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ARTICLE

Chitosan Reduces Gluconeogenesis and Increases Glucose Uptake in Skeletal Muscle in Streptozotocin-Induced Diabetic Rats

SHING-HWA LIU,[†] YU-HAN CHANG,[‡] AND MENG-TSAN CHIANG*,[‡]

[†]Institute of Toxicology, College of Medicine, National Taiwan University, Taipei 100, Taiwan, Republic of China, and [‡]Department of Food Science, College of Life Science, National Taiwan Ocean University, Keelung 202, Taiwan, Republic of China

Chitosan is a natural and versatile biomaterial with a blood-glucose-lowering effect in diabetic animals, but the mechanism of action is still unknown. This study was designed to investigate the possible mechanisms involved in the hypoglycemic activity of chitosan in rats with streptozotocin (STZ)-induced diabetes. Male Sprague−Dawley (SD) rats were divided into non-diabetic with cellulose (control), diabetic with cellulose (DM), and diabetic with low- (DM + LCS) and high- (DM + HCS) molecular-weight chitosan groups. After a 4 week feeding study, plasma glucose and fructosamine levels were increased while plasma leptin was decreased in the DM group when compared to the control group. These alternations caused by diabetes could be effectively reversed by both chitosan treatments. The increased gluconeogenesis-related signals including phospho-enolpyruvate carboxykinase (PEPCK) expression and phosphorylations of p38 and AMP-activated kinase (AMPK) in the livers of diabetic rats were attenuated by chitosans. Moreover, chitosan significantly increased muscle glucose uptake-related signals including Akt phosphorylation and glucose transporter-4 (GLUT4) translocation from the cytosol to membrane in the soleus muscles of diabetic rats. These results indicate that chitosan may possess a potential for alleviating type-1 diabetic hyperglycemia through the decrease in liver gluconeogenesis and increase in skeletal muscle glucose uptake and use.

KEYWORDS: Chitosan; streptozotocin; glucose metabolism; liver; skeletal muscle

INTRODUCTION

Chitin is a polysaccharide based on the *N*-acetyl-glucosamine monomer and is found in shellfish, clams, oysters, krill, squid, fungi, and insects (*1, 2*). Chitin is insoluble in water but is converted to a more soluble chitosan after deacetylation and partial hydrolysis. Chitosan is a versatile biomaterial that has been widely employed as a dietary supplement and also used for pharmacological and biomedical applications (*1−3*). It has been well-known for its cholesterol-lowering effects. Increased fecal cholesterol excretion by interfering interestinal micelle was proposed to be the mechanism response for the hypocholesterolemic properties of chitosan(*4−6*). Recently, scientists have focused on the effect of chitosan on glucose metabolism. A 24 week randomized, double-blind, placebo-controlled trial in overweight and obese adults has shown that the chitosan treatment loses more body weight ($p = 0.03$), circulating total and low-density lipoprotein (LDL) cholesterol, and glucose than the placebo treatment (*7*). In addition, several animal studies demonstrated

the potential of low-molecular-weight (MW) chitosan on hypoglycemic activity in non-insulin-dependent diabetes mellitus (NIDDM, type-2 diabetes) (*8−10*). However, the studies of chitosan on type-1 diabetes are relatively rare. Decreased intestinal disaccharidases and prolonged glucose absorption in the small intestines in diabetic rats were suggested to be partly involved in reducing plasma glucose levels in streptozotocin-induced diabetic rats (*5*). Nevertheless, the precise action and mechanism of chitosan on diabetes are still not well-understood.

Type-1 diabetes [insulin-dependent diabetes mellitus (IDDM)] is characterized by autoimmune-mediated destruction of pancreatic *β* cells culminating in absolute insulin deficiency. It has been predicted that between 2005 and 2020, new cases of type-1 diabetes in European children younger than 5 years will double and that the prevalence of cases in those younger than 15 years will increase by 70% (*11*). People with type-1 diabetes require daily insulin treatment to sustain life. The treatment by injection of insulin usually incurs pain and may negate the quality of life of diabetic patients. The alternative routes for insulin delivery, such as oral and nasal pathways have been explored over the years. Chitosan and its derivatives have been investigated as functional excipients of delivering insulin via oral, nasal, and transdermal pathways (*12, 13*). However, ongoing efforts to test individual agents with potential to ameliorate type-1 diabetes are needed.

*To whom correspondence should be addressed: Department of Food Science, College of Life Science, National Taiwan Ocean University, Keelung 202, Taiwan, Republic of China. Telephone: +886-2-24622192 ext. 5117/5118. Fax: +886-2-24634203. E-mail: a0071@mail.ntou.edu.tw.

In the present study, we investigate the effects and possible mechanisms of chitosan with high (average MW of 8.60×10^5) and low (average MW of 1.77×10^4) MW as a dietary supplement on glucose metabolism in streptozotocin (STZ)-induced type-1 diabetic rats. When chitosan supplementation was applied to diabetic rats, the hyperglycemia, increased liver gluconeogenesis, and decreased muscle glucose use were effectively alleviated. Moreover, AMP-activated protein kinase (AMPK) regulates phosphoenolpyruvate carboxykinase (PEPCK, a key enzyme of gluconeogenesis) gene expression (*14*). It has also been found that cAMP-dependent activation of genes involved in gluconeogenesis is dependent upon the p38 pathway (*15*). Glucose transporter-4 (GLUT4) is a primary mediator for glucose uptake into skeletal muscle, which can be regulated by phosphatidyl inositol 3-kinase (PI3K) and Akt (protein kinase B) signaling (*16*). Therefore, in this study, the roles of AMPK, p38, Akt, and GLUT4 signalings in chitosan-alleviated glucose metabolism in diabetic animals are also investigated.

MATERIALS AND METHODS

Materials. High-MW chitosan, which was obtained from shrimp shell chitin by alkali fusion, was purchased from Taiwan Applied Chemistry Co. (Kaohsiung, Taiwan). Cellulose was purchased from Sigma Chemical Co. (St. Louis, MO). The low-MW chitosan was prepared from high-MW chitosan by the method by Varum et al. (*17*), with a modification. In brief, the high-MW chitosan powder was dissolved in 8 N HCl with gentle shaking, and the dissolved sample was hydrolyzed to 55 °C for 3 h. The reaction was stopped by cooling the solution to 0 °C and then adding an equal volume of 8 N NaOH (0 °C) to reach a final pH of 9−10. The resulting low-MW chitosan sample was washed with deionized water and then dried at 50 °C.

Determination of the Deacetylation Degree (DD), Average MW, and Viscosity of Chitosan Samples. The average MW and DD of the chitosan were determined using high-performance liquid chromatography and Fourier transform infrared spectroscopy, respectively, as described by Chen and Liu (*18*). Viscosity measurement was performed on chitosan dissolved in 0.1 N HCl solution using a Haake viscometer CV20 (Haake Mess-Technik GmbHu Co., Karlsruhe, Germany), as described by Chen and Chen (*19*).

Animals and Diets. Male, 7-week-old Sprague−Dawley (SD) rats were purchased from BioLASCO Taiwan Co., Ltd. (Taipei, Taiwan). Rats were fed a chow diet (Rodent Laboratory Chow, Ralston Purina, St. Louis, MO) for 2 weeks, and then the animals were divided into four groups: (1) non-diabetic rats with 5% cellulose (control), (2) diabetic rats with 5% cellulose (DM), (3) diabetic rats with 5% high-MW chitosan (DM + HCS), (4) diabetic rats with 5% low-MW chitosan (DM + LCS). Each group contains 10 animals. The physiochemical properties of chitosan samples and composition of the experimental diet given to test animals are shown in **Table 1**. The diabetes in rats was induced by a single subcutaneous injection (60 mg/kg) of streptozotocin (STZ, Sigma Co., MO) dissolved in freshly prepared 0.05 M citrate buffer (pH 4.5). After 1 week, the STZ-injected rats were checked for fasting plasma glucose concentration (> 300 mg/dL) to confirm the status of diabetes before feeding the experimental diet. Although we did not measure the real values of other parameters in addition to the fasting plasma glucose level in diabetic rats before dietary treatment, to simplify the study, the end-point data of non-diabetic rats was regarded as the "blank control group". The changes of all data in plasma, liver, feces, and enzyme activity were fully examined between dietary treatment and disease status. Rats were housed in individual stainless-steel cages in a room kept at 23 ± 1 °C and $60 \pm 5\%$ relative humidity with a 12 h light and dark cycle (lighting from 8:00 a.m. to 8:00 p.m.). Food and drinking water were available *ad libitum* and measured daily for 4 weeks. The body weight was measured every week. After the 4 week feeding study, the animals were sacrificed. This study was approved by the Animal House Management Committee of the National Taiwan Ocean University. The animals were maintained in accordance with the guidelines for the care and use of laboratory animals as issued by the Animal Center of the National Science Council.

Table 1. Composition of Experimental Diets (%)

ingredients[a]	control	DM	DM + LCS	DM + HCS
casein	20	20	20	20
lard	8	8	8	8
corn oil	2	2	2	2
vitamin mixture[b]	1	1	1	1
salt mixture[c]	5	5	5	5
cholesterol	0.5	0.5	0.5	0.5
choline chloride	0.2	0.2	0.2	0.2
cholic acid	0.3	0.3	0.3	0.3
cellulose	5	5		
low-MW chitosan[d]			5	
high-MW chitosan[e]				5
corn starch	58	58	58	58

[a] Control, non-diabetic with 5% cellulose group; DM, diabetic with 5% cellulose group; DM + LCS, diabetic with 5% low-MW chitosan group; M + HCS, diabetic with 5% high-MW chitosan group. [b] AIN 93 vitamin mixture procured from ICN Biochemicals (Costa Mesa, CA). [c] AIN 93 mineral mixture procured from ICN Biochemicals (Costa Mesa, CA). [d] The average MW and viscosity of chitosan were about 1.77×10^4 Da and 27.4 cm/s (cps), respectively. The degree of deacetylation was about 94.8%. [e] The average MW and viscosity of chitosan were about 8.60×10^5 Da and 538.0 cps, respectively. The degree of deacetylation was about 92.7%.

Collection of Blood and Tissue Samples. At the end of the experimental period, animals fasted for 12 h prior to being sacrificed (at 10:00 a.m.) by exsanguinations via the abdominal aorta while under diethyl ether anesthesia. Heparin was used as the anticoagulant. Plasma was separated from the blood by centrifugation ($1750g$) at 4 °C for 20 min. The liver and soleus muscle from each animal were excised and weighed.

Determination of Plasma Glucose, Insulin, Fructosamine, and Leptin. Plasma glucose was determined with a kit purchased from Audit Diagnostics Co. (Cork, Ireland). Plasma fructosamine was measured using a kit purchased from Sigma Chemical Co. (St. Louis, MO). Plasma insulin was determined using a rat insulin enzyme-linked immunosorbent assay (ELISA) kit (Mercodia AB, Uppsala, Sweden) Plasma leptin was determined using a rat leptin ELISA kit (Assay Designs, Inc., Ann Arbor, MI).

Determination of the Glycogen Content in Liver. The glycogen content was analyzed according to the method by Murat and Serfaty (*20*). Samples of excised tissues were homogenized in ice-cold citrate buffer (0.1 M, pH 4.2), followed by centrifugation at $10600g$ for 30 min at 4 °C. The free glucose content in the supernatant was then measured by a test kit (Audit Diagnostics). Amyloglucosidase (2 mg, Sigma) was added with the homogenate and incubated for 4 h at 37 °C. The total glucose content in incubation was measured as described above. The glycogen content in the liver was calculated as the differences between total and free glucose. The initial free glucose value was subtracted, with the difference being the value used to calculate the glycogen content of the tissue.

Western Blot Analysis. Tissues were homogenized in buffer that contained 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 0.25 M sucrose, 0.5 mM ethanesulfonic acid (EDTA), 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10μg/mL leupeptin, 10μg/mL aprotinin, and 1 mM sodium orthovanadate (Na_3VO_4) (pH 7.5). The cell homogenates were precleared from nuclei and cell debris by centrifugation at $10000g$ for 15 min at 4 °C. The supernatant (total cell lysate) was then ultracentrifuged at $100000g$ for 1 h at 4 °C, which resulted in a supernatant, referred to as the "cytosolic fraction". In some experiments, the pellets were resuspended in 250μL of homogenizing buffer and 1% (v/v) nonidet P-40 and incubated on ice for 30 min, followed by centrifugation at $100000g$ for 30 min at 4 °C. The supernatant fraction was termed the "membrane fraction". Total protein containing $30−80 \mu$g was separated on 8% sodium dodecyl sulfate (SDS)−polyacrylamide minigels and transferred to nitrocellulose membranes (Amersham). After blocking, blots were incubated with antibodies for PEPCK, p38, phospho-p38, AMPK, Akt, phospho-Akt, and GLUT4 in phosphate-buffered saline (PBS)/Tween-20 for 1 h, followed by two washes in PBS/Tween-20, and then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG for 30 min. Moreover, β-actin served as a control for sample loading and integrity. The antibody-reactive bands were revealed by the enhanced chemiluminescence kit (Amersham) and were used to expose to Kodak radiographic film. The amount of polypeptide was quantitated by
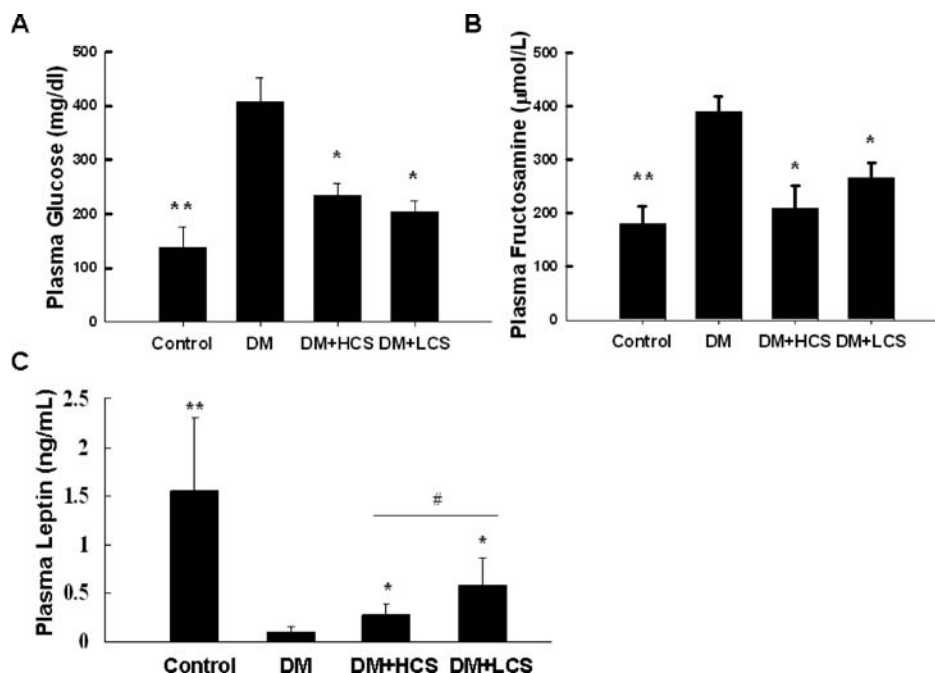
Figure 1. Effects of chitosan on the changes of plasma glucose, fructosamine, and plasma leptin levels in STZ-induced diabetic rats. Control and diabetic (DM) rats were fed diets with or without chitosan supplements (low-MW chitosan, LCS; high-MW chitosan, HCS) for 4 weeks, and then the (A) plasma glucose, (B) fructosamine, and (C) plasma leptin levels were measured. Results are expressed as mean ± SD (n = 10). (∗) $p < 0.05$ DM versus LCS + DM and HCS + DM. (∗∗) $p < 0.05$ control versus DM. (#) $p < 0.05$ LCS + DM versus HCS + DM.

integrated densitometric analysis of the film (Kodak Gel Logic-100 Imaging System).

Statistical Evaluation. Results are given as the means ± standard deviation (SD). Statistical differences among groups were calculated by analysis of variance (ANOVA) (SAS Institute, Cary, NC), and group means were considered to be significantly different at $p < 0.05$ as determined by Duncan's multiple range test.

RESULTS

Effects of Chitosan on Body Weight and Adipose Tissue in STZ-Induced Diabetic Rats. At the end of the study, a significant decrease in body weight and adipose tissue weight was observed in diabetic rats when compared to non-diabetic rats (body weight: control, 517.8 ± 38.9 g; DM, 348.7 ± 49.3 g and adipose tissue: control, 20.9 ± 3.70 g; DM, 1.94 ± 1.45 g). No significant difference in body weight was observed in diabetic rats regardless of diet (DM + LCS, 357.4 ± 50.9 g; DM + HCS, 340.9 ± 24.7 g), but low-MW chitosan feeding significantly increased ($p < 0.05$) the adipose tissue weight (DM + LCS, 3.83 ± 1.90 g; DM, 1.94 ± 1.45 g). No significant difference in adipose tissue weight was observed between the two chitosan groups (DM + LCS, 3.83 ± 1.90 g; DM + HCS, 2.10 ± 1.44 g).

Effects of Chitosan on Blood Glucose, Fructosamine, Leptin, and Liver Glycogen in STZ-Induced Diabetic Rats. It is well-known that the levels of blood glucose and fructosamine (a glycated serum protein complex that reflects the average blood glucose concentration over the previous 1−3 weeks) are increased in diabetes. As shown in **Figure 1**, the blood glucose and fructosamine levels were markedly increased in rats treated with STZ. Dietary supplementation with both high- (average MW of 8.60×10^5) and low- (average MW of 1.77×10^4) MW chitosan for 4 weeks effectively reversed the increased blood glucose and fructosamine levels in STZ-induced diabetic rats. The blood insulin level was markedly reduced in STZ-induced diabetic rats, which was not affected by chitosan (data not shown). Moreover, the plasma leptin level was markedly decreased in STZ diabetic rats
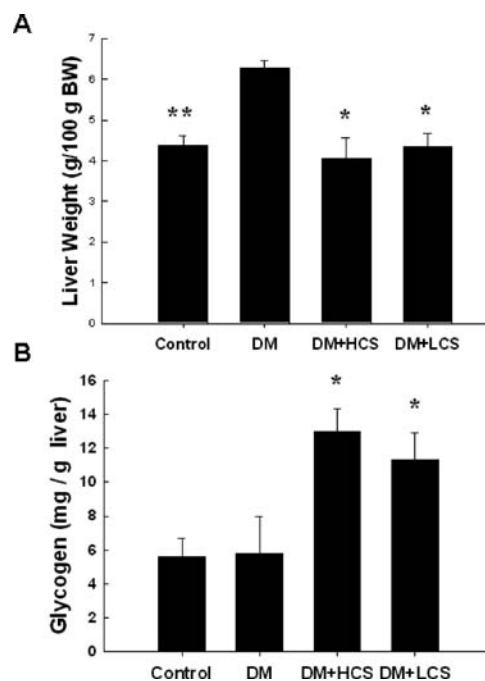

Figure 2. Effects of chitosan on the changes of weight and glycogen content in the livers of STZ-induced diabetic rats. Control and diabetic (DM) rats were fed diets with or without chitosan supplements (low-MW chitosan, LCS; high-MW chitosan, HCS) for 4 weeks, and then the (A) liver weight and (B) glycogen content were measured. Results are expressed as mean ± SD (n = 10). (∗) $p < 0.05$ DM versus LCS + DM and HCS + DM. (∗∗) $p < 0.05$ control versus DM.

(**Figure 1C**). Chitosan significantly reversed the decrease in the plasma leptin level in STZ diabetic rats; the effect of low-MW chitosan was higher than that of high-MW chitosan (**Figure 1C**). The relative liver weight was also increased in STZ-induced
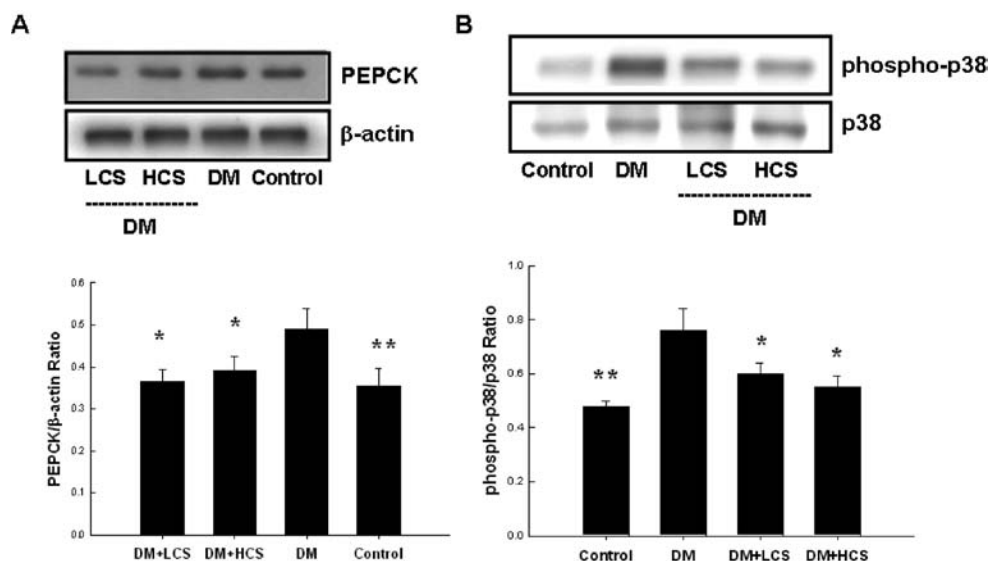
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Figure 3. Effects of chitosan on the PEPCK protein expression and p38 protein phosphorylation in the livers of STZ-induced diabetic rats. Control and diabetic (DM) rats were fed diets with or without chitosan supplements (low-MW chitosan, LCS; high-MW chitosan, HCS) for 4 weeks, and then the (A) PEPCK protein expression and (B) p38 protein phosphorylation were analyzed by western blotting. Quantification of protein expression was performed by densitometric analysis. Results are expressed as mean $\pm$ SD ($n = 9$). ($*$) $p < 0.05$ DM versus LCS + DM and HCS + DM. ($**$) $p < 0.05$ control versus DM.

diabetic rats (**Figure 2**). Both high- and low-MW chitosan markedly reduced the increase in the liver weight but significantly enhanced the liver glycogen content in STZ-induced diabetic rats (**Figure 2**).

Effects of Chitosan on Liver PEPCK, p38, and AMPK and Muscle Akt and GLUT4 Signals in STZ-Induced Diabetic Rats. As shown in **Figure 3**, the PEPCK protein expression and p38 protein phosphorylation were increased in the livers of STZ-induced diabetic rats. Both high- and low-MW chitosan supplementation effectively reversed the increase in diabetic liver PEPCK and phospho-p38 protein expressions (**Figure 3**). Moreover, the phosphorylation of AMPK was markedly enhanced in livers of diabetic rats fed with both high- and low-MW chitosan-containing diets for 4 weeks (**Figure 4**).

On the other hand, the phosphorylation of Akt protein in the soleus muscle was decreased in STZ-induced diabetic rats (**Figure 5A**). GLUT4 translocation evaluated by the difference in cytosol and membrane GLUT4 protein levels in the soleus muscle were also decreased in STZ-induced diabetic rats (**Figure 5B**). Dietary supplementation with both high- and low-MW chitosan significantly reversed these alterations in Akt phosphorylation and GLUT4 translocation in diabetic soleus muscles (**Figure 5B**).

DISCUSSION

Chitosan has been shown to possess the hypolipidemic and hypoglycemic (major in type-2 diabetic model) effects *in vitro* and *in vivo* (*4*, *5*, *8−10*). The precise action and mechanism of chitosan on diabetes are still not well-understood. Kondo and colleagues have reported that administration of low-MW chitosan (average MW of 20000) prevents the progression of low-dose STZ-induced slowly progressive diabetes mellitus in mice (NIDDM model) (*8*). Hayashi and Ito have shown that daily administration of chitosan solutions as drinking water prevents the progression of diabetes in an obese diabetic KK-Ay mice model (NIDDM model) (*9*). The anti-diabetic effect of low-MW chitosan (oligosaccharide) was also found in a neonatal NIDDM rat model (*10*). Here, we found that dietary supplementation with both high- (average MW of 8.60×10^5) and low- (average MW of 1.77×10^4) MW chitosan effectively reversed the hyperglycemia and hyperfructosaminemia
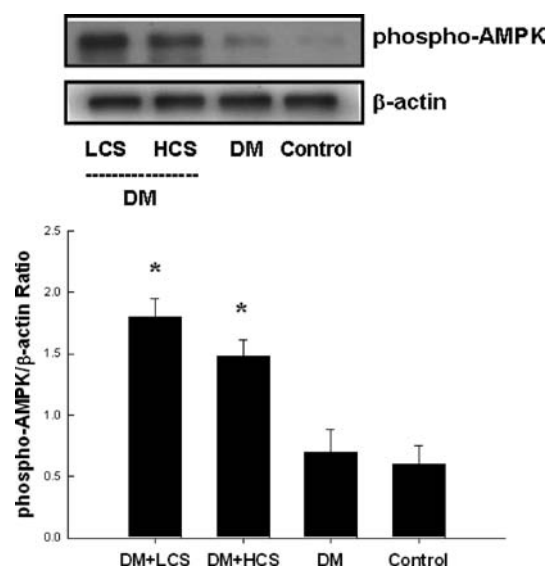

Figure 4. Effect of chitosan on the AMPK protein phosphorylation in the livers of STZ-induced diabetic rats. Control and diabetic (DM) rats were fed diets with or without chitosan supplements (low-MW chitosan, LCS; high-MW chitosan, HCS) for 4 weeks, and then the AMPK protein phosphorylation was analyzed by western blotting. Quantification of protein expression was performed by densitometric analysis. Results are expressed as mean $\pm$ SD ($n = 7$). ($*$) $p < 0.05$ DM versus LCS + DM and HCS + DM.

in STZ-induced type-1 (IDDM) diabetic rats. The recent study of Zeng and colleagues has shown that high- (average MW of 7.60×10^5) and low- (average MW of 3.27×10^4) MW chitosan give the high liver and kidney distribution in mice after oral administration (*21*). This finding indicates that both high- and low-MW chitosan can be absorbed and distributed in the body after oral administration.

The liver is an important organ for regulating and maintaining the blood glucose concentration. Gluconeogenesis is an anabolic pathway of glucose formation from non-hexose precursors. Gluconeogenesis is highly responsible for hepatic glucose production,
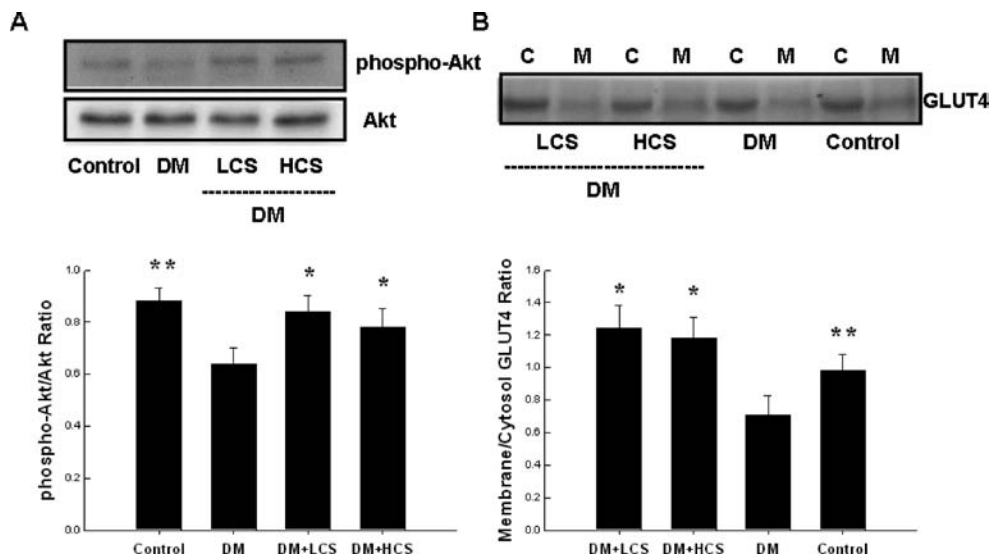
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Figure 5. Effects of chitosan on the Akt protein phosphorylation and GLUT4 translocation in the soleus muscles of STZ-induced diabetic rats. Control and diabetic (DM) rats were fed diets with or without chitosan supplements (low-MW chitosan, LCS; high-MW chitosan, HCS) for 4 weeks, and then the (A) Akt protein phosphorylation and (B) GLUT4 translocation evaluated by the difference in cytosol and membrane GLUT4 protein levels were analyzed by western blotting. Quantification of protein expression was performed by densitometric analysis. Results are expressed as mean $\pm$ SD ($n = 7$). ($*$) $p < 0.05$ DM versus LCS + DM and HCS + DM. ($**$) $p < 0.05$ control versus DM.

which is an essential mechanism for the maintenance of circulating blood glucose levels (*22, 23*). Insulin can inhibit the rate of gluconeogenesis in the liver. PEPCK is one of the important gluconeogenic enzymes. It has been shown that activation of p38 signaling enhances expression of hepatic PEPCK (*24, 25*). Activation of AMPK (an intracellular energy sensor) by 5-aminoimidazole-4-carboxamide riboside (AICAR) has been shown to inhibit hepatic gluconeogenesis through a p38-activated PEPCK signaling pathway (*26*). On the other hand, excessive hepatic gluconeogenesis and glucose production are important contributors to diabetic hyperglycemia. The liver gluconeogenic pathway has been recognized as a target for treating diabetes mellitus (*23, 27*). In the present study, we found that both high- and low-MW chitosan markedly reduced the increase in liver weight and liver PEPCK and phospho-p38 protein expressions but significantly enhanced the phosphorylation of liver AMPK and liver glycogen content in STZ-induced type-1 diabetic rats. These results indicate that chitosan possesses the ability to reduce liver gluconeogenesis, which may further contribute to the decrease in diabetic hyperglycemia.

Insulin has been known to maintain glucose homeostasis largely by enhancing glucose uptake into skeletal muscle and adipose tissues, which is a process mediated by GLUT4 (*28*). Protein kinase Akt/PKB has been demonstrated to be a central mediator of insulin-regulated translocation of GLUT4 (*29*). van Dam and colleagues have indicated that Akt activation is required at a late stage of insulin-induced GLUT4 translocation to the plasma membrane (*30*). It has been suggested that impaired translocation or activation of glucose transporters in the skeletal muscle is shown in IDDM subjects (*31*). Intensive insulin therapy has shown that no significant alterations occur in the amount of GLUT4 protein and mRNA in the skeletal muscle of type-1 diabetic patients (*32*). It has also been shown that Akt phosphorylation and GLUT4 translocation in the skeletal muscles were significantly less in STZ-induced diabetic rats than in the normal control rats (*33*). In the present work, we found that chitosan significantly reversed the alterations in Akt phosphorylation and GLUT4 translocation in STZ diabetic soleus muscles, indicating that the increase in muscle glucose uptake and use by chitosan

may contribute to the improvement in hyperglycemia in this type-1 diabetic rat model.

Leptin is a hormone secreted by adipocytes that regulates multiple functions, including food intake, energy homeostasis, thermoregulation, pro-inflammatory immune responses, bone metabolism, and endocrine (*34, 35*). Leptin has been shown to stimulate fatty acid oxidation by activating AMPK (*36*). It has been observed that leptin possesses the blood-glucose-lowering effect in normal rodents (*37*) and in rodents with partial insulin deficiency induced by STZ (*38*). A recent study has also shown that leptin reverses the catabolic consequences of the total lack of insulin in a type-1 diabetic rodent model, suggesting it may be a strategy for making type-1 diabetes insulin-independent (*39*). In the present study, we found that leptin levels in the plasma were markedly reduced in STZ-induced type-1 diabetic rats. Dietary supplementation of chitosan significantly reversed the decrease in plasma leptin in STZ diabetic rats. The increased leptin levels by low-MW chitosan feeding in diabetic rat groups may be related to the increased adipocytes. It is possible that chitosan treatment may exhibit a reduction in lipolysis in adipose tissue and an increase in the glucose uptake into the glucose target tissue (adipose, liver, and muscle), thereby affecting the glucose concentration. These findings imply that increased leptin may contribute, at least in part, to the ameliorative effect of chitosan on glucose metabolism in this type-1 diabetic rat model.

In conclusion, we found, in the present study, that both high- and low-MW chitosan markedly reduced the increases in blood glucose and fructosamine levels and liver weight in STZ-induced type-1 diabetic rats. Both high- and low-MW chitosan could also effectively activate AMPK phosphorylation, increase glycogen content, reverse the increases in diabetic liver PEPCK and phospho-p38 protein expressions, and reverse the decreases in diabetic skeletal muscle Akt protein phosphorylation and GLUT4 translocation. These results imply that chitosan with high- and low-MW possesses a potential for alleviating type-1 diabetic hyperglycemia through the decrease in liver gluconeogenesis and increase in skeletal muscle glucose use.

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