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The sphingosine-1-phosphate receptor modulator, FTY720, prevents the incidence of diabetes in Spontaneously Diabetic Torii rats

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Short title

Effects of FTY720 in type 2 diabetic rats

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Abstract

The sphingosin-1-phosphate (S1P) receptor modulator regulates lymphocyte trafficking, resulting in its depletion from circulation which ultimately cause immunosuppression. In this study, we investigated the preventive effect of fingolimod (FTY720) in the non-obese type 2 diabetic model, Spontaneously Diabetic Torii (SDT) rats. The S1P receptor modulator, FTY720 (0.3 mg/kg p.o.), was administered for 12 weeks to SDT rats from 5 to 17 weeks of age. Based on our findings, FTY720 could suppress the incidence of diabetes in SDT rats. Further, glucose intolerance was improved in FTY720 treated-SDT rats at 14 weeks of age. Based on the haematological and histological analyses performed at 17 to 18 weeks of age, a decrease in lymphocytes and monocytes in the peripheral blood and a decrease in lymphocyte and atrophy in spleen occurred in the FTY720-treated SDT rats. Furthermore, the pancreatic changes, such as inflammation, atrophy, and fibrosis in islets observed in SDT rats were improved by FTY720 treatment. These findings suggest that the immunomodulatory effects of FTY720 reduced the pancreatic lesion in SDT rats, thereby demonstrating its preventive effect against diabetes. The development of diabetes in SDT rats is related to disorders of the immune system. However, the S1P receptor modulator may be useful for treating type 2 diabetes.

Key words: diabetes, immunomodulation, SDT rat, sphingosin-1-phosphate receptor

modulator

1. Introduction

Type 2 diabetes is a polygenic disorder characterized by insulin deficiency and insulin resistance. Owing to excessive calorie intake and sedentary lifestyles, the number of patients with this disorder has increased worldwide.[1,2] The reduction of functional pancreatic β-cell mass is an important factor in the incidence and progression of diabetes.[3] Although an initial compensatory increase in β-cell mass is induced by insulin resistance, diabetes occurs when the functional β-cell mass fails to sufficiently increase.[4,5] Therefore, preserving or increasing β-cell mass is a pivotal step in the treatment of type 2 diabetes.[6,7]

Fingolimod (FTY720) is an immunomodulatory drug approved for the treatment of multiple sclerosis. FTY720 acts via the sphingosine-1-phosphate (S1P) receptors, exhibiting potential for the treatment of several autoimmune diseases, such as multiple sclerosis, type 1 diabetes, and systemic lupus erythematosus via animal models.[8-10] FTY720 is activated following phosphorylation by sphingosine kinase 2 to form the phosphate, which binds to the S1P receptors (S1P1, S1P3, S1P4, and S1P5) and inhibits the release of lymphocytes from lymphoid tissues.[11,12] FTY720 is reported to reduce hyperglycaemia by enhancing β-cell regeneration via the phosphoinoside-3-kinase (PI3K)-dependent regulation of cyclin D3 and p57^{KIP2} in *db/db* mice.[13]

The Spontaneously Diabetic Torii (SDT) rat is a non-obese type 2 diabetes model that displays hypoinsulinemia followed by severe hyperglycaemia after ~15 weeks of age and diabetic complications at ~40 weeks of age.[14,15] SDT rats also exhibit glucose intolerance

prior to the onset of diabetes,[16,17] with pancreatic β-cell injury observed in the pre-diabetes stage.[18]

FTY720 is reported to exhibit pancreatic protective effects in type 1 diabetic animal models, including non-obese diabetic (NOD) mice and LEW.1AR1-iddm rat; however, only few reports have been published on its use in type 2 diabetic animal models. In this study, we sought to determine whether FTY720, an S1P modulator, exhibits preventive effects against type 2 diabetes in SDT rats. Such finding would promote the consideration of the diabetic aetiology in SDT rats and the establishment of a new treatment for type 2 diabetes.

2.   Results

The chronic effects of FTY720 on the biological parameters are shown in Figure 1. Body weights of SDT rats at 13 to 17 weeks of age were lower than those of Sprague-Dawley (SD) rats. However, at 17 weeks of age, the body weight of FTY720-treated SDT rats was significantly increased relative to that of the control SDT rats (Figure 1A). The non-fasted serum glucose levels in SDT rats increased after 13 weeks of age relative to those in SD rats, with a remarkable elevation observed at 17 weeks of age. Further, hyperglycaemia was significantly reduced by FTY720 treatment (at 17 weeks of age, control SDT rats: 662.2 ± 66.8 mg/dL, FTY720-treated SDT rats: 207.7 ± 186.1 mg/dL, SD rats: 138.6 ± 12.6 mg/dL) (Figure 1B). Non-fasted serum insulin level in SDT rats

was significantly decreased at 17 weeks of age relative to that in SD rats. However, the reduction in insulin level was inhibited in FTY720-treated SDT rats (Figure 1C). After 13 weeks of age, non-fasted serum triglyceride (TG) levels in the FTY720 group showed a tendency to decrease relative to those in the control group; however, there was no significant change (Figure 1D). The non-fasted serum total cholesterol (TC) levels in each group were comparable during the experimental period (data not shown). However, non-fasted serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels increased after 9 weeks of age in SDT rats compared to SD rats, with a remarkable elevation observed at 17 weeks of age. These increases were found to be inhibited by FTY720 treatment at 17 weeks of age (Figures 1E and 1F). Leukocyte levels in control SDT rats were significantly higher than those in SD rats at 17 weeks of age. However, FTY720 treatment suppressed this increase in SDT rats (Table 1). Based on the differential counts of leukocytes, the neutrophil, lymphocyte, monocyte, and basophil counts were significantly higher in SDT rats; however, the lymphocyte and monocyte counts in the FTY720 treatment group were significantly decreased.

The cumulative incidence of diabetes (>250 mg/dL in non-fasted serum glucose level) in each group is shown in Figure 2. The onset of diabetes in control SDT rats occurred at 13 weeks of age, and its incidence was 33.3%. All SDT rats developed diabetes at 17 weeks of age, with the cumulative incidence reaching 100%. FTY720 treatment suppressed the incidence of diabetes. Further, the cumulative incidence at 13 and

17 weeks of age was 0 and 16.7%, respectively.

The results of oral glucose tolerance test (OGTT) for rats at 14 weeks of age are shown in Figure 3. SDT rats in the control group showed glucose intolerance; however, at 60 minutes after glucose loading, glucose level was significantly decreased in the FTY720 group (Figure 3A). The insulinogenic index in the control group was lower than that in the SD group. Conversely, the FTY720-treated group had a higher level than the control group; however, no significant changes were identified (Control SDT rats: 0.0132 ± 0.0096, FTY720-treated SDT rats: 0.0180 ± 0.0055, SD rats: 0.218 ± 0.0171) (Figure 3C).

The changes in the absolute and relative weights of the pancreas and spleen are shown in Table 2. The absolute weight of the pancreas in the control group was significantly reduced relative to that in the SD group; however, its weight in the FTY720 group was higher than that in the control group. The relative weight of the spleen in the control group was significantly increased relative to that in the SD group. Additionally, the absolute and relative weight of the spleen in the FTY720 group was significantly decreased relative to that in the control group.

The histopathological changes in the pancreas and spleen are shown in Figure 4 and Table 3. Pancreatic abnormalities, such as inflammation, fibrosis, and atrophy, were observed in control SDT rats; however, treatment with FTY720 could improve these abnormalities. There were no overt abnormalities in the spleen of control SDT rats.

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Nonetheless, a decrease in lymphocyte and atrophy in white pulp, and extramedullary haematopoiesis were observed in the spleen of FTY720-treated SDT rats.

3. Discussion

FTY720, an immunomodulatory drug, is a synthetic sphingosine analogue of myriocin, a metabolite of *Isaria sinclarii*,[19,20] that exhibits great potential in different animal models, such as transplantation, immunological disease, and diabetes and its complications.[10,21,22] Many reports using NOD mice, an autoimmune type 1 diabetic model, have been previously published. In a study using NOD mice, FTY720 inhibited the progression of β-cell destruction and exhibited pancreatic protection.[10] The SDT rat is a type 2 diabetic model; however, the pancreatic lesions, including β-cell destruction and mass reduction, the mechanisms of which are unknown, may be related to the incidence and progression of diabetes.[17,18]

SDT rats has achieved the diabetic conditions at 17 weeks of age in this study. Original report of SDT rats introduces that this animal model develops diabetes at around 20-25 weeks of age.[23] However, as generation progress, the onset of diabetes in SDT rats is getting earlier without any other differences in its final phenotype. Recent report shows that SDT rats develop diabetes between 15 and 20 weeks of age.[24] Therefore, we consider that the early onset of diabetes in SDT rats in this study does not affect the result. We tested the OGTT at 14 weeks of age because our previous study showed that SDT rats

develop insulin resistance at 14-16 weeks of age but not at 8 weeks of age.[16,17] We considered that SDT rats develop insulin resistance around 14 weeks of age. Also, we assumed that the evaluation of pharmacological effect of FTY720 on insulin resistance in this animal model becomes difficult at age after 17-weeks because the diabetes progresses rapidly.

In this study, FTY720 suppressed the development of diabetes and exhibited pancreatic protection in SDT rats. We have not monitored food intake and water consumption in this study. However, according to the report by Peters, 0.3 mg/kg/day of FTY720 does not affect food intake and water consumption on rat for 20 weeks.[25] Therefore, we consider that the effects of FTY720 on SDT rat was not due to food or water intake. Based on the haematological examination, the number of circulating lymphocytes, monocytes, and neutrophils were significantly increased in SDT rats compared to SD rats. However, FTY720 suppressed the number of lymphocytes and monocytes. Such finding aligns with the suppression of experimental autoimmune encephalomyelitis in mice and rats administered FTY720, which correlated with the reduced number of lymphocytes and monocytes, but not neutrophils in the peripheral blood.[26] In SDT rats, the number of lymphocytes was increased at 6 to 8 weeks of age, which is the early pre-diabetes stage. Thereafter, monocyte count increased at 12 weeks of age.[18] Based on the histopathological analyses, we observed atrophy, a decrease in lymphocyte, and extramedullary haematopoiesis in the spleen, with improvements in the pancreatic

abnormalities in the FTY720 group. Additionally, FTY720 was found to inhibit lymphocyte infiltration into the pancreas. FTY720 inhibits S1P signalling by inducing the internalization and degradation of the receptors, thereby suppressing the immune response by sequestering the circulating mature lymphocytes from blood and peripheral tissues to secondary lymphoid tissues and thymus.[11,27,28] The depletion of circulating lymphocyte with FTY720 treatment in SDT rats is considered to induce pancreatic protection, resulting in the suppression of diabetes incidence. The increase in serum insulin level in the FTY720 group might be caused by the pancreatic protective effects. As the serum ALT and AST levels in the FTY720 group were suppressed, this might be due to an improvement in liver dysfunction with the suppression of diabetes development and the regulation of the immune system by FTY720 treatment.

FTY720 is reported to exhibit pancreatic protective effects in several type 1 diabetic animal models, such as NOD mice and LEW.1AR1-iddm rats. Lymphocytic infiltration is observed to surround the islets in NOD mice, and this infiltration continues to amass and invade the islet, ultimately destroying this tissue. The chronic administration of FTY720 prevented the development of diabetes by blocking islet invasion.[10,29] Additionally, in the LEW.1AR1-iddm rat, FTY720 treatment prevented the incidence of diabetes by promoting the retention of activated immune cells in the lymph nodes.[30]

Only few studies have investigated the pharmacological effects of FTY720 using mice with type 2 diabetes. In diet-induced obese (DIO) mice, FTY720 treatment prevented

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weight gain, improved insulin sensitivity, and reduced lymphocytes and macrophages in the adipose tissue.[22] In FTY720-treated *db/db* mice, the pancreatic β-cell mass increases with cyclin D3 and p57^{KIP2} regulation.[13] Although FTY720 treatment may control β-cell regeneration via the S1P signalling pathway in SDT rats, no reports have analysed the lymphocyte dynamics induced by FTY720 in *db/db* mice. Nonetheless, FTY720 may exhibit pancreatic protection by depleting the levels of circulating lymphocytes in *db/db* mice. Moreover, pancreatic abnormalities in SDT rats are reported to be related to failure of the immune and/or inflammatory system, including enhanced vascular endothelial growth factor signalling.[18] The pancreatic protective effects of FTY720 in SDT rats might be caused by an increase in the functional β-cell mass and inhibition of β-cell destruction. T lymphocytes are also crucial for the development of metabolic inflammation and insulin resistance and the immunomodulatory system is associated with the progression of type 2 diabetes.[31] Blood lymphocytes controlled via treatment with the S1P modulator is a new strategy for diabetic treatment.

In conclusion, the S1P modulator, FTY720, might exhibit pancreatic protection in SDT rats by depleting the circulating lymphocytes, ultimately suppressing the incidence of diabetes. The development of diabetes in SDT rats is related to a disorder of the immune system. However, an S1P receptor modulator may be useful for the treatment of type 2 diabetes.

4. Materials and methods

4.1 Animals and chemicals

Male SDT rats and SD rats (CLEA Japan, Tokyo, Japan) were employed in the present study, with SD rats employed as the normal rats. All animal procedures and the protocol complied with the guidelines for animal experimentation set by the Ethics Committee for Animal Use at JT and Niigata University. Rats were maintained at 23 ± 3 °C on a 12 h/12 h light-dark cycle with *ad libitum* access to a standard diet (CRF-1; Oriental Yeast, Tokyo, Japan) and water. FTY720 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was suspended in 0.5% methyl cellulose and orally administered (0.3 mg/kg) for 12 weeks to animals in the FTY720-treated group once daily from 5 to 17 weeks of age. 0.5% methyl cellulose was administered to animals in the control group and SD rats to serve as the vehicle. Oral administration of FTY720 (0.3 mg/kg) shows sufficient immunosuppressive effects on rats, and therefore commonly used for various disease models of rats.[11,21,25,32] The initial average weights of control SDT rats, FTY720-treated SDT rats and SD rats were 210.2 ± 4.4 g, 205.9 ± 13.7 g and 178.2 ± 6.6 g, respectively.

4.2 Biochemical parameters

During the experimental period, body weight and biochemical parameters were monitored every 4 weeks. Blood samples were collected from the tail vein under non-fasting conditions. Plasma glucose, TG, TC, ALT, and AST levels were measured using

commercial kits (Roche Diagnostics, Basel, Switzerland) and an automatic analyser (Hitachi 7180; Hitachi High-Technologies, Tokyo, Japan). Commercial ELISA kits were used to measure serum insulin (Rat insulin ELISA Kit; Morinaga Institute of Biological Science, Yokohama, Japan). Haematological examination of leukocyte count was performed at 17 weeks of age. The differential counts of leukocytes, such as neutrophils, lymphocytes, monocyte, and basophil, were determined. The levels were measured using an automatic analyser (ADVIA 120 Hematology System; Siemens AG, Erlangen, Germany). An OGTT was performed at 14 weeks of age. The glucose solution (2 g/5 mL/kg) was administered to overnight-fasted rats. Blood samples were collected from the tail vein before and 30, 60, and 120 minutes after glucose-loading. The serum glucose and insulin levels were measured as described. The insulinogenic index (Δinsulin/Δglucose) was calculated using incremental serum insulin and glucose levels for 0-30 minutes after glucose loading.

4.3 Tissue sampling and histopathology

Necropsy was performed at 18 weeks of age. All animals were sacrificed via exsanguination under isoflurane anaesthesia. The pancreas and spleen of rats were immediately removed and fixed in 10% neural-buffered formalin. Additionally, the weights of the pancreas and spleen were measured. After resection, thin sections (3 to 5 μm) of the tissue were paraffin-embedded using standard techniques. The sections were

then stained with haematoxylin and eosin (HE). Histopathological findings were scored in a blind manner based on the area or number of degenerated sites as follows[33-37]: – negative, ± very slight; approximately <5%, + slight; approximately 5% to 25%, ++ moderate; approximately 25% to 50%, +++ severe; generally >50%.

4.4 Statistical analysis

Data are expressed as mean ± standard deviation. The following statistical analyses were performed to derive the differences between the mean values: homogeneity of variance was evaluated by the F-test followed by the Student's *t*-test or Aspin-Welch's *t*-test for homoscedastic data or heteroscedastic data, respectively. To compare the cumulative incidence of diabetes, the log-rank test was employed. All statistical analyses were performed using Statlight 2000 (Yukms Corp., Tokyo, Japan) or GraphPad Prism® 6.07 (GraphPad Software, San Diego, CA, USA). Differences were defined as significant at *P* < .05.

Conflict of interests

Kazuma Kobayashi, Tomohiko Sasase, Yukihito Ishii, and Yoshiaki Katsuda are employees of Japan Tobacco Inc. Katsuhiro Miyajima, Takahisa Yamada, and Takeshi Ohta have no conflict of interest.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure Legends

Figure 1 Effect of FTY720 on body weight (A), and the levels of glucose (B), insulin (C), TG (D), ALT (E), and AST (F) in SDT rats. Rats were administered FTY720 for 12 weeks, from 5 to 17 weeks of age. Data represent mean ± standard deviation (n = 5-6). *P < .05, **P < .01; significantly different relative to the control SDT rats. #P < .05, ##P < .01; significantly different relative to the SD rats.

Figure 2 Effect of FTY720 on the cumulative incidence of diabetes in SDT rats. Rats were administered FTY720 for 12 weeks, from 5 to 17 weeks of age. Significant difference between two groups was detected by log-rank test (P < .0001).

Figure 3 Effect of FTY720 on the levels of glucose (A) and insulin (B) in glucose-loaded SDT rats at 14 weeks of age. Data represent mean ± standard deviation (n = 5-6). *P < .05; significantly different relative to the control SDT rats. #P < .05; significantly different relative to the SD rats. The insulinogenic index (C) was calculated using incremental insulin and glucose levels at 0-30 minutes after glucose-loading. N.S.; not significant vs. SD rats or control SDT rats (Student's t-test).

Figure 4 Histological changes in the pancreas and spleen owing to FTY720. Pancreas (A-C) and spleen (D-F) from 18 weeks old control SDT rats, FTY720-treated SDT rats,

and SD rats were histopathologically evaluated by HE staining. Bar = 100 μm.

Table 1　Haematological examination of FTY720-treated SDT rats at 17 weeks of age

Group	Leukocyte ($\times 10^3$ cells/μL)	Neutrophil ($\times 10^3$ cells/μL)	Lymphocyte ($\times 10^3$ cells/μL)	Monocyte ($\times 10^3$ cells/μL)	Basophil ($\times 10^3$ cells/μL)
SDT (control)	$16.25 \pm 1.11^{\#\#}$	$3.83 \pm 0.91^{\#\#}$	$11.39 \pm 0.88^{\#\#}$	$0.49 \pm 0.12^{\#\#}$	$0.05 \pm 0.01^{\#\#}$
SDT (FTY720)	$6.51 \pm 1.06^{**}$	3.31 ± 0.54	$2.54 \pm 0.49^{**}$	$0.25 \pm 0.10^{**}$	0.04 ± 0.01
SD	8.09 ± 1.15	1.65 ± 0.98	5.90 ± 0.65	0.20 ± 0.09	0.02 ± 0.01

Data represent mean ± standard deviation (n = 5-6).

$\#\#P < .01$ vs. SD rats, $**P < .01$ vs. control SDT rats.

Table 2   Absolute and relative weights of the pancreas and spleen at 18 weeks of age

Group		Pancreas	Spleen
SDT (control)	Absolute weight (mg)	$1041.3 \pm 212.8^{\#}$	1219.7 ± 74.2
	Relative weight (mg/g)	2.0016 ± 0.4158	$2.3416 \pm 0.0915^{\#}$
SDT (FTY720)	Absolute weight (mg)	1351.0 ± 147.2	$908.5 \pm 87.4^{**}$
	Relative weight (mg/g)	2.3921 ± 0.1912	$1.6112 \pm 0.1508^{**}$
SD	Absolute weight (mg)	1515.0 ± 297.9	1203.2 ± 213.5
	Relative weight (mg/g)	2.2301 ± 0.4459	1.7845 ± 0.4033

Data represent mean ± standard deviation (n = 5-6).

#P < .05; ##P < .01 vs. SD rats, *P < .05; **P < .01 vs. control SDT rats.

Table 3 Histopathological findings in the pancreas and spleen at 18 weeks of age

Organ		Findings	SDT (control)						SDT (FTY720)					SD				
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Pancreas	islet																		
		fibrosis	++	++	+++	+++	++	+	+	±	−	±	±	−	−	−	−	−	−
		atrophy	+	+	+	+	+	+	+	±	−	−	−	−	−	−	−	−	−
		hemosiderin deposition	+	++	++	+	+	+	±	±	−	−	−	−	−	−	−	−	−
		inflammation	++	++	++	++	+	+	+	+	±	±	±	−	−	−	−	−	−
		hypertrophy	+	+	++	+	+	+	±	±	−	±	−	−	−	−	−	−	−
	acinus																		
		atrophy	+	+	+	+	+	+	±	+	±	−	−	−	−	−	−	−	−
		infiltration of inflammatory cells	+	+	++	+	+	+	±	±	+	−	−	±	−	−	−	−	−
		haemorrhage	+	+	+	+	+	+	−	±	±	−	−	−	−	−	−	−	−
Spleen		atrophy in white pulp	−	−	−	−	−	−	+	++	++	++	+	±	−	−	−	−	−
		decrease number in lymphocyte	−	−	−	−	−	−	+	+	+	++	+	±	−	−	−	−	−
		decrease number of lymphocytes in red pulp	−	−	−	−	−	−	+	+	−	+	−	−	−	−	−	−	−
		extramedullary haematopoiesis	−	−	−	−	−	−	++	++	++	++	++	++	−	−	−	−	−

SDT (control) group (animal #1-6); SDT (FTY720) group (animal #7-12); SD group (animal #13-17)

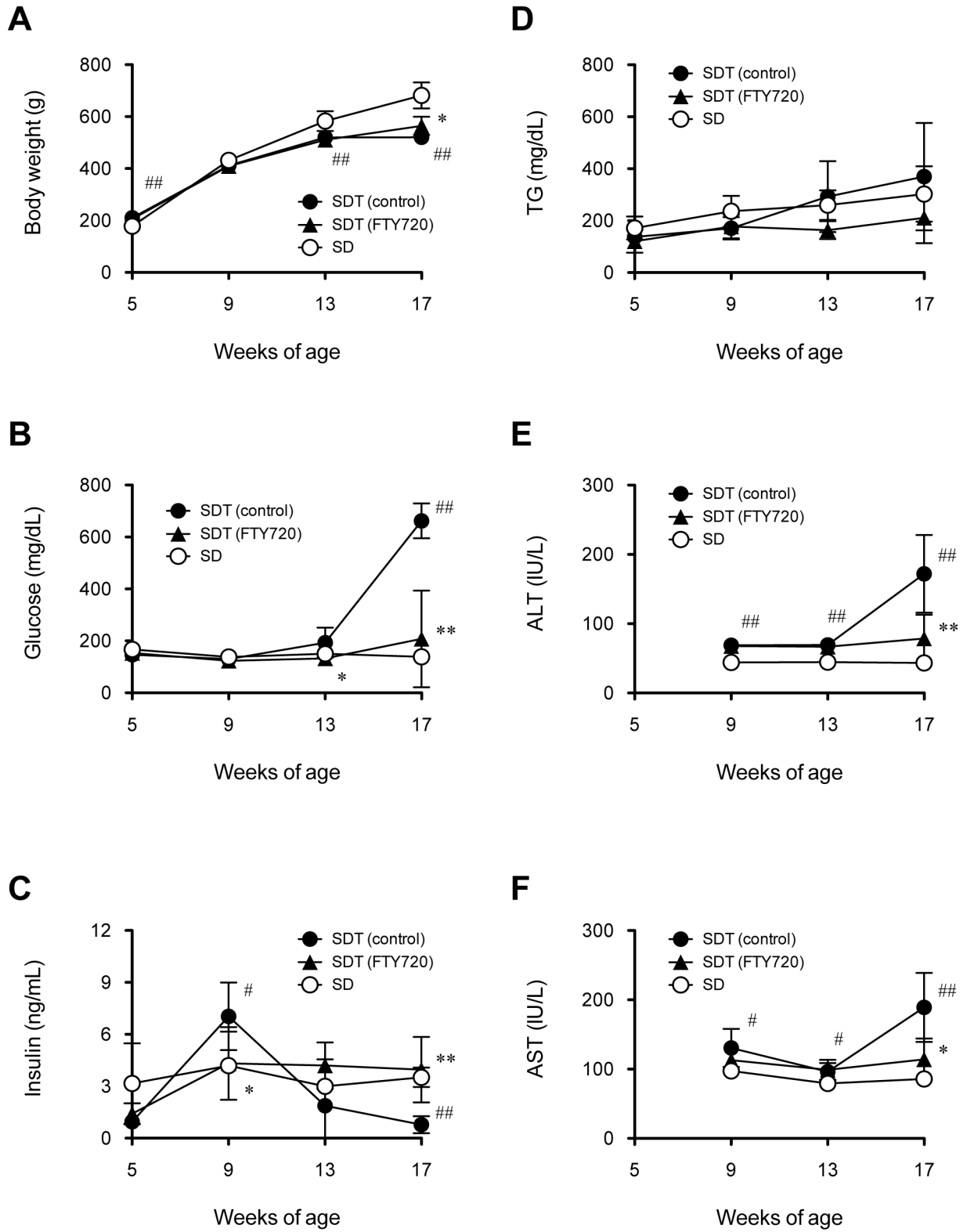- Negative; ± Very slight; + Slight; ++ Moderate; +++ Severe
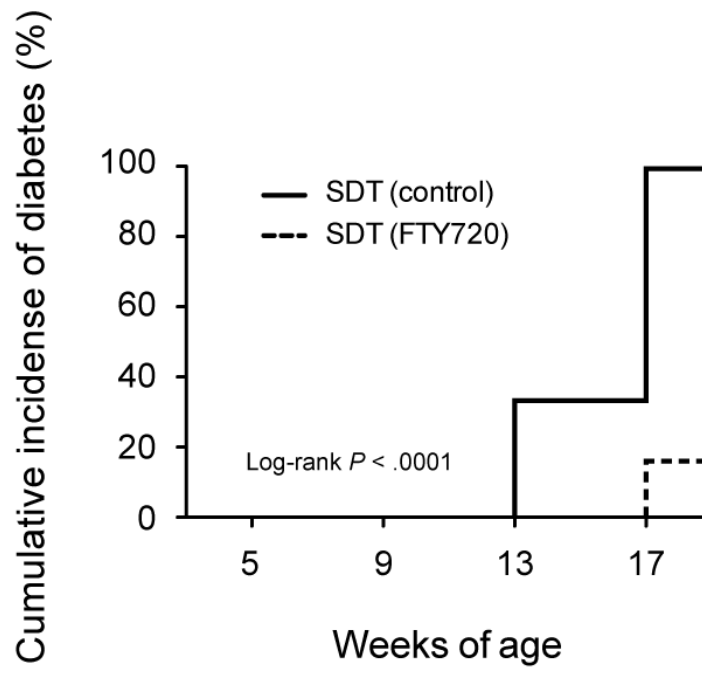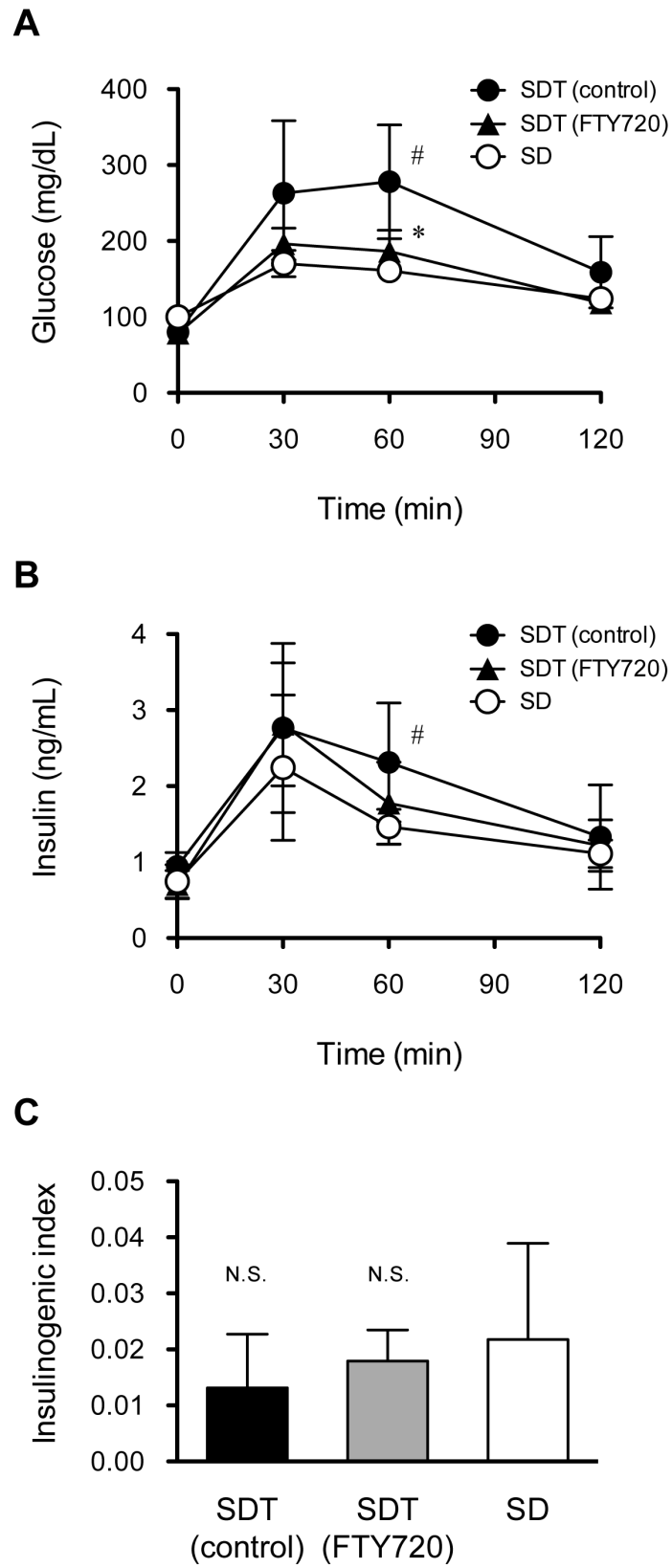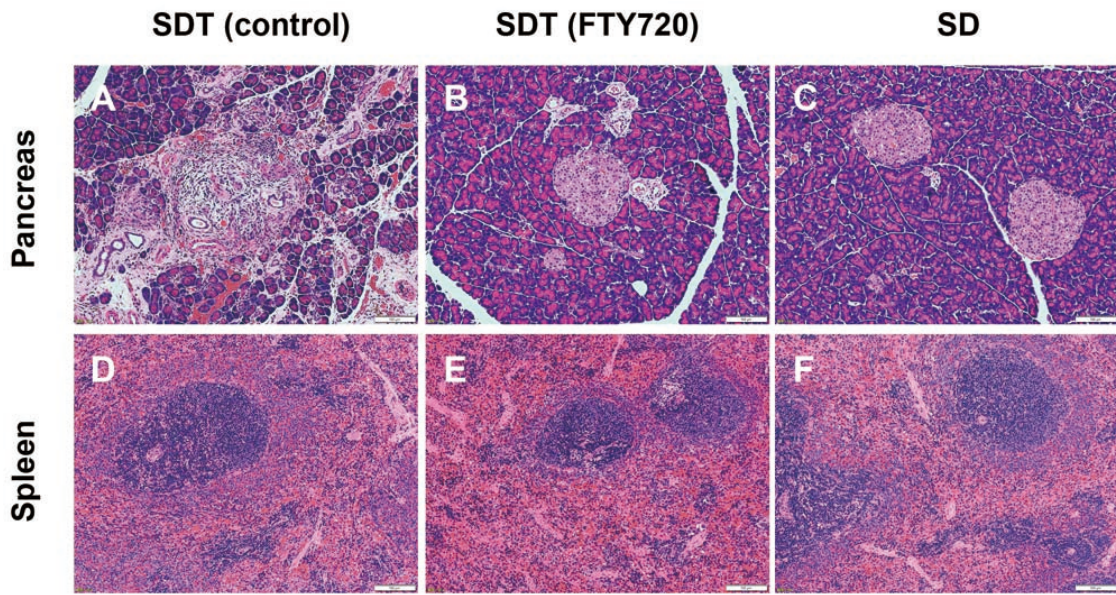
Figure 1

Figure 2

A

B

C

Figure 3

Figure 4