-1c is preferentially involved in fatty acid and TG synthesis; SREBP-2 is preferentially to activate cholesterol synthesis in the liver [26]. AMPK could also regulate liver PPARα activity [27]. PPARα regulates the activities of fatty acid oxidation systems including mitochondrial and peroxisomal β-oxidation and microsomal ω-oxidation that are involved in energy expenditure [28]. In PPARα-deficient ob/ob mice, the inhibition of fatty acid oxidation resulted in increasing weight gain and severe fatty liver [29]. Impairment of mitochondrial fatty acid β-oxidation capacity has been found to cause hepatic diacylglycerol accumulation and insulin resistance [30]. In the present study, HF diet inhibited the AMPKα phosphorylation and PPARα protein expression and promoted the protein expressions of
SREBP1c, SREBP2, and PPARγ and increased the TC and TG synthesis in the livers, which could be significantly reversed by both high- and low-MW chitosan supplementations. High-MW chitosan was more efficient than low-MW chitosan on the upregulation of AMPKα phosphorylation and PPARα protein expression.

Kuipers et al. (1997) have demonstrated that apoE deficiency impairs hepatic VLDL-TG assembly and secretion in mice [31]. Maugeais et al. (2000) have found that the secretion of hepatic VLDL-TG can be promoted by apoE expression through the increase in the production rate of VLDL-apoB in a mouse model [32]. The patients of apoB-defective genetic form of familial hypobetalipoproteinemia frequently exerted fatty liver [33]. MTTP has been shown to increase hepatic VLDL-TG assembly and secretion under apoB100 background in ob/ob mice [34]. In the present study, feeding HF diet inhibited the protein expressions of apoE and MTTP in the liver, resulting in liver lipid accumulation, which could be reversed by high- and low-MW chitosan supplementation. High-MW chitosan was more efficient than low-MW chitosan on the upregulation of apoE and MTTP protein expression.

The intestinal chylomicron and liver VLDL secretion and the utilization of blood triglyceride by muscle, heart and adipose tissues influence the changes of blood TG levels. The previous studies have observed the decreased circulating TG levels in HF diet-fed mice [35,36]. It may be due to the reduced dietary carbohydrate. Chiu et al. (2015) have also observed the decreased blood TG levels in HF diet-fed rats that could be reversed by high-MW chitosan supplementation [37]. In the present study, we found that HF diet feeding inhibited the blood Angptl4 and hepatic MTTP and ApoE protein expressions and decreased the blood TG levels, which could be reversed by both high- and low-MW chitosan supplementations. These results indicate that both high- and low-MW chitosan are capable of ameliorating the alteration in lipid metabolism induced by high fat diet feeding.
4. Materials and Methods

4.1. Materials

High-MW chitosan prepared from crab shell chitin was obtained from Charming & Beauty Co. (Taipei, Taiwan). The high-MW chitosan (MW: 740 kDa) was used to prepare the low-MW chitosan (MW: 91 kDa) as described previously [38]. In brief, the powder of high-MW chitosan was dissolved in 8 N HCl at 55 °C for 3 h. The cooling solution was used to stop the reaction and added equal NaOH (8 N) to adjust the pH to 9-10. The sample was then washed and dried. The high-performance liquid chromatography and Fourier transform infrared spectroscopy were used to detect the average molecular weight and deacetylation degree, respectively. The measurement of viscosity was performed by a Haake viscometer (CV20; Haake Mess-Technik GmbHu, Karlsruhe, Germany).

4.2. Animals and Diets

Male Sprague-Dawley rats (six-week old) were purchased from BioLASCO (Taipei, Taiwan) acclimatized with a chow diet (Rodent Laboratory Chow, Ralston Purina, St. Louis, MO) for one week. Rats were divided into four groups (n=8 of each group): (1) normal control (NC) group, (2) high-fat (HF) diet group, (3) HF diet + 5% high-MW (740 kDa) chitosan (HC) group, (4) HF diet + 5% low-MW (91 kDa) chitosan (LC) group. The diet formulation was shown in Table 5. Rats were individually housed in stainless-steel cages and maintained in the rooms controlled with temperature (23 ± 1°C), light (12 h light/dark cycle), and humidity (40-60% relative humidity). After 8 weeks of experimental intervention, rats were fasted for 12 h and then sacrificed by exsanguination under anesthesia. Blood samples were harvested for biochemical analysis. The liver, adipose, and intestine tissues were isolated, weighted, and stored at -80°C until the analysis for lipid profile. Feces were collected for 3 consecutive days prior to sacrifice and stored at -80°C until the analysis for fecal lipid contents. The animal experimental procedures were performed in accordance with
the guidelines for the care and use of laboratory animals [39] and approved by the Animal House Management Committee of the National Taiwan Ocean University.

Table 5. Composition of experimental diets (%).

<table>
<thead>
<tr>
<th>Ingredient (%)</th>
<th>NC</th>
<th>HF</th>
<th>HC</th>
<th>LC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Lard</td>
<td>3</td>
<td>13</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Vitamin mixture¹</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Minerals²</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Cholic acid</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Corn starch</td>
<td>64.8</td>
<td>54.1</td>
<td>54.1</td>
<td>54.1</td>
</tr>
<tr>
<td>Cellulose</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>High molecular weight chitosan³</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low molecular weight chitosan⁴</td>
<td></td>
<td>5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NC: normal control (3% Lard + 2% soybean oil) + 5% cellulose; HF: High-fat diet (13% Lard + 2% Soybean oil) + 5% cellulose; HC: High-fat diet + 5% High molecular weight chitosan; LC: High-fat diet + 5% Low molecular weight chitosan. ¹AIN-93 vitamin mixture; ²AIN-93 mineral mixture; ³The average MW and DD of High molecular weight chitosan about 7.4×10⁵ Dalton and 91%, respectively; ⁴The average MW and DD of Low molecular weight chitosan about 9.1×10⁴ Dalton and 92%, respectively.

4.3. Measurement of Triglyceride (TG), Cholesterol (TC), Lipoproteins, and Activities of Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT)

The TG and TC levels in samples from blood, liver, and adipose tissues and feces were measured by using Audit Diagnostics Enzymatic Assay kits (Audit Diagnostics, Cork, Ireland). The plasma lipoproteins [high-density lipoprotein (HDL), low-density lipoprotein (LDL), and very-low-density lipoprotein (VLDL)] were isolated through a density gradient performed by an ultracentrifuge (Hitachi, Tokyo, Japan) with 194000 x g at 10°C for 3 h. The levels of AST and ALT were determined by the AST and ALT enzymatic kits (Randox, Antrim, UK). The change of absorbance at 340 nm was detected by a time scan
spectrophotometer (U-2880A; Hitachi, Tokyo, Japan).

4.4. Measurement of Lipolysis Rate

Lipolysis rate was measured as described previously [37]. Briefly, 0.2 g adipose tissues were minced and added into a N-tris-(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (25 mM) buffer containing isoproterenol (1 μM). A glycerol detection kit (Randox, Amtrim, U.K.) was used to measure the glycerol levels in samples after 1, 2, and 3 h of incubation at 37°C and the absorbance was recorded at 520 nm. The lipolysis rate was expressed by nano-moles glycerol/gram adipose tissue/h.

4.5. Measurement of Lipoprotein Lipase (LPL) Activity

LPL activity was measured as described previously [37]. The 0.1 g adipose tissues were minced and added into a Krebs-Ringer bicarbonate buffer (pH 7.4) with 10 units/mL heparin for 60 min at 37°C, and then samples were mixed with equal volume of p-nitrophenyl butyrate (2 mM). LPL activity was measured by the levels of p-nitrophenol formation over 10 min incubation. The absorbance was recorded at 400 nm.

4.6. Histological Analysis of Liver

Five micrometer thick of liver paraffin sections were used for histological examination. The hematoxylin and eosin (H&E)-stained tissue sections were examined by a Nikon Eclipse TS100 microscope equipped with a Nikon D5100 digital camera.

4.7. Protein Expression Analysis

The protein expression was determined by Western blotting as described previously [40]. The 50-100 μg proteins were used and loaded into SDS-PAGE gel (8%-12%), and then transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked for at least 1 h, and then probed with primary antibodies for AMP-activated protein kinase (AMPK)α and its phosphorylated form (p-AMPKα) (Cell Signaling Technology, Danvers, MA, USA), peroxisome proliferator-activated receptor
(PPAR)-γ, PPAR-α, angiopoietin-like 4 protein (Angptl4), sterol-regulatory-element-binding protein (SREBP)1c, SREBP2, microsomal triglyceride transfer protein (MTTP), β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and apolipoprotein E (ApoE) (Bioss Antibodies, Woburn, MA, USA) at 4°C overnight. Next, the membranes were immuno-probed again with horseradish peroxidase-conjugated secondary antibodies. The antigen-antibody complexes were visualized by using an enhanced chemiluminescence kit (Bio-Rad) and exposed to X-ray film (Fujifilm, Tokyo, Japan). The protein bands were analyzed densitometrically using Image J 1.51 software (National Institutes of Health, Bethesda, MD, USA).

4.8. Statistical Analysis

All results are presented as the Mean ± Standard Deviation (SD). The difference among experimental groups is assessed by one-way analysis of variance (ANOVA) followed by Dunnett’s test with the IBM SPSS Statistics 22.0 software.

5. Conclusions

In conclusion, the present study showed that in a HF diet-fed rat model, both high- and low MW chitosan (1) inhibit MTTP and increase Angptl4 protein expressions in the intestine and increase the small intestine length and fecal lipid excretion; (2) activate AMPK and inhibit downstream lipogenesis transcription factors (SREBP2, SREBP1c, and PPARγ) protein expressions and promote PPARα protein expression in the liver; (3) promote liver VLDL secretion-related proteins (ApoE and MTTP) expressions; leading in reducing fatty synthesis, increasing β-oxidation, and improving fatty liver. Comparison of high- and low-MW chitosan, high-MW chitosan may be more effective than low-MW chitosan on the inhibition of intestinal lipid absorption and the enhancement of hepatic lipid oxidation and the prevention of fatty liver.
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Conflict of Interest: The authors declare no competing financial interest.

References


