Original Research

Chitosan oligosaccharides promote reverse cholesterol transport and expression of scavenger receptor BI and CYP7A1 in mice

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Abstract

Chitosan oligosaccharides (COS) are beneficial in improving plasma lipids and diminishing atherosclerotic risks. In this study, we examined the effects of COS on reverse cholesterol transport (RCT) in C57BL/6 mice. ^{3}H-cholesterol-laden macrophages were injected intraperitoneally into mice fed with various dosage of COS (250, 500, 1000 mg/kg mouse weight, respectively) or vehicle by gastric gavages. Plasma lipid level was determined and ^{3}H-cholesterol was traced in plasma, liver, bile and feces. The effects of COS on hepatic cholesterol 7 alpha-hydroxylase (CYP7A1) and scavenger receptor BI (SR-BI) expression were also investigated. COS administration led to a significant decrease in plasma total cholesterol and low-density lipoprotein (LDL) cholesterol and a significant increase in peritoneal macrophage-derived ^{3}H-cholesterol in liver and bile as well as in feces. Liver protein expressions of CYP7A1, SR-BI and LDL receptor (LDL-R) were improved in a dosage-dependent manner in COS-administered mice. Our findings provide the first *in vivo* demonstration of a positive role for COS in RCT pathway and hepatic CYP7A1 and SR-BI expression in mice. Additionally, the LDL cholesterol lowering effect might be relative to hepatic LDL-R expression stimulated by COS in mice.

Keywords: chitosan oligosaccharides, reverse cholesterol transport, scavenger receptor BI, cholesterol 7 alpha-hydroxylase

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Introduction

Atherosclerotic cardiovascular disease is the leading cause of morbidity and mortality in modern societies.[1] Increasing evidence indicates that reverse cholesterol transport (RCT) is a very important defense mechanism of atherosclerotic diseases. RCT is a cholesterol metabolism pathway that transports cholesterol from the macrophage foam cell in atherosclerotic plaque to the liver for excretion in the bile.[2] Cholesterol efflux from macrophage to high-density lipoprotein (HDL) is mediated by ATP-binding cassette transporters A1 and G1 (ABCA1 and ABCG1) on the membrane of macrophages. HDL particles transfer free cholesterol or cholesterol ester (CE) to hepatocytes through scavenger receptor BI (SR-BI) for bile acid synthesis catalyzed by cholesterol 7 alpha-hydroxylase (CYP7A1). The secretion of bile acid is mediated by ATP-binding cassette transporters G5 and G8 (ABCG5 and ABCG8).[2] Various antiatherosclerosis medications or bioactive components may be beneficial in the expression of certain transporters or key enzymes involving RCT.

SR-BI, a well-established HDL receptor, is widely expressed in hepatocytes, macrophages, endothelial cells and other cells or tissues.[3] Over-expression of hepatic SR-BI can promote macrophage RCT to the feces[4] and attenuate atherosclerosis lesions.[5] Previous studies have indicated that hepatic SR-BI plays a key role in RCT and might be a therapeutic target in atherosclerotic disease.[5]

Another essential enzyme involving RCT is CYP7A1, the rate-limiting enzyme of bile acid synthesis. Expression of CYP7A1 is down-regulated by sterol regulatory element-binding proteins when plasma cholesterol levels are low and up-regulated by liver X receptor (LXR) when cholesterol levels are high.[6,7] Many compounds or medications might have a positive effect on RCT or plasma lipids by stimulating CYP7A1 expression or activity.[7,8]

Accumulating literatures reported that chitosan exerts beneficial lipid-regulating properties.[9–11] Dietary chitosan enhances hepatic CYP7A1 activity and reduces plasma and liver cholesterol concentrations in diet-induced hypercholesterolemia in rats.[8] Administration of chitosan oligosaccharide (COS), the degradation product of chitosan, raised HDL cholesterol (HDL-C) and reduced low-density lipoprotein (LDL) cholesterol (LDL-C) in high-fat-diet-fed rats.[12] It is therefore conceivable that COS might play a

positive role in RCT. To investigate the impact of COS on *in vivo* macrophage-to-feces RCT, a classic mouse model was employed.[2] In this study, we examined the effects of COS on RCT in mice and investigated the change of certain enzymes and transporters involved in RCT.

Materials and methods

Materials

COS (pharmaceutical grade) with a molecular weight not more than 1000 Da was purchased from Shanghai U-sea Biotech Co, Ltd (Shanghai, China). Acetylated LDL (Ac-LDL) was prepared following a previous study with partial modifications.[13] [1,2-^{3}H]-cholesterol was obtained from PerkinElmer (Waltham, MA, USA). Antibody of CYP7A1 was obtained from Abcam (Cambridge, MA, USA). Antibodies of SR-BI,LDL receptor (LDL-R) and β-actin were obtained from Novus (Littleton, CO, USA) and Cell Signaling Technology (Danvers, MA, USA), respectively.

Animals

Six-week-old female C57BL/6 mice and chow diet were obtained from the Experimental Animal Center of Peking University, Beijing, China. The mice accessed diet and water *ad libitum* and were kept in a temperature- and humidity-controlled room with a 12/12 h light–dark cycle. 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.

^{3}H-cholesterol-laden cell preparation

^{3}H-cholesterol-laden cells were prepared as described previously,[14] briefly introduced as follows. RAW264.7 cells were co-cultured with 5 μCi/mL ^{3}H-cholesterol and 100 μg/mL of Ac-LDL for 48 h, then equilibrated and harvested for mice intraperitoneal injection. The ratios of intracellular ^{3}H-cholesterol and total ^{3}H-cholesterol were determined by a liquid scintillation counter. In this study, more than 95% of the ^{3}H-cholesterol was taken up by cultured macrophages.

In vivo experiments

Twenty-four female mice were daily fed by gastric gavage with COS (250, 500, 1000 mg COS/kg mouse weight, dissolved in distilled water at a dose of 10 mL/kg mouse weight for eight mice, respectively) or distilled water (10 mL/kg mouse weight) only as control. After four weeks, ^{3}H-cholesterol-labeled foam cells (6.0×10^6 cells containing 6.1×10^5 counts per minute in 0.5 mL minimum essential medium) were injected intraperitoneally to each mouse. Blood was collected at 0, 6 and 24 h after injection. Plasma was isolated for ^{3}H-cholesterol quantification by liquid scintillation counting and lipid (total cholesterol, TC; triglyceride, TG; HDL-C; LDL-cholesterol; LDL-C) analysis by enzymatic method. At 24 h after injection, mice were anesthetized and sacrificed. Liver tissue, bile and feces were removed and stored at -70°C for ^{3}H-cholesterol quantification by liquid scintillation counting and for protein expression analysis by realtime polymerase chain reaction (PCR) and Western blot.

Realtime PCR

Total RNA was isolated by TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA). cDNA synthesis was performed using MuLV reverse transcriptase (Applied Biosystems, Carlsbad, CA, USA) as described previously.[15] Realtime PCR was performed using a SYBR-green PCR master mix kit (TianGen Biotech, Beijing, China). The primers used for realtime PCR are listed below: SR-BI sense primer: 5′ ATCTGGTGGA CAAATGGAA 3′, antisense primer: 5′ GAAGCGATACG TGGGAAT 3′; ABCA1 sense primer: 5′ CGTTTCCGGG AAGTGTCCTA 3′, antisense primer: 5′ GCTAGAGATGA CAAGGAGGATGGA 3′; ABCB4 sense primer: 5′ CCCCA CAGAGGGTAAGAT 3′, antisense primer: 5′ CCAACCA GGGTGTCAAAT 3′; ABCB11 sense primer: 5′ CAAATAA GGTTGTGGGTAA 3′, antisense primer: 5′ AGGACTGACA GCGAGAAT 3′; ABCG1 sense primer: 5′ GGGAAGTTGA TAAAGGATGT 3′, antisense primer: 5′ GATTCGGGCTA TGTATGG 3′; ABCG5 sense primer: 5′ AGCGTCAGCAA CCGTGTC 3′, antisense primer: 5′ AGCAGCGTGGTC TTCCCT 3′; ABCG8 sense primer: 5′ TTAAGCCACTCCC AATACA 3′, antisense primer: 5′ GTTGCTCCAAGAA TAAATGA 3′; apoAI sense primer: 5′ GGCACGTATGGCA GCAAGAT 3′, antisense primer: 5′ CCCAGAAGTCCCGA GTCAAT 3′; mouse CYP7A1 sense primer: 5′ TGGGCA TCTCAAGCAAACAC 3′, antisense primer: 5′ TCATTGC TTCAGGGCTCCTG 3′; 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA-R) sense primer: 5′ TTTGAGCAGC GTCCATTC 3′, antisense primer: 5′ GCAGTCAGTGAGCC TTCG 3′; and LXRα sense primer: 5′ TTTGAGCAGCGTCC ATTC 3′, antisense primer: 5′ GCAGTCAGTGAGCCTTCG 3′. The data were analyzed by using Rotor-gene Q software version 1.7 (Qiagen China, Shanghai, China). Relative mRNA levels were calculated by the method of 2^{-DDCt}.

Western blot

Total protein of tissue was isolated and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The proteins were transferred on polyvinylidene fluoride membranes. Protein levels of SR-BI, CYP7A1 and LDL-R were evaluated and referenced with β-actin by Western blot. The target bands were quantified using Image-Pro Plus software version 6.0 (Media Cybernetics Corp, Bethesda, MD, USA).

Statistics

Data were reported as mean $\pm$ standard deviation (SD) and subjected to analysis-of-variance analysis. *F*-test and the Student–Neuman–Keuls *post hoc* test analyses were performed on these data to analyze the variances and

significances between groups. Probability values <0.05 were considered significant.

Results

Effects of COS on mice body weight and plasma cholesterol

After six weeks of COS treatment, no difference was observed in mice body weight between the control and COS groups (Table 1).

As shown in Figure 1, COS treatment (250, 500 or 1000 mg COS/kg mouse weight) significantly lowered plasma TC concentrations by 20%, 21% and 19% than the control group (mice fed vehicle), respectively (95.51, 94.48 and 96.62 mg/dL in the COS groups, versus 118.62 mg/dL in control). Also, plasma LDL-C from the COS groups were significantly decreased by 27%, 27% and 32%, respectively (13.94, 13.98 and 12.34 mg/dL in COS groups, versus 18.97 mg/dL in control), while plasma HDL-C and TG were not changed.

Effects of COS on RCT

To evaluate the effects of COS on RCT, a classic isotope-labeled cholesterol-tracing assay was employed. As shown in Figure 2a, plasma ^{3}H-labeled cholesterol remained unchanged between the control and COS groups at 6, 12 and 24 h after foam cell injection. In liver (Figure 2b), ^{3}H-cholesterol levels were increased significantly by 31%, 41% and 83%, respectively, in the COS groups compared with the control. As shown in Figure 2c, ^{3}H-cholesterol accumulations in bile were augmented significantly by 27%, 48% and 59%, respectively, in the COS groups compared with control. Fecal ^{3}H-tracer levels were amplified significantly by 57%, 80% and 102%, respectively, in the COS groups compared with the control. (Figure 2d).

Effects of COS on gene expression involving RCT in liver and in small intestine

To investigate the potential mechanism of COS on RCT, expression of enzymes, transporters and transcription factors in liver (ABCA1\G1\G5\G8, apoAI, CYP7A1, LDL-R, HMG-CoA-R, SR-BI and LXRα) or in small intestine (ABCB4\B11\G5\G8) were determined by realtime PCR and Western blot. As shown in Figures 3a and b, COS treatment enhanced mRNA expression of SR-BI and CYP7A1 in a dosage-dependent manner (by 5%, 28% and 49% in SR-BI, and by 27%, 192% and 320% in CYP7A1, respectively) compared with the control. Moreover, no difference in ABCA1\G1\G5\G8 expressions were observed between the COS groups and control (Figures 3c, d and Supplementary Figure 1a, which can be found at http://ebm.rsmjournals.com/lookup/suppl/doi:10.1258/ebm.2011.011275/-/DC1).

Protein expressions of hepatic SR-BI and CYP7A1 were also determined. As shown in Figures 3e and f, COS treatment increased SR-BI and CYP7A1 protein levels in a dosage-dependent manner (by 37%, 42% and 45% for SR-BI, and by 15%, 31% and 64% for CYP7A1, respectively) compared with the control. To elucidate the LDL-C lowering effect of COS, LDL-R expression was examined. As shown in Figure 4, LDL-R protein expression increased significantly (by 12%, 20% and 32%, respectively) in the COS groups compared with control.

Table 1 Mouse body weight comparison

	Mouse weight		
Group	Week 0	Week 6	*P*
Control	17.4 ± 1.4	16.8 ± 2.1	0.22
250 mg/kg	17.2 ± 0.8	17.5 ± 0.7	0.22
500 mg/kg	17.5 ± 0.7	17.6 ± 0.7	0.25
1000 mg/kg	17.4 ± 2.4	16.9 ± 2.1	0.57

Mice weight of each group in week 0 and week 6 were analyzed. Data (means ± SD, $n = 6$) were expressed as grams. Weight difference between week 0 and week 6 were calculated and probability values were listed

Discussion

Accumulating evidences has shown that RCT is a defensive mechanism to attenuate atherosclerotic lesions.[2,4,6] The RCT pathway includes a series of steps beginning in peripheral tissues with the efflux of cholesterol or CE to HDL and ending in the liver and gastrointestinal tract with the secretion of cholesterol into bile and their clearance through feces. In these steps, various enzymes and cholesterol transporters play crucial roles. In this study, we found that COS promoted the cholesterol transport from intraperitoneal macrophages to feces. The results suggested that COS promoted RCT in the mice model. Our novel findings also indicated that COS enhanced the expression of hepatic CYP7A1 and SR-BI, which suggested that COS might improve cholesterol uptake of liver and utilization of cholesterol for bile acid synthesis, thus leading to the promotion of RCT.

Our previous studies reported that COS improved the plasma lipid profile in high-fat-diet-fed rats [12] and decreased plaque size in the aorta of apolipoprotein E-deficient mice (unpublished data). But whether chitosan or COS is beneficial to RCT in the animal model remains unsolved. In this study, our result provided evidence for the first time that COS promotes RCT in the murine model.

Researchers have found that peripheral ABCA1 and ABCG1 are the main transporters for transferring cholesterol to plasma HDL, and hepatic SR-BI plays a critical role in cholesterol uptake from plasma to the liver.[16,17] Furthermore, plasma HDL-C is closely related to ABCA1 and ABCG1 expression.[17] In this study, our results have shown that no differences in HDL-C and plasma ^{3}H-cholesterol clearance were observed between the COS groups and control, which is because COS moderately improved hepatic SR-BI expression without affecting expression of ABCA1 and G1 (Figure 3a, e and Supplementary Figure 1a).

We reported that COS might play a positive role in hepatic SR-BI expression for the first time in the current study. Distribution and regulation of SR-BI are more

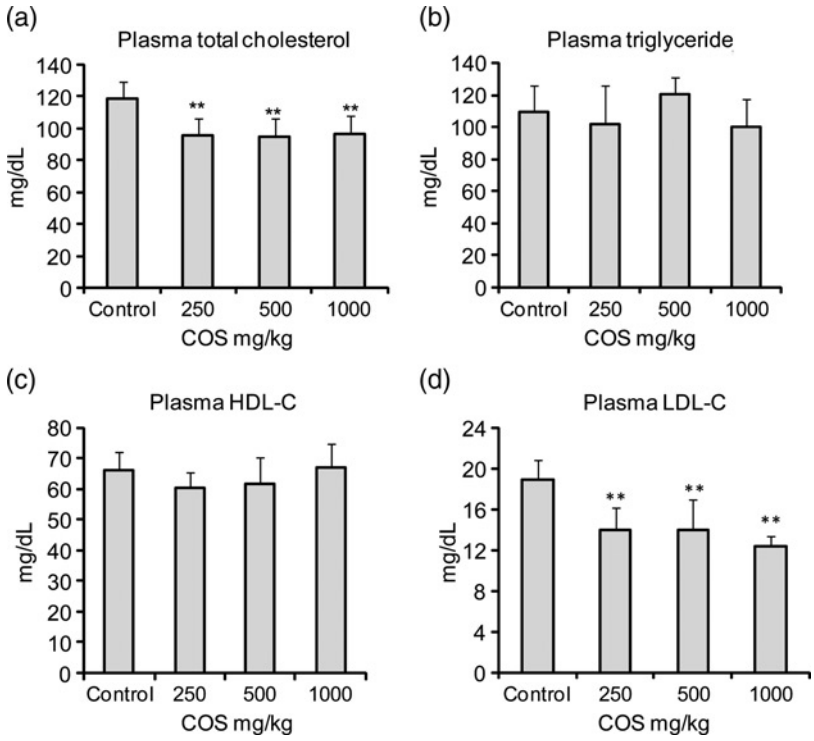

Figure 1 Plasma total cholesterol and low-density lipoprotein cholesterol (LDL-C) were decreased in the chitosan oligosaccharide (COS) group. Plasma total cholesterol (a), triglyceride (b), high-density lipoprotein cholesterol (HDL-C; c) and LDL-C (d), respectively, were determined by enzyme methods. Data (means $\pm$ SD, $n = 6$) are expressed as mg per deciliter. $^{*}P < 0.05$ versus control; $^{**}P < 0.01$ versus control

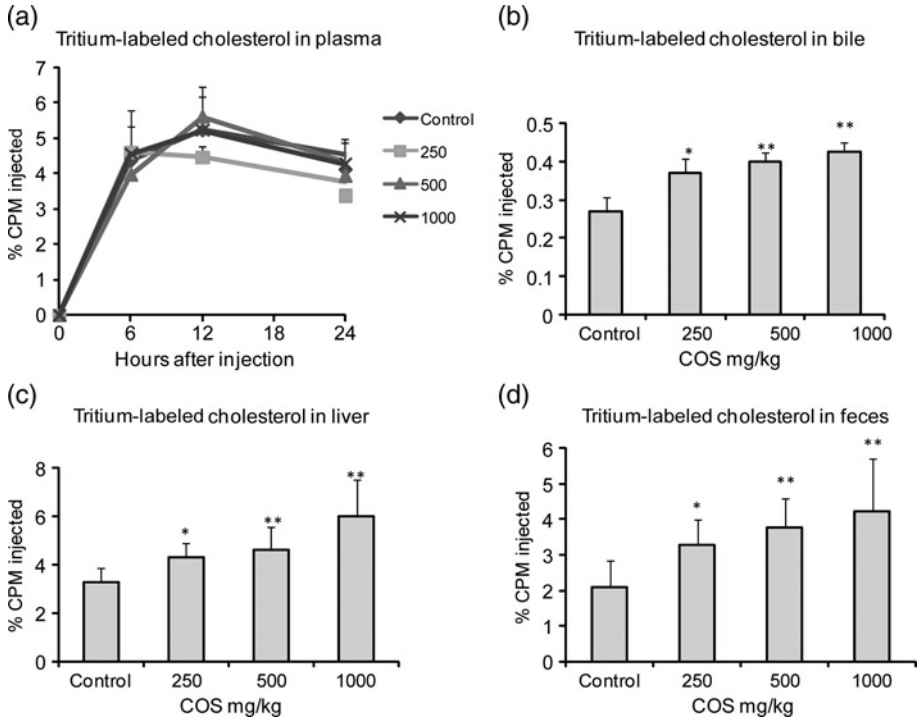

Figure 2 Chitosan oligosaccharide (COS) promoted reverse cholesterol transport in mice. ^{3}H-cholesterol-laden macrophages were harvested and counted. Into each mouse, 6.0×10^{6} cells (containing 5.9×10^{5} counts per minute in 0.5 mL minimum essential medium) were injected intraperitoneally. Plasma was collected at 0, 6 and 24 h after injection for liquid scintillation counting and the levels were expressed as the percentage of total counts per minute. Liver tissue, bile and feces were harvested and stored at -70°C for ^{3}H-cholesterol quantification by liquid scintillation counting (means $\pm$ SD, $n = 6$). $^{*}P < 0.05$; $^{**}P < 0.01$ versus control

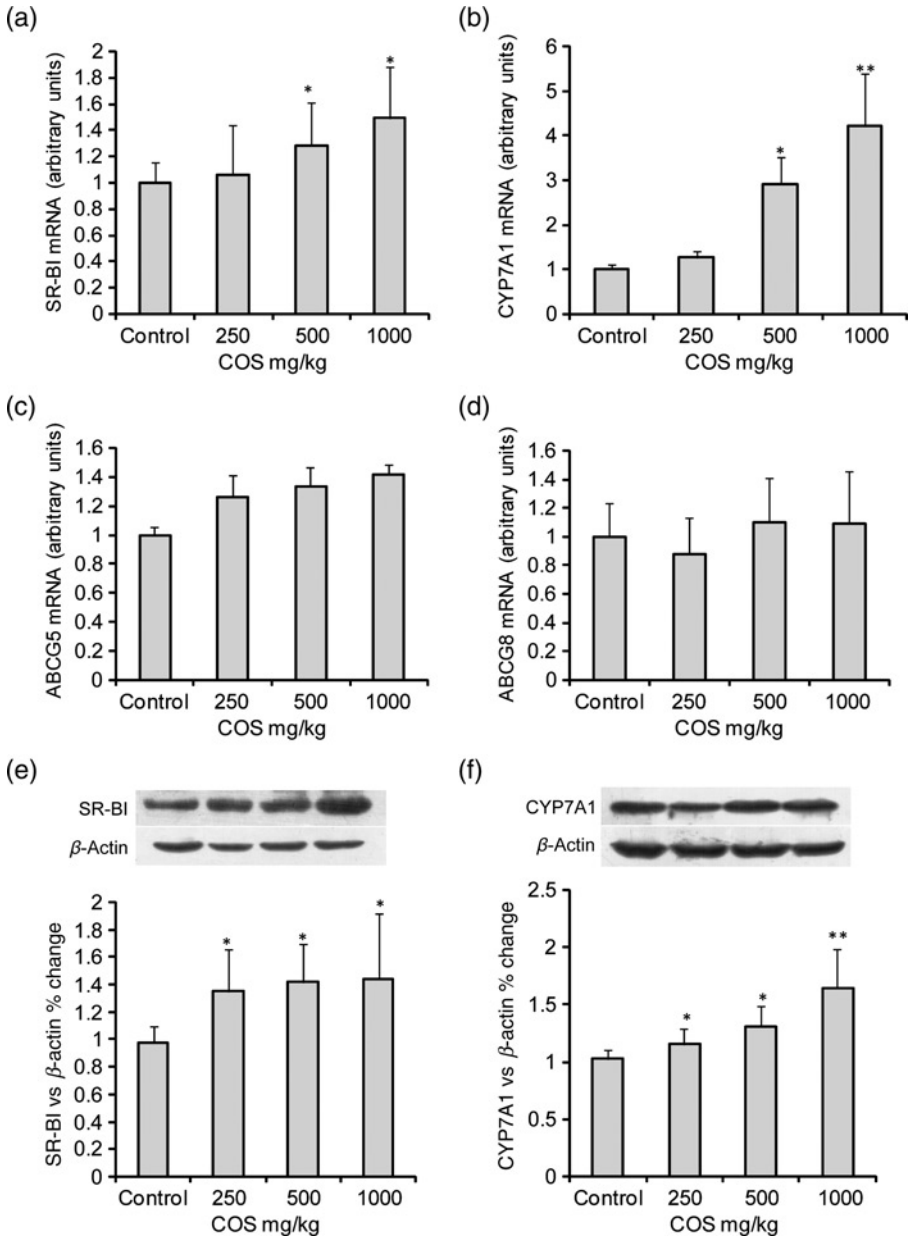

Figure 3 Chitosan oligosaccharide (COS) enhanced the expression of scavenger receptor BI (SR-BI) and cholesterol 7 alpha-hydroxylase (CYP7A1) in mice liver. (a–d) Effect of COS on the mRNA expression of hepatic genes by realtime polymerase chain reaction. Expression levels of mRNA are indicated as fold differences compared with control mice. (e–f) Effect of COS on the protein expression of SR-BI and CYP7A1 by Western blots. Protein of liver tissue from COS or control groups were conducted by sodium dodecyl sulfate polyacrylamide gel electrophoresis and the proteins were transferred on polyvinylidene fluoride membranes for Western blot analysis and quantified by densitometry (see details in Materials and methods). A representative Western blot result from four independent experiments is shown in the upper panel and the quantification of these proteins is shown in the lower panel. All values are presented as mean ± SD of four independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ versus control

complex than those of ABCA1 or ABCG1. Hepatic expression of SR-BI is modulated by many factors including LXR, farnesoid X receptor (FXR) and peroxisome proliferator-activated receptor γ (PPARγ).[18] LXR is a member of a nuclear receptor family of transcription factors and is closely related to PPAR and FXR. SR-BI expression is regulated by heterodimer formation and activation of LXR with FXR.[19] In this study, we found that hepatic LXRα expression increased only in the high-dosage COS group (Supplement Figure 1a), whereas COS increased SR-BI expression in a dosage-dependent manner. It is suggested that COS treatment might participate in the expression of LXR subunits as well as LXR–FXR heterodimer formation or activation. Therefore, further study is necessary to investigate the effect of COS on SR-BI and LXR.

The main way of liver cholesterol consumption is through bile acid synthesis, and the primary rate-limited enzyme of bile acid synthesis pathway is CYP7A1.[20] In this study, we reported for the first time that administration of COS could improve the expression of CYP7A1 in a dosage-dependent manner in mice. Similarly, chitosan-fed rats showed a higher CYP7A1 activity due

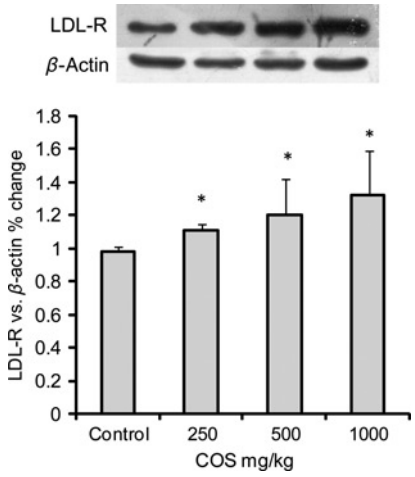

Figure 4 Chitosan oligosaccharide (COS) increased low-density lipoprotein (LDL) receptor expression in mice liver. Protein of liver tissue from COS or control groups were conducted by sodium dodecyl sulfate polyacrylamide gel electrophoresis and the proteins were transferred on polyvinylidene fluoride membranes for Western blot analysis and quantified by densitometry (see details in Materials and methods). A representative Western blot result from four independent experiments is shown in the upper panel and the quantification of these proteins is shown in the lower panel. All values are presented as mean ± SD of four independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ versus control

to the augmentation of fecal neutral acid excretion.[8] Therefore, the positive effect of COS on CYP7A1 expression might be the primary reason of ^{3}H-cholesterol accumulation in bile and in feces (Figures 2c and d, 3b and f). Subsequently, increased HMG-CoA-R expression might be a compensatory effect to maintain cholesterol homeostasis in the liver (Supplementary Figure 1a).

Dietary cholesterol absorption and bile acid secretion are important determinants of cholesterol metabolism. ABCG5 and ABCG8 are two ABC half-transporters that form a heterodimer in liver and in small intestine.[21] ABCG5–ABCG8 heterodimers efflux dietary cholesterol out of the intestinal cells, whereas in hepatocytes, they efflux those sterols into the bile.[21] In this study, expression of ABCG5\G8 was not observed in COS groups, which suggested that cholesterol secretion was not affected by COS treatment in mice. Small intestine data indicated that COS treatment did not affect the expression of genes involving cholesterol absorption and bile acid secretion (Supplementary Figure 1b, please see http://ebm.rsmjournals.com/lookup/suppl/doi:10.1258/ebm.2011.011275/-/DC1).[15]

LDL-C lowering effects of COS on rat or on mice were observed in many groups.[22] Our findings in this study were consistent with the results reported by Shen *et al.*,[22] who demonstrated that COS can significantly reduce the level of LDL-C in mice. However, there is a slight difference with the results reported by Zhou *et al.*,[23] which might have originated from the complexity of the component and metabolism of lipoproteins, as well as species differences. In the current study, we reported for the first time that COS treatment improved hepatic expression of LDL-R in mice. The reduced TC level by COS might be attributable to the decreased LDL-C level, not HDL-C. In the present study, we found that COS decreased plasma TC and LDL-C without affecting HDL-C, which subsequently enhanced the ratio of HDL-C in plasma. As we know, the most potent antiatherogenic factor is HDL, which independently predicts the epidemiological risk of cardiovascular events.[1] Therefore in this study, COS treatment might improve plasma lipid by increasing the HDL-C percentage of total plasma cholesterol in mice.

Cholesterol homeostasis is critical for cell function and metabolism. LXR sense intracellular cholesterol content and initiate various adaptive mechanisms, protecting the cell from imbalance of cholesterol homeostasis. RCT is one of the key mechanisms for keeping cholesterol homeostasis and re-distributing cholesterol *in vivo*.[24] Many genes involving RCT (ABCA1\G1, SR-BI, CYP7A1, ABCG5\G8, HMGCoA-R, etc.) are regulated by LXR. In liver, cholesterol pool homeostasis is vital for hepatocyte function, including cholesterol *de novo* synthesis and bile formation.[24] Previous studies and our current findings pointed out that COS treatment promoted cholesterol elimination from liver to feces via bile. Therefore, the expression of CYP7A1 was up-regulated to retain the bile acid and cholesterol in bile, which subsequently enhanced cholesterol uptake from circulation to the liver via SR-BI and LDL-R pathways.

Plasma TG level is closely related to carbohydrate and lipid metabolism. Our plasma TG data indicated that COS treatment plays a positive role in plasma cholesterol without affecting other plasma lipid metabolisms in the normolipidemia C57BL/6 mouse model. As we know, many lipid-regulating drugs, such as statins or fibrates, can have adverse effects on liver and kidney functions.[25,26] Our previous study showed that COS had no obvious effect on liver and kidney functions and present no morphological abnormity in rat.[12] Therefore, COS treatment might be a possible safer, lipid-regulating medication.

In summary, our findings showed that COS promoted RCT, lowered plasma LDL-C and increased expression of hepatic SR-BI, CYP7A1 and LDL-R in mice. These data suggested that COS is an effective substance in improving RCT, one of the contributors to the inhibition of atherosclerosis. The present study adds to the accumulating evidence for bioactive components in cholesterol metabolism. To further understand the promotion of COS on RCT in mice, investigations are required to focus on enzymes or transporters, such as CYP7A1 and SR-BI which involved in RCT. In this study, COS showed a significant decreasing LDL-C effect without affecting HDL-C in plasma, which suggests that COS may be beneficial in developing medications for atherosclerotic diseases.

Author contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript. CZ and YY were responsible for animal maintenance, killing, blood and tissue collection, acquisition of isotope tracing assay data, analysis and manuscript preparation. CZ and GS were responsible for RNA and protein analysis, acquisition of data and analysis. CZ, TL and LL assisted in animal gastric gavage, tissue collection and protein analysis. TL and XW assisted in plasma analysis. SQ was responsible for the funding, study design, data acquisition and analysis, interpretation and manuscript preparation. CZ and YY contributed equally to this study.

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