

Antidiabetic Effects of Chitosan Oligosaccharides in Neonatal Streptozotocin-Induced Noninsulin-Dependent Diabetes Mellitus in Rats

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The antidiabetic effect of chitosan oligosaccharide (COS) was investigated in neonatal streptozotocin (STZ)-induced noninsulin-dependent diabetes mellitus rats. The fasting glucose level was reduced by about 19% in diabetic rats after treatment with 0.3% COS. Glucose tolerance was lower in the diabetic group compared with the normal group. After diabetic rats had been treated with 0.3% COS for 4 weeks, glucose tolerance increased significantly *versus* the diabetic control group, and glucose-inducible insulin expression increased significantly. In addition, fed-triglyceride (TG) levels in diabetic rats drinking 0.3% COS were reduced by 49% compared with those in diabetic control rats. The cholesterol levels of animals treated with COS were reduced by about 10% in fed or fasting conditions versus the corresponding controls, although the difference was not statistically significant. It was found that COS has a TG-lowering effect in diabetic rats, and that COS reduces signs of diabetic cardiomyopathy such as vacuolation of mitochondria and the separation and degeneration of myofibrils. In conclusion, these results indicate that COS can be used as an antidiabetic agent because it increases glucose tolerance and insulin secretion and decreases TG.

Key words diabetes; chitosan oligosaccharide; glucose tolerance; streptozotocin

Diabetic patients experience various vascular complications, such as atherosclerosis^{1,2)} and diabetic blindness.^{3,4)} The major cause of death in diabetic patients is coronary heart disease resulting from premature atherosclerosis.⁵⁾ Diabetes mellitus is classified as type 1 (insulin-dependent diabetes mellitus) or type 2 (noninsulin-dependent diabetes mellitus), which are further subdivided into the obese type and lean type. Neonatal streptozotocin (STZ)-induced diabetes is a well-recognized type 2 diabetic animal model.⁶⁾ Glucose-inducible insulin expression is impaired in STZ-induced type 2 diabetic rat islets.⁷⁾ The concentrations of plasma glucose, triglyceride (TG), and cholesterol are elevated, but blood insulin is reduced in STZ-induced diabetic rats.⁸⁾ Hyperglycemia is known to contribute to defective β -cell function in lean-type diabetes.⁷⁾ Moreover, although glucose levels may be well controlled, atherosclerosis can develop in diabetic patients,⁹⁾ because high fatty acid levels in plasma increase free radical levels, which are a cause of endothelial cell damage.¹⁰⁾ In addition, antioxidant levels are decreased in diabetic patients. Previous reports have suggested that improvements in defective lipid, glucose, and antioxidant levels should be useful in the prevention of diabetic complications.¹¹⁾

Chitosan is prepared by the alkaline deacetylation of chitin.¹²⁾ Biological activities of chitosan, such as its antitumor effects,^{13,14)} cholesterol-lowering effects,^{15,16)} and antibacterial effects^{17,18)} are well known. However, few reports are available on the antidiabetic effects of chitosan.^{19–21)} The first, by Miura *et al.* reported that polymeric chitosan had blood glucose-lowering and lipid-lowering effects in neonatal STZ-induced diabetic mice.¹⁹⁾ In addition, Kondo *et al.*²⁰⁾ and Hayashi and Ito²¹⁾ reported that low molecular-weight chitosan (chitosan lactate average MW: 20000) had an antidiabetic effect in STZ-induced diabetic mice and obese diabetic KK-A^y mice. The form of chitosan absorbed was not resolved, although it was suggested that chitosan polymer is absorbed as an oligomer after digestion. Moreover, lysozyme

can hydrolyze partially deacetylated chitosan.^{22,23)} The effects of fully deacetylated chitosan oligomer in diabetic rats have not been reported, and the chitosans used in previous reports were not clearly defined in terms of their degrees of polymerization or deacetylation.

The main objective of this study was to investigate the antidiabetic effect of chitosan oligosaccharide (COS) in STZ-induced diabetic rats using a defined COS. The COS was prepared by enzymatic hydrolysis, isolated by size-exclusion chromatography, and analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. We also examined the effects of COS on glucose tolerance, insulin secretion, and blood glucose, TG, and cholesterol levels in STZ-induced diabetic rats.

MATERIALS AND METHODS

Compounds and Chemicals Chitosan polymer (99.9% deacetylated), and chitosanase derived from *Bacillus* sp. were kindly provided by Kunpoong Bio Co., Ltd. (Seoul, Korea). STZ, a diabetogenic agent, was obtained from Sigma (St. Louis, MO, U.S.A.), ascorbic acid from F. Hoffmann-La Roche Ltd. (Basel, Switzerland), and Veckman glucose reagent was purchased from Veckman instruments, Inc. (Galway, Ireland). Other chemicals were obtained from Sigma, except where otherwise stated.

Preparation and Composition of Chitosan Oligomer To obtain chitosan oligomer, chitosan polymer was hydrolyzed using chitosanase. Chitosan (2 g) was dissolved in 100 ml of 1.5% ascorbic acid in water, and incubated with 2 units of chitosanase at 50 °C for 4 h. The hydrolysate was applied to a Bio-gel P10 column (2×80 cm) and eluted with distilled water. A portion of the oligomer was dried using a freeze-dryer, and the white powder obtained was analyzed using MALDI-TOF mass spectrometry (Voyager DE, Perkin-Elmer PerSeptive Biosystem), and used as COS in this study. The composition of the oligomer was calculated from peak

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intensities in the MALDI-TOF mass spectrum. The COS was found to be composed of 33.6% disaccharide, 16.9% trisaccharide, 15.8% tetrasaccharide, 12.4% pentasaccharide, 8.3% hexasaccharide, 7.1% heptasaccharide, and 5.9% octasaccharide. COS ascorbate was composed of COS 55% and ascorbic acid 45%.

Animals Healthy Sprague-Dawley rats were kept for breeding. The animals were maintained in a temperature- and humidity-regulated room (22±2 °C, 55±15%, respectively) with controlled lighting (12-h light/dark cycle). To induce type 2 diabetes, STZ (80 mg/kg, i.p.) in 0.1 M citrate buffer (pH 5.0) was administered to a group of 3-d-old pups.²⁴⁾ The normal control group received only citrate buffer. The pups were weaned when 4 weeks old, and glucose tolerance was tested 8 weeks after the STZ injection.

Blood Sample Collection and Glucose Tolerance Test For the glucose tolerance test (GTT), glucose (2 g/kg, i.p.) was administered to rats that had been fasted for 18 h. Blood samples were collected from a tail vein at 0, 1, and 2 h. Plasma was separated immediately and analyzed for glucose content, and residual plasma was preserved at -70 °C in a deep freezer. Glucose was measured using Veckman glucose reagent with a glucose analyzer 2 (Beckman Coulter Inc., CA, U.S.A.), and plasma insulin was assayed using commercial rat insulin ELISA kits (Crystal Chem Inc., U.S.A.). The results of GTT are expressed as integrated area under the curve over the first 2 h after glucose administration ($GAUC_{0-2}$). The concentrations of serum cholesterol and TG were measured using commercially available kits, *i.e.*, a cholesterol-E kit and a TG kit (Yeongdong Pharmaceutical Co., Korea).

Experimental Procedure Experimental animals were divided into the following groups: normal control group; normal group drinking 0.3% COS; diabetic control group; and diabetic group drinking 0.3% COS. COS-treated animals were allowed water containing 0.3% COS ad libitum for 4 weeks, and the normal and diabetic control groups were allowed water containing 0.1% ascorbic acid for 4 weeks. Fed and fasting plasma glucose, TG, and total cholesterol levels were measured when the animals were 8 weeks old (*i.e.*, immediately before treatment) and at 4 weeks after treatment.

Electron Microscopy Hearts were removed after 8 weeks of treatment, fixed overnight in 0.1 M cacodylate buffer (pH 7.4) containing 10% formalin, washed three times with fresh buffer, and postfixed in 1% osmium tetroxide for 1 h. After fixation, heart tissues were washed three times with the same buffer and dehydrated in an ethanol series. The dehydrated tissues were treated with propylene oxide for 1 h, embedded in Epon 812 resin, and hardened at 60 °C for 2 d. The Epon block was sectioned using an ultramicrotome and stained with uranyl acetate and lead nitrate for 30 min before being examined under an electron microscope (LEO 906, LEO Electron Microscopy Ltd., Oberkochen, Germany).

Statistical Analysis The data were analyzed using the Mann-Whitney test using GraphPad Prism (version 3.00 for Windows, GraphPad Software, San Diego, CA, U.S.A., www.graphpad.com). In all cases, a value of $p < 0.05$ was considered significant. The data were expressed as mean ± S.E.

RESULTS

Characteristics of STZ-Induced Diabetic Rats Diabetic rats were confirmed by the GTT 8 weeks after STZ injection. Before treatment (at 8 weeks), body weight and TG in the diabetic animal groups were almost the same as those in the normal groups. However, the glucose levels and $GAUC_{0-2}$ of the diabetic rats were higher than those of the corresponding normal rats (Table 1). The glucose level was 114±18.6 mg/dl in the diabetic group and 87.5±10.1 mg/dl in the normal group. The $GAUC_{0-2}$ was 583±101.4 mg/dl in the diabetic group and 274±41.3 mg/dl in the normal controls.

Effect of COS on Glucose Level of Diabetic Rats Fasting plasma glucose levels remained within the normal range after 4 weeks of treatment in the nondiabetic groups, whether COS fed or not. The fasting plasma glucose level was lowered by 19% in diabetic rats fed COS (Table 2). The plasma glucose level was higher in the nontreated diabetic group than in the nontreated controls. In treated diabetic rats, the fasting glucose level was similar to that on the nontreated controls, although COS treatment did not lower fed-glucose levels in diabetic rats (Table 2).

Effect of COS on Glucose Tolerance in Diabetic Rats COS treatment in the diabetic group had a significant effect on reducing the $GAUC_{0-2}$ induced by glucose loading (2 g/kg, i.p.) (Fig. 1). The $GAUC_{0-2}$ level of the treated group was 430±50.9 mg/dl but the $GAUC_{0-2}$ level of the control group was 554.0±93.0 after 4 weeks' treatment. When diabetic rats were treated with 0.3% COS for 4 weeks, the $GAUC_{0-2}$ level was reduced from 583±101.4 mg/dl (be-

Table 1. Characteristics of Sprague-Dawley Rats before COS Treatment

	Normal rats (n=20)	Diabetic rats (n=20)
Body weight (g)	138.7±15.1	139.0±17.2
Plasma glucose (mg/dl)	87.5±10.1	114.0±18.6*
Cholesterol (mg/dl)	120.1±20.1	142.5±24.6
Triglyceride (mg/dl)	116.5±20.3	104.5±13.7
Glucose tolerance (mg/dl) ($GAUC_{0-2}$)	274.5±41.3	583.0±101.4*

Diabetes was induced by intraperitoneal injection of STZ (80 mg/kg) to 3-d-old Sprague-Dawley rat pups.²⁴⁾ Blood samples were collected from 18 h-fasted rats 8 weeks after STZ injection. Each value represents mean±S.E. * Significantly different from normal rats ($p < 0.05$).

Table 2. Effect of COS Treatment on Various Parameters in Normal and Diabetic Sprague-Dawley Rats

	Normal rats		Diabetic rats	
	Control	Treated	Control	Treated
In fasting rats				
Glucose	90.0±10	102.0±14	111.0±15 ^{a)}	90.0±16 ^{b)}
Triglyceride	81.3±19.9	69.5±30.4	78.9±26.9	65.9±22.4
Cholesterol	145.0±21.7	133.2±34.8	150.0±26.2	122.0±33.2
In fed rats				
Glucose	120.0±11.5	125.0±10	135.0±18	133.0±14
Triglyceride	198.6±134.9	124.0±21.4	222.2±80.5	114.0±32.6 ^{b)}
Cholesterol	140.1±12.4	124.0±10.7	126.8±18.4	111.9±13.4

Normal and diabetic rats were treated with 0.3% COS for 4 weeks. Each value (mg/dl) represents mean±S.E. from 6 rats. a) Significantly different from the normal control rats ($p < 0.05$); b) significantly different from diabetic control rats ($p < 0.05$).

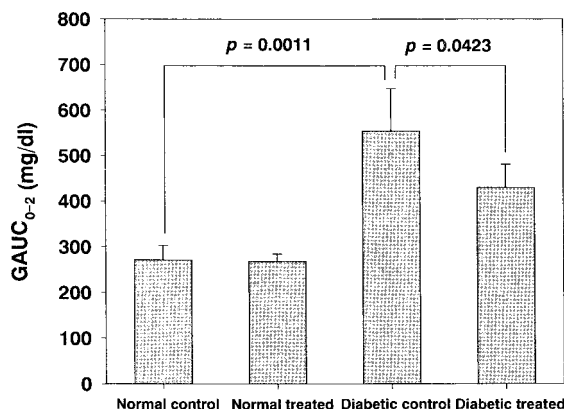


Fig. 1. Effect of COS on the Plasma Glucose Levels of 18 h-Fasted Rats after an Intraperitoneal Glucose Loading (2 g/kg)

The values represent the integrated area under the plasma glucose curve over 2 h ($GAUC_{0-2}$). Each value represents mean \pm S.E. of 6 rats.

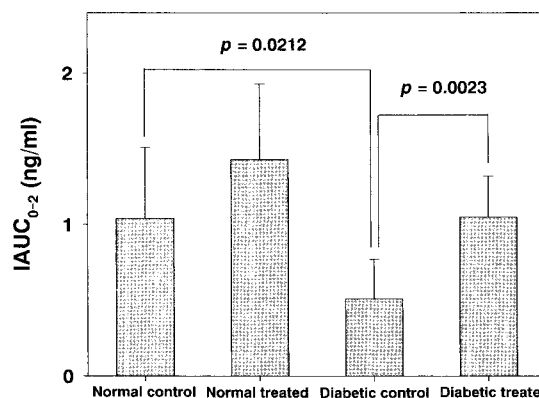


Fig. 2. Effect of COS on Plasma Insulin Levels during GTT of 18 h-Fasted Rats after Intraperitoneal Glucose Loading (2 g/kg)

The values represent the area integrated under the plasma insulin curve over 2 h ($IAUC_{0-2}$). Each value represents mean \pm S.E. of 6 rats.

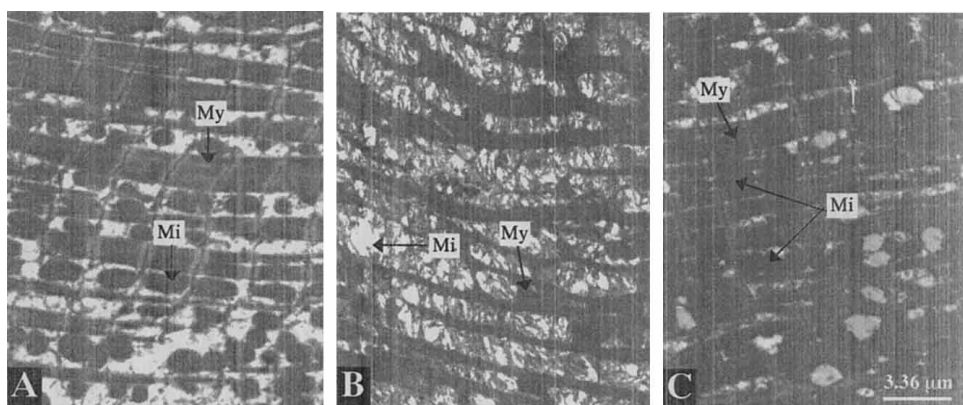


Fig. 3. Protective Effect of COS against Heart Injury in Diabetic Rats

Diabetic rats were treated with 0.3% COS for 8 weeks, and heart tissues were examined by electron microscopy. A: In the myocardium of a diabetic treated rat, the structure of myofibrils (My) was well preserved and mitochondrial (Mi) swelling and vacuolation were improved. B: In the myocardium of a diabetic control rat, myofibrils were separated and degenerated and mitochondria were swollen and vacuolated. C: In the myocardium of a normal control rat, myofibrils were densely packed and aligned and mitochondria were intact.

fore treatment) to 430 ± 50.9 mg/dl (after treatment) (Table 1, Fig. 1). However, nontreated diabetic rats fed water containing 0.1% ascorbic acid for 4 weeks were found to have unaltered glucose tolerance. The glucose tolerance of nontreated normal rats did not alter throughout the experiment, whether they were treated with COS or not.

Effect of COS on Insulin Secretion Induced by Glucose in Diabetic Rats The integrated area under the curve for insulin ($IAUC_{0-2}$) was significantly higher in the treated diabetic rats than in the nontreated diabetic rats. Although the $IAUC_{0-2}$ of the nontreated diabetic animals was one-half that in the nontreated controls, the $IAUC_{0-2}$ in the treated diabetic rats was similar to that in the nontreated controls (Fig. 2).

Effect of COS on Serum TG Level of Diabetic Rats After the normal and diabetic animals had been treated with 0.3% COS, the fed-TG levels decreased significantly (Table 2). Fed-TG levels in COS-treated animals were 38% lower than those in normal control rats and 49% lower than in diabetic control rats, respectively. However, fasting-TG and cholesterol levels were not different between these groups. Cholesterol in the 0.3% COS-fed animals were about 10% lower than in the corresponding nontreated animals, regardless of feeding or fasting, although the difference was not statisti-

cally significant.

Protective Effect of COS on Diabetes-Induced Heart Injury To investigate the preventive effect of COS on heart injury induced by diabetes, diabetic rats were treated with 0.3% COS for 8 weeks, and heart tissues were compared with those of the diabetic controls under the electron microscope (Fig. 3). In animals treated with COS for 8 weeks, alterations in myocardial structure were prevented. However, electron microscopic morphometry of heart samples from diabetic control rats revealed diabetic cardiomyopathy, which consisted of the vacuolation and swelling of mitochondria and the separation and degeneration of myofibrils.

DISCUSSION

The COS used in this study was prepared by enzymatic hydrolysis and size-exclusion chromatography. MALDI-TOF mass spectroscopy showed that the oligomer was effectively completely deacetylated, and that it was composed of 2–8 dp oligomers (data not shown). Chitosan polymer has a high molecular weight and viscosity, and it is insoluble at pH values above 6.3 (the pK_a of chitosan). On the other hand, chitosan oligomers have low viscosities and are freely soluble at neutral pH. The properties of COS may be usefully ap-

plied in food. Several enzymatic and chemical methods of producing chitosan oligomer have been described.^{22,25)} However, the chemical methods involve long harsh treatments, and thus the enzymatic hydrolysis of chitosan offers many advantages for food additive preparation. Many different chitosanases, including *Streptomyces*²⁶⁾ and *Bacillus* chitosanase,²⁷⁾ have been studied.

A few reports have claimed that chitosan has antidiabetic effects.^{19–21)} However, the degree of acetylation and the molecular weight of the chitosan oligomers used in the previous reports were not defined. Moreover, the mechanism of chitosan absorption in the small intestine is not known. Although it is generally believed that chitosan polymer is primarily absorbed after it has been transformed into oligosaccharides by chitosanase, which is secreted by intestinal bacteria or by lysozymes in the intestinal fluid, it has been reported that fully deacetylated chitosan was not digested by lysozymes, while a partially acetylated chitosan was.^{22,23)} In the present study, fully deacetylated COS composed of 2–8 oligomers was used. Many investigators have reported that hyperglycemia and hyperlipidemia may be induced in STZ-diabetic rats.²⁸⁾ In addition, diabetes has these same cardiovascular disease risk factors. Therefore the atherosclerotic risk is great in poorly controlled diabetic patients, possibly because of associated hyperglycemia, hypercholesterolemia, and hypertriglyceridemia,⁵⁾ which illustrates the importance of the control of these three conditions to prevent diabetic complications.

In the present study, glucose tolerance was increased in diabetic rats administered COS (Fig. 1). The $IAUC_{0-2}$ was significantly increased in diabetic animals treated with COS (Fig. 2). These results mean that the observed increase in glucose tolerance may be attributed to the normalization of insulin secretion. In addition, Kondo *et al.* reported that long-term (22-week) administration of low molecular-weight chitosan (mean MW 20000) prevents the progression of slowly progressive diabetes mellitus in mice.²⁰⁾ They suggested that the putative mechanism of the antidiabetic action of chitosan was the prevention of the decrease in β -cells in pancreatic islets. Hayashi and Ito reported that the antidiabetic action of chitosan was not due to D-glucosamine, a monosaccharide contained in chitosan, in the obese diabetic KK-A^y mice model.²¹⁾ In this study, glucose tolerance and insulin secretion were increased when diabetic animals were treated for 4 weeks with low molecular-weight chitosan (MW < 1500). These data indicate that one of the putative mechanisms of the antidiabetic action of COS is the induction of glucose-inducible insulin secretion in diabetic rats, although the exact mechanisms of the antidiabetic action of chitosan have not been clarified. Furthermore, COS treatment was found to prevent diabetic alterations in heart tissues in diabetic rats (Fig. 3). This protective effect against myocardial structural alteration may be caused by the glucose-lowering and TG-lowering effects of COS. Diabetes is associated with vascular destruction caused by increased oxidative stress, and this study shows that COS can reduce plasma glucose and TG levels, thus increasing free radical production. It was re-

ported that signs of diabetic cardiomyopathy²⁹⁾ such as mitochondrial swelling and myofibril degeneration could be prevented by insulin treatment.³⁰⁾ In this study, COS increased glucose tolerance and insulin secretion (Figs. 1, 2). Therefore COS may be useful for preventing diabetic cardiomyopathy.

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