

Risk of cardiovascular disease is suppressed by dietary supplementation with protamine and chitooligosaccharide in Sprague-Dawley rats

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Abstract. Protamine from salmon spermaries is a novel dietary protein. Chitooligosaccharide (COS) is an oligosaccharide derived from chitin or chitosan, a long-chain polymer, by chemical or enzymatic hydrolysis. These two compounds are known to enhance lipid metabolism by interrupting the digestion and absorption of fat in the body. Cardiovascular disease (CVD) refers to any type of specific disease that affects the heart and circulatory system. Dyslipidemia, a condition involving high levels of low-density lipoprotein (LDL) cholesterol and low levels of high-density lipoprotein (HDL) cholesterol, is generally known to be a primary cause of CVD development. The risk of CVD is usually associated with the atherogenic index (AI) and cardiac risk factor (CRF). The CVD risk is also closely associated with serum levels of total cholesterol (T-CHO), LDL cholesterol and HDL cholesterol. In the present study, we evaluated alterations in serum lipid contents following the administration of protamine, COS and mixtures of these two compounds to male Sprague-Dawley (SD) rats, and their ability to reduce CVD risk. Based on the results of a serum lipid assay, protamine, COS and their mixtures were found to significantly reduce AI, CRF and CVD risk by decreasing serum levels of TG, T-CHO and LDL cholesterol and increasing serum HDL cholesterol levels. By contrast, TG and T-CHO concentrations in feces were markedly increased. Accumulation of lipids in the liver tissues of the SD rats fed high-fat diets was also inhibited by the intake

of protamine and COS. Our findings suggest that protamine, COS and combinations of the two compounds may be used as a dietary therapy for preventing CVD due to their suppressive effects on hyperlipidemia and hypercholesterolemia.

Introduction

Cardiovascular disease (CVD) refers to any disorder that affects the heart and/or circulatory system. This includes coronary heart disease, cardiomyopathy, ischemic heart disease, atherosclerosis and congestive heart failure. CVD has been the most common cause of mortality worldwide over the last two decades (1) and has increased the economic burden due to the high costs of medical treatment (2,3). The causative factors of CVD include obesity, diabetes, high blood pressure, high blood cholesterol, smoking and family history. Among these factors, hypercholesterolemia and unhealthy ratios of the two smallest lipoprotein cholesterols, low-density lipoprotein (LDL) cholesterol and high-density lipoprotein (HDL) cholesterol, resulting from a high-fat diet and impaired lipid metabolism have been closely linked to CVD incidence and mortality (4,5). LDL cholesterol is often referred to as bad cholesterol since, while circulating through the bloodstream, it tends to form deposits in the walls of the arteries, thereby decreasing artery diameter and resulting in atherosclerosis. Conversely, HDL cholesterol is referred to as good cholesterol since it is able to maintain the healthy state of the inner blood vessel walls by scavenging and recycling cholesterol by transporting it to the liver where it is reprocessed (6,7). Accordingly, the blood levels of LDL and HDL cholesterol are closely associated with the development of CVD and the LDL cholesterol/HDL cholesterol ratio is used to calculate the CVD risk factor (4,5). To reduce the incidence of CVD by achieving the desirable balance between HDL and LDL cholesterol levels, dietary therapy may be considered as a first line treatment.

In the present study, we evaluated dietary supplementation with protamine and chitooligosaccharide (COS) as a potential anti-CVD therapy. Protamine is a protein from salmon sperm which contains high levels of arginine, a basic amino acid, and helps to prevent DNA damage (8). In addition, protamine

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restricts fat absorption in the intestine (9) by markedly inhibiting triglyceride (TG) hydrolysis (10), a process required for the absorption of lipids in the intestine (11). COS is an oligosaccharide made from chitin or chitosan, a long-chain polymer of N-acetylglucosamine, by chemical or enzymatic hydrolysis (12). Chitin and chitosan have limited use as nutrient sources due to their low solubility and high viscosity resulting from their polymeric structures (12,13). By contrast, COS is widely used in food, pharmaceutical and medicinal formulations as a biomaterial due to its high solubility, low molecular weight, low viscosity and non-toxicity (14-16). According to a recent study, COS restores healthy blood pressure, reduces cholesterol levels and prevents alcoholic liver disease (17). In addition, COS exerts protective effects against infections and enhances antitumor activity (18). COS has also been shown to reduce plasma lipid levels in healthy men and TG levels in obese diabetic mice (19-21).

The aim of this study was to evaluate the abilities of protamine, COS and a mixture of the two compounds to reduce CVD risk *in vivo*. We measured the TG, total cholesterol (T-CHO), LDL cholesterol and HDL cholesterol levels in serum and feces following the dietary administration of protamine, COS and combinations of the two to male Sprague Dawley (SD) rats fed a high-fat diet. We also examined their effects on lipid accumulation in the rat liver tissues by histochemical analysis. Alterations in lipid metabolism resulting from the experimental diets including protamine and COS demonstrated that these two compounds help to regulate hyperlipidemia and hypercholesterolemia, thereby decreasing CVD risk *in vivo*.

Materials and methods

Animal adaptation. Healthy male SD rats were purchased from Central Laboratory Animal, Inc. (Seoul, Korea). The 7-week-old male SD rats were housed in a conventional animal facility at the Laboratory Animal Research Center of Chungbuk National University (Cheongju, Korea). The animals were allowed to acclimate for 1 week after arrival. The rats were used for *in vivo* experiments in accordance with the approved institutional guidelines of Chungbuk National University.

High-fat and experimental diet preparation. Salmon protamine (98% purity; hydrochloride salt; Maruha Nichiro Foods, Tokyo, Japan) and COS (88% purity; CNA Biotech, Cheongwon, Korea) were provided by LG Household and Health Care Ltd. (Daejeon, Korea). A high-fat diet was prepared as a corn oil suspension by mixing 6 ml corn oil (Sigma-Aldrich, St. Louis, MO, USA), 80 mg cholic acid (Sigma-Aldrich), 2 mg cholesteryl oleate (Sigma-Aldrich) and 1 mg margarine (Seoul Milk Ltd., Seoul, Korea) in 6 ml distilled water (22). The experimental diets were made by adding protamine, COS and a mixture of the two to the corn oil suspension in high and low doses.

Administration of experimental diets to rats. Male SD rats weighing 323.78 ± 4.80 g were divided into seven groups; 3 ml of each experimental diet was orally administered once to each rat in the relevant group via a Zonde needle. The vehicle

group (n=5) was treated with the corn oil suspension alone. The experimental groups (each group, n=5) were administered the experimental diets of protamine, COS or a mixture of the two with the corn oil suspension. The treatment groups were: i) P100, 100 mg protamine/kg body weight (bw); ii) O300, 300 mg COS/kg bw; iii) PO100/300, 100 mg protamine/kg bw and 300 mg COS/kg bw; iv) P8.3, 8.3 mg protamine/kg bw; v) O25, 25 mg COS/kg bw; and vi) PO8.3/25, 8.3 mg protamine/kg bw and 25 mg COS/kg bw. Prior to the oral administration of the experimental diets, the rats were starved for 18 h and blood samples were collected from the tail vein (0 h). Following the oral administration of the diet, blood samples were collected at 3, 9 and 24 h. Feces were collected from each group at the same time points and stored at -20°C until analysis.

Analysis of serum and fecal lipids. Blood (0.5-1 ml) was collected using a Vacuum Serum Separation Tube (SST; Green Cross Corp., Yongin, Gyeonggi, Korea) and left at room temperature for 1 h. Serum was isolated from the blood samples by centrifuging at 3,000 rpm at 4°C for 20 min and then stored at -20°C . Serum analysis was conducted using a Hitachi Clinical Analyzer 7080 (Hitachi Korea, Ltd., Seoul, Korea) to measure the serum concentrations of various lipid components, including TG, T-CHO, HDL cholesterol and LDL cholesterol. The atherogenic index (AI), cardiac risk factor (CRF) and CVD risk were determined based on serum LDL cholesterol, HDL cholesterol and T-CHO concentrations. The AI was calculated as $(\text{T-CHO} - \text{HDL cholesterol})/\text{HDL cholesterol}$, the CRF was calculated as $(\text{T-CHO}/\text{HDL cholesterol})$ and the CVD risk was calculated as $(\text{LDL cholesterol}/\text{HDL cholesterol})$.

Rat feces from each group was collected for fecal lipid analysis, and then fecal crude fat was extracted according to the Rose-Gottlieb method (23). For this procedure, 5 g feces were placed into a Mojonnier fat extractor, 6 ml NH_4OH (OCI Co., Ltd., Ulsan, Korea) was added and the mixture was left for 3 min. This mixture was combined with 12 ml 95% alcohol (OCI Co., Ltd.), 25 ml diethyl ether (OCI Co., Ltd.) and 25 ml petroleum ether (OCI Co., Ltd.) and then left at room temperature for 1-2 h. Finally, fecal crude fat was collected by withdrawing the ether phase at 75°C and analyzed to determine the TG and T-CHO levels. Fecal TG was measured with a triglyceride assay kit (Cayman Chemical Co., Ann Arbor, MI, USA) and fecal T-CHO was analyzed with an Enzychrome cholesterol assay kit (BioAssay Systems, Hayward, CA, USA) according to the manufacturer's instructions.

Histological analysis by Oil Red O staining. Twenty-four hours after the oral administration of the experimental diets, liver tissues were harvested from the sacrificed rats and immediately frozen in a deep freezer at -80°C . The frozen liver tissues were cryo-sectioned ($6\text{-}\mu\text{m}$ thick), fixed in a 10% formalin solution (OCI Co., Ltd.) at 4°C for 5 min and then rinsed three times with distilled water. A 5% Oil Red O working solution was prepared by dissolving Oil Red O powder (Sigma-Aldrich) in propylene glycol (OCI Co., Ltd.) and used to stain the sectioned tissues according to the manufacturer's instructions. Counterstaining was conducted with hematoxylin (Sigma-Aldrich) and the sections were then mounted in glycerine (OCI Co., Ltd.).

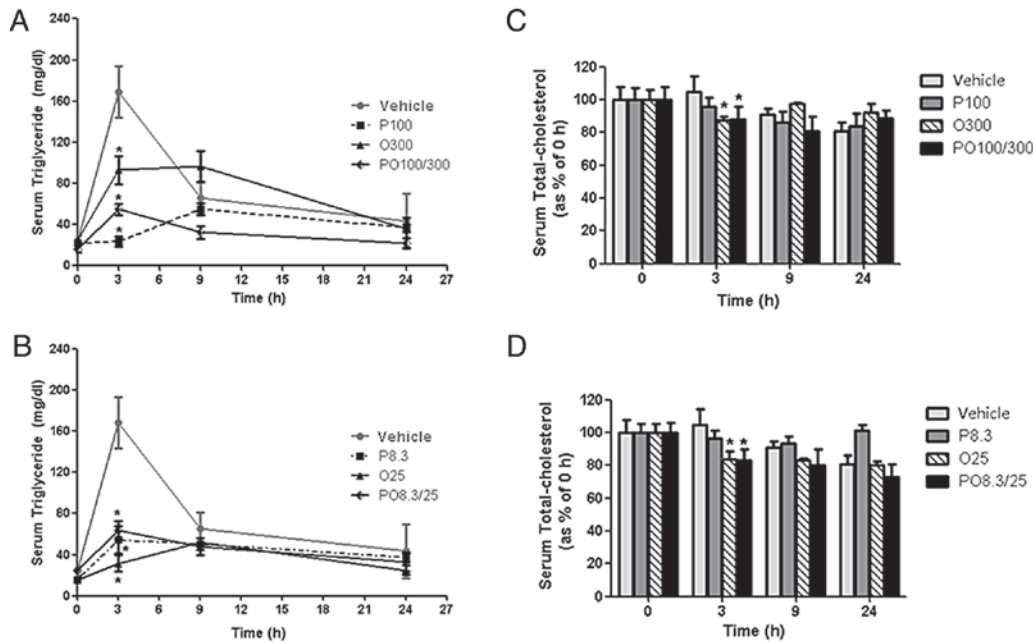


Figure 1. Analysis of triglyceride (TG) and total cholesterol (T-CHO) serum concentrations. Following the oral administration of protamine, chitoooligosaccharide (COS) and mixtures of the two compounds in corn oil suspensions, blood samples were collected and left at room temperature. Serum was isolated from the blood samples by centrifuging and the serum concentrations of various lipids were measured. Serum concentrations of (A and B) TG and (C and D) T-CHO in high- and low-dose treatment groups. The treatment groups were: P100, 100 mg protamine/kg body weight (bw); O300, 300 mg COS/kg bw; PO100/300, 100 mg protamine/kg bw + 300 mg COS/kg bw; P8.3, 8.3 mg protamine/kg bw; O25, 25 mg COS/kg bw; PO8.3/25, 8.3 mg protamine/kg bw + 25 mg COS/kg bw. Values represent the mean \pm SEM. *Significant elevation compared to vehicle (corn oil) at the same time, $P < 0.05$ (Dunnett's multiple comparison test).

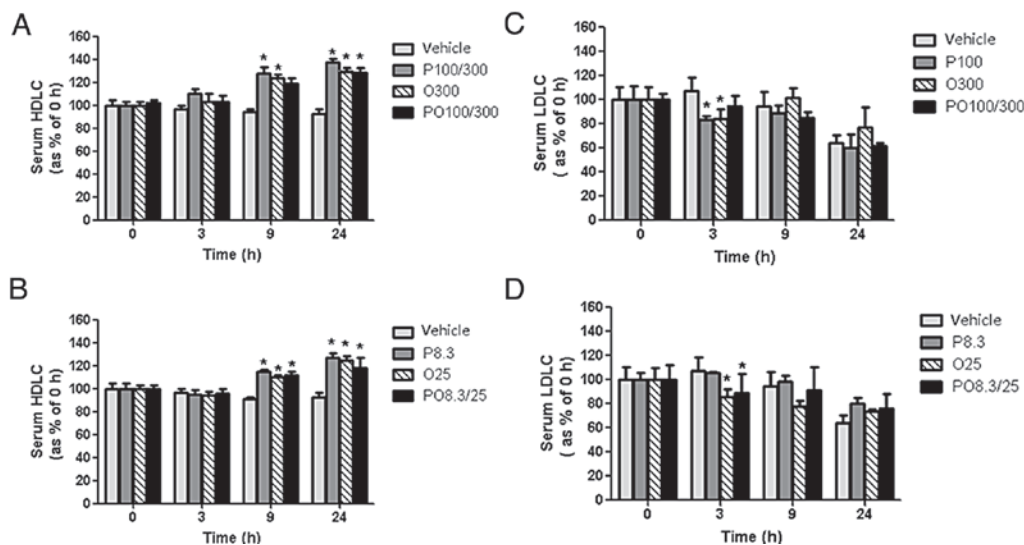


Figure 2. Serum high-density lipoprotein cholesterol (HDLC) and low-density lipoprotein cholesterol (LDLC) analysis. Serum levels of (A and B) HDLC and (C and D) LDLC for the high- and low-dose treatment groups. The animals were treated as described in Fig. 1. Values represent the mean \pm SEM. *Significant elevation compared to vehicle (corn oil) at the same time, $P < 0.05$ (Dunnett's multiple comparison test).

Lipid-containing cells were detected as those containing red inclusions using a light microscope (BX51 U-LH100HGWI, Olympus, Tokyo, Japan; magnifications, x40 and x400).

Statistical analysis. Data were analyzed with GraphPad Prism software (San Diego, CA, USA). *In vitro* data were presented as the mean \pm SEM. One-way ANOVA was performed followed by Dunnett's multiple comparison test. $P < 0.05$ was considered to indicate a statistically significant result (24-26).

Results

Serum lipid concentrations. Serum TG concentrations in the vehicle control group rapidly increased following the intake of a high-fat diet within 3 h. By contrast, treatment with high and low doses of protamine, COS and their mixtures (P100, O300, PO100/300, P8.3, O25 and PO8.3/25 groups) significantly inhibited serum TG concentrations by 84.3, 47.1, 51.0, 50.7, 71.0 and 63.0% at 3 h, respectively, compared with those of the

Table I. Atherogenic index (AI) of Sprague-Dawley (SD) rats fed a high-fat diet containing protamine, chitooligosaccharide (COS) and a mixture of these two compounds.

Group	Vehicle ^a	P100	O300	PO100	P8.3	O25	PO8.3
AI ^b	1.79±0.11	1.14±0.07 ^c	1.20±0.19 ^c	1.25±0.11 ^c	1.49±0.05 ^c	1.24±0.08 ^c	1.29±0.11 ^c

^aVehicle, high-fat diet; P100, high-fat diet + 100 mg protamine/kg body weight (bw); O300, high-fat diet + 300 mg COS/kg bw; PO100, high-fat diet + 100 mg protamine/kg bw + 300 mg COS/kg bw; P8.3, high-fat diet + 8.3 mg protamine/kg bw; O25, high-fat diet + 25 mg COS/kg bw; PO8.3, high-fat diet + 8.3 mg protamine/kg bw + 25 mg COS/kg bw. ^bAI = (total cholesterol - HDL cholesterol)/HDL cholesterol. Results are presented as the mean ± SEM of three rats per group. ^cP<0.05 based on one-way ANOVA (Dunnett's multiple comparison test) results. HDL, high density lipoprotein.

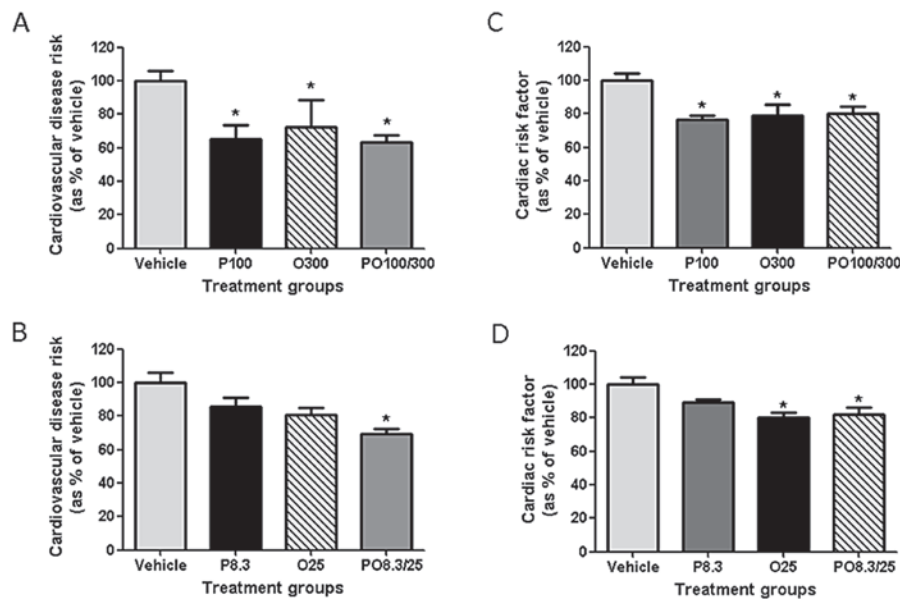


Figure 3. Cardiovascular disease (CVD) risk and cardiac risk factor (CRF) analysis. After measuring serum lipid concentrations, the values of each factor were calculated as described in Materials and methods. (A and B) CVD risks for the high- and low-dose treatment groups. (C and D) CRF for the high- and low-dose treatment groups. Values are the mean ± SEM. *P<0.05 (Dunnett's multiple comparison test). LDL, low-density lipoprotein; HDL, high-density lipoprotein; T-CHO, total cholesterol.

vehicle group (Fig. 1A and B). Serum T-CHO concentrations were less effectively reduced compared with the serum TG concentrations. However, the serum T-CHO levels of the O25 and PO8.3/25 groups were significantly decreased by 19.7 and 20.6%, respectively, at 3 h compared with those of the vehicle group (Fig. 1C and D). Serum HDL cholesterol concentrations of the P100, O300, PO100/300, P8.3, O25 and PO8.3/25 groups were significantly increased by 32.8, 27.3, 26.5, 25.1, 23.5 and 19.1%, respectively, at 24 h compared with those of the vehicle group (Fig. 2A and B). Conversely, the P100, O300, O25 and PO100/300 treatments decreased serum LDL cholesterol levels by 30.5, 37.7, 35.6 and 44.4%, respectively, after 3 h compared with those of the vehicle control (Fig. 2C and D).

CVD risk factors. The CVD risk, CRF and AI of rats fed protamine, COS or their mixtures were significantly decreased compared with those of the vehicle group (Fig. 3 and Table I). The CVD risk values of the high-dose treatment groups (P100, O300 and PO100/300) were decreased by 34.8, 27.5 and 36.6%, respectively, at 24 h. Among the rats treated with

low doses, only the PO8.3/25 group revealed a significantly decreased CVD risk (30.8%; Fig. 3A and B). The CRF values of the P100, O300 and PO100/300 groups were decreased by 23.4, 21.2 and 19.5%, respectively, at 24 h. The CRF values of the O25 and PO8.3/25 groups were decreased by 19.7 and 17.8%, respectively (Fig. 3C and D). The AI values of all treatment groups were significantly decreased (Table I).

Fecal TG and cholesterol concentrations. Protamine, COS, and mixtures of the two significantly increased the TG and T-CHO concentrations in the feces compared with those of the vehicle group in rats fed a high-fat diet. The fecal TG concentrations generally increased over time within 24 h. In particular, the P100 and PO100/300 groups revealed highly increased levels of fecal TG at 9 h. The fecal TG concentrations of the O25 group also increased significantly at 24 h (494.9%) compared with those of the vehicle group (Fig. 4A and B). The fecal T-CHO concentrations of the rats treated with the experimental diets were increased to a lesser degree. The fecal T-CHO concentrations of the P100, O300 and O25

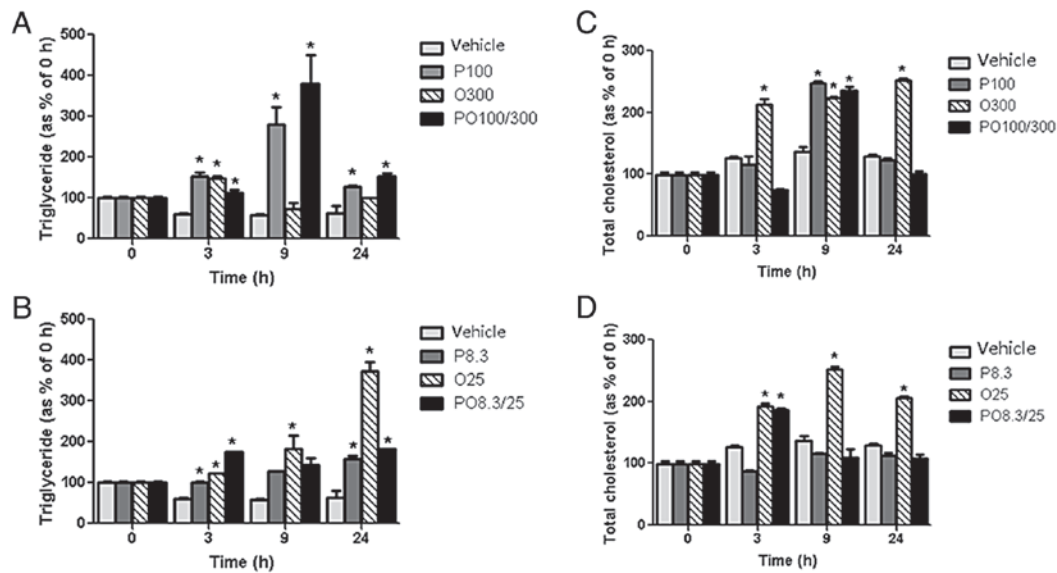


Figure 4. Analysis of fecal lipid concentrations. Following the oral administration of protamine, chitoooligosaccharide (COS) and mixtures of the two in corn oil suspension, feces from the rats in each group were collected. Fecal crude fat was then extracted and analyzed for triglyceride (TG) and total cholesterol (T-CHO) content. (A and B) Fecal TG concentrations in the high- and low-dose treatment groups. (C and D) Fecal T-CHO concentrations in the high- and low-dose treatment groups. Values are the mean \pm SEM. *Significant elevation compared to vehicle (corn oil) at the same time, $P < 0.05$ (Dunnett's multiple comparison test).

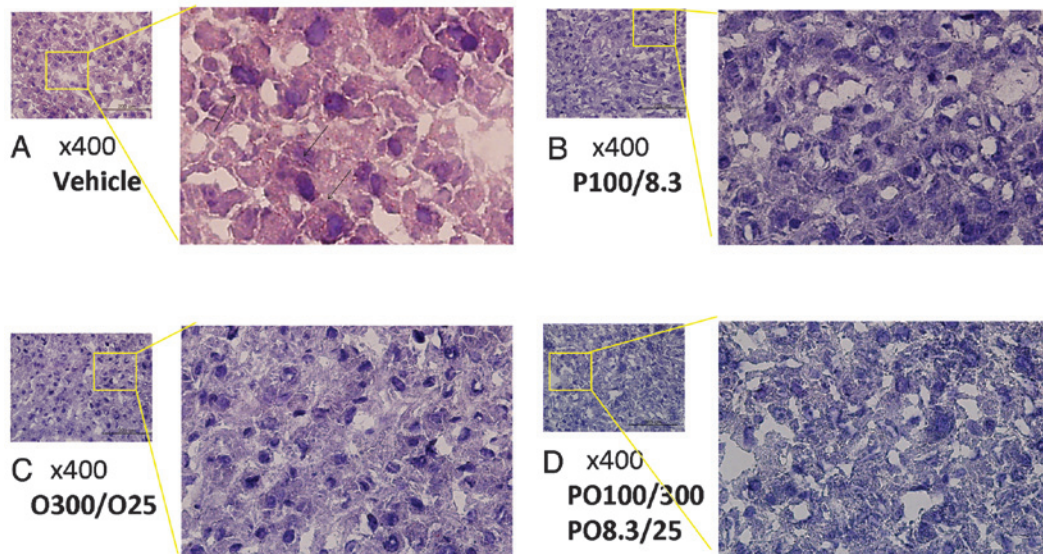


Figure 5. Histological analysis of liver tissues. Following the oral administration of protamine, chitoooligosaccharide (COS) and mixtures of these two compounds, liver tissues were harvested from the rats, frozen, cryo-sectioned and stained with an Oil Red O working solution. Counter-staining was conducted with hematoxylin and the tissue sections were then mounted with glycerine. Lipid in the cells appeared as red inclusions under a light microscope (magnification, x400). Liver tissues from (A) vehicle control, (B) protamine-treated, (C) COS-treated and (D) protamine- and COS-treated rats.

groups were increased by 82.2, 64.4 and 84.5%, respectively, at 9 h (Fig. 4C and D).

Lipid accumulation in liver tissues. Lipid accumulation in the rat liver tissues was detected by Oil Red O staining and histological analysis. The red inclusions corresponded to lipids derived from TG and T-CHO from the high-fat diet. Significant levels of lipid accumulation were observed in the vehicle group, but increases in lipid accumulation were not observed in all experimental diet groups treated with protamine, COS or a mixture of the two compounds (Fig. 5).

Discussion

A high-fat diet and dysfunctional lipid metabolism are closely associated with the increasing prevalence of being overweight and of obesity, and are influential causes of CVD (27,28). In particular, dyslipidemia, characterized by high levels of very low-density lipoproteins (VLDL) and TG and decreased HDL and LDL levels in the serum, is generally known to be a strong predictor for the development of CVD (29). In the present study, we evaluated the effects of a dietary therapy including protamine and COS, two prominent candidates for

positively affecting lipid metabolism, on CVD risk in rats. We measured the improvements of lipid metabolism following the administration of protamine, COS and their mixtures *in vivo* by analyzing serum and fecal lipid levels as well as hepatic lipid accumulation within 24 h of the oral administration of a high-fat diet along with protamine and COS to SD rats. The dose-dependent effects of protamine (30,31) and COS were also examined. The factors CVD risk, CRF and AI were then calculated based on serum T-CHO, HDL cholesterol and LDL cholesterol concentrations.

Protamine, COS and mixtures of the two compounds effectively reduced the serum levels of TG, T-CHO and LDL cholesterol compared with the vehicle. By contrast, these compounds significantly increased the serum levels of HDL cholesterol. When serum cholesterol levels were translated into risk factor values, protamine, COS and their mixtures were shown to decrease AI, CVD risk and CRF. The AI was significantly reduced in all experimental diet groups compared with the vehicle control group. CVD risk and CRF were also significantly reduced in all rats treated with high doses as well as in the low-dose PO8.3/25 group, indicating that high doses of protamine and COS were more effective than low doses for reducing these risk factors. Additionally, a combination of protamine and COS had greater effects than treatment with protamine or COS alone at low doses. However, the additive effect of protamine and COS was not evident at high doses. We therefore conclude that protamine and COS have effects that are beneficial for the prevention of diseases associated with arteriosclerosis and cardiac failure by improving lipid metabolism.

An unhealthy serum cholesterol profile (higher levels of LDL cholesterol and lower levels of HDL cholesterol) is known to promote atheroma development in atherosclerosis, which is strongly associated with CVD (32,33). Improvement of an unfavorable serum cholesterol profile by dietary supplementation with protamine and COS was achieved by reversing the unhealthy ratio of LDL cholesterol to HDL cholesterol. Serum HDL cholesterol concentrations were significantly increased by protamine and COS. However, the two compounds effectively reduced the serum levels of LDL cholesterol. HDL cholesterol is considered to have a variety of useful actions that tend to reduce the risk for CVD. HDL cholesterol removes and recycles LDL cholesterol by transporting it to the liver, an action which is generally thought to produce a central anti-atherogenic effect (34). A previous study has shown that there is an inverse correlation between HDL levels and the risk of CVD in which the risk of CVD is increased by 14% with 5 mg/dl decrements in HDL levels (35).

Unlike in serum, TG and T-CHO concentrations in feces were markedly increased by protamine, COS and their mixtures over the same period of time, indicating that TG and T-CHO were effectively excreted from the body by rats fed the experimental diets. When treated with high doses, the fecal TG concentrations were maximized at 9 h. However, low-dose treatments resulted in the maximum amount of TG being excreted in the feces at 24 h. Fecal T-CHO concentrations were also considerably increased by protamine and COS. Specifically, high-dose COS treatment increased fecal T-CHO levels by the greatest amount. These results demonstrated that protamine, COS and mixtures of the two interrupted lipid

accumulation in the liver and blood vessels. This was further shown by histological analysis using Oil Red O staining. Liver tissues obtained from rats fed high-fat diets revealed a large number of lipid inclusions stained with the Oil Red O working solution, but little lipid accumulation was observed in rats treated with protamine, COS and their mixtures.

In summary, our results demonstrated that protamine, COS and mixtures of the two compounds effectively reduced CVD risk, CRF and AI by decreasing serum levels of TG, T-CHO and LDL cholesterol and enhancing serum HDL cholesterol levels. The reduction of serum TG and T-CHO levels may be explained by the finding that protamine and COS promoted the excretion of TG and T-CHO in feces, thereby preventing accumulation in the body. However, future studies are required to elucidate the mechanism(s) underlying the reduction of CVD risk through beneficial changes in HDL cholesterol/LDL cholesterol ratios due to treatment with protamine, COS and their mixtures. In conclusion, our results suggest that protamine and COS are promising dietary supplements for preventing CVD by alleviating hyperlipidemia and hypercholesterolemia.

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