

# Sphingosine-1-phosphate induces islet $\beta$ -cell proliferation and decreases cell apoptosis in high-fat diet/streptozotocin diabetic mice

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Received December 23, 2018; Accepted August 8, 2019

DOI: 10.3892/etm.2019.7999

**Abstract.** Sphingosine-1-phosphate (S1P) has been reported to enhance the function of islet  $\beta$ -cells, providing a potential therapeutic target for diabetes mellitus. In the present study, the effects of S1P on the proliferation and apoptosis of  $\beta$ -cells in type 2 diabetic mice were investigated. The mice were administered intraperitoneal S1P solution daily at a dose of 20  $\mu$ g/kg for three weeks. The intraperitoneal glucose tolerance test (IPGTT) and homeostatic model assessment of insulin resistance (HOMA-IR) index determination were carried out. Immunohistochemical staining was used to detect the protein expression of insulin, antigen Ki-67 and S1P receptor isoforms (S1PR1/S1PR2/S1PR3) in pancreatic islets. Compared with the diabetic control (DC) group, the IPGTT results and HOMA-IR index in the S1P treatment group were decreased. The islets in the S1P group exhibited higher insulin immunostaining intensity than the DC group, as well as higher proliferation ( $P < 0.05$ ) and lower apoptosis rates ( $P < 0.05$ ). Positive staining for the S1P receptors S1PR1, S1PR2 and S1PR3 was observed in the cytoplasm and membrane of the islet cells. S1PR1 and S1PR2 proteins showed increased expression in the S1P and DC groups compared with the normal control group ( $P < 0.01$  and  $P < 0.05$ , respectively), whereas no significant difference was observed in the expression of S1PR3 among these groups. In conclusion, extracellular S1P can induce islet  $\beta$ -cell proliferation and decrease cell apoptosis in diabetic mice. S1P function may be mediated via S1PR1 and S1PR2; therefore, targeting S1P/S1PR signalling pathways may be a novel therapeutic strategy for diabetes mellitus.

## Introduction

Diabetes mellitus has become the public health threat with the highest incidence rate. The latest estimated overall prevalence of total diabetes was 10.9% and the prevalence of prediabetes was 35.7% in China (1). Overall, diabetes is broadly classified into 2 major types: Type 1 and type 2. Type 2 diabetes mellitus (T2DM) is the most common and complex disease, characterised by insulin resistance, which can lead to a relative state of hyperinsulinaemia to maintain normal glycaemia, eventually resulting in  $\beta$ -cell failure (2). T2DM is considered to be influenced by multifactorial nature, including genetic, environmental and lifestyle factors (3). However, the molecular mechanisms underlying insulin dysfunction have not been completely elucidated yet. Previous studies indicated an association between sphingosine-1-phosphate (S1P) and T2DM (4,5). S1P was reported to activate key signalling cascades responsible for the maintenance of sphingolipid metabolism, which contributed to the promotion of cell proliferation and suppression of apoptosis (6). Ceramide and S1P, products of sphingomyelin metabolism, appeared to represent opposing signalling cascades, the former signifying cell growth arrest, and the latter proliferation and survival, the so-called sphingolipid rheostat (7-10). The sphingolipid rheostat was first coined in the mid-90's to describe the repression of ceramide-mediated programmed cell death through the conversion of sphingosine to S1P (10-13). Strong evidence suggested that in balancing the sphingolipid rheostat, S1P signalling was crucial in the prevention and treatment of diabetes (2). S1P regulates insulin synthesis, insulin resistance, reduced blood glucose levels, maintenance of blood vessels and decreased inflammation (2,13). Therefore, the pharmacological roles of S1P involvement in glucose metabolism remains unclear, particularly in terms of islet functions. In the present study, S1P is proposed as a key regulatory factor of islet  $\beta$ -cell proliferation, potentially influencing insulin secretion.

S1P is a unique bioactive lipid mediator, which is generated from sphingomyelin metabolites and has been found to play an important role in DNA synthesis,  $Ca^{2+}$  mobilization,

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**Key words:** sphingosine-1-phosphate, sphingosine-1-phosphate receptor, proliferation, apoptosis, diabetes mellitus

MAPK pathway activation (6,14), and, more recently, the proliferation and survival function of islet  $\beta$ -cell regulation (13). Extracellular S1P was reported to act as a primary messenger, or ligand receptor, either inside or outside the cell via the activation of five specific G-protein-coupled receptors, denoted as S1P receptors 1-5 (S1PR1-5), located at the cell surface and characterized by the cell type-specific expression pattern (6,14-22). Each S1PR is involved in distinct and overlapping intracellular signalling pathways (13,23). S1PRs activate a variety of different G-proteins, namely Gi (S1PR1-5), Gq (S1PR2/3) and G12/13 (S1PR2-5), which in turn results in partly diverse functional properties of a single S1PR (22,24). S1PR1 preferentially couples with Gi and regulates numerous cell reactions, such as lymphocyte trafficking and angiogenesis (19,25). In contrast, S1PR2 and S1PR3 couple with several G proteins, including Gi, Gq and G12/13, and mediate other pathways (21,26). S1PR1-3 are widely distributed, whereas S1PR4 is expressed in lymphoid tissues and lung tissue, and S1PR5 expression is restricted to tissue of the nervous system. A total of four S1PR isoforms (S1PR1-4) have been identified in rat and mouse islets and INS-1 cells (27). This indicates that S1P, by signalling through S1PR2, plays a key role in pancreas development, linking lineage allocation and specification, using a combination of genetic approaches (22,28). However, the involvement of specific S1PR subtypes for S1P-induced islet  $\beta$ -cell proliferation remains controversial.

To the best of our knowledge, little is known about the effects of S1P on islet  $\beta$ -cell proliferation and the role of S1PR subtypes. Therefore, the aim of the present study was to investigate the potential effects of S1P on the proliferation and apoptosis of pancreatic islet  $\beta$ -cell in T2DM mice, established by a high-fat diet (HFD) with low-dose streptozotocin. The effects of S1P on blood glucose, the intraperitoneal glucose tolerance test (IPGTT), homeostatic model assessment of insulin resistance (HOMA-IR) index and insulin secretion were investigated, and the expression and localization of S1PR1-3 were observed in diabetic mice islet cells. This study aimed to explore whether exogenous S1P induced islet  $\beta$ -cell proliferation and decreased cell apoptosis, and to evaluate the role of relevant S1PR subtypes in diabetic mice. The significance of this study was to build further information on the protective effects of S1P on islet  $\beta$ -cell injury and the role of S1P in preventing the process of diabetes, and to provide novel ideas for the prevention and treatment of diabetes.

## Materials and methods

**Test materials.** Streptozotocin (STZ), S1P and normal rabbit IgG (cat. no. NI01) were purchased from Sigma-Aldrich; Merck KGaA. Mouse anti-Ki-67 monoclonal antibody (cat. no. BD550609) was purchased from BD Biosciences; Becton, Dickinson and Company. Rabbit anti-insulin (cat. no. sc-9168) and anti-S1P3 (cat. no. sc-30024) polyclonal antibodies and normal mouse IgG (cat. no. sc-2025) were purchased from Santa Cruz Biotechnology, Inc. The TUNEL kit (cat. no. 11684817910) was purchased from Roche Diagnostics. Rabbit anti-S1P1 polyclonal antibody (cat. no. ab11424) was purchased from Abcam. Rabbit anti-S1P2 polyclonal antibody (cat. no. BS2594) was purchased from Bioworld Technology, Inc. The biotin-streptavidin horseradish peroxidase detection

system include the secondary antibody of 1% biotin-labelled goat anti-mouse/rabbit IgG (cat. no. SP-9000) and diaminobenzidine (DAB) kit (cat. no. ZLI-9017) were purchased from Beijing ZSGB-BIO Technologies, Inc.

**Establishment of HFD/STZ diabetic mice and experimental design.** A total of 30 male C57BL/6J mice (age, 4 weeks; weight, 16-20 g) were obtained from the Experimental Animal Centre of Xi'an Jiaotong University, Xi'an, China [license no. SCXK (Shan) 2012-003] and were randomly assigned to 2 groups: Normal control (NC) on a normal diet ( $n=10$ ), and low-dose-STZ-treated diabetic group on an HFD, under standard animal housing conditions ( $n=20$ ). Food intake and body weight were monitored weekly. Following feeding on an HFD for 4 weeks, a single low dose of STZ (120 mg/kg in 0.1 M citrate buffer; pH 4.5) was administered via intraperitoneal injection after a 14-h fasting to induce diabetes, while the NC group was treated with the same volume of citrate buffer solution. A total of two weeks after the STZ treatment, blood glucose levels were measured to confirm diabetes. Values of  $>11.1$  mmol/l were considered to indicate diabetes in the animals. A total of two mice did not fulfil the conditions to be considered diabetic and one mouse died during the aforementioned process. The remaining 17 diabetic mice were randomly divided into two groups using a random number table: Vehicle diabetic control (DC) group ( $n=8$ ) and S1P-treated group (S1P group;  $n=9$ ). S1P storage solution (1 mg S1P dissolved in 1 ml methanol) was preserved in the dark at  $-20^{\circ}\text{C}$  and diluted in PBS buffer to make the working concentration. The S1P group animals were administered 20  $\mu\text{g/kg}$  working concentration S1P, whereas the vehicle diabetic control group mice received an equal volume of methanol in PBS by intraperitoneal injection daily for 3 weeks. The DC and S1P groups continued to be fed with an HFD, while the non-diabetic NC group was fed with the normal diet. Fasting blood glucose, body weight, food intake and drinking volume were measured weekly. All mice were housed under conditions of a 12-h light/dark cycle at a temperature of  $25^{\circ}\text{C}$  and a humidity of 55%. All mice were allowed free access to food and water throughout the entire experiment. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Xi'an Jiaotong University of Health Sciences, Xi'an, China.

**IPGTT.** Glucose tolerance was evaluated using a conventional IPGTT and conducted 21 days after S1P administration. Mice were weighed after fasting for 8 h, before being administered an intraperitoneal glucose injection (2 g/kg). Blood samples ( $\sim 0.6$   $\mu\text{l}$ ) were collected from the tail before and at 30, 60 and 120 min after glucose administration. The blood glucose level was determined with an Accu-Check Active digital glucose meter (Roche Diagnostics). The areas under the curves (AUCs) of the IPGTT results were calculated for the blood glucose levels at 0 ( $\text{BG}_0$ ), 30 ( $\text{BG}_{30}$ ), 60 ( $\text{BG}_{60}$ ) and 120 ( $\text{BG}_{120}$ ) min, using the following equation:  $\text{AUC} = 1/4 (\text{BG}_0) + 1/2 (\text{BG}_{30}) + 3/4 (\text{BG}_{60}) + 1/2 (\text{BG}_{120})$ . Fasting insulin was determined by radioimmunoassay. The HOMA-IR index was calculated using Matthew's equation:  $\text{Fasting insulin level } (\mu\text{IU/ml}) \times \text{fasting glucose level (mmol/l)} / 22.5$  (29). A HOMA-IR index value of  $>2.5$  was considered to indicate the insulin resistance (29).

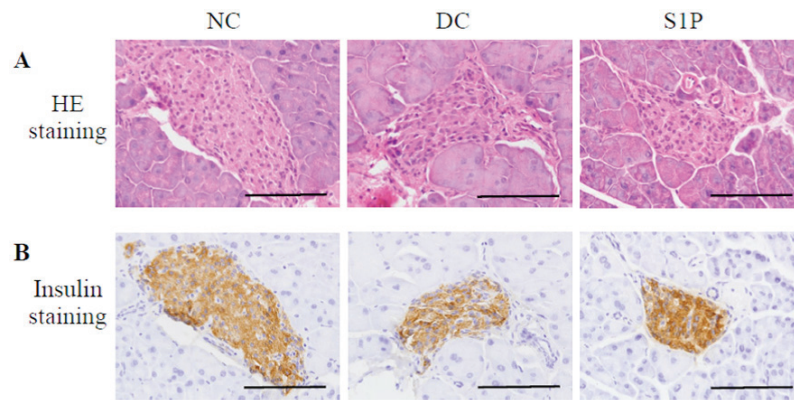


Figure 1. Staining results from mouse islets. Representative images of (A) hematoxylin and eosin staining and (B) immunohistochemical insulin staining in the normal control (n=10), diabetic control (n=8) and sphingosine-1-phosphate (n=9) groups. Insulin expression is indicated by the brown staining in the cells. Magnification, x400; scale bar, 100  $\mu$ m.

**Histology and immunohistochemistry.** Each mouse had one pancreas and in total the mice in 3 groups had a total of 27 pancreases. The tail of the pancreas was taken to make paraffin slides. Pancreatic tissues were carefully excised, fixed in 10% neutral buffered formalin for 24 h at room temperature, dehydrated, embedded in paraffin and cut into 5- $\mu$ m sections. Overall,  $\geq 7$  consecutive sections were cut from the largest cross-sectional area in the middle of each pancreas tissue specimen and were used for hematoxylin and eosin (HE) staining, immunohistochemical staining (insulin, Ki-67, S1PR1, S1PR2 and S1PR3) and TUNEL staining. Each pancreas had one slide for each protein immunohistochemical staining by strictly following the same time and conditions in the process of immunohistochemical staining.

For histological examination, sections were stained with hematoxylin for 5 min and with eosin for 2 min at room temperature. For immunohistochemical staining, the samples were routinely dewaxed, hydrated and incubated with 3%  $H_2O_2$  at room temperature for 10 min. Then, a microwave was used to retrieval the antigen by heating to 95-100°C for 10 min in 0.01 mol/l sodium citrate buffer (pH, 6.0). The slices were then blocked with 0.1% goat serum for 15 min at room temperature. The sections were incubated with anti-insulin (1:1,400), anti-Ki-67 (1:200), anti-S1PR1 (1:400), anti-S1PR2 (1:150) and anti-S1PR3 (1:40) primary antibodies at 4°C overnight, followed by incubation with secondary antibody (1% biotin-labelled goat anti-mouse/rabbit IgG) at 37°C for 15 min. This was followed by incubation with horseradish peroxidase-labelled streptomyces ovalbumin at 37°C for 15 min and staining with DAB at room temperature. Slides were counterstained with hematoxylin for 1 min at room temperature to identify the cell nuclei. As a negative control (Fig. S1A), the insulin, S1PR1, S1PR2 and S1PR3 primary antibodies were replaced with rabbit non-specific IgG, whereas the Ki-67 primary antibody was replaced with mouse non-specific IgG (Ki-67) at the same dilution at 4°C overnight. Myocardial (S1PR1-positive), kidney (S1PR2- and S1PR3-positive) and tumour (Ki-67-positive) mouse tissue was used for positive controls (Fig. S1B). The staining was observed using a DP71 fluorescence microscope (magnification, x400; Olympus Corporation). The images were analysed using Adobe Photoshop CS4 extended software (Adobe, Inc.).

Table I. The average optical density and the maximum islet area of insulin immunohistochemistry in all groups.

| Group | n  | IOD/area            | Area ( $\mu m^2$ )            |
|-------|----|---------------------|-------------------------------|
| NC    | 10 | 0.2246 $\pm$ 0.0414 | 1,4236 $\pm$ 7906             |
| DC    | 8  | 0.1896 $\pm$ 0.0376 | 8,404 $\pm$ 5217 <sup>a</sup> |
| S1P   | 9  | 0.2251 $\pm$ 0.0366 | 9,992 $\pm$ 4955 <sup>b</sup> |

Values are expressed as the mean  $\pm$  standard deviation. Parametric one-way analysis of variance followed by Least Significant Difference post hoc test was performed for the comparison of the groups. <sup>a</sup>P<0.01 and <sup>b</sup>P<0.05 vs. NC. NC, normal control; DC, diabetic control; S1P, sphingosine-1-phosphate; IOD, integrated optical density.

Image pro Plus 6.0 software (Media Cybernetics, Inc.) was used for semi-quantitative analysis of insulin, Ki-67 and S1PR expression in pancreatic tissue. A total of three to five unique fields of view were randomly selected in each slice (magnification, x400) and averages were calculated for analysis. The integrated optical density (IOD) of positively stained cells and the corresponding islet area in each islet were measured and calculated automatically, and the average values were calculated for analysis. The average optical density (IOD/area) was calculated for the relative amount of protein. After insulin staining, the number of islets in each slide was counted and the area of the maximum islet on each slide was measured in order to assess islet size. Following Ki-67 staining, the proliferating nuclei were stained dark brown. The cells whose nuclei were dyed dark brown were considered to be proliferating islet cells and the proliferation rate in each islet (%) was calculated as the number of proliferating cells/the total number of cells x100%. Overall, three to five islets were counted for each section and average values were taken.

**TUNEL analysis.** The formalin-fixed and paraffin-embedded pancreas tissue sections were stained with HE and immunohistochemical staining of insulin. Following the HE and insulin staining, images of at least 5 fields on each slide were captured using a fluorescence microscope (magnification,



x400; Olympus Corporation). Apoptosis was demonstrated using a TUNEL assay kit (Roche Diagnostics), according to the manufacturer's protocols. After TUNEL staining, apoptotic nuclei were stained dark brown and cells with dark brown nuclei were considered to be apoptotic islet cells. The apoptosis rate in each islet (%) was calculated as the number of apoptotic cells/the total number of cells  $\times 100\%$  in each islet. Overall, three to five islets were counted for each section and average values were taken.

**Statistical analysis.** Statistical analysis was performed using SPSS version 21.0 software (IBM Corp.). Continuous variables are expressed as the mean  $\pm$  standard deviation or median (interquartile) from at least three independent experiments. Intervention and control groups were compared with one-way analysis of variance followed by Least Significant Difference post hoc test for parametric analysis and Kruskal-Wallis H test for nonparametric analysis.  $P < 0.05$  was considered to indicate statistically significant differences.

## Results

**Islet  $\beta$ -cell morphology and structural changes.** The islets of the NC mice were round or oval, with clear boundaries and complete film. In addition, the islets were evenly distributed between the pancreatic acinus; the islet cells were round or oval, of uniform size, strong staining in the cytoplasm and a visible centred nucleus. The number of islets in the DC group was decreased and they were unevenly distributed, with different degrees of atrophy, and vacuoles appeared within them. A further loss of  $\beta$ -cells was noted in the diabetic group within irregularly shaped islets. Ill-defined  $\beta$ -cells were loosely arranged, the cell volume was increased and the nucleus exhibited an abnormal distribution (Fig. 1A). The number of positively stained cells decreased and the insulin staining was decreased compared with in the NC group (Fig. 1B), suggesting that islet  $\beta$ -cells showed marked damage after establishment of the animal model. Due to the limitations of tissue size and the specific parts of sections, the number of the islets was not identical on different slides, and each slide could count ~three to ten islets. A number of the small islets only appear to be small as they were produced by cutting through the edge of an islet, the maximum islet area was used as an indicator in each slide to assess the islet area. Compared with that in the NC group, the islet area in the DC group was significantly smaller ( $P < 0.01$ ; Table I). The S1P administration group exhibited less damage; the number and volume of islet cells was increased, and the islet cells had a clearer structure, and a more regular shape and arrangement compared with the DC group (Fig. 1A). Immunohistochemistry on the pancreas of the NC mice revealed intense insulin staining, whereas weak staining was observed in the core of the islets in the DC group. The islets in the S1P administration group displayed stronger insulin immunostaining intensity compared with the DC group (Fig. 1B). The islet area in the S1P group was significantly decreased compared with that in the NC group ( $P < 0.05$ ) and appeared larger than that in the DC group, but the difference compare with the NC group was not statistically significant (Table I). An increase in positive insulin staining granules suggested that S1P could decrease islet damage and

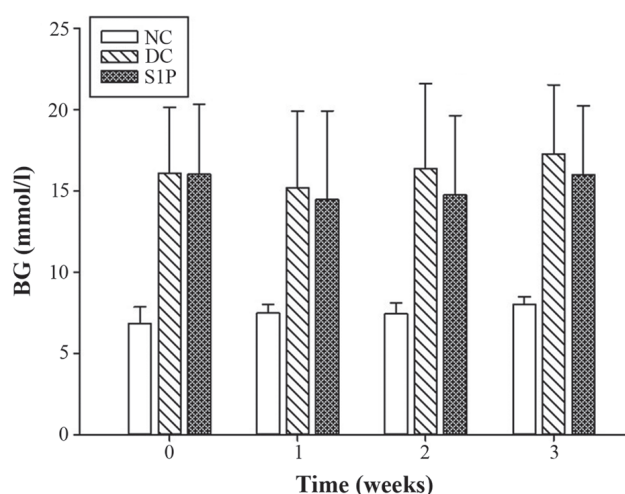


Figure 2. Effect of S1P on mouse fasting BG. The fasting BG levels of the NC (n=10), DC (n=8) and S1P (n=9) groups at 0, 1, 2 and 3 weeks after S1P administration. Values are expressed as the mean  $\pm$  standard deviation. Parametric one-way analysis of variance followed by Least Significant Difference post hoc test was performed for the comparison of the groups. NC, normal control; DC, diabetic control; S1P, sphingosine-1-phosphate; BG, blood glucose.

improve  $\beta$ -cell morphology. The average optical density of the insulin immunohistochemistry (IOD/area) and maximum islet area were calculated using Image Pro Plus 6.0 image analysis system (Table I).

**Effect of S1P on fasting blood glucose.** The blood glucose level was measured before and 1, 2 and 3 weeks after S1P administration to observe the effect of S1P on the fasting blood glucose levels of T2DM mice. The fasting blood glucose in the DC mice was significantly increased compared with the NC group ( $P < 0.01$ ; Table II); 1 week after S1P administration, the fasting levels in the S1P group were decreased slightly compared with the diabetic model group, but the difference was not significant ( $P > 0.05$ ). The same trend was observed at weeks 2 and 3 after the administration of S1P. No statistically significant difference was observed in the fasting blood glucose between the DC and S1P group (Fig. 2; Table SI).

**Effect of S1P on mouse IPGTT results.** IPGTT was conducted 3 weeks after S1P administration to observe the effect of S1P on pancreatic  $\beta$ -cell function and metabolic regulation in T2DM mice. Compared with that in the NC group, the blood glucose in the DC mice reached its peak 30 min after glucose injection and maintained a high level throughout the whole experiment. The blood glucose in the S1P mice appeared to decrease 1 and 2 h after glucose injection, compared with in the DC group, but the difference was not significant ( $P > 0.05$ ; Table II).

**Effect of S1P on fasting serum insulin levels and the HOMA-IR index.** Fasting serum insulin levels were not significantly different among groups (all  $P > 0.05$ ; Table III) and were consistent with insulin levels in T2DM. The AUC and HOMA-IR index were calculated in all groups. Compared with that measured in the NC group, the HOMA-IR index in the DC mice was significantly increased ( $P < 0.01$ ), whereas

Table II. IPGTT in all groups (mmol/l).

| Group | n  | 0 min                   | 30 min                  | 60 min                  | 120 min                 |
|-------|----|-------------------------|-------------------------|-------------------------|-------------------------|
| NC    | 10 | 8.03±0.47               | 16.54±1.96              | 11.56±1.29              | 8.61±0.93               |
| DC    | 8  | 17.26±4.27 <sup>a</sup> | 29.76±4.14 <sup>a</sup> | 29.59±2.84 <sup>a</sup> | 24.35±3.52 <sup>a</sup> |
| S1P   | 9  | 16.01±4.26 <sup>a</sup> | 29.70±3.09 <sup>a</sup> | 28.51±4.49 <sup>a</sup> | 22.93±4.44 <sup>a</sup> |

Values are expressed as the mean ± standard deviation. Parametric one-way analysis of variance followed by Least Significant Difference post hoc test was performed for the comparison of the groups. <sup>a</sup>P<0.01 vs. NC. NC, normal control; DC, diabetic control; S1P, sphingosine-1-phosphate; IPGTT, intraperitoneal glucose tolerance test.

Table III. FINS (mIU/L), HOMA-IR and AUC in all groups.

| Group | n  | FINS (mIU/L) | HOMA-IR                | AUC                     |
|-------|----|--------------|------------------------|-------------------------|
| NC    | 10 | 7.51±2.60    | 2.68±0.98              | 23.25±1.68              |
| DC    | 8  | 8.37±2.31    | 6.56±2.80 <sup>a</sup> | 53.56±5.76 <sup>a</sup> |
| S1P   | 9  | 8.39±1.93    | 5.82±1.44 <sup>a</sup> | 51.70±6.45 <sup>a</sup> |

Values are expressed as the mean ± standard deviation. Parametric one-way analysis of variance followed by Least Significant Difference post hoc test was performed for the comparison of the groups. <sup>a</sup>P<0.01 vs. NC. NC, normal control; DC, diabetic control; S1P, sphingosine-1-phosphate; FINS, fasting insulin; HOMA-IR, homeostatic model assessment of insulin resistance; AUC, areas under the curve.

that in S1P group decreased slightly compared with that in the DC group, but the difference was not statistically significant (P>0.05; Table III). The HOMA-IR exhibited a trend similar to that observed with the AUC. Compared with that calculated in the NC group, the AUC was significantly increased in the DC and S1P groups (both P<0.01). The AUC calculated for the S1P group appeared to decrease compared with the DC group, but with no statistical significance (P>0.05; Table III).

**Mouse pancreatic  $\beta$ -cell proliferation assay.** Next, an investigation into whether exogenous S1P promoted  $\beta$ -cell proliferation was carried out. Ki-67-positive staining appeared as a brownish yellow colour in the nucleus, whereas the cell membrane and cytoplasm displayed no staining (Fig. 3A and C). A total of three to five unique fields of view of the islets were selected randomly and the cell proliferation rate of each islet was calculated separately, then the mean value was taken for analysis. The rates of Ki-67 expression were <3% in all groups of mouse islets (Fig. 3D). Fewer Ki-67-positive cells were observed within the S1P administration group of mouse islets compared with the NC group (Fig. 3A and C), whereas almost no Ki-67-positive cells were noted in the diabetic model control group (Fig. 3B). Ki-67-positive cells increased significantly in the S1P group compared with the DC group (P<0.05; Fig. 3D; Table SII).

**S1P inhibits apoptosis in islet  $\beta$ -cell.** Islet  $\beta$ -cell dysfunction and insulin resistance are multifaceted with their interdependence for triggering the pathogenesis of T2DM. TUNEL

staining of mouse islet  $\beta$ -cell was used to determine the percentage of  $\beta$ -cells that were undergoing apoptosis (Fig. 4). The apoptotic islet cell nuclei were stained brown, whereas normal nuclei were blue (Fig. 4A-C). A significantly increased number of apoptotic cells were observed in the islets of the DC mice, compared with those counted in the NC mouse islets (P<0.01; Fig. 4D; Table SIII). A significantly increased number of TUNEL-positive cells was observed in the diabetic model group compared with the S1P administration group (P<0.05; Fig. 4D; Table SIII), suggesting that S1P serves a positive role in protecting islet cells against apoptosis.

**S1P may enhance islet  $\beta$ -cell proliferation and survival via S1PR1 and S1PR2.** As most of the S1P-induced effects were mediated via S1PR, it was of interest to investigate the differences of protein expression of the S1PR subtypes in the mouse pancreas. S1P signalling is mediated via the activation of specific S1PRs (S1PR1-5), of which mouse islet  $\beta$ -cell express mainly S1PR1-3 (22). To determine which S1PR subtypes mediate the S1P-induced protective response in islet cells, normal and diabetic mouse islets were incubated with anti-S1PR1-3 antibodies. The positive S1PR1, S1PR2 and S1PR3 protein staining was localised in the cytoplasm and membrane of the islet cells, with almost no expression in the exocrine pancreas, consistent with the insulin staining results (Fig. 5A). Compared with that in the NC group, the positive staining of the S1PR1 protein in the diabetic mice was stronger and exhibited more uneven distribution (Fig. 5A). The Image Pro Plus 6.0 software image analysis system was used to calculate the average density of immunohistochemical S1PR1 protein staining (IOD/area) and significant differences were observed between the two diabetic groups, and the NC group (P<0.01; Fig. 5B; Table SIV). S1PR2 exhibited a similar expression trend to S1PR1 (Fig. 5C; Table SIV). No statistical difference was observed in the expression of S1PR3 protein between the diabetic and NC group (P>0.05; Fig. 5D; Table SIV). These results indicate that S1PR1, S1PR2 and S1PR3 proteins were expressed in the pancreatic  $\beta$ -cells. The protein expression of S1PR1 and S1PR2 in the diabetic mice was significantly increased compared with the NC group (P<0.01 and P<0.05, respectively; Fig. 5B and C), whereas no significant difference was observed in the expression of S1PR3 (Fig. 5D). Overall, the findings of the present study indicate that extracellular S1P induces islet  $\beta$ -cell proliferation and inhibits cell apoptosis, possibly via S1PR1 and S1PR2 activation, leading to enhanced islet  $\beta$ -cell protection.

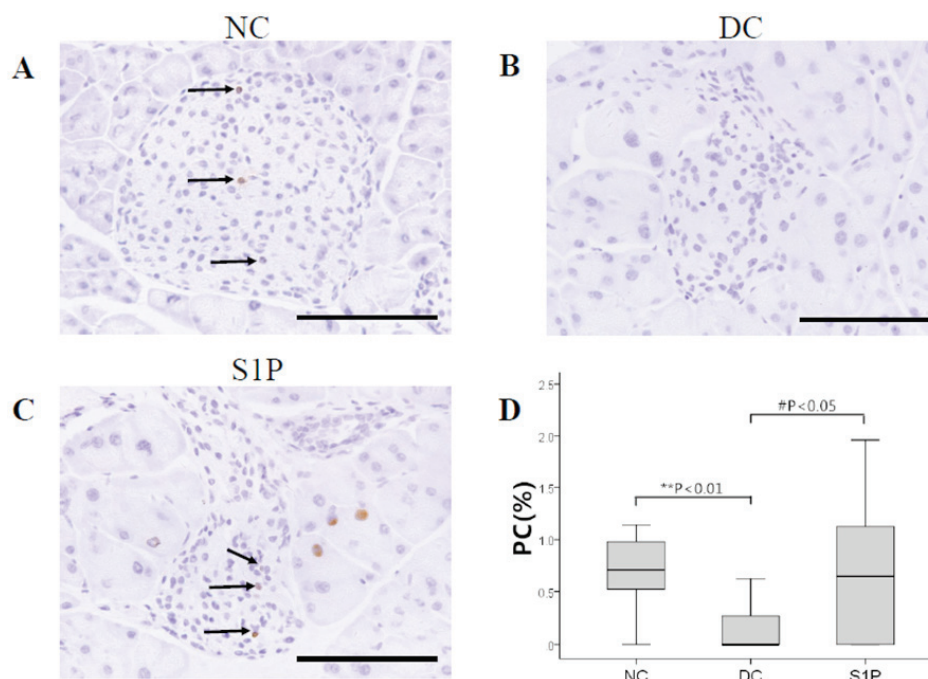


Figure 3. Proliferation rate of islet cells in all groups. Immunohistochemical staining of Ki-67 in mouse islets cells of the (A) NC (n=10), (B) DC (n=8) and (C) S1P (n=9) groups. Arrows indicate the PC. Magnification, x400; scale bar, 100  $\mu$ m. (D) Proliferation rate of islet cells in all groups. Values are expressed as the median (interquartile). Statistical analysis was performed using the Kruskal-Wallis H test. \*\*P<0.01; #P<0.05. NC, normal control; DC, diabetic control; S1P, sphingosine-1-phosphate; PC, proliferating cells.

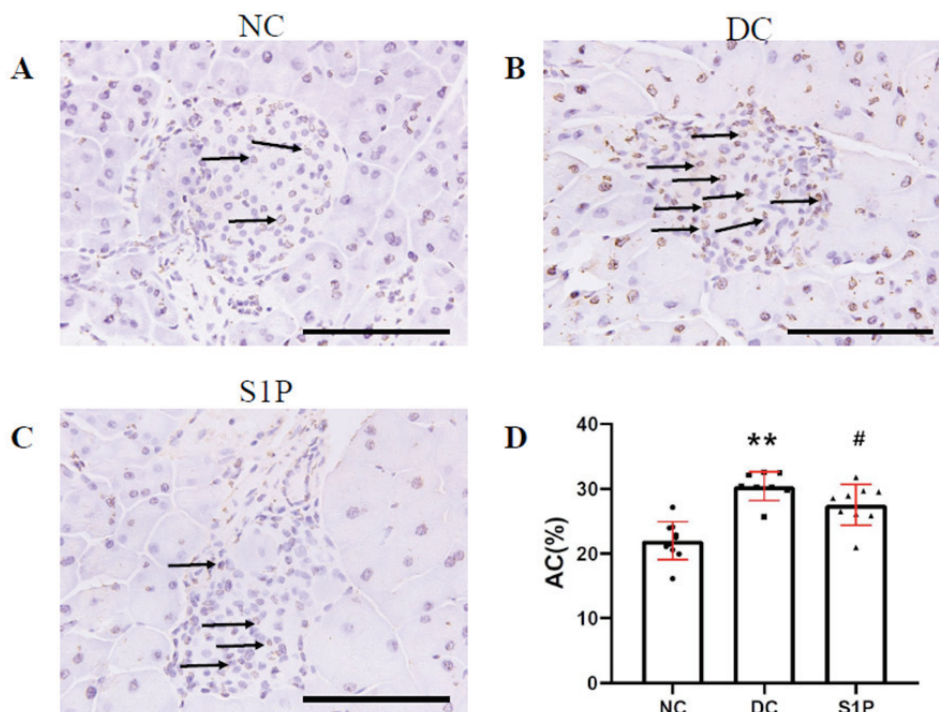


Figure 4. Apoptosis rate of islet cells in all groups. TUNEL assay detection of apoptotic cells in the mouse islets of the (A) NC (n=10), (B) DC (n=8) and (C) S1P (n=9) groups. Arrows indicate the AC. Magnification, x400; scale bar, 100  $\mu$ m. (D) Apoptosis rate of islet cells in all groups. Values are expressed as the mean  $\pm$  standard deviation. Parametric one-way analysis of variance followed by Least Significant Difference post hoc test was performed for comparison of the groups. \*\*P<0.01 vs. NC; #P<0.05 vs. DC. NC, normal control; DC, diabetic control; S1P, sphingosine-1-phosphate; AC, apoptotic cells.

## Discussion

T2DM is characterized by insulin resistance in target tissues and deficiency in the production of insulin from pancreatic

$\beta$ -cells, caused by decreased  $\beta$ -cell mass (30). Multiple signalling mechanisms have been demonstrated to influence the islet  $\beta$ -cell mass, of which  $\beta$ -cell apoptosis emerged as a key mechanism causing decompensation of  $\beta$ -cells and the



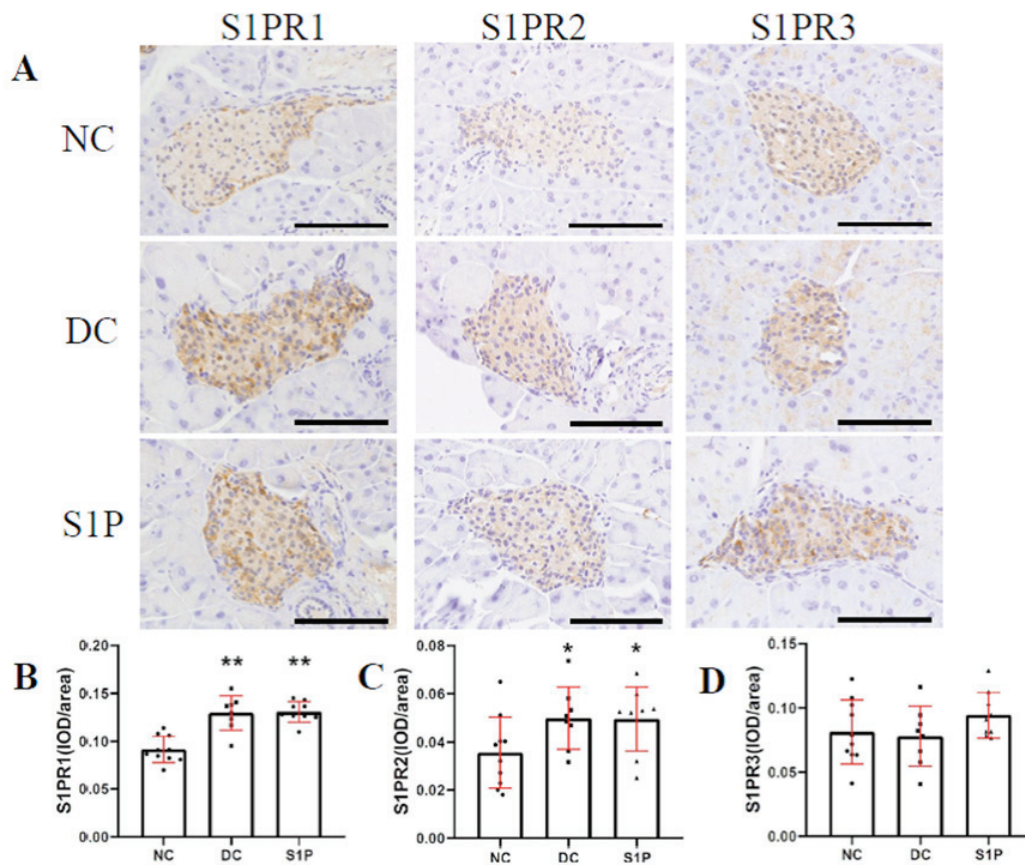


Figure 5. Immunohistochemical staining of S1PR1-3 subtypes in the mouse islets. (A) Representative images of immunohistochemical staining of S1PR1, S1PR2 and S1PR3 in the NC (n=10), DC (n=8), and S1P (n=9) groups. S1PR1-3 expression is indicated by the brown staining in the cells. Magnification, x400; scale bar, 100  $\mu$ m. IOD/area of (B) S1PR1, (C) S1PR2 and (D) S1PR3 in all groups. Values are expressed as the mean  $\pm$  standard deviation. Parametric one-way analysis of variance followed by Least Significant Difference post hoc test was performed for the comparison of the groups. \*P<0.05 and \*\*P<0.01 vs. NC. NC, normal control; DC, diabetic control; S1P, sphingosine-1-phosphate; S1PR, S1P receptor; IOD, integrated optical density.

development of diabetes (31,32). Apoptosis of  $\beta$ -cell plays an important role in the occurrence and development of diabetes, mediated by glucose, free fatty acids, sulfonylurea, amylin and ceramide (33). The sphingolipid rheostat signalling pathway is a highly conserved balanced system comprising ceramide and pro-apoptotic functions on the one hand, and S1P-induced cell proliferation and survival on the other (7-10,34). Until now, there are only few studies on S1P signaling pathway in diabetes which are in the initial stage and not fully understood, and not much is known about the effect of S1P on islet  $\beta$ -cell proliferation. In the present study, the effects of S1P on the proliferation and apoptosis of pancreatic islet  $\beta$ -cells in type 2 diabetic mice were focused on, and the expression and localization of S1P receptors S1PR1-3 in the pancreatic of type 2 diabetic mice was observed. On the one hand, the present study tried to indicate that S1P promotes proliferation and decreases apoptosis of islet  $\beta$ -cell in diabetes. On the other hand, this study first observed the expression and localization of S1P receptors S1PR1-3 from histomorphology *in situ* in the pancreatic islets of T2DM mice. The present study demonstrated that compared with in the DC group, the islets in the S1P administration group showed a greater insulin immunostaining intensity, higher proliferation rate and increased numbers of Ki-67-positive cells, whereas the apoptosis rates were lower. Currently, apoptosis is categorized into two pathways: Extrinsic and intrinsic pathways, while

intrinsic pathways includes the mitochondrial pathway and endoplasmic reticulum pathway, extrinsic pathways includes the death receptor pathway (35). Apoptosis can be induced through apoptosis inducing factor without relying on caspases in the mitochondrial pathway. Therefore, caspase 3 staining was not chosen but TUNEL staining to determine apoptosis of  $\beta$ -cells (36). These results suggest that S1P leads to an improvement in the morphology of islet  $\beta$ -cells, the promotion of their proliferation and inhibition of apoptosis in diabetic mice, indicating that this compound serves a role in protecting islet cells via S1PRs. Previous studies investigated the role of S1P on islet  $\beta$ -cell proliferation, insulin secretion and apoptosis, and the subsequent prevention of diabetes development in obese mice (37) as well as its effect on other cell types (10,38-42). Furthermore, addition of exogenous S1P at nanomolar levels significantly protected  $\beta$ -cell against cytokine-induced cell death (43), as well as MIN6 cells against palmitate-induced apoptosis (37).

A previous study indicated that S1P was able to significantly stimulate glucose-independent insulin secretion through the activation of phospholipase C in the clonal hamster  $\beta$ -cell HIT-T15 line, as well as in isolated mouse islets (44). The present study demonstrated that S1P had an effect on improving blood glucose control and IPGTT outcomes, suggesting the protective effect of S1P on  $\beta$ -cells in diabetes. Although the results of immunohistochemical staining with Ki67 showed that the

proliferation of  $\beta$ -cells in islets was increased after S1P treatment compared with the DC group, the number of proliferating  $\beta$ -cells was still small and the proliferation rate of Ki67 in islets was <3%, which may still not be enough to secrete enough insulin to reduce blood glucose significantly. So even though the result of blood glucose and some other results showed no statistical significance, the therapeutic and protective effects of S1P on diabetes cannot be denied. In agreement with the results of the present study, S1P has been shown to be important for insulin synthesis and secretion in a rat insulinoma cell line (45). Strong evidence exists supporting the critical roles of S1P on the progression of diabetes mellitus, including insulin sensitivity and secretion, pancreatic  $\beta$ -cell apoptosis, and the development of diabetic inflammatory state (46).

The results of the present study suggested that S1P lead to an improvement in the morphology of islet  $\beta$ -cells, the promotion of their proliferation and inhibition of apoptosis in diabetic mice, indicating that this compound serves a role in protecting islet cells via S1PRs. S1P has been reported to regulate insulin resistance through receptor-mediated pathways in pancreatic  $\beta$ -cells, but the specific S1PR subtypes involved remain unknown. Among the five cognate receptors (S1PR1-5), a previous study reported that islet and INS-1 cells expressed S1PR1, S1PR2, S1PR3 and S1PR4 subtypes (27). S1P signalling is mediated via activation of specific S1PRs (S1PR1-5), of which mouse islet  $\beta$ -cell express mainly S1PR1-3 (22). Mechanistically, S1P decreased and inhibited adipocyte proliferation and differentiation via the downregulation of S1PR1, and decreased the activity of the peroxisome proliferator activator receptor  $\gamma$  in the adipose tissues of a serine palmitoyltransferase 2 knockout mouse model (47). In addition, S1PR1, S1P seemed to counteract insulin signalling and confer insulin resistance via S1PR2 in pancreatic  $\beta$ -cells (4,48). Specific S1PR2 antagonist JTE-013-treatment in S1PR2-deficient mice attenuated  $\beta$ -cell apoptosis, ameliorated blood glucose elevation, rescued  $\beta$ -cell damage and decreased the incidence of diabetes in STZ-induced wild-type models (49,50). In agreement with these findings, the present study demonstrated that extracellular S1P induced proliferation and decreased apoptosis in pancreatic  $\beta$ -cells. The present study also indicated that the expression of S1PR1 and S1PR2 proteins in diabetic mice group were increased compared with the normal control group, while S1PR3 showed no difference. S1PR1 and S1PR2 may play a certain role in the pathogenesis and pathophysiological changes in T2DM, whereas the S1PR3 subtype may not be involved in diabetes. All these results confirmed that the S1P signaling pathway was involved in the development of T2DM.

However, the study has certain limitations. The sample size of the animals included was relatively small and the course, and observation time of S1P administration was short. The protein expression of the S1PR subtypes was detected in pancreatic islets using immunohistochemical staining, but not further confirmed by isolation of the mouse islet  $\beta$ -cells and detection using reverse transcription PCR and western blot analysis. Each step in the process of immunohistochemical staining may affect the final result of staining. The influencing factors include the fixed time of tissue samples, the thickness of the slide, the time and method of antigen retrieval process, the time of antibody incubation, the time of DAB color rendering

and the time of hematoxylin redyeing. Therefore, the present study strictly followed the same time and conditions in the process of immunohistochemical staining, so as to minimize the differences in staining conditions. Although immunohistochemistry is only a semi-quantitative analysis, the results can explain certain problems under strict experimental operation. Finally, further animal and cell experiments to investigate the mechanism of S1P signalling in diabetes are required.

In conclusion, the present study demonstrated that S1P serves a positive role in protecting islet  $\beta$ -cells against apoptosis, suggesting the physiological significance of S1P in preserving  $\beta$ -cell mass in diet-induced diabetic mice, indicating a potential novel therapeutic strategy for delaying the occurrence and development of diabetes. However, to date, the role of S1P on  $\beta$ -cell function and diabetes mellitus is still not fully understood, this study has laid a foundation for the further research on the relationship between S1P and diabetes. Further studies are required to address the function of S1P and S1PR subtypes in islet  $\beta$ -cells.

### Acknowledgements

The authors thank Dr Gang Tian, The First Affiliated Hospital of Xi'an Jiaotong University, for assisting with helpful suggestions and a review of the manuscript.

### Funding

The present study was supported by The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China (grant no. 2013YK20), the Clinical Research Award of the First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China (grant no. XJTU1AF-CRF-2016-016) and the Chinese Medical Association (grant no. 13040470432).

### Availability of data and materials

The datasets used and/or analysed during the present study are available from the corresponding author on reasonable request.

### Authors' contributions

JS designed and conceived the study. YH designed and performed the experiments. BS and XZ analysed the data. JS and YH wrote the manuscript. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

All animal procedures were approved by the Institutional Animal Care and Use Committee of the Xi'an Jiaotong University of Health Sciences, Xi'an, China.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.



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