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Molecules and Cells



# Amelioration of Streptozotocin-Induced Diabetes by *Agrocybe chaxingu* Polysaccharide

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**The aim of this study was to investigate the preventive effect of *Agrocybe chaxingu* polysaccharide on streptozocin (STZ)-induced pancreatic β-cells destruction. *Agrocybe chaxingu* polysaccharide markedly reduced nitric oxide (NO) production and iNOS expression levels in RINm5F cells in a dose-dependent manner. In addition, *Agrocybe chaxingu* polysaccharide significantly inhibited iNOS expression and blood glucose levels in STZ-induced diabetic mice. Moreover, immunohistochemical analysis revealed that it enhanced pancreatic β-cells resistance to destruction by STZ. These results suggest that *Agrocybe chaxingu* polysaccharide may have value as a therapeutic agent against diabetes mellitus.**

## INTRODUCTION

Insulin-dependent diabetes mellitus (IDDM) results from the destruction of insulin-producing pancreatic β cells by number of causes, including viruses, chemical toxins, diet, and autoimmune responses (Atkinson and Eisenbarth, 2001; Bach, 1994; Jaeckel et al., 2002). However, the cellular mechanisms of β-cells destruction remain unclear, though it has been established that locally produced reactive oxygen species (ROS) and nitric oxide (NO) induced after cytokine stimulation are involved (Kubisch et al., 1997; Lakey et al., 2001; Rabinovitch et al., 1996; Sjoholm, 1988). NO is formed by inducible NO synthase (iNOS), which is involved in β-cell destruction. NO is a highly reactive radical, which inhibits the electron transport chain complexes and leads to decreased glucose oxidation rates, ATP generation and insulin production (Corbett and McDaniel, 1994; Corbett et al., 1992; Kwon et al., 2003).

Diabetes mellitus (DM) is a significant global heath concern characterized by hyperglycemia and associated with disturbances in carbohydrate, protein and fat metabolism leading to metabolic imbalances in tissues especially the pancreas (Alberti et al., 1998; Jin et al., 2008). It is well known that diabetic animal models exhibit high oxidative stress in pancreatic islets and oxidative stress plays a role in DM. Streptozotocin (STZ), an antibiotic produced by *Streptomyces achromogenes*, is a common used agent in experimental diabetes and the diabetogenic capacity of STZ may depend on its ability to damage β-cells and induce ROS (Coskun et al., 2005; Ohkuwa et al., 1995; Rakieten et al., 1963). A number of reports have explained increasing antioxidant enzyme expression in insulin-producing cells to enhance their resistance to the cytotoxic challenges caused by ROS, NO, and cytokines. Also, it is known that overexpression of antioxidant enzymes in the pancreas increased the resistance of insulin-producing cells against cytokine-mediated toxicity by inactivating ROS (Eum et al., 2004; Lortz et al., 2000; Tiedge et al., 1999).

Mushrooms are well known for their medically potent effects and have been widely used in China and other oriental countries for hundreds of years in treatments of various diseases including cancer. Different mushrooms are reported to have a number of biological activities including anti-tumor, anti-bacterial, and anti-viral activities (Eo et al., 2000; Wang et al., 1997; Yoon et al., 1994). In addition, they have been reported to have anti-inflammatory and protective effects in the livers of rats (Lin et al., 1993; 1995). It is well established from *in vitro* and *in vivo* studies that the polysaccharides fraction of mushrooms is largely responsible for their anti-tumor efficacy (Wang et al., 1997). Other studies have shown that the molecular mechanism underlying the anti-tumor and anti-inflammatory effects of some compounds derived from edible or traditional medicinal plants is to inhibit COX-2 and iNOS expression (Surh et al., 2001). In a previous study, we showed that polysaccharide from *Agrocybe chaxingu* inhibited LPS-induced iNOS and COX-2 expression levels in Raw 264.7 cells. Also, it prevented TPA-induced skin inflammation (Lee et al., 2009a).

In the present study, we examined polysaccharide from edible mushroom *Agrocybe chaxingu* for inhibitory effects against NO expression in oxidative stress-induced insulin-producing RINm5F cells and STZ-induced diabetic mice. Our results showed that the *Agrocybe chaxingu* polysaccharide efficiently protect against oxidative stress-induced β-cell damage *in vitro*

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and *in vivo*. These results indicate that *Agrocybe chaxingu* polysaccharide may have value as a therapeutic agent against diabetes mellitus.

## MATERIALS AND METHODS

### Materials

Fetal bovine serum (FBS), RPMI 1640, and penicillin-streptomycin antibiotics were purchased from Gibco BRL (USA). Primary antibodies against iNOS and actin were purchased from the Santa Cruz Biotechnology company (USA). RINm5F cells, an insulin-producing cell line, were purchased from the American Type Culture Collection (ATCC; USA). Sodium nitroprusside (SNP) was obtained from Sigma (USA). All other chemicals and reagents were the highest analytical grade available.

### Preparation of mushroom polysaccharide

The mushroom polysaccharide preparation as described previously (Angeli et al., 2006; 2009; Gonzaga et al., 2005; Yoshioka et al., 1985). Briefly, the polysaccharides were extracted from aqueous suspension of mushroom, heated, and acidification of the medium. After neutralization (0.1 mol/L NaOH), followed by the addition of 1% NaCl and precipitated in ethanol (1:5 v/v). The precipitated was separated by centrifugation in an ethanol-hydrogen peroxide solution (1:1 v/v) and second extraction with ethanol (1:4 v/v). The soluble fraction was lyophilized, dissolved in water and it was performed by FTIR and NMR spectroscopy. And then, the polysaccharide fraction was shaker and centrifugation. The precipitate was wash with NaCl/thymol (1:1) solution and dialysis in water. Then the precipitate polysaccharide structure was confirmed.

### Cell culture and MTT assay

The insulin-producing RINm5F cells were cultured in RPMI 1640 medium containing 2 mM of glutamine, 10% fetal bovine serum (FBS) and antibiotics (100 μg/ml streptomycin, 100 U/ml penicillin) at 37°C under humidified conditions of 95% air and 5% $CO_2$.

The biological activity of the *Agrocybe chaxingu* polysaccharide was assessed by measuring the cell viability of RINm5F cells treated with SNP (0.5 mM) for 12 h. The cells were then seeded into a 6-well plate at 70% confluence. The cells were first pre-treated with polysaccharides (5-50 μg) for 1 h, followed by the SNP. Cell viability was estimated with a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheyltetrazolium bromide (MTT) staining (Lee et al., 2007).

### Determination of NO production

RINm5F cells were incubated in 24-well plates at 70% confluence for 12 h. After incubation, the cells were pretreated with the polysaccharide (5-50 μg) for 1 h before treatment with SNP (0.5 mM) for 12 h, and the culture medium was harvested. NO production was determined by measuring nitrite which is the metabolite of NO oxidation, as described previously (Lee et al., 2009b; Misko et al., 1993). Briefly, 100 μl of cell culture medium was mixed with an equal volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride) incubated at room temperature for 10 min, and then the absorbance at 540 nm was measured with a microplate reader. $NaNO_2$ was used as the standard.

### Western blot analysis

Lysates from RINm5F cells were prepared by incubating cells in lysis buffer 4°C for 30 min. Protein concentration was determined using a Bio-Rad protein assay. Proteins (40 μg) were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and the proteins were electrophoretically transferred to a nitrocellulose membrane. The membrane was blocked in 5% nonfat milk in Tris-buffered saline (TBS; 20 mM Tris, 0.2 M NaCl, pH 7.5) containing 0.05% Tween-20 (TBST) for 2 h and was then incubated for 1 h at room temperature with anti-iNOS, and β-actin antibodies (Santa Cruz Biotechnology, USA; dilution 1:500) in TBST. After washing, the membrane was incubated for 1 h with a proper secondary antibody conjugated to horseradish peroxidase diluted 1:10,000 in TBST. The membrane was incubated with a chemiluminescent substrate and exposed to Hyperfilm ECL (Amersham Biosciences, USA).

### Determination of iNOS protein expression

RINm5F cells were incubated in 6-well plates at 70% confluence for 12 h. After incubation, the cells were pretreated with polysaccharide (10-50 μg) for 1 h before treatment with SNP (0.5 mM) for 12 h, and the culture medium was harvested. Then, the expression of iNOS protein levels were determined by Western blotting used anti- iNOS antibodies.

### RT-PCR analysis

Total RNA was isolated from RINm5F cells using a Trizol reagent kit (Invitrogen, USA) according to the manufacturer's instructions (Park et al., 2008; Zhang et al., 2005). The RNA (2 μg) was reversibly transcribed with 10,000 U of reverse transcriptase and 0.5 μg/μl oligo-(dT) primer. PCR amplification of cDAN aliquots was performed with the following sense and antisense primers: iNOS antisense, 5′-CTGTCAGAGCCTCGTGGCTTT-3′; sense, 5′-ATGGCTCGGGATGTGGCTAC-3′. beta-actin antisense, 5′-GGACAGTGAGGCCAGGATGG-3′; sense, 5′-AGTGTGACGTTGACATCCGTAAAGA-3′. PCR was performed in 50 μl of 10 mM Tris-HCl (pH 8.3), 25 mM $MgCl_2$, 10 mM dNTP, 100 U of *Taq* DNA polymerase, and 0.1 μM of each primer, The process was terminated by heating to 70°C for 15 min. PCR products were resolved on a 1% agarose gel and visualized with UV light.

### Animals and immunohistochemistry

Male 6-8 week old ICR mice were purchased from the Experimental Animal Center, at Hallym University, Chunchon, Korea. The animals were housed at a constant temperature (23°C) and relative humidity (60%) with a fixed 12-h light/12-h dark cycle and free access to food and water. The animals used in this experiment were treated according to the "Principles of Laboratory Animal Care" (NIH Publication No. 86-23).

We examined whether polysaccharide ameliorate the diabetic status in STZ-induced diabetic mice. Male ICR mice were divided into three groups of five animals. Mice in group 2 were given a single intraperitoneal (i.p.) injection of 120 mg/kg STZ freshly dissolved in 50 mM citrate buffer (pH 4.5) to induce diabetes, whereas mice in group 1 were injected with the equivalent volume of citrate buffer. Mice in group 3 were injected five times (beginning 2 days prior to induction of diabetes and 1, 3 days after STZ injection) with 10 mg/kg of polysaccharide. The mice were killed by cervical dislocation 1, 3, and 7 days after STZ injection, and the pancreas was dissected for subsequent assay and histological examinations. Blood glucose from the tail plexus was assayed using AccuChek active glucose strips and a refractance meter (Roche, Germany). To minimize the effects of diurnal fluctuations, blood samples were collected at the same time every day. For observation of insulin-positive β-cells, tissue sections were incubated with an anti-mouse insulin IgG (InnoGenex, USA; dilution 1:300) and stained with a peroxidase/DAB system kit (Dako EnVision Kit, Glostrup, Denmark).

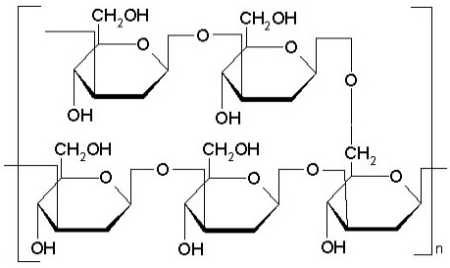

**Fig. 1.** The structure of a polysaccharide.

The number of insulin positive cells in the islet was counted manually under light microscopy (Carl Zeiss, Germany).

**Statistical analysis**

Statistical analysis was performed using the student's *t*-test and ANOVA. *P* values of < 0.05 were considered statistically significant.

## RESULTS AND DISCUSSION

For hundreds of years, mushrooms have been widely used as a tonic to promote health and longevity in oriental countries. There is a continuous demand for health-aids and natural drugs in the increasingly aged population. In a previous study, we showed that *Agrocybe chaxingu* polysaccharide markedly inhibited LPS-induced nitric oxide (NO) and cyclooxgenease-2 (COX-2) production in macrophage Raw 264.7 cells (Lee et al., 2009a). In this study, we examined the protective effects of the edible mushroom *Agrocybe chaxingu* β-glucan (polysaccharide) on NO production *in vitro* and *in vivo*. It is well known that a variety of oligosaccharides, including the β-glucans found in mushrooms, and it has various roles (Eo et al., 2000; Lin et al., 1993; 1995; Surh et al., 2001; Wang et al., 1997; Yoon et al., 1994). However, the effect of *Agrocybe chaxingu* polysaccharide (Fig. 1) on diabetes is unknown.

To determine whether polysaccharide have cytotoxic effects on RINm5F cells, the cells were treated with various (5-50 μg/ml) concentrations of polysaccharide and were incubated for 12 h. As shown in Fig. 2, the polysaccharide did not affect the cell viability at the various concentrations. Moreover, the polysaccharide alone did not affect the viability even at higher (0.1-1 mg/ml) concentrations (data not shown).

It is well known that NO plays a key role in the pathophysiology of many diseases. Also, sodium nitroprusside (SNP) is generating intracellular NO. To determine the effects of polysaccharide on NO production, RINm5F cells were incubated for 12 h with SNP (0.5 mM) in the presence or absence of various (5-50 μg/ml) concentrations of polysaccharide after which cell culture media were collected and nitrite levels determined. The polysaccharide reduced NO production in a dose-dependent manner (Fig. 3A). In order to determine the cytoprotectctive effect of polysaccharide on RINm5F cells, we examined the effect of polysaccharide on cell death induced by SNP. As shown in Fig. 3B, only 41% of cultured RINm5F cells remained viable after the cells had been exposed to 0.5 mM SNP for 12 h. However, cell viability increased significantly with pretreatment of polysaccharide in a dose-dependent manner reaching over 70% at the highest concentration of polysaccharide. Biological macromolecules are known to be major target of oxidative stress and NO. As shown in Fig. 4, DNA fragmentation in RINm5F cells was induced excessively by SNP treatment. Moreover, elevated levels of DNA fragmentation were signify-

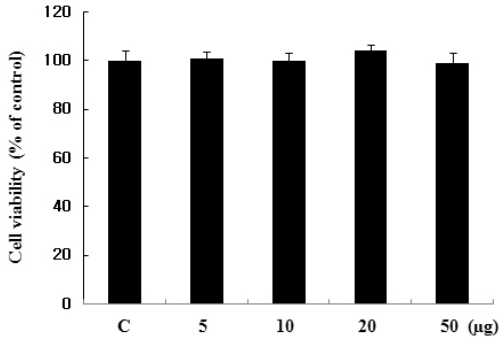


**Fig. 2.** Effect of polysaccharide on cell viability. RINm5F cells were incubated with polysaccharide (5-50 μg/ml) for 12 h and cell viabilities were estimated by a colorimetric assay using MTT.

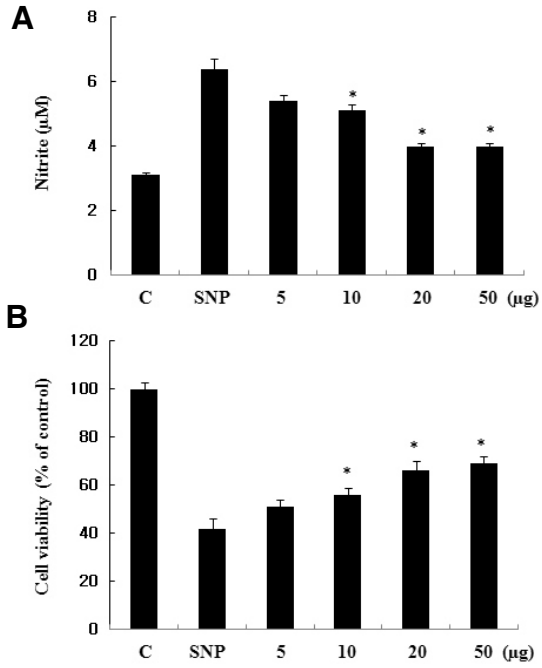


**Fig. 3.** Effects of polysaccharide on SNP-induced NO production (A) and cell viability (B). RINm5F cells were pretreated with the polysaccharide (5-50 μg/ml) for 1 h before incubation with SNP (0.5 mM) for 12 h. Nitrite levels were measured in the culture media of SNP-stimulated cells by the Griess reaction (A) and cell viabilities were estimated by a colorimetric assay using MTT (B). Each bar represents the mean ± SEM obtained from five experiments. Asterisks denote statistically significant difference from SNP treated group.

cantly decreased by polysaccharide. These results indicate that polysaccharide play a defensive role in cells under oxidative stress by removing NO.

We then examined the effects of polysaccharide on iNOS expression levels under SNP exposure. RINm5F cells were incubated for 12 h with SNP (0.5 mM) in the presence or absence of polysaccharide (10-50 μg/ml). Polysaccharide suppressed SNP-induced iNOS protein expression levels in a dose-dependent manner (Fig. 5A). We further examined the effects of polysaccharide on iNOS mRNA expression levels in SNP-induced cells by RT-PCR. As shown in Fig. 5B, polysaccharide inhibited SNP-induced mRNA expression levels of

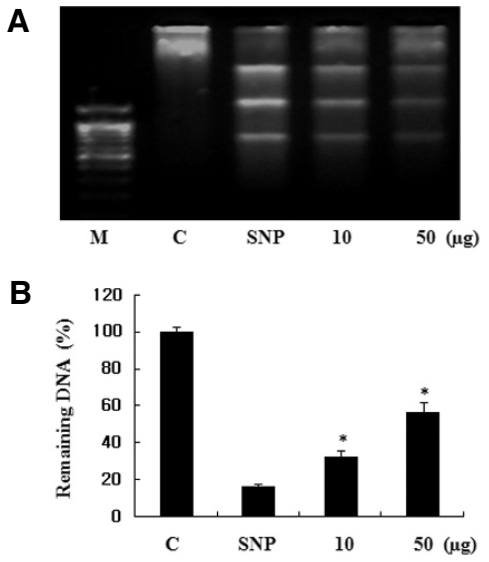


**Fig. 4.** Effects of polysaccharide on SNP-induced DNA fragmentation. RINm5F cells were pretreated with polysaccharide (10-50 μg/ml) for 1 h before incubation with SNP (0.5 mM) for 12 h. After DNA was extracted, DNA samples were separated by 1.5% agarose gel and stained with ethidium bromide. The level of remained DNA detected by agarose gel was quantified by densitometer. Asterisks denote statistically significant difference from SNP treated group.

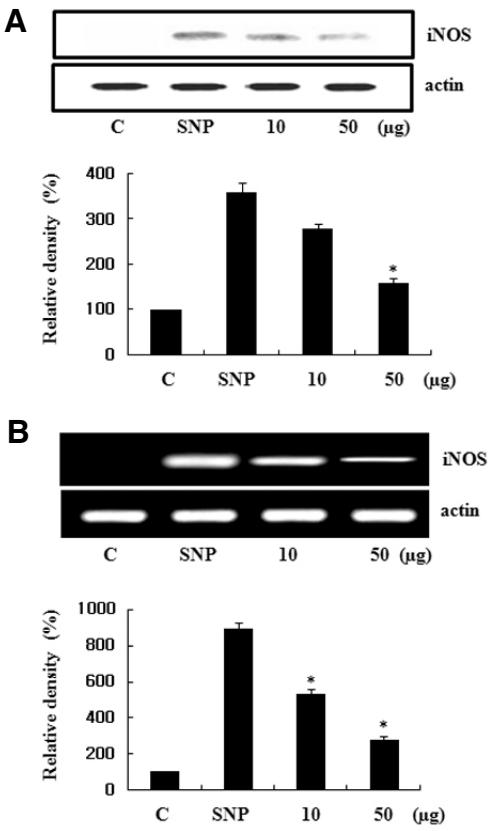


**Fig. 5.** Inhibitory effects of polysaccharide on SNP-induced iNOS protein (A) and mRNA (B) levels in RINm5F cells. The cells were pretreated with the polysaccharide for 1 h before incubation with SNP (0.5 mM) for 12 h. Cells lysates were prepared and analyzed for iNOS protein expression levels by Western blotting (A). Total RNA was extracted. iNOS mRNA was analyzed by RT-PCR using specific primers (B). The level of iNOS detected by Western blot and agarose gel was quantified by densitometer. Asterisks denote statistically significant difference from SNP treated group.

iNOS in a dose-dependent manner. These results suggest that the inhibition of iNOS mRNA expression by polysaccharide was responsible for the inhibition of NO production. Kwon et al. (2003) suggest that excessive NO production by overexpression of iNOS gene is seen in acute and chronic disease. Therefore, down regulation of iNOS gene expression may be an effective therapeutic strategy for preventing acute and chronic diseases including inflammatory reactions. We previously study show that the protective effect of polysaccharide against lipopolysaccaride (LPS)-induced NO and cyclooxygenase-2 (COX-2) expression in murine macrophage Raw 264.7 cells as well as 12-O-tetradecanoylphorbol 13-acetate (TPA)-induced ear edema in mice (Lee et al., 2009a). Other studies suggest that the inhibition of iNOS expression is important for alleviating diabetes mellitus (DM) progression (Coskun et al., 2005; Jin et al., 2008; Kwon et al., 2003). Thus, the inhibition of iNOS expressions may constitute an effective new therapeutic strategy for the treatment of DM.

Steptozotocin (STZ) has a β-cell cytotoxic and it is one of the most commonly used substances to induce diabetes in experimental animals (Kim et al., 2008; Szkudelski, 2001). Baynes and Thorpe et al. (1996) suggest that in STZ-induced type I diabetes mellitus (IDDM), hyperglycemia and oxidative stress have been implicated in the etiology and pathology of disease complications. Although the precise mechanism of pancreatic β-cell destruction has not been fully characterized, ROS and NO are regarded as major mediators of the cytotoxic actions of cytokines in the pathogenesis of DM (Kubisch et al., 1997; Lakey et al., 2001; Rabinovitch et al., 1996; Sjoholm, 1988).

As shown in Fig. 6, the blood glucose levels of control mice were about 120 mg/dl through the experimental period. In the diabetes-induced group, a single injection of 120 mg/kg STZ markedly increased blood glucose levels from day 1, reaching a maximum level of about 510 mg/dl at 7 days after the initial STZ injection. These high glucose levels were maintained throughout the experimental period. However, these increased glucose levels were considerably lowered by multiple injection of polysaccharide. The blood glucose levels of the polysaccharide-treated mice decreased significantly to 53-70% of those of the STZ-injected mice. In addition, morphological studies revealed that polysaccharide efficiently protected pancreatic β-cells in STZ-induced diabetic mice. As shown in Fig. 7, a single injection of 120 mg/kg STZ induced severe pancreatic β-cells destruction. As a result of cells, the masses of Langerhans' islets were markedly reduced time dependently and their overall shape becomes non-spherical and irregular in appearance. This cellular destruction was prevented by five times injections of polysaccharide (10 mg/kg). We also counted insulin-positive cells in the relatively large islets (n = 12) near the periductal and perivascular regions to estimate the effects of the polysaccharides after 7 days. In normal control mice group, the numbers of insulin-positive cells were 67.3 ± 7.4 cells/islet. The numbers of insulin-positive cells in STZ-injected groups were markedly decrease 21.3 ± 3.7 cells/islet. However, when diabetic animals were injected with polysaccharide, the number of insulin-positive cells increased significantly from 37.3 ± 5.8 cells/islet. These observations demonstrated that polysaccharide efficiently protected pancreatic β-cells.

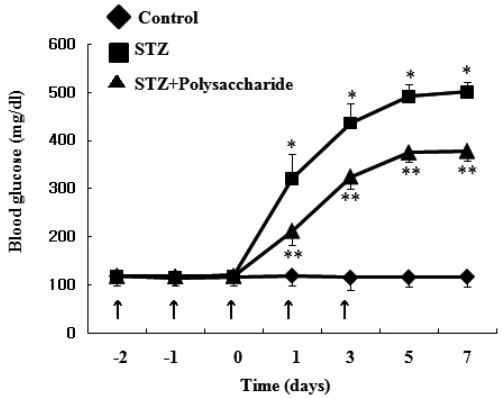


**Fig. 6.** Effects of polysaccharide on blood glucose levels in streptozotocin (STZ)-induced diabetic mice. Diabetes was induced by a single i.p. injection of 120 mg/kg STZ in mice. STZ-induced diabetic mice were injected five times (beginning 2 days prior to induction of diabetes and 1, 3 days after STZ injection) with 10 mg/kg of polysaccharide. Each bar represents the mean ± SEM obtained from four experiments. *P < 0.01 versus untreated control group. **P < 0.01 versus the relevant STZ-induced diabetic group.

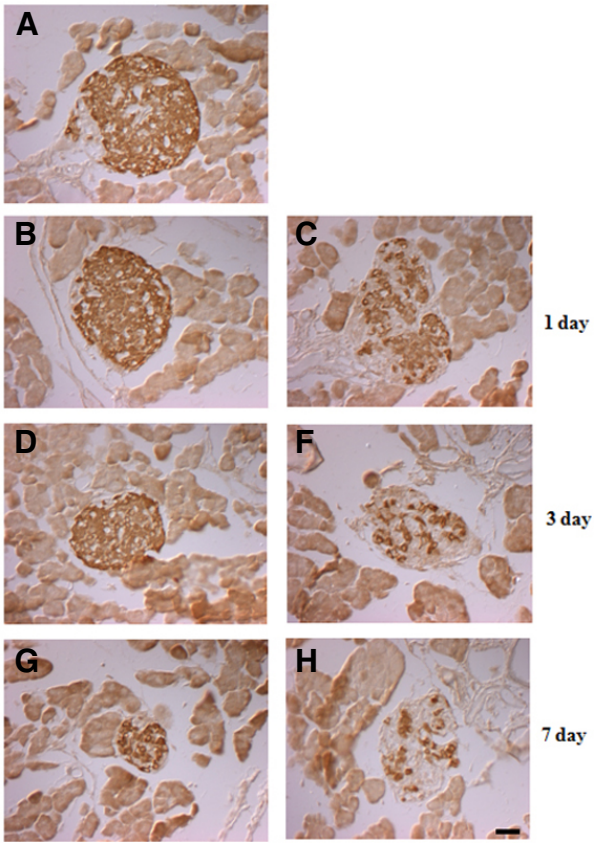


**Fig. 7.** Light micrographs of mouse pancreas showing insulin-positive cells of (A) untreated control mice, (B, D, G) polysaccharide-injected diabetic mice, and (C, F, H) STZ-induced diabetic mice. Tissue sections were incubated with an anti-mouse insulin antibody, stained with a peroxidase/DAB system, and observed under light microscopy (original magnification ×200). Bar = 50 μm. These are representatives of insulin immunehistochemistry obtained from animals for each group.

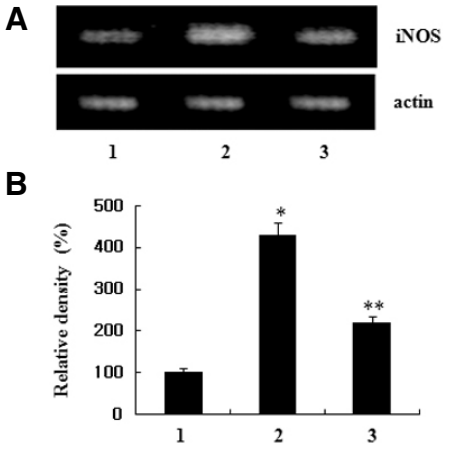


**Fig. 8.** Inhibitory effect of polysaccharide on iNOS mRNA expression levels in STZ-induced diabetic mice pancreas. Diabetes was induced by a single i.p. injection of 120 mg/kg STZ in mice. STZ-induced diabetic mice were injected five times (beginning 2 days prior to induction of diabetes and 1, 3 days after STZ injection) with 10 mg/kg of polysaccharide. Total RNA was extracted. iNOS mRNA was analyzed by RT-PCR using specific primers (B). Lanes in A and B are as follows: lane 1, Normal control mice; lane 2, STZ-induced diabetic mice; lane 3, polysaccharide treated mice. Each bar represents the mean ± SEM obtained from four experiments. *P < 0.01 versus untreated control group. **P < 0.01 versus the relevant STZ-induced diabetic group.

Under the same conditions, we examined whether polysaccharide inhibit iNOS mRNA expression levels in pancreas tissue. In STZ-injected mice group, the levels of iNOS mRNA was about 4.3-fold higher than those in control mice. However, STZ induced iNOS mRNA expression levels were significantly decreased by treatment with polysaccharide (Fig. 8). These results suggest that iNOS mRNA expression levels were induced by STZ injection, and that polysaccharide treatment inhibits this induction. In addition, polysaccharide efficiently protected pancreatic β-cells and thereby ameliorates the diabetic status of STZ induced diabetic mice. Kwon et al. (2006) demonstrate that *Cortex cinnamomi* extract inhibits SZT induced NO production and iNOS mRNA expression *in vitro* and *in vivo* and suggest that *Cortex cinnamomi* extract be used to prevent the progress of DM.

In summary, we demonstrated for the first time that polysaccharides can efficiently inhibit SNP-induced NO expression levels and cell death in RINm5F cells. Also, we have shown that polysaccharide inhibit STZ-induced β-cells destruction in experimental diabetic mice. Although the detailed mechanism remains to be further elucidated, polysaccharide may have value as a therapeutic agent against diabetes mellitus progression.

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