Original Article

Lipid-lowering and anti-oxidation effects of chondroitin sulfate prepared from squid cartilage in hypercholesterolemia mice

Yin Wang, Lin-Hong Ye, Sun-Zhong Ye, Shu-Ji Liu, Zhi-Yu Liu, Cheng-Ye Wu

Fisheries Research Institute of Fujian, Key Laboratory of Cultivation and High-Value Utilization of Marine Organisms in Fujian Province, Xiamen, Fujian, China

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Abstract: Atherosclerosis is one of the most common diseases in elderly and may lead to fatty liver, renal failure, myocardial infarction, coronary heart disease and other cardiovascular diseases, and high cholesterol is one of the main factors causing atherosclerosis. So far, proven clinical lipid-lowering drugs for atherosclerosis are mainly statins and fibrates which have side effects on kidney and liver when used in long-term, hence the necessity to explore efficient but low-side effect drug candidate for atherosclerosis. The present study used a hypercholesterolemia mouse model to assess a wide spectrum of effects of chondroitin sulfate (CS) prepared from squid cartilage on lipid-lowering and anti-oxidation. The results showed that CS could control weight gain in mice, reduce liver index, coronary heart disease (R-CHD) value and atherosclerosis index (AI) and increase kidney index, indicating its potential in preventing atherosclerosis and coronary heart disease. Meanwhile, CS effectively reduced cholesterol (TC) and triglycerides (TG) in both serum and liver, lowered levels of low-density lipoprotein cholesterol (LDL-C) and increased high-density lipoprotein cholesterol (HDL-C) in serum. Further investigation evidenced that CS enhanced the activities of liver lipoprotein lipase (LPL), hepatic lipase (HL), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px). CS prepared from squid cartilage is an efficient therapeutic avenue to improve hyperlipidemia and could exert a preventive effect in fatty liver, atherosclerosis, coronary heart disease and other diseases due to its excellent lipid-lowering and antioxidant properties.

Keywords: Chondroitin sulfate, lipid-lowering, antioxidant, hypercholesterolemia

Introduction

Hypercholesterolemia is one of the main factors causing atherosclerosis by way of long-term accumulation of excess cholesterol (TC), triglycerides (TG) and lipid peroxides in the blood stream and their subsequent depositions into the vessel wall resulting in decreased arterial elasticity, luminal diameter and finally arterial atherosclerosis [1]. Atherosclerosis does not only significantly increase the incidences of diabetes and hypertension, but may also lead to fatty liver, renal failure, myocardial infarction, coronary heart disease and other cardiovascular diseases [2]. Up to now, the commonly used clinical lipid-lowering drugs majorly include statins, fibric acid derivatives, niacin, cholesterol binding resins and cholesterol absorption inhibitors which achieve the lipid-lowering effect by reducing the intracellular TC, accelerating the removal of low-density lipoprotein (LDL) in blood circulation and inhibiting the synthesis of very low-density lipoprotein (VLDL) in liver [3-5]. However, subjects at high risk for cardiovascular disease, those requiring more intensive treatment for cholesterolemia reduction, often do not reach the desired LDL cholesterol (LDLC) target, either because of the lack of efficiency of the available therapies or because of the side effects, especially on liver and kidney functions, consequently to the high drug dosages required [6, 7]. Therefore, it is important to explore novel candidates with efficient lipid-lowering properties and, preferably, with low side effects [8].

Chondroitin sulfate (CS) belongs to the vast family of glycosaminoglycans which represent
linear polysaccharides constituted of disaccharide motifs [9, 10]. Experimental and clinical data indicate that CS is able to exert therapeutic effect against a variety of diseases including Parkinson’s and Alzheimer’s diseases, inflammatory bowel diseases, sclerosis, rheumatoid arthritis, systemic lupus erythematosus and a diversity of cancers [11-20]. Previous clinical and pre-clinical studies have equally shown that commercial CS exhibits prophylactic effects against a variety of cardiac events such as ischemic coronary disease [21] and atherosclerosis in sub-human primates [22]. However, although CS has been demonstrated as an anti-inflammatory agent in atherosclerosis, its function in atherosclerosis, especially for what concerns its anti-oxidant and lipid-lowering ability has not been fully studied hitherto. In previous studies, Yao [23] treated mice on high-fat diet with CS extracted from either eel or sheep cartilage and found that TC and LDL-C levels were significantly reduced and TG level was elevated in mice fed with high-fat-diet and subsequently treated with CS, which suggests that CS could exert lipid-lowering effect.

In parallel, it was demonstrated that hypercholesterolemia can instigate oxidant stress [24] because the excessive accumulation of free radicals in cells can easily initiate changes in cell metabolism, structure and function due to the radical-induced changes in membrane permeability and cell mobility, which is closely related to high blood cholesterol disease [3]. There is emerging evidence that CS exert antioxidant effects [25-27] and its application as a therapeutic drug for atherosclerosis and other cardiovascular diseases could reduce oxidation complications linked to hypercholesterolemia.

Therefore, better scrutiny of CS therapeutic and prophylactic capacities, its safety and adequate dosage in animal models would be a paramount contributive step in treating cardiovascular diseases in human. Previously, we have extracted CS from squid cartilage in our laboratory [28] but its bio-functional role in the treatment of human diseases, especially its lipid-lowering and its antioxidant capacity has not been investigated so far. In addition, studies suggest that squid cartilage oversulfated CS-E exerts numerous biological activities, including lymphoid regulatory activities, anticoagulant activities and neuroregulatory activities, which appear to reflect the biological activities of mammalian CS chains containing the so-called E disaccharide unit [29, 30]. In view of the above, we hypothesized that CS could exhibits low side effects in human if validated as lipid lowering compound.

Therefore, in this study, we have established a KM mouse model of hypercholesterolemia in order to explore the functional lipid-lowering and anti-oxidation role of CS prepared from squid, which may provide some evidence for using CS from squid as lipid-lowering drug in the future.

Materials and methods

Reagents

CS from squid cartilage (CS content of 70.11% as measured by phloroglucinol method, protein content of 4.97% as determined by the Coomassie brilliant blue method) was laboratory-made as described in our previous publication [28]; high-fat diet and normal diet were purchased from Suzhou Double Lions Laboratory Animal Feed Science and Technology Co., Ltd. Simvastatin was purchased from Beijing Peking University WBL Biotech Co., Ltd. Triglyceride assay kit (Zhejiang Dong Ou Diagnostic Products Co., Ltd), total cholesterol kits, LDL cholesterol kits, HDL cholesterol measurement kit (Beijing Beihua Kangtai clinical reagents Limited), superoxide dismutase (SOD) kit, glutathione peroxidase (GSH-Px) test kit, total lipase test kit, protein quantification kit (Nanjing Jincheng Bioengineering Institute) were used according to the vendors’ protocols.

Diet composition

The normal diet consisted of 3.0% (w/w) butter, 41.5% corn starch, 20% casein, 5.0% sucrose, 3.0% cellulose, 1.0% vitamin mixture (AIN-76), 3.5% mineral mixture (AIN-76), 0.4% choline chloride and 22.6% water. The high-fat diet consisted of basal diet 80.8%, 0.2% bile salt, 5% lard, 10% egg yolk powder, and 4% cholesterol (total kcal: 535.2) with the composition of the basal diet as follows: Corn (26%), soybean meal (24.7%), wheat flour (34%), fish meal (5%), vegetable oil (2.3%), alfalfa meal (3%), 5% of premix (vitamins and minerals) composed of 21.72% crude protein, crude fat 4.57% and 52.96% of carbohydrates.
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**Mouse model of hypercholesterolemia**

A total of 108 3-month-old males SPF grade Kunming mice (KM) (weight between 18 and 22 grams) were purchased from Lake Hayes animals Limited. The KM species was selected according to the State Food and Drug Administration’s implementation of “Health Food Inspection and Evaluation of Technical Specifications” (2003 edition), for example experiment P518, for lowering functional verification experiment. Mice were kept in a 12 h light/dark cycle facility under 21°C–25°C with humidity of 45% to 55% and weighed every week. After the mice were allowed to acclimatize for 1 week in this laboratory environment, their blood was collected from the caudal artery for the measurement of total cholesterol. Thereafter, mice were randomly divided into normal control (n = 36) and model (n = 72) groups. The animals in the model group were fed high-fat diet for 2 weeks to induce hypercholesterolemia (HM group). The normal mice were fed normal diet and served as controls. All the mice were weekly weighed and recorded. Two weeks later all mice were anesthetized with ether and orbital venous blood were collected for detections of TC and LDL-C levels in serum. The hypercholesterolemia model was successfully established if the level of TC or LDL-C in the serum of model animals was significantly increased compared with that in normal control animals. All animal studies and protocols were performed in accordance with standard guidelines as described in the ‘Guide for the Care and Use of Laboratory Animals’ (US National Institutes of Health 85-23, revised 1996). The study was reviewed and approved by the Animal Experiments Committee of Fisheries Research Institute of Fujian.

**Scheme of drug administration**

The model mice on high fat diet were divided into 6 groups (n = 12 in each group) among which there was no significant difference of weight or cholesterol level, and then orally treated with 8 mg/kg Simvastatin (drug control, HMD), 300 mg/kg CS (CS low dose, CSL), 600 mg/kg CS (CS medium dose, CSM), 1200 mg/kg CS (CS high dose, CSH), water (Hyperlipidemia model, HM) or a combination of simvastatin and high concentration of CS (DCSL) one time daily for 5 weeks. The normal control animals were distributed into three groups (n = 12 in each group) treated with high concentration of CS (NCCSH), simvastatin (DC) or water (untreated control, NC), respectively. The doses of CS were selected based on the results of our preliminary studies on the comparison of CS and simvastatin effects and the selected doses did not exhibit harmful effects on mice.

**Measurement of serum indicators**

After the last drug treatment, mice were starved for 16 hours, anesthetized with ether and the body weight recorded. Blood samples were collected and kept at room temperature for 3 h followed by centrifugation at 4000 rpm for 10 min to obtain serum. TC, TG, HDL-C, LDL-C, SOD and GSH-Px levels in serum were measured using corresponding kit as instructed.

**Measurement of lipid index and organ index**

Liver, kidney and spleen of mice were removed, rinsed with saline and weighed immediately after mice sacrifice by cervical dislocation. TC, HDL-C and LDL-C were measured using corresponding kits, the Atherosclerosis Index (AI), Coronary Heart Disease Index (R-CHD) and Viscera Index were calculated as following:

Equation 1: \( \text{AI} = \frac{\text{TC} - \text{HDL-C}}{\text{HDL-C}} \)

Equation 2: \( \text{R-CHD} = \frac{\text{LDL-C}}{\text{HDL-C}} \)

Equation 3: \( \text{Viscera Index} = \frac{\text{Visceraweight}}{\text{Body weight}} \times 100 \)

**Measurement of liver Index**

0.2 g liver was accurately weighed, grinded with 2 mL saline and then centrifuged at 2500 rpm for 10 min. Supernatant was collected and measured for LPL, HL, SOD and GSH-Px as instructed. Another 0.1 g liver was accurately weighed, grinded with the same volume of chloroform-methanol solution and centrifuged at 4000 rpm for 10 min. Supernatant was collected and measured for liver TC and TG level as described in the manufacturers’ guidelines.

**Statistical analysis**

The generation of graphs and statistical analysis were carried out using the GraphPad Prism software version 6.01 for windows. Two-way
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ANOVA was followed by multiple comparison tests to evaluate the significance between groups regarding the SOD and GSH-Px activities. For statistical analysis of other studied variables, One-way ANOVA, equally followed by multiple comparison tests, was applied. The difference was considered significant at P<0.05.

Results

Effect of CS on the body weight of hypercholesterolemia mice

The body weight of mice in CSL, CSM, CSH, HMD and DCSL groups was significantly decreased compared with that in HM group (P<0.05, Figure 1). There was no significant difference between CSH, HDM and DCSL groups compared to the NC, DC and NCCSH groups (P>0.05), indicating that CS could reduce body weight of mice with high cholesterol level while not affecting the natural growth of mice.

Effect of CS on the physiological indexes of hypercholesterolemia mice

After 5 weeks high fat diet, the liver index was significantly increased while the kidney index was reduced compared with NC group (Figure 2A, 2B), indicating that the liver and kidney had been damaged to some extent, maybe because the high fat diet caused disruption of metabolism in mice and increased metabolic burden on the liver and kidney which finally resulted in the swelling of the liver and atrophy of kidney. Notably, the liver index of all CS treated groups, HMD and DCSL group was significantly decreased (Figure 2A, P<0.05) when compared with the model group. Interestingly, both liver and kidney indexes in CSH and DCSL groups were comparable to that of the normal control group (Figure 2A, 2B). All these data indicated that CS could improve the function of liver and kidney, with better performance when combined with simvastatin. Although CS improved the spleen index in a dose-dependent manner when compared with the HM group, no significant difference was found among control and experimental animals.

The results (Figure 3A) equally indicated that atherosclerosis index (AI) was significantly increased in HM group compared with NC, DC and NCCSH groups (P<0.01), but CS and simvastatin treatments significantly reduced the AI compared with HM group (P<0.05), suggesting the protective effect of CS on atherosclerosis.

As shown in Figure 3B, R-CHD in HM group was significantly increased compared with NC, DC and NCCSH groups (P<0.01). On the contrary, after CS treatment, R-CHD was significantly reduced compared with HM group (P<0.05), showing that CS could significantly reduce the risk of coronary heart disease and can be more efficient when combined with simvastatin.

Effect of CS on the blood lipid level in hypercholesterolemia mice

The measurements of blood lipids showcased that TC and LDL-C levels in the serum of mice in HM group were dramatically elevated compared with that in NC, DC and NCCSH groups (P<0.0001, Figure 4A, 4B), while decreased HDL-C level was recorded (P<0.0001, Figure 4C), indicating that high-fat diet interrupted the lipid metabolism in mice, which indicated that the hypercholesterolemia KM mouse model was successfully established.

As shown in Figure 4A, the TC level in mice from CSH, HMD and DCSL groups was significantly lower than that in HM group (P<0.0001), and the TG level in mice from CSM, CSH, HMD and DCSL groups was also significantly lower than that in HM group (Figure 4D, P<0.0001), show-
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ing that CS as well as simvastatin could effectively lower TC and TG level in hypercholesterolemia mice. As indicated in Figure 4B, the HDL-C level in mice from CSM, CSH, HMD and DCSL groups was significantly increased than that in HM group (P<0.01), while LDL-C level in all treated group was remarkably decreased than that in HM group (Figure 4D, P<0.01), demonstrating that CS could decrease LDL-C level and increase HDL-C level, and therefore could exert protective effects against or ameliorate cardiovascular diseases such as atherosclerosis and coronary heart disease.

Effect of CS on lipid levels and LPL and HL activities in the liver of hypercholesterolemia mice

The measurements of lipids in liver (Figure 5), revealed that the TC and TG level in liver of HM group were higher than that in NC, DC and NCCSH groups (P<0.0001, Figure 5C), while medium and high dose of CS as well as simvastatin and combination of simvastatin and CS significantly improved LPL activity compared with HM mice (P<0.0001, Figure 5C), showing that CS could increase LPL activity in high cholesterol diet mice.

The HL activity in the liver of HM mice was obviously lower than that in NC, DC and NCCSH groups (P<0.0001, Figure 5C), while medium and high dose of CS treatment improve HL activity compared with HM mice (P<0.0001, Figure 5D), and the improvement of HL activity by CS was dose-dependent, indicating that CS possibly enhanced HL activity, which is beneficial for the treatment of atherosclerosis. Furthermore, combination of CS and simvastatin restored the HL and LPL activities close to normal.
Effect of CS on the antioxidant index in high cholesterol diet mice

The determination of superoxide dismutase (SOD) activity in the serum and liver of HM mice was dramatically decreased compared with NC, DC and NCCSH mice (P<0.01, Figure 6A, 6B), while SOD activity in both serum and liver of all CS-treated mice was significantly elevated compared with HM mice (P<0.05, Figure 6A). Additionally, SOD activity in CSH mice had no significant difference compared with NC mice or HMD mice. All these data showed that CS was remarkably effective on promoting SOD activity.

GSH-Px activity in both serum and liver of HM mice was dramatically decreased compared with NC, DC and NCCSH mice (P<0.01, Figure 6B), while GSH-Px activity in both serum and liver of all CS-treated mice was significantly improved compared with HM mice (P<0.05, Figure 6B) except for CSL group. Similarly to SOD activity, GSH-Px activity in CSH mice had no difference with NC mice or HMD mice. All these data showed that CS had evident effect on improving GSH-Px activity in serum and liver.

Discussion

Hypercholesterolemia, which modifies cholesterol metabolism, is widely recognized as a major risk factor and mortality. Natural compounds have been used in the treatment of various chronic human pathological conditions. Thus, the therapeutic benefits of plant extracts without side effects have been the focus of many extensive studies.

In this study, we established hypercholesterolemia KM mouse model to study the effect of CS extracted from squid cartilage on lowering lipid level and its antioxidant function. Mice in HM group were treated with high fat diet and the body weight of mouse in HM group was significantly increased compared with NC group, showing that high-fat diet caused an increase of energy intake or abnormality of metabolism in mice. In addition, both TC and LDL-C level in the serum of mice in HM group were dramatically elevated compared with that in NC group (P<0.01), while the HDL-C were lowered (P<0.01), indicating that high-fat diet instigated abnormal lipid metabolism in mice and allowed the successful establishment of KM mice hypercholesterolemia model. These results were similar to those of previous studies showing the effects of high-fat diets in inducing mice obesity [31-33]. High levels of cholesterol in the blood, essentially LDL-C, are the main factors leading to atherosclerosis. LDL-C is the principal target for risk-reduction therapy of coronary heart disease, as demonstrated by numerous primary and secondary prevention trials [34, 35]. In the present study, treatment of hypercholesterolemia mice with different doses of CS or simvastatin significantly decreased the weight gain caused by the
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Viscera index, as the ratio of a weight of the given organ and body weight, can reflect the status of nutrition and lesions in a specific organ. Viscera index has its own pattern at different developmental stages, and will behave abnormally if there is damage in a specific organ. Our findings revealed that CS significantly reduced liver index and increased kidney index, indicating its ability to modulate the metabolic burden of liver or kidney and to prevent these organs from subsequent swelling or atrophy due to hypercholesterolemia.

Atherosclerosis index (AI) reflects the distribution of lipoproteins and cholesterol all over the body and a greater AI value indicates a higher degree of atherosclerosis and a higher risk of cardiovascular diseases [36]. Coronary Heart Disease (CHD) is characterized by the myocardial ischemia resulting from functional or structural changes occurring in the coronary circulation and subsequent imbalance between coronary flow and myocardial demand. Elevated LDL-C levels and decreased HDL-C levels are two independent risk factors of coronary heart disease and R-CHD can reflect the status of lipid metabolism in the body [37]. Our findings showed that administration of CS considerably decreased AI and R-CHD value compared with hypercholesterolemia mice, demonstrating that CS could be effective in the prevention and treatment of atherosclerosis and coronary heart disease.

The significantly increased levels of TC and/or LDL-C in animal serum are the markers of a successful hypercholesterolemia model. There is an increasing evidence that hyperlipidemia caused by high levels of TC, especially hypercholesterolemia, is an important risk factor for atherosclerosis, stroke and coronary heart disease [38]. HDL-C is mainly synthesized in the liver and helps fulfill the reverse cholesterol transport (RCT) process, which transports cholesterol into the liver through blood circulation and finally expels it from the body as bile acids. Therefore, increased HDL-C helps remove excess cholesterol and suppresses the occurrence of atherosclerosis [39]. It has been reported that HDL-C levels is negatively correlated with the incidence of coronary heart disease and that there is a significant linear relationship between the level of LDL-C and incidence of coronary heart disease [40]. In this study, CS significantly lowered TC, TG and LDL-C levels and increase HDL-C level in serum compared to that in high-fat diet and control mice, indicating that CS could regulate the level of these molecules and help maintain a balanced lipid metabolic environment in the body.

Liver is usually the first affected organ when lipid metabolism is abnormal, with excessive TG accumulation in liver and formation of fatty

Figure 5. Effects of CS on lipid levels, LPL and HL activities in liver of hypercholesterolemia mice. TC (A) and TG (B) level in HM liver were significantly elevated compared with that in normal control mice, but reduced in that from all dose of CS treated mice. CS rescued the suppression of both LPL (C) and LDL-C (D) activities caused by high-fat diet. *P<0.05, **P<0.01 and ****P<0.0001 compared with the NC group, #P<0.0001 compared with HM group. ns = non-significant, ns$ = non-significant compared with the CSH group.
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Liver [41, 42]. We investigated the effects of CS on the levels of TC and TG in liver of hypercholesterolemia mice and found that CS treatment could also regulate levels of TC and TG and LPL and HL activities in liver, showing that it could prevent or treat fatty liver through improving LPL and HL activity and reducing TC and TG accumulation in liver. Lipoprotein lipase (LPL) is expressed on the endothelial cells in the capillary and plays an important role in lipoprotein cycle [43] by catalyzing hydrolysis of plasma lipoproteins such as TG. Hepatic lipase (HL), mainly synthesized in the liver cells, is one of the key enzymes involved in the TG metabolism. It binds with TC to subsequently decrease the plasma concentration of TC and TG and thus regulates lipid metabolism, and has been proven to exert clinical anti-atherosclerosis effect [44, 45].

The role of oxidative stress in the development of cardiovascular injuries has been reliably established [46, 47]. It has been accepted that the generation of antioxidant enzymes protects cells by removing oxides faster, and increase of activity of antioxidant enzymes can indirectly reflect the improvement of antioxidant capacity in vivo [48]. SOD (Superoxide dismutase) is one of the major endogenous antioxidant enzymes and closely related to cell oxidative metabolism. It ameliorates liver damage by catalyzing superoxide anion radical disproportionation, reducing or removing the free radicals in the body and inhibiting lipid peroxidation [49]. Similarly, GSH-Px (Glutathione peroxidase) is an antioxidant enzyme able to scavenge or inhibit free radical reaction. It specifically catalyzes glutathione (GSH) to react with hydrogen peroxide or organic hydroperoxide, removes malondialdehyde (MDA) and other peroxidation products, prevents the free radical-induced membrane lipid peroxidation reaction, and protects the integrity and function of the cell membrane [50]. Herein, we found that CS significantly improved SOD and GSH-Px activities in the serum and liver of hypercholesterolemia mice, and maintained oxidation and antioxidant balance in vivo. But it is not clear how anti-oxidation acts on the process of lipid metabolism and in lowering blood lipids; the underlying mechanism still needs further investigation.

In conclusion, we successfully established a KM mouse model of hypercholesterolemia and evaluated the potential of CS extracted from squid cartilage on lowering lipid level and its antioxidant function. Similarly to simvastatin,
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CS showed the potential to reduce the weight gain caused by the high-fat diet, significantly improved liver and decreased AI and R-CHD value and displayed a great antioxidant activity. These findings demonstrated that CS could be an effective drug for prevention and treatment of atherosclerosis and coronary heart disease.

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Disclosure of conflict of interest

None.

Address correspondence to: Cheng Ye Wu, Fisheries Research Institute of Fujian, Key Laboratory of Cultivation and High-value Utilization of Marine Organisms in Fujian Province, 7 Haishan Road, Xiamen 361000, Fujian, China. Tel: +86-13906059263; E-mail: wuchengye2362@126.com

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