Chitosan Oligosaccharides Attenuate Atherosclerosis and Decrease Non-HDL in ApoE-/- Mice

Yang Yu, Tian Luo, Shuai Liu, Guohua Song, Jiju Han, Yun Wang, Shutong Yao, Lei Feng and Shucun Qin

Yang Yu and Tian Luo contributed equally to this work.

Key Laboratory of Atherosclerosis in Universities of Shandong; Institute of Atherosclerosis, Taishan Medical University, Taian, China

Aim: Chitosan-oligosaccharides (COS) treatment showed lipid lowering effects in rats and reverse cholesterol transport (RCT) promotion in mice, suggested that COS might be a potential atheroprotective material. In this study, we investigated the effects of COS treatment on atherosclerosis (AS) in apolipoprotein E deficient mice (apoE-/-).

Methods: After feeding high fat (HF) diet for 12 weeks with the gastric gavages administration of COS or vehicle, respectively, the mice were sacrificed for the assessment of atherosclerosis, plaque stability, and the mechanism investigation.

Results: Cholesterol and TG in non-high density lipoprotein (non-HDL) fractions were reduced dramatically in COS groups. The COS treatment decreased the lesion areas of aortic enface, plaque areas in aortic root, and increased plaque stability in apoE-/-.

Furthermore, the COS treatment significantly enhanced the expression of liver low density lipoprotein receptor (LDL-R), scavenger receptor BI (SR-BI) as well as the expression of macrophage SR-BI and ATP binding cassette transporter A1(ABCA1). We also found that the COS treatment did not affect the plasma lipid level in LDL-R deficient mice and cholesterol absorption in wild type mice.

Conclusions: COS treatment attenuated AS and decreased plasma non-HDL level in apoE-/-, and the potential mechanism might be involved with enhanced expression of hepatic LDL-R and SR-BI, and macrophage ABCA1.


Key words: Chitosan oligosaccharides, Atherosclerosis, Scavenger receptor BI, Low density lipoprotein receptor, ATP binding cassette transporter A1

Introduction

Low-density lipoprotein (LDL) and very low density lipoprotein (VLDL), also referred to as non-high density lipoprotein (non-HDL), are the main constituents of pro-atherosclerosis (AS) lipoproteins. The current research is focused on natural materials for lipid-regulating compounds for AS disease treatment.

Chitosan, the deacetylated form of chitin, has been demonstrated to be a functional lipid-lowering material. Chitosan oligosaccharides (COS), the soluble degradation product of chitosan, may be absorbed more readily by animals and humans than chitosan. Plasma COS levels have been shown to reach the maximal concentration at 30 min after the oral administration of COS and decrease to undetectable levels after 4-5 hours in rats, which suggested that COS may be a potential candidate for pharmaceutical application. Furthermore, reports have shown that COS displayed similar properties as chitosan in improving immunity, antitumor, and anti-infection. Some studies indicated that chitosan had beneficial lipid-regulating effects in animal and humans.

In addition, we previously reported that COS administration increased HDL cholesterol (HDL-C) and reduced LDL choles-
terol (LDL-C) in rats on a high-fat (HF) diet. Furthermore, the COS treatment promoted reverse cholesterol transport (RCT), a defensive mechanism against AS, in wild type mice. Moreover, our data showed that LDL-C was decreased and the hepatic LDL receptor (LDL-R) expression was enhanced in the COS treated wild type mice. Therefore, we hypothesized that COS displayed anti-AS effects.

The apolipoprotein E deficient (apoE/-) mouse is a putative model for AS research with a non-HDL dominant lipoprotein profile. In this study, we demonstrate that COS administration decreased the plasma non-HDL cholesterol (non-HDL-C) and triglyceride (TG) levels and attenuated AS lesions in apoE/- mice fed a HF diet.

**Methods**

**Reagents**

COS (pharmaceutical grade) with a molecular weight not more than 1000 Dal was purchased from Shanghai u-sea Biotech Co., Ltd. Cholesterol was obtained from Shanghai Lanji Science and Technology Co., Ltd (Shanghai, China). The HF diet was prepared as reported previously. Assay kits for total cholesterol (TC), TG, and HDL-C were from Biosino Bio-technology and Science Inc. (Beijing, China). Primary antibodies against LDL-R, scavenger receptor BI (SR-BI), ATP binding cassette transporter A1 (ABCA1), ABCG5, ABCG8, Niemann-Pick C1-Like1 (NPC1L1), MYLIP (IDOL), α unit of liver X receptor (LXRα), and tubulin were obtained from Abcam (Cambridge, MA, USA). Primary antibody of sterol regulatory element binding proteins (SREBP) 1 and 2 were purchased from Santa Cruz (Dallas, TX, USA). Primary antibody of MOMA-2 was purchased from Serotec (Langford Lane, Kidlington, UK). Primary antibody of apolipoprotein B (apoB) and matrix metalloproteinase-9 (MMP-9) was purchased from Novus Biologicals (Littleton, CO, USA).

**Mice, Diet and COS Treatment**

Thirty-six male 12-week-old apoE/- mice were obtained from the laboratory animal center of the Academy of Military Medical Sciences (Beijing, China). Experimental subjects were housed in a temperature and humidity controlled room with a 12/12 h light-dark cycle. The mice were randomly divided into 3 groups, the Control group (n=12, distilled water treated group) and two COS treated groups (n=12, 250 mg/kg/day or 1000 mg/kg/day designated as COS-L and COS-H, respectively). All groups were fed a HF diet (15.8% fat and 1.25% cholesterol) for 12 weeks before being sacrificed. For the plasma lipid assay, apoE/-, wild type, and LDL receptor knockout mice (LDL-R/-) were fed a chow diet or HF diet for 4 weeks before fasting blood harvesting. This study was approved by the Laboratory Animal Care Committee of Taishan Medical University, and all animal experiments were conducted in accordance with the Guidelines of Care and Use of Laboratory Animals at the Taishan Medical University.

**Lipid Analysis**

The fasting blood was collected and TC, HDL-C and TG were assayed. Non-HDL-C was calculated by subtracting the amount of HDL-C from TC. Lipoprotein profiles were obtained by fast protein liquid chromatography (FPLC). Briefly, 100 μL pooled plasma was loaded onto a SuperoseTM 6 column (10/300) connected with the ÄKTA FPLC on a purifier-900 system to separate the lipoprotein fractions and eluted with the mobile phase (0.15 M NaCl, 0.01 M Na2HPO4 and 0.1 M EDTA, pH 7.5) at a flow rate of 0.3 mL/min. Fifty fractions (0.5 mL each) of eluate were collected and the cholesterol and TG content from each fraction were determined. The liver tissue was homogenized and lipid was extracted for TC and TG determinations.

**Atherosclerosis Analysis**

The aortas were dissected and the arches were exposed for photographing. A quantitative analysis of an aortic lesion en face assay was performed as previously described. The plaque morphological histomorphometric characters were analyzed via oil red O staining, Masson’s staining, or hematoxylin/eosin (H&E) staining. The total intimal lesion area per cross section was quantified by taking the average of 6 sections spaced 30 μm apart from the base of the aortic root. A quantitative analysis of the plaque area was performed as described previously.

**Immunostaining of Macrophage and MMP-9 in the Aorta Roots**

Sequential sections were stained with specific antibodies and staining areas were quantified as described previously.

**Determination of the Plasma Inflammatory Cytokine Expressions**

Plasma tumor necrosis factor α (TNFα) and interleukin 6 (IL-6) levels were determined by enzyme-linked immunosorbent assay (ELISA) kits (R&D, McKinley Place NE, Minneapolis, USA).
and real-time PCR analysis were performed as described previously\(^{11}\). The primers are listed in Table 1.

### Western Blot

The total protein extraction from tissue or cultured cells and a Western Blot analysis were performed as described previously\(^{11}\).

### Statistics

Each experiment was conducted at least 3 times. The data were presented as the means±standard deviations (SD) and subjected to an ANOVA analysis or the Mann-Whitney \(U\) test (nonparametric test) as appropriate. Statistical significance was considered to be present at \(p<0.05\).
HDL fractions in a dose-dependent manner in apoE-/- fed high-fat diet (Fig. 1E, F). These findings indicated that COS possesses anti-atherosclerotic properties.

COS Administration Decreased Plasma ApoB100 and ApoB48

Apolipoprotein B (apoB) is the primary apolipoprotein in LDL and VLDL and the largest apolipoprotein synthesized in the liver (apoB100) and small intestine (apoB48). To investigate the effect of the COS treatment on the plasma apoB content, 0.5 μL combined fasting plasma from each group was detected by Western blot. The COS treatment significantly decreased the plasma apoB100 and apoB48 levels in a dose-dependent manner in apoE-/- mice fed a HF diet or chow diet (Fig. 2A, B). Additionally, the COS treatment reduced the plasma apoB100 and apoB48 levels in wild type mice fed a chow diet (Fig. 2C). Our findings indicated that COS administration consistently decreased the plasma apoB100 and apoB48 levels in mice.

COS Administration Attenuated AS Lesions

To evaluate the effect of the COS treatment on AS lesions, we dissected aortas and photographed them (Fig. 3A). In addition, we measured the proximal and whole aortic lesion areas. After 12 weeks of COS administration on a HF diet, we found a reduction of lesions in the aortas (Fig. 3B, C). We additionally discovered a 27% and 38% reduction of the whole aortic lesion areas in the COS-L and COS-H groups, respectively, and a 33% and 53% reduction in the aortic root areas in the COS-L and COS-H groups, respectively, compared with the Control group (Fig. 3D, E). These findings indicated that COS possesses anti-atherosclerotic properties.

Table 2. Effect of COS on plasma lipid levels in apoE-/- and wild type mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Plasma lipid levels after COS administration for 10 weeks (mg/dL)</th>
<th>TC</th>
<th>HDL-C</th>
<th>Non-HDL-C</th>
<th>TG</th>
</tr>
</thead>
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<tr>
<td>apoE-/- fed</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>high-fat diet</td>
<td>Control (n=11)</td>
<td>843.6±74.2</td>
<td>59.1±15.3</td>
<td>689.5±75.1</td>
<td>168.1±35.3</td>
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<td></td>
<td>COS-L (n=12)</td>
<td>746.4±144.3*</td>
<td>65.8±10.9</td>
<td>605.3±76.1*</td>
<td>118.9±14.7**</td>
</tr>
<tr>
<td></td>
<td>COS-H (n=12)</td>
<td>642.0±57.6**</td>
<td>61.1±18.7</td>
<td>501.2±69.8**</td>
<td>116.1±22.4**</td>
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<tr>
<td>apoE-/- fed</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>chow diet</td>
<td>Control (n=11)</td>
<td>392.1±46.2</td>
<td>47.3±13.9</td>
<td>344.8±41.6</td>
<td>154.7±26.6</td>
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<td></td>
<td>COS-L (n=12)</td>
<td>356.8±29.7*</td>
<td>42.5±15.1</td>
<td>314.3±25.5</td>
<td>135.4±31.5</td>
</tr>
<tr>
<td></td>
<td>COS-H (n=12)</td>
<td>348.5±61.6*</td>
<td>39.6±20.4</td>
<td>308.9±47.9*</td>
<td>151.2±19.8</td>
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<tr>
<td>Wild type fed</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>chow diet</td>
<td>Control (n=11)</td>
<td>118.1±11.9</td>
<td>66.3±5.7</td>
<td>51.8±8.5</td>
<td>68.5±27.2</td>
</tr>
<tr>
<td></td>
<td>COS-L (n=10)</td>
<td>95.5±11.0*</td>
<td>60.4±5.2</td>
<td>35.1±12.3*</td>
<td>56.1±16.9</td>
</tr>
<tr>
<td></td>
<td>COS-H (n=10)</td>
<td>97.5±19.8*</td>
<td>66.9±7.6</td>
<td>30.6±9.1**</td>
<td>53.8±27.4</td>
</tr>
</tbody>
</table>

Values are presented as the means ± SD. TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; TG, triglyceride; apoE-/-, apolipoprotein E deficient mouse. *, compared with wild type, p < 0.05. **, compared with wild type, p < 0.01.

Results

COS Treatment Decreased Plasma Cholesterol and TG

As shown in Table 2, plasma TC and non-HDL-C were significantly decreased in the COS treatment groups in a dose-dependent manner (11% and 23% of TC, and 12% and 27% of non-HDL-C in the COS-L and COS-H groups, respectively), whereas the HDL-C level was not changed. The plasma TG level decreased remarkably in the COS treatment groups (29% and 31% in the COS-L and COS-H groups, respectively). Cholesterol and TG are the main lipid components in lipoproteins, therefore our results indicated that the compositions of the lipoproteins were altered in the COS groups.

To further investigate the lipid distribution among the lipoproteins affected by the COS treatment, we employed FPLC to fractionize the lipoproteins and measured cholesterol and TG in each fraction. Our results indicated that the COS treatment significantly decreased cholesterol and TG in the non-HDL fractions in a dose-dependent manner in apoE-/- mice fed a HF diet or chow diet, whereas no change was observed in the HDL fractions (Fig. 1A-D). We additionally performed the same experiments in wild type mice fed COS. Consistent with the plasma lipid level, the COS treatment decreased non-HDL TG, however, no difference in the cholesterol or TG contents was observed in the HDL fractions from wild type mice (Fig. 1E, F).
apoE-/- mice were collected after being fed a HF diet for 8 weeks and sectioned at the aorta sinus level for a histological analysis. H&E staining of these sections demonstrated a blank area (indicating lipid deposit...
Effects of COS Administration on the TNFα and IL-6 Levels

During AS, the plasma pro-inflammatory cytokine levels are much higher than during normal conditions. Yoon reported that COS may suppress the inflammation of macrophages induced by LPS in vitro. However, our data showed that the plasma TNFα and IL-6 levels were unchanged (Supplementary Fig. 1), which indicated that the COS treatment did not lead to significant anti-inflammatory effects in this study.

COS Administration Improved the Expression of Hepatic LDL-R and SR-BI

The uptake of contents from lipoprotein particles by the liver is through LDL-R and SR-BI. COS administration significantly increased the SR-BI expression at the transcriptional level (Fig. 5A, C). In addition, the Western blot analyses showed that COS administration significantly enhanced the SR-BI expression by 32% and 87% in the COS-L group and the COS-H group, respectively. COS administration significantly improved the LDL-R expression at the transcriptional level in the plaque from the Control group, whereas no obvious lipid deposition was observed in the COS groups (Fig. 4A). The results suggested that the plaque content may have been altered following the COS treatment. Therefore, the plaque composition was analyzed. The data demonstrated that the COS treatment significantly decreased the percentage of lipid (30% in the COS-L group and 45% in the COS-H group, respectively, Fig. 4B). The number of macrophages in the plaques was also significantly reduced (32% in the COS-L group and 55% in the COS-H group, respectively). However, no obvious change was observed in the collagen content, which suggested that the primary effect of COS was on lipid regulation, but not on smooth muscle cells. These results indicated that the COS treatment may provide a beneficial effect to the plaque stability.

MMP-9 promotes the deterioration of AS plaques ultimately leading to rupture. In this study, we also detected the MMP-9 distribution in the plaques. However, no obvious change was observed in the MMP-9 expression in the plaques from each group (Fig. 4D).
Cholesterol-laden macrophages, also referred to as "foam cells," are the main cellular compositions of AS plaque. The macrophage and lipid deposition were reduced in the COS groups (Fig. 3B, C), therefore we studied the cholesterol transporters in the peripheral macrophages. The peritoneal macrophage is a useful and easily harvested model for peripheral macrophage mechanism research. The SR-BI expression was significantly increased at the transcriptional level by 13-fold and 14-fold in the COS-L and COS-H groups, respectively (Fig. 5B, D). In addition, the expression was significantly increased in the transcriptional level by 6-fold and 9-fold in the COS-L and COS-H groups, respectively. Moreover, the expression was increased by 13-fold and 14-fold in the COS-L and COS-H groups, respectively (Fig. 5B, D). The mRNA expression of ABCA1 was dramatically increased by 7-fold and 9-fold in the COS-L and COS-H groups, respectively (Fig. 5B, D). Additionally, the Western blot results showed that the mRNA level (2.1-fold and 2.3-fold in the COS-L and COS-H groups, respectively, Fig. 5A, C) and at the protein level (4.1-fold and 3.9-fold in the COS-L group and COS-H group, respectively) were significantly enhanced. To clarify the role of LDL-R in the COS treatment assay, we also determined the plasma lipid levels in LDL-R-/- mice fed a HF diet and COS, however, no changes in the cholesterol level and a moderate reduction of TG were observed (Table 3), which strongly suggested that the cholesterol-lowering effect was via the LDL receptor. These findings indicate that the COS treatment improved the expression of hepatic SR-BI and LDL-R in apoE-/- mice fed a HF diet.

Fig. 3. COS attenuate the AS lesion formation in apoE-/- mice fed a HF diet. A, Mice were euthanized, and the aortas were dissected and photographed. B and C, Representative images of oil red O stained whole aorta en face and aortic sections. D, Quantitative display of the en face assay. E, Quantitative display of the root assay. All values were expressed as the mean ± SD. *p < 0.05, **p < 0.01 VS. the Control group.
Fig. 4. COS administration decreased the lipid and macrophage content in apoE−/− mice fed a HF diet. Sectioned aorta sinus plaques were stained by H&E staining (A), oil red O staining for lipids (B), MOMA-2 for macrophages (C), Masson’s staining for collagen (D), and anti-matrix metalloproteinase (MMP)-9 for MMP-9 (E). F, Plaques from the COS treatment groups exhibited decreased percentages of lipid (30% and 45%) and macrophages (32% and 55%) compared with those from the Control group. No change was observed in the collagen and MMP9 levels. *p<0.05, **p<0.01 for the COS group VS. the Control group (n=10 for each group). Bars: 60 μm (A) and 0.3 mm (B, C, D, and E).
Fig. 5. Effects of COS administration on the expression of genes involved in lipid metabolism in the liver, peritoneal macrophages, and cell lines. A, Hepatic mRNA was determined by real-time PCR. B, mRNA from peritoneal macrophages was determined by real-time PCR. C, The hepatic protein expression levels of ATP binding cassette transporter A1 (ABCA1), LDL-R, and scavenger receptor BI (SR-BI) were detected by Western blot. D, The peritoneal macrophage protein expression levels of ABCA1 and SR-BI were detected by Western blot. E, mRNA expression of HepG2 cells was detected by real-time PCR. F, mRNA expression of Raw264.7 cells was detected by real-time PCR. G, Hepatic SREBP-1 and SREBP-2 expression levels were detected by Western blot. H, Effects of COS administration on the expression of LXRα during lipid metabolism in the liver. The hepatic protein expression of α unit of liver X receptor (LXRα) was detected by Western blot and normalized to tubulin. For the Western blot results, a representative Western blot from four independent experiments was shown (upper panel) and the quantification of these proteins were shown on the lower panel. All values were presented as the mean ± SD of four independent experiments. *, p<0.05 VS. the Control group. **, p<0.01 VS. the Control group.
ABCA1 expression significantly increased by 4-fold and 5-fold in the COS-L group and the COS-H group, respectively. These findings indicated that the COS treatment may play a positive role in the macrophage ABCA1 and SR-BI expression in apoE-/- mice fed a HF diet.

Effects of the COS Treatment on Cultured HepG2 Cells and RAW264.7 Cells

To further investigate the effects of the COS treatment on genes related with cholesterol transport metabolism in hepatocytes or macrophages, the HepG2 cell line or RAW264.7 cell line were employed for a gene expression analysis, respectively. The expression of ABCA1 and LDL-R in HepG2 cells were significantly increased (50% and 52% of ABCA1 and LDL-R, respectively) in the cells treated with 50 mg/L COS (Fig.5E). The SR-BI expression was increased with the treatment of COS (30% and 70% enhancement in 10 mg/L and 50 mg/L groups, respectively), whereas no change in the ABCG1 expression was observed. In addition, we used a higher dose treatment (75-200 mg/L) of COS on the HepG2 cells and cell death dramatically increased. This phenomenon was consistent with Shen’s report, which may be attributable to the inhibition effects of COS on ABCA1 and SR-BI.

The ABCA1 expression in RAW264.7 was significantly increased (2.5-fold and 6.6-fold) with the treatment of 75 mg/L and 100 mg/L COS, respectively (Fig.5F), whereas no change was observed in the expression of the other genes. We previously reported that the COS treatment may promote the expression of LXRα in the liver14). In the present study, we additionally found that the LXRα expression was significantly enhanced with the treatment of COS (Fig.5F, H). These findings indicated that the COS treatment may promote the expression of cholesterol transporters in hepatic cells and macrophages, and this effect may be related to the expression and activation of LXRα. To further clarify this mechanism, we investigated the translocation of SREBP-1 and 2. COS administration significantly enhanced the nuclear SREBP-1 expression, whereas no change was found in the expression of nuclear SREBP-2 (Fig.5G).

These data indicated that the SREBP-1 pathway may be involved in the enhanced hepatic SR-BI and LDL-R expression after COS administration. However, the SREBP-1 activation may be the main reason of liver lipid accumulation (Table 4), however, further investigation is necessary.

COS Administration Increased the Expression of ABCG5 and ABCG8 in the Small Intestine

Previous data showed that COS administration decreased the plasma apoB48 after food intake, suggesting that COS may also play a positive role in the small intestine. We found that COS administration could increase the expression of ABCG5 and ABCG8 (Fig.6), however, no change was observed in the expression of ABCA1, NPC1L1, and IDOL (Supplementary Fig.3). To exclude the effect of COS on cholesterol in the intestine, we conducted a cholesterol absorption assay. The COS treatment did not affect the cholesterol absorption in wild type mice (Fig.6C). Therefore, our finding indicated that COS may promote cholesterol secretion in the small intestine.

Discussion

In this study, we demonstrated for the first time that the oral administration of COS resulted in the following: 1) the attenuation of AS lesions; 2) the significant reduction of the plasma non-HDL levels in a dose-dependent manner; 3) the enhancement of plaque stability by reducing the plaque lipid and macrophage; 4) the increased expression of hepatic LDL-R and SR-BI; 5) the increased expression of ABCA1 and SR-BI in the peripheral macrophages; and 6) the promotion of ABCG5/8 expression in the small intestine.

A high non-HDL level in circulation is one of the most important causes inducing AS diseases due to oxidative non-HDL particles, which are prone to

Table 3. Effect of COS on plasma lipid levels in LDLR-/- mice fed a high-fat diet

<table>
<thead>
<tr>
<th></th>
<th>TC</th>
<th>TG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>912.6±118.2</td>
<td>206.3±36.2</td>
</tr>
<tr>
<td>COS (1000 mg/kg/day)</td>
<td>908.5±39.8</td>
<td>123.9±23.8 **</td>
</tr>
</tbody>
</table>

Values are presented as the means±SD. TC, total cholesterol; TG, triglyceride. ** , compared with the vehicle group, p<0.01.

Table 4. Effect of COS on hepatic lipid content in apoE-/- mice fed a high-fat diet

<table>
<thead>
<tr>
<th></th>
<th>TC (mg/g)</th>
<th>TG (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=9)</td>
<td>5.3±0.6</td>
<td>10.3±0.7</td>
</tr>
<tr>
<td>COS-L (n=9)</td>
<td>7.0±0.6*</td>
<td>12.2±1.3*</td>
</tr>
<tr>
<td>COS-H (n=9)</td>
<td>8.2±0.8*</td>
<td>14.5±1.6*</td>
</tr>
</tbody>
</table>

Liver lipids from apoE-/- mice fed a high-fat diet or high-fat diet + COS for six weeks were extracted and triglyceride (TG) and total cholesterol (TC) were measured enzymatically. Data represent the mean values (n=9) ± SD normalized to the wet tissue weight.
Fig. 6. COS administration increased the ABCG5 and ABCG8 expression levels but did not affect the cholesterol absorption in the small intestine. A, mRNA from the small intestine was determined by real-time PCR. B, The hepatic protein expression levels of ABCA1, ABCG5, ABCG8, and Niemann-Pick C1-Like1 (NPC1L1) were detected by Western blot. C, An intestinal absorption study in C57 mice fed a chow diet with or without COS treatment for 4 weeks. For the Western blot results, a representative Western blot result from four independent experiments was shown (upper panel) and the quantification of these proteins were shown on the lower panel. For the absorption assay, 6-7 animals were used for the detection. All values were presented as the mean ± SD of four independent experiments. *, p < 0.05 VS. the Control group. **, p < 0.01 VS. the Control group.
uptake by macrophages in the subendothelial space of the aortic wall and subsequently lead to the formation of foam cells in the artery walls. The accumulation of macrophage-derived foam cells in AS plaque may enlarge the plaque size and decrease the stability of plaques. Thus, an effective lipid-lowering therapy may attenuate the AS disease by decreasing the non-HDL levels in circulation. Ormrod et al. found that chitosan, the precursor of COS, showed anti-AS effects in apoE/-/- mice fed a HF diet after 18 weeks administration. Therefore, we similarly used adult apoE/-/- mice under a long-term COS treatment. In this study, we found that the role of the COS treatment may effectively lower the cholesterol and TG in the non-HDL particles without affecting HDL, and the findings were consistent with our previous studies. Another effect of the COS treatment is the reduction of plasma apoB100 synthesized by the hepatocytes, and the reduction of apoB-containing particles may be one mechanism of its atheroprotective effects. We herein proved for the first time that the non-HDL cholesterol-lowering effects of COS administration were mainly mediated by the increased expression of LDL-R, possibly because the COS treatment was able to decrease the plasma lipid in apoE/-/- and wild type mice fed a HF diet or Chow diet, respectively, but not in LDL-R/-/- mice. Therefore, we further investigated the intracellular molecular mechanisms of COS on the synthesis and secretion of apoB in the hepatocytes.

Improving the plaque stability is another important therapy target of AS diseases. The lipid content and macrophages was found to be negatively related with the plaque stability. Our data suggested that COS showed a positive role on the plaque stability. Moreover, the reduction of macrophage and lipid accumulation in the plaque strongly suggested that the cholesterol efflux may be involved in the anti-AS effect of COS. The cholesterol efflux from peripheral tissues to HDL is mediated by ABCA1 and G1. Afterward, cholesterol or cholesterol ester in HDL is taken up by the hepatocytes through SR-BI for bile acid synthesis. This finding was consistent with our previous finding that the COS treatment promoted RCT in wild type mice. In the present study, we additionally found that the enhanced expression of hepatic LDL-R in the COS groups, which suggested pro-AS lipoprotein clearance, was promoted via the LDLR-mediated pathway. Therefore, our data indicated that the reduction of the macrophage content and lipid composition in the plaque was mainly due to the decreased cholesterol accumulation in circulation in the COS treatment groups. Conversely, we conducted a cholesterol absorption assay and clarified the effects of COS administration on the cholesterol absorption, which was consistent with the data of cholesterol transportation in the small intestine. Thus, the targets of the COS treatment may have focused on the non-HDL cholesterol uptake in the liver and cholesterol secretion in the small intestine.

In the small intestine, the localization of ABCA1 is in the basolateral side, while ABCG5/8 is primarily located in the apical side, and both of them facilitate the cholesterol efflux through enterocytes. Although ABCA1, G5, and G8 are all the target genes of LXR, the regulation of these cholesterol transporters depends on the cholesterol loading status of hepatocytes or enterocytes. In this study, the COS treatment increased the expression of ABCG5/8 in the small intestine, which may have been due to: 1) similar to chitosan, COS promotes cholesterol excretion from enterocytes via the ABCG5/8 pathway or 2) a high calorie diet induced hyperlipidemia, leading to a concentration gradient of cholesterol, which subsequently drove the plasma cholesterol transportation from the bloodstream to the lumen via the induction of ABCG5/8, but not ABCA1. Conversely, increased ABCG5/8 expression without ABCA1 alteration was reported previously, suggesting that the regulation of the LXR target genes is complicated and some post-translational mechanisms, such as transporter trafficking, may be involved. Further investigation utilizing enterocytes under COS treatment, particularly via promoter assays, will therefore be necessary.

Furthermore, in vitro studies indicated that the COS treatment increased the ABCA1 mRNA expression in HepG2 cells and RAW264.7 cells, whereas no hepatic ABCA1 expression change was observed in the COS treated apoE/-/- mice. The effects may be relative to a higher expression of LXR in the COS groups. However, LXR sense intracellular cholesterol content and initiate various adaptive mechanisms protecting the cell from an imbalance of cholesterol homeostasis. LDL-R, ABCA1, SR-BI, and apoE are one subset of proteins under the regulation of the LXR pathway. To further understand the regulation of LXR, we investigated the COS effect on hepatic SREBP the finding suggested that SREBP-LXR axis activation is the leading role in the atheroprotective and lipid-lowering effect of COS administration in apoE/-/- mice. However, we additionally noticed that the activation of SREBP-1 may induce the hepatic lipid accumulation with high-dose COS administration (see Table 4). As a result, these findings indicated that in order to clarify the mechanism of COS on cholesterol metabolism, further studies on the activation of LXR are required.
In conclusion, we herein demonstrate that COS treatment not only represses AS induced by a HF diet, but also improves the plaque stability in apoE-/- mice. The present study accumulates positive evidence for bioactive components in cholesterol metabolism. Further investigation is necessary to elucidate the potential mechanism of bioactive components.

Acknowledgements

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Conflicts of Interest

None.

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Supplementary Fig. 1.
Serum TNFα and IL-6 level determinations by ELISA. The serum TNFα and IL-6 levels in both of the COS groups and the Control group were detected by ELISA after being fed a 12-week HCHF diet. A, Serum TNFα levels of each group. B, Serum IL-6 levels of each group. Results are expressed as the means ± SD (n=6).

Supplementary Fig. 2.
Transcription of hepatic genes involved in cholesterol metabolism. mRNA from the liver was determined by real-time PCR. All values were presented as the means ± SD of four independent experiments. *, p<0.05 VS. the Control group. **, p<0.01 VS. the Control group.
Supplementary Fig. 3.
Effects of COS administration on the expression of the Inducible degrader of LDL-R (Idol) involved in lipid metabolism in the small intestine. The protein expression levels of α unit of IDOL were detected by Western blot and normalized to actin. All values were presented as the means ± SD of four independent experiments. *, p<0.05 VS. the Control group. **, p<0.01 VS. the Control group.