

Low Molecular Weight Chitosan Prevents the Progression of Low Dose Streptozotocin-Induced Slowly Progressive Diabetes Mellitus in Mice

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The present study was designed to clarify the effect of low molecular weight (LMW) chitosan (chitosan lactate, average MW: 20000) on the progression of slowly progressive non-insulin-dependent diabetes mellitus (NIDDM) induced by a single i.p. injection of low dose (100 mg/kg) streptozotocin (STZ) to 8-week-old male ICR mice. The non-fasting serum glucose levels of STZ-treated control mice continued to rise throughout the experimental period until 23 weeks after STZ treatment. The 0.2% or 0.8% chitosan (water solution), given as drinking water from prediabetic stage (2 weeks after STZ treatment), markedly prevented the time course-related rise of serum glucose levels of diabetic mice. In addition, the reduction of relative numbers of insulin-immunoreactive cells (β -cells) in the islets of diabetic mice at 24 weeks after STZ treatment was markedly prevented by 0.2% or 0.8% chitosan administration. However, the progression of hyperglycemia in diabetic mice was not affected by 0.2% glucosamine, a monosaccharide of chitosan. The glucose levels of normal mice were not affected by 0.8% chitosan administration. When 0.2% chitosan administration was stopped at 20 weeks, these animals had still maintained significantly lower serum glucose levels, compared to control animals, even at 5 weeks after stopping the administration. These results indicate that LMW chitosan prevents the progression of low dose STZ-induced slowly progressive NIDDM.

Key words chitosan; hypoglycemic action; streptozotocin; diabetes

Chitosan is chemically a polymeric D-glucosamine, a basic polysaccharide, and is produced by deacetylating chitin, a polymeric N-acetyl- β -D-glucosamine, with 40–45% NaOH at 120 °C (Fig. 1).¹⁾ Chitin is insoluble in water, acid or alkaline solution. However, chitosan is easily solubilized in acid solution, because it has amino groups in its chemical structure. It has been reported that chitosan has a lot of pharmacological actions such as immunopotentiating,^{2,3)} anti-hypertensive,⁴⁾ serum cholesterol-lowering,^{5–7)} anti-bacterial,^{8,9)} and wound healing-promoting actions.^{10–12)} In the previous study, we have reported that chitosan (MW: 25000–50000) has potent gastric cytoprotective and ulcer-healing promoting actions in rats.¹³⁾ Miura *et al.*¹⁴⁾ have first shown that chitosan given as a 5% food mixture produces consistent hypoglycemic and hypolipidemic effects in normal mice and neonatal streptozotocin (STZ)-induced diabetic mice, one of the animal models of non-obese type non-insulin-dependent diabetes mellitus (NIDDM). Recently, we succeeded in making a new mouse model of slowly progressive NIDDM by only a single i.p. injection of a subdiabetogenic small dose (100 mg/kg) of STZ to 8-week-old male ICR mice.¹⁵⁾ Therefore, in the present study, we examined the effect of a long-term administration of low molecular weight (LMW) chitosan (chitosan lactate, average MW: 20000) given as drinking water on serum glucose levels and on the number of insulin-immunoreactive cells (β -cells) in pancreatic islets in slowly progressive diabetic mice induced by a low dose STZ. The mechanism of the absorption of chitosan from the small intestine has not been well defined. It is believed that chitosan may be primarily absorbed after it has been transformed into oligosaccharides and a monosaccharide, D-glucosamine, by chitosanase secreted from intestinal bacterias or by lysozymes in intestinal fluid. Therefore, in the present study, we further examined the effect of D(+)-glucosamine HCl (glucosamine), a monosaccharide of chitosan, given daily as drinking water, on serum glucose levels in these

slowly progressive diabetic mice.

MATERIALS AND METHODS

Animals Eight-week-old male ICR mice (Nippon SLC, Shizuoka, Japan) were used in the experiment. They were housed an isolator caging system in an air-conditioned animal room at 23 \pm 1 °C.

Compounds The compounds employed were LMW chitosan (Yaizu Suisankagaku Ind., Co., Ltd., Shizuoka, Japan) and glucosamine (Sigma St. Louis, MO, U.S.A.). These test compounds were used as water solutions. As a diabetogenic agent, STZ was obtained from Sigma (St. Louis, MO, U.S.A.).

Induction of Diabetes Mice were fasted for 20 h before inducing diabetes with STZ. STZ (100 mg/kg) freshly dissolved in 0.05 M citrate buffer, pH 4.5, was intraperitoneally injected to the fasted mice. For comparing STZ-treated dia-

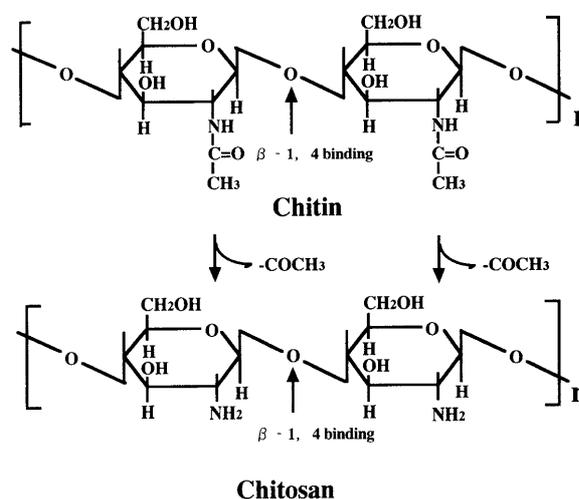


Fig. 1. Chemical Structures of Chitin and Chitosan

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betic mice with normal mice, normal mice were injected with an equivalent volume of citrate buffer.

Experimental Procedure In the first experiment, in order to evaluate the effects of LMW chitosan and glucosamine given from a prediabetic stage on serum glucose levels in slowly progressive diabetic mice, 0.2% LMW chitosan, 0.8% LMW chitosan or 0.2% glucosamine was given as drinking water for 21 weeks, from 2 weeks after STZ treatment. In addition, to evaluate the effect of LMW chitosan on non-fasting serum glucose levels of normal mice, 0.8% LMW chitosan was given as drinking water for 21 weeks from 2 weeks after normal mice were divided into each group. Normal or diabetic control mice were given distilled water instead of test compounds. Blood samples were withdrawn from the cavernous sinus with a capillary under ether anesthesia at 0 (just before treatment), 2, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21 and 23 weeks after STZ or citrate buffer treatment for the determination of non-fasting serum glucose. Serum glucose was determined using commercial agents of Glucose CII-test Wako (Wako Pure Chemical Industries). The body weight of each mouse was measured immediately before blood collection. At 12, 18 and 22 weeks after STZ or citrate buffer treatment, these animals were kept in individual metabolic cages for 24 h, and the consumption of food and drinking water, as well as urine volume per 24 h was measured. At the beginning of 24 weeks after STZ treatment, all animals were sacrificed by decapitation and their pancreata were taken for glucagon and insulin immunostaining.

In the second experiment, in order to evaluate the effect of withdrawal of LMW chitosan, given from a prediabetic stage, on non-fasting serum glucose levels, 0.2% LMW chitosan was given as drinking water for 18 weeks, from 2 weeks after STZ treatment, and the administration of the compound was then stopped for 5 weeks. During the withdrawal of LMW chitosan, distilled water was given as drinking water. Control animals were continuously given distilled water before and after the withdrawal. Blood samples were taken as described in the first experiment at 0 (just before withdrawal), 6 h, 24 h, 3 d and 1, 3 and 5 weeks after the withdrawal of LMW chitosan for serum glucose assay. In addition, the food and drinking water consumption, as well as urine volume per 24 h, was measured using metabolic cages at 1 and 5 weeks after the withdrawal.

In the third experiment, in order to assess the effect of LMW chitosan given from the diabetic stage on non-fasting serum glucose levels, 0.2% LMW chitosan was administered as drinking water for 6 to 13 weeks after STZ treatment. Blood samples were taken at 0, 2, 3, 5, 6, 7, 9, 11 and 13 weeks after STZ treatment for serum glucose assay.

Immunocytochemistry The pancreata taken from animals in the first experiment were fixed in 10% buffered formalin and then embedded in paraffin. Two consecutive sections (section thickness, 1 μ m) were cut from the paraffin block.

Statistical Analysis Results obtained were expressed as the mean \pm S.E. The data were analyzed by one-way analysis and Duncan multiple range test or non-parametric statistics. In all cases, $p < 0.05$ was considered significant.

RESULTS

Effects of LMW Chitosan and Glucosamine Given from a Prediabetic Stage Figure 2 shows the effect of LMW chitosan administered from a prediabetic stage on non-fasting serum glucose levels and on food and drinking water consumption and urine volume per 24 h in normal and low dose STZ-induced slowly progressive diabetic mice. The non-fasting serum glucose levels of STZ-treated control mice continued to rise throughout the experimental period until 23 weeks (0 week: 198 ± 3 mg/dl; 2 weeks: 273 ± 47 mg/dl; 5 weeks: 440 ± 66 mg/dl; 9 weeks: 518 ± 71 mg/dl; 13 weeks: 584 ± 58 mg/dl; 23 weeks: 788 ± 53 mg/dl) (Fig. 2A). The time course-related increase in serum glucose levels in diabetic mice was markedly prevented by giving 0.2 or 0.8% LMW chitosan as drinking water beginning from 2 weeks from STZ treatment (23 weeks: 0.2% LMW chitosan, 316 ± 63 mg/dl; 0.8% LMW chitosan, 427 ± 83 mg/dl vs. diabetic control, 788 ± 53 mg/dl, $p < 0.01$).

There was no significant difference in the time course-related gain in body weight after STZ treatment between 0.2 or 0.8% LMW chitosan-given mice and diabetic control mice throughout the 23-week-observation period (0 week: 0.2% LMW chitosan, 34.4 ± 0.5 g; 0.8% LMW chitosan, 34.2 ± 0.4 g vs. diabetic control, 34.0 ± 0.3 g; 23 weeks: 0.2% chitosan, 45.0 ± 0.9 g; 0.8% LMW chitosan, 44.0 ± 1.1 g vs. diabetic control, 44.7 ± 1.1 g).

At 12, 18 and 22 weeks after STZ treatment, the food and drinking water consumption, as well as urine volume in the diabetic control mice was significantly greater than those in the normal control mice (Fig. 2B). The food consumption in 0.2 or 0.8% LMW chitosan-administered mice was significantly less than that in diabetic control mice at 12 weeks (0.2% LMW chitosan: 6.1 ± 0.7 g, 0.8% LMW chitosan: 6.2 ± 0.9 g vs. diabetic control: 9.1 ± 1.6 g, $p < 0.05$), although no significant difference was observed at 18 and 22 weeks. The drinking water consumption and urine volume in 0.2 or 0.8% LMW chitosan-administered mice were markedly reduced, compared with those in the diabetic control mice at all weeks measured (drinking water consumption and urine volume at 22 weeks: 0.2% LMW chitosan, 7.9 ± 1.2 ml and 2.4 ± 0.9 ml, respectively; 0.8% LMW chitosan, 12.8 ± 4.4 ml and 6.0 ± 2.9 ml, respectively vs. diabetic control, 28.4 ± 5.1 ml and 20.6 ± 4.5 ml, respectively, $p < 0.05$ or $p < 0.01$). However, the effects of daily administration of 0.2 or 0.8% LMW chitosan as drinking water on the serum glucose levels, food and drinking water consumption and urine volume were not dose-dependent. Therefore, we examined the effects of LMW chitosan at concentrations less than 0.2% on these parameters (Fig. 3). The daily administration of 0.05, 0.1 or 0.2% LMW chitosan as drinking water prevented the progression of an increase in the glucose levels in a dose-dependent manner. In addition, LMW chitosan at three different concentrations dose-dependently prevented polyuria, overdrinking and overeating in diabetic mice at 6 and 12 weeks after STZ treatment (Fig. 3).

The serum glucose levels in normal mice were not affected by a long-term administration of 0.8% LMW chitosan (Fig. 2A). No significant difference in the food and drinking water consumption and urine volume between 0.8% LMW chitosan-administered mice and normal control mice was ob-

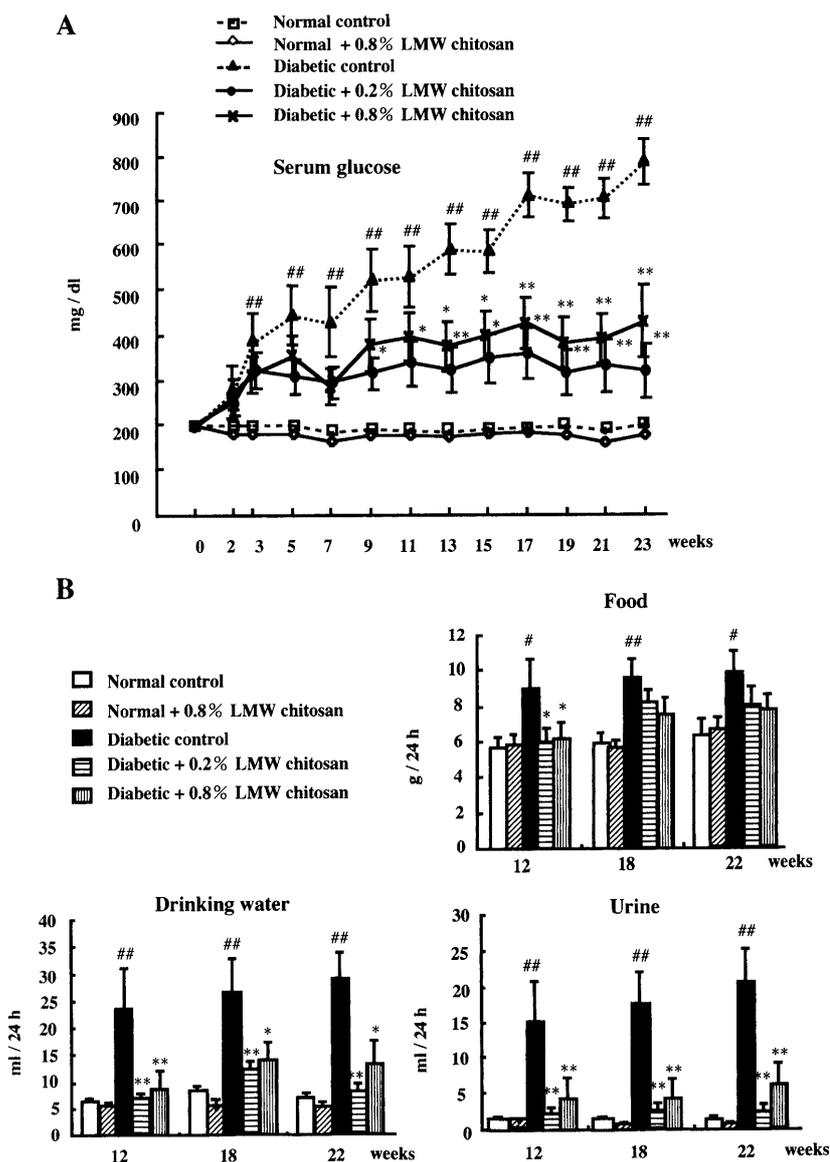


Fig. 2. Effect of 0.2% or 0.8% LMW Chitosan Given from a Prediabetic Stage on Non-Fasting Serum Glucose Levels (A) and on Food and Drinking Water Consumption and Urine Volume per 24 h (B) in Normal and Low Dose STZ-Induced Slowly Progressive Diabetic Mice

LMW chitosan was given as drinking water for 21 weeks, from 2 weeks after a single i.p. injection of 100 mg/kg STZ. Each plot denotes the mean \pm S.E. for 8 to 10 mice. Significantly different from the respective normal control, # $p < 0.05$, ## $p < 0.01$. Significantly different from the respective diabetic control, * $p < 0.05$, ** $p < 0.01$.

served.

Figure 4 shows the effect of LMW chitosan, given from a prediabetic stage, on the relative numbers of insulin (β -cells)- and glucagon-immunoreactive cells (α -cells) in pancreatic islets of diabetic mice. At 24 weeks after STZ treatment, there was a marked reduction of relative numbers of β -cells in the islets of control diabetic mice, as compared to those of normal mice. The reduction of relative numbers of β -cells of diabetic mice was markedly prevented by the drinking administration of 0.2 or 0.8% LMW chitosan from 2 weeks after STZ treatment. Representative photomicrographs of glucagon- and insulin-immunoreactive cells in pancreatic islets from normal and diabetic control mice and diabetic mice given 0.2 or 0.8% LMW chitosan at 24 weeks after STZ treatment are shown in Fig. 5.

The effect of glucosamine, given from a prediabetic stage, on non-fasting serum glucose levels in diabetic mice is shown in Fig. 6. The drinking administration of 0.2% glucosamine from 2 weeks after STZ treatment failed to prevent

a time course-related increase in serum glucose levels in diabetic mice.

Effect of the Withdrawal of LMW Chitosan Given from a Prediabetic Stage Figure 7 shows changes in non-fasting serum glucose levels following the cessation of LMW chitosan administration in diabetic mice.

Daily drinking administration of 0.2% LMW chitosan for 18 weeks, from 2 weeks after STZ treatment, markedly prevented the progressive increase in serum glucose levels, and significantly lower serum glucose levels were still maintained, even at 5 weeks following the cessation of LMW chitosan administration. In addition, the suppressive action of LMW chitosan on overeating, overdrinking and polyuria in diabetic mice was also maintained throughout the observation period after the withdrawal of LMW chitosan (data not shown).

Effects of LMW Chitosan Given from a Diabetic Stage Figure 8 shows the effect of LMW chitosan, administered from a diabetic stage, on non-fasting serum glucose levels in

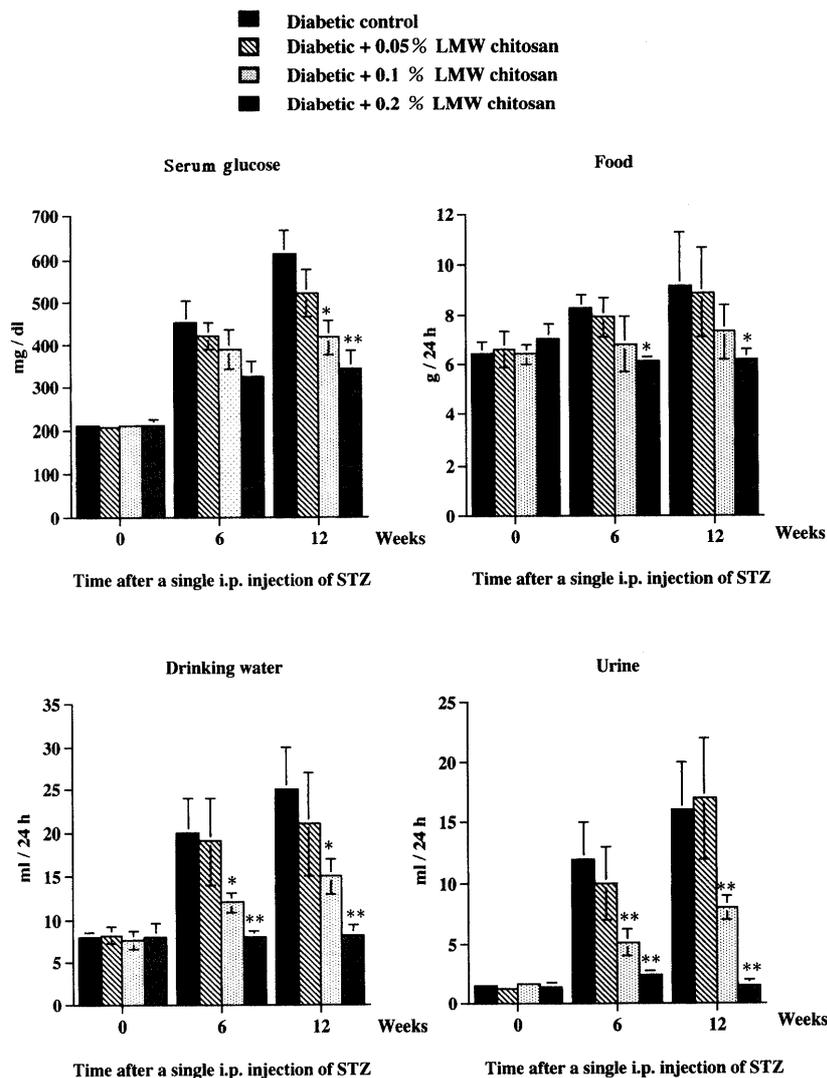


Fig. 3. Effect of 0.05%, 0.1% or 0.2% LMW Chitosan Given from a Prediabetic Stage on Non-Fasting Serum Glucose Levels and on Food and Drinking Water Consumption and Urine Volume per 24 h in Low Dose STZ-Induced Slowly Progressive Diabetic Mice. LMW chitosan was given as drinking water for 10 weeks, from 2 weeks after a single i.p. injection of 100 mg/kg STZ. Each plot denotes the mean±S.E. for 10 mice. Significantly different from the respective diabetic control, **p*<0.05, ***p*<0.01.

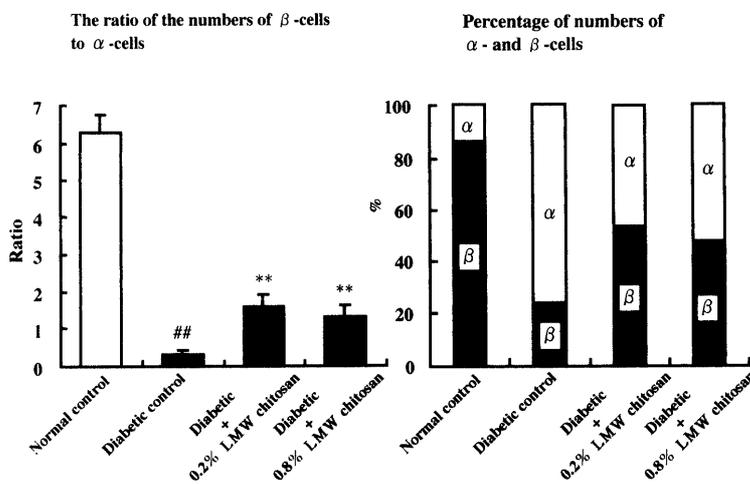


Fig. 4. Effect of 0.2% or 0.8% LMW Chitosan Given from a Prediabetic Stage on the Relative Numbers of Glucagon (α -Cells)- and Insulin-Immunoreactive Cells (β -Cells) in Normal and Low Dose STZ-Induced Slowly Progressive Diabetic Mice. LMW chitosan was given as drinking water for 21 weeks, from 2 weeks after a single i.p. injection of 100 mg/kg STZ. At the beginning of 24 weeks, pancreata were taken from these animals for glucagon and insulin immunostaining. Each column denotes the mean±S.E. for 8 to 10 mice. Significantly different from the normal control, ##*p*<0.01. Significantly different from the diabetic control, ***p*<0.01.

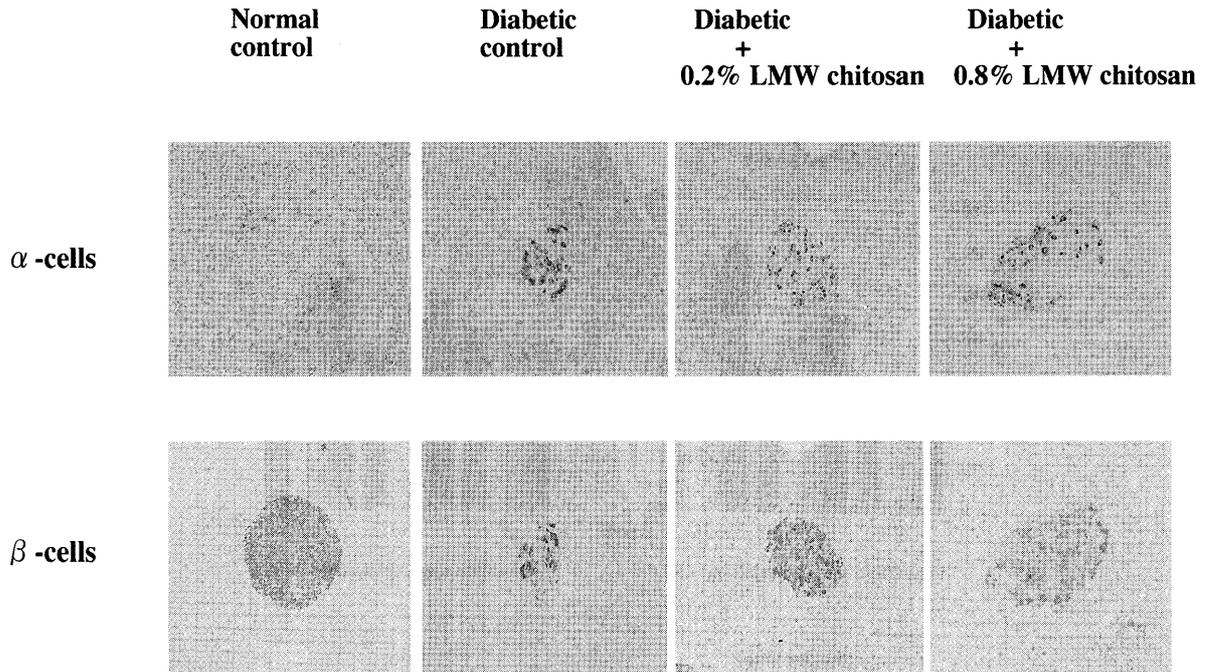


Fig. 5. Light Micrographs of Glucagon (α -Cells)- and Insulin-Immunoreactive Cells (β -Cells) in the Islets of Pancreata from Normal and Diabetic Control Mice and Diabetic Mice Given a Water Solution of 0.2 or 0.8% LMW Chitosan at 24 Weeks after STZ Treatment ($\times 100$)

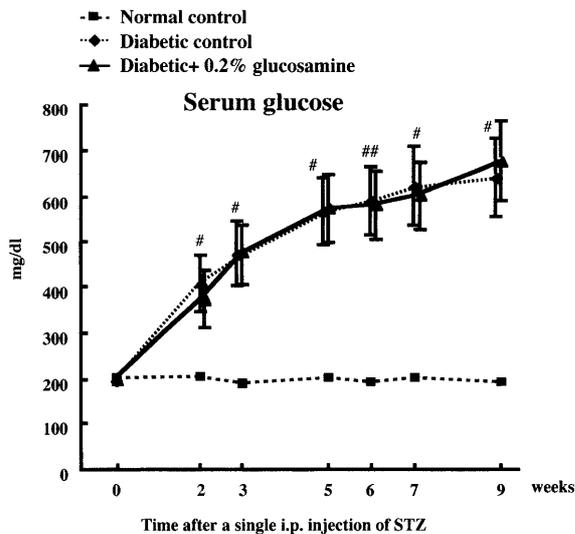


Fig. 6. Effect of 0.2% Glucosamine from a Prediabetic Stage on Non-Fasting Serum Glucose Levels in Low Dose STZ-Induced Slowly Progressive Diabetic Mice

Glucosamine was given as drinking water for 7 weeks from 2 weeks after a single i.p. injection of 100 mg/kg STZ. Each plot denotes the mean \pm S.E. for 10 mice. Significantly different from the respective normal control, # $p < 0.05$, ## $p < 0.01$.

progressive diabetic mice. The daily drinking administration of 0.2% LMW chitosan for 7 weeks, from 6 weeks after STZ treatment, slightly lowered non-fasting serum glucose levels from 1 week of LMW chitosan treatment. However, no significant difference in the glucose levels between chitosan-given mice and control diabetic mice was recognized throughout the observation period.

DISCUSSION

The present study indicates that long-term administration

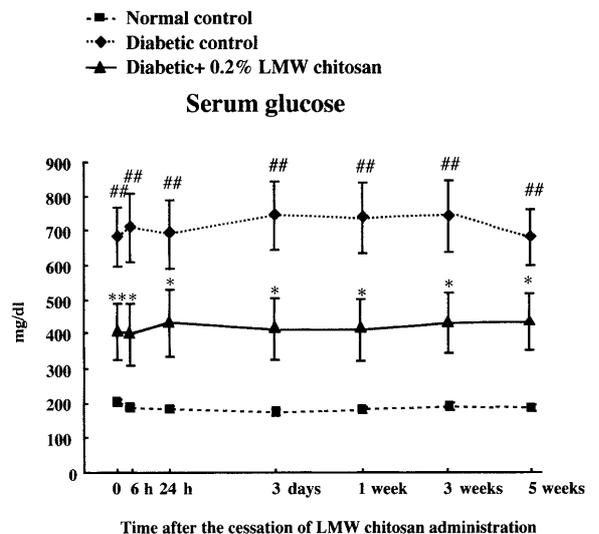


Fig. 7. Effect of the Withdrawal of 0.2% LMW Chitosan, Given from a Prediabetic Stage, on Non-Fasting Serum Glucose Levels in Low Dose STZ-Induced Slowly Progressive Diabetic Mice

LMW chitosan was given as drinking water for 18 weeks, from 2 weeks after a single i.p. injection of 100 mg/kg STZ, and the administration of LMW chitosan was then stopped for 5 weeks. Each plot denotes the mean \pm S.E. for 8 to 10 mice. Significantly different from the respective normal control, ## $p < 0.01$. Significantly different from the respective diabetic control, * $p < 0.05$, ** $p < 0.01$.

of LMW chitosan prevents the progression of slowly progressive NIDDM in mice induced by a subdiabetogenic low dose of STZ.

STZ (*N*-nitroso derivative of glucosamine) is a broad-spectrum antibiotic extracted from *Streptomyces acromogenes*.¹⁶ STZ is a pancreatic β -cell toxin that induces rapid and irreversible necrosis of β -cells,¹⁷ and has been widely used for creating the experimental animal models of insulin-dependent diabetes mellitus (IDDM).¹⁸⁻²¹ STZ has also been used for making models of NIDDM with hypoinsulinemia by

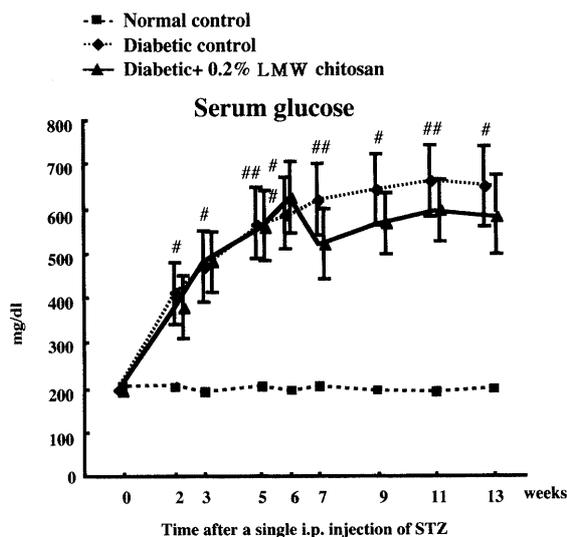


Fig. 8. Effect of 0.2% LMW Chitosan Given from a Diabetic Stage on Non-fasting Serum Glucose Levels in Low Dose STZ-Induced Slowly Progressive Diabetic Mice

LMW chitosan was given as drinking water for 7 weeks, from 6 weeks after a single i.p. injection of 100 mg/kg STZ. Each plot denotes the mean \pm S.E. for 10 mice. Significantly different from the respective normal control, # $p < 0.05$, ## $p < 0.01$.

neonatal STZ administration.^{22,23} Recently, we have reported that a single i.p. injection of a subdiabetogenic dose (100 mg/kg) of STZ to 8-week-old male ICR mice is able to produce slowly progressive NIDDM.¹⁵ In our further study concerning this diabetic mouse model, the area of pancreatic islets and the number of insulin-immunoreactive cells (β -cells) continued to decrease gradually in inverse proportion to the increase in serum glucose as the day went on throughout a 24-week observation period after STZ treatment (Ito *et al.*, unpublished data). NIDDM in humans worsens slowly and progressively and converts into IDDM after long-term chronic hyperglycemia. Therefore, this new diabetic mouse model may be useful for clarifying the mechanism of the progression of NIDDM in human or to assess agents to block the progression of NIDDM. In the present study, we used this slowly progressive diabetic model to examine the antidiabetic action of LMW chitosan. In this experiment, 0.2 or 0.8% LMW chitosan given as drinking water from the prediabetic stage of 2 weeks after STZ treatment markedly prevented the time course-related increase in non-fasting serum glucose levels of low dose STZ-induced slowly progressive diabetic mice. In addition, polyurea, overdrinking and overeating in diabetic mice observed at 12, 18 and 22 weeks after STZ treatment were markedly prevented by the drinking administration of 0.2 or 0.8% LMW chitosan. However, these effects of daily administration of LMW chitosan at both concentrations were not dose-dependent. Therefore, we furthermore examined the effect of LMW chitosan at concentrations less than 0.2% as an additional experiment. As a result, LMW chitosan at concentrations of 0.05, 0.1 or 0.2% given as drinking water prevented the progression of the increase in the serum glucose levels in a dose-dependent manner. Moreover, polyurea, overdrinking and overeating observed in diabetic mice were suppressed as the concentrations of LMW chitosan increased. Therefore, the preventive actions of LMW chitosan on polyuria, overdrinking and overeating may be a secondary action of this compound *via*

the prevention of the progression of diabetes.

Long-term administration of 0.8% LMW chitosan to normal mice did not affect non-fasting serum glucose levels, food or drinking water consumption or urine volume. Miura *et al.*¹⁴ have reported that chitosan given as a 5% food mixture for 4 weeks is significantly effective in lowering blood glucose levels in normal and neonatal STZ-induced diabetic mice. Our results, obtained with the administration of LMW chitosan to normal mice, are not in agreement with their results. They did not describe the degree of deacetylation and molecular weight of chitosan, or the food consumption of normal mice in their report. Therefore, the reason for the discrepancy is not clear at this time.

In the present experiment, the drinking administration of 0.2% LMW chitosan solution from a diabetic stage of 6 weeks after STZ treatment showed no apparent effect on non-fasting serum glucose levels. These results strongly suggest that LMW chitosan may be a preventive agent on slowly progressive NIDDM but not a curative agent.

In the present experiment, we gave LMW chitosan as drinking water. The consumption of 0.2 or 0.8% LMW chitosan/diabetic mouse/24 h varied from 6 to 13 ml throughout the experimental period until 23 weeks. If a mouse weighing 40 g drinks 10 ml of LMW chitosan solution per 24 h, the dosages of this compound are 500 mg/kg/d in 0.2% solution and 2 g/kg/d in 0.8% solution.

As described in the introduction, it is believed that chitosan may be primarily absorbed after it has been transformed into oligosaccharides and a monosaccharide by chitosanase secreted from intestinal bacteria or by lysozymes in intestinal fluid. In our experiment, the drinking administration of 0.2% glucosamine, a monosaccharide of chitosan, failed to prevent the progression of hyperglycemia in diabetic mice, although it was given from a prediabetic stage. This result suggests that LMW chitosan may exert a beneficial effect with the oligosaccharides, but not with the monosaccharide.

Daily drinking administration of 0.2% LMW chitosan from a prediabetic stage prevented the progression of an increase in serum glucose levels. It is of interest that significantly lower serum glucose levels were still maintained even at 5 weeks following the cessation of LMW chitosan administration.

As mentioned above, the area of pancreatic islets and the number of insulin-immunoreactive cells (β -cells) in a slowly progressive diabetic model of mice, used in this experiment, continued to decrease gradually as the day went on throughout the 24-week observation period after STZ treatment. Interestingly, however, in this model, non-fasting serum insulin levels were maintained at normal levels for this observation period, in spite of a time course-related reduction in the number of β -cells (Ito *et al.*, unpublished data). These results suggest that the remaining surviving β -cells which escaped attack by STZ in this diabetic model may cause the oversecretion of insulin to maintain normoglycemia. Therefore, we assumed that the time course-related decrease in the number of β -cells may be attributed to the death due to oversecretion of the remaining β -cells. In the present study, the relative decrease in the number of β -cells was markedly prevented by the administration of LMW chitosan from a prediabetic stage. Kobayashi *et al.*²⁴ have reported that insulin

therapy from an early stage in islet cell antibody-positive patients with apparent NIDDM prevents the progression of the disease by improving the insulin response to glucose. They postulated from their results that exogenously administered insulin may improve the insulin response of the diabetic patient by providing relief for exhausted β -cells or by stimulating β -cell proliferation. In our experiment, the mechanism by which LMW chitosan prevents the reduction of the number of β -cells in slowly progressive diabetes in mice remains unclear this time. LMW chitosan, like insulin, may stimulate β -cell proliferation. Further study is needed to clarify this.

In summary, the present study indicated that a long-term administration of LMW chitosan from a prediabetic stage prevents the progression of slowly progressive NIDDM in mice by preventing the decrease in β -cells in pancreatic islets. NIDDM is a typical disease relative to life style. Chitosan administration in combination with a therapy of diet and exercise training may be effective in preventing the induction and progression of slowly progressive NIDDM.

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