Bee Pollen Polysaccharide From *Rosa rugosa* Thunb. (Rosaceae) Promotes Pancreatic β-Cell Proliferation and Insulin Secretion

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Insufficient pancreatic β-cell or insulin-producing β-cell are implicated in all types of diabetes mellitus. Our previous studies showed bee pollen polysaccharide RBPP-P improves insulin resistance in type 2 diabetic mice by inhibiting liver fat deposition. However, its potential of regulating β-cell function and integrity is not fully known. Herein, we observed that β-cell proliferation (*n*/equals 10), insulin synthesis (*n*/equals 5, *p*/equals 0.01684) and insulin incretion (*n*/equals 5, *p*/equals 0.02115) were intensely activated in MIN6 cells when treatment with RBPP-P. In alloxan-induced diabetic mice, oral administration of RBPP-P (*n*/equals 10) effectively decreased the blood glucose (*p*/equals 0.0326), drink intake (*p*/equals < 0.001) and urine (*p*/equals < 0.001). It directly stimulated phosphorylation of p38 (*p*/equals 0.00439), ERK (*p*/equals 0.02951) and AKT (*p*/equals 0.0072) to maintain the islet function and mass. Thus, our data suggest that RBPP-P is a natural compound to regulate β-cell proliferation and function, indicating it might have therapeutic potential against type 1 diabetes.

Keywords: bee pollen polysaccharides, insulin secretion, β-cell proliferation, T1DM, antidiabetic activity

INTRODUCTION

Type 1 diabetes (T1DM) is an autoimmune disorder characterized by the destruction of insulin-producing pancreatic β-cell and resultant hyperglycemia (Wilcox et al., 2016; Katsarou et al., 2017). The treatment of diabetes is mainly based on administration of insulin, the replacement or regeneration of insulin-producing cells, pancreas transplantation (Atkinson et al., 2014). Near perfect control of blood glucose levels can be restored by pancreas or islet transplantation, without the risk of serious hypoglycemic episodes that are associated with intensive insulin therapy. However, current protocols are inadequate to prevent islet rejection long-term, caused harmful side effects and some of them are expensive (Hirshberg et al., 2003). Around 80% of the world’s population uses natural plants and their bioactive compositions for effective, less expensive and low-toxicity treatment (World Health Organization, 2017). These compounds have relative ability to reduce blood glucose while maintaining their growth and secreting properties targeting the endogenous β-cell. Thus, increasing the number of pancreatic β-cell or enhancing the function of islets is an effective way to treat T1DM (Mustafa et al., 2012; Vetere et al., 2014; Alejandro et al., 2015).

The proliferation of β-cell is exquisitely regulated to meet metabolic demand through complex mechanisms that involve the integration and interaction of multiple factors (Bernal-Mizrachi et al., 2014), but the mechanisms are still unclear. Insulin is synthesized by pancreatic β-cell and plays a predominant role in glucose homeostasis. Insulin or insulin-like growth factor (IGF) knockout...
non-diabetic mice exhibited the decrease of β-cell mass, suggesting that their important roles in regulation of β-cell proliferation (Sabir et al., 2018). Mice have two genes encoding insulin, Ins1 and Ins2. The absence of Ins1 hinders the progress of T1DM, but the absence of Ins2 accelerates the development of T1DM (Babaya et al., 2006). Besides that, the transcription of the insulin gene is regulated by many factors, including Mafa, Pdx1 and Neuro D1 (Zhang et al., 2020). Several signaling pathways also participated in β-cell proliferation. Recent studies have suggested crosstalk between AKT and islet-enriched transcription factors (Humphrey et al., 2010; Weng et al., 2020). MAPKs, including ERKs, JNKs and p38-MAPKs, have been found to be associated with cell survival, proliferation and stress response (Trempolec et al., 2013).

Bee pollen is the most popular natural health food in the world, and polysaccharide is the main bioactive components. Studies have shown that bee pollen polysaccharides have different functions, such as immunomodulation (Li et al., 2009), hypoglycemic (Gong et al., 2017), hypolipidemic (Denisow et al., 2016), anti-aging (Graikou et al., 2008) and anti-tumor (Wang et al., 2013). In our previous work, bee pollen polysaccharide from Rosa rugosa Thunb. (Rosaceae) were isolated and fractionated into three fractions (RBPP, RBPP-N, RBPP-P), RBPP-P could reduce the levels of blood glucose and lipid in type 2 diabetes (T2DM) mice by alleviating liver steatosis and insulin resistance (Gong et al., 2017). However, the mechanism of RBPP-P in the treatment of protecting the pancreas functions and promoting insulin secretion in T1DM is not fully elucidated. Thus, in this study we evaluated the effects of RBPP-P on β-cell proliferation and pancreas function in vitro and in vivo.

**MATERIALS AND METHODS**

**Bee Pollen Polysaccharides Preparation and Fractionation**

Bee pollen obtained from Rosa rugosa Thunb. (Rosaceae) was isolated and fractionated based on our previous report (Gong et al., 2017). Briefly, bee pollen powder was extracted with hot water and polysaccharides (RBPP) were precipitated by ethanol. RBPP was further fractionated by DEAE-cellulose chromatography into the acidic fraction (RBPP-P).

**Bee Pollen Polysaccharides Characterization**

The total carbohydrate content was determined using the phenol sulfuric acid method with glucose as the standard. The sugar compositions were analyzed by HPLC, and the structural feature of RBPP-P was analyzed by FT-IR and 13C-NMR as described in previous publications (Gong et al., 2017).

**Cell Culture**

MIN6 cells (mice pancreatic β-cell line) were obtained from the American Type Culture Collection. MIN6 cells were maintained in DMEM supplemented with 15% fetal calf serum, 2 mM L-glutamine (Gibco, 35050-061), 1 mM pyruvate (Aladdin, S104174) and 285 μM β-mercaptoethanol (Gen-view, GM195) at 37°C in a 5% CO2 incubator.

**Cell Viability Assay**

For MTT assay, MIN6 cells were seeded at the density of 1.5 × 10^4 cells on 96-well plate for 24 h. They were grown in pretreatment medium without (as control) or with 0.01, 0.1, and 1.0 mg/ml of polysaccharides for 24 h. The media were removed and 100 μl MTT (0.5 mg/ml) was added and incubated for additional 4 h. For dissolving formazan, 50 μl of 20% SDS/0.04% HCl solution was added to each well, and incubated in 37°C overnight. The absorbance at 570 nm was measured using a microplate reader (Biotek, United States). Cell proliferation under all the conditions was expressed as a percentage of the control, which was set at 100%.

**Ki-67 Immunostaining**

MIN6 cells were seeded on 24-well tissue culture plate at 1.5 × 10^5 cells and incubated for 24 h. The cells were grown for 24 h in pretreatment medium without (as control) or with 0.1 mg/ml of polysaccharides. After that, cells were gently washed with PBS and then fixed for 20 min at room temperature in 4% paraformaldehyde. Fixed cells were permeabilized in 0.1% Triton X-100/PBS for 15 min and then blocked with 2% BSA/5% bovine serum in PBS at room temperature for 20 min. Afterward, cells were incubated with a rabbit monoclonal antibody against Ki-67 (CST, 9129S, 1:500) for 1 h. After washing with PBS, cells were incubated with Alexa Flur™ 594-conjugated goat anti rabbit antibody (Invitrogen, A11072, 1:100) and DAPI (1:500) for 40 min. The images were taken using epifluorescence microscope (Olympus BX51) with ×40 objective.

**Insulin Secretion Assay**

MIN6 cells were seeded in 48-well plate at 5 × 10^5 cells for 24 h, followed by 0.1 mg/ml polysaccharides or 30 mM KCl for 24 h. Then cells were incubated in KRB balanced buffer (115 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 20 mM NaHCO3, and 16 mM HEPES; pH 7.4) containing 0.2% BSA for 2 h. Medium was then replaced with KRB containing 5.5 mM glucose, 5.5 mM glucose plus 30 mM KCl or 5.5 mM glucose plus 1.0 mg/ml of different polysaccharides for 1 h, respectively. Supernatant was collected and insulin content was measured by ELISA (Innovation Beyond Limits, Germany). The cells were lyzed with lysis buffer (Boster, AR0102) for measurement of total protein content. The insulin secretion was defined as insulin content/protein content.

**qRT-PCR Assay**

qRT-PCR was established as described previously (Yang et al., 2020). MIN6 cells were isolated using TRIZOL (Invitrogen) and cDNA was generated from 1 μg of RNA using M-MLV reverse transcriptase (Promega, Fitchburg, WI, United States). The 20 μl of PCR mixture contained 1 μl of cDNA. Real-time PCR assays were conducted with a LC480 Light Cycler (Roche, Germany) using the applied primer sequences listed Table 1. PCR amplification was conducted as following: denaturation at 95°C
for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 45 s. GAPDH were used as internal standards for mRNAs. Relative expression of genes was determined using a comparative method (2^−ΔΔCT).

Western Blot
Pancreases were lysed in lysis buffer, containing 50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM EGTA, 1% TritonX-100, 100 mM NaF, 10 mM Na3P2O7 and 1 mM PMSF. The supernatants were collected after centrifugation, and the protein concentration was determined using the Coomassie Brilliant Blue (BBI, A610037-0025) assay. 30 μg tissue lysate was detected by western blot and probed for antibodies against phospho-p38 (Thr180/Tyr182) antibody (CST, 9215), p38 antibody (CST, 9212), phospho-ERK1/2 (Thr202/Tyr204) antibody (CST, 9101), ERK1/2 antibody (CST, 9107), phospho-Akt (Ser473) antibody (CST, 9271), Akt antibody (CST, 9272), actin (BD Biosciences, 612,657). Quantification was performed using the ImageJ software (Rockville, MD, United States).

Animal Treatment
Male C57BL/6J mice were obtained from GemPharmatech Co., Ltd. (Nanjing, China). All experiments were approved by the Animal Care and Use Committee of Northeast Normal University (SYXK 2018-0015). The mice were housed in a temperature-controlled facility (21°C, 12 h light/12 h dark cycle, 60–70% humidity), provided standard laboratory chow and water, and were subjected to treatment at 5 weeks of age.

Induction T1DM
To induce T1DM, the animals in the experimental group were fasted for 24 h, able to freely access water. Mice were injected intraperitoneally with fresh prepared alloxan (ALX, 150 mg/kg; sigma, A7413) saline solution for consecutive five days. Fasting blood glucose of the animals were measured and only the mice with blood glucose levels above 200 mg/dl were used for the experiments.

Experimental Design
Thirty mice were randomly divided into three groups (n = 10) and treated oral daily for 28 days. Group I: normal control; Group II: ALX control; Group III: ALX mice treated with RBPP-P (100 mg/kg). Fasting blood glucose (FBG), body weight and food intake were measured each week of the experiment. Urine volume and water consumption of animals were evaluated on the 28th days of the experiment. At the end of study, blood and organ samples were collected for the determination of biochemical parameters.

Statistical Analysis
The results were expressed as the means ± s.d. Statistical analysis of the data was performed using Student’s t-test and two-way repeated-measures ANOVA with Dunnett’s post-hoc test (IBM SPSS Statistics 17.0, Armonk, NY, United States). All experiments were repeated at least three times. The level of significance was set at *p < 0.05, **p < 0.01, ***p < 0.001.
RESULTS

Preparation and Characterization of RBPP-P

Bee pollen polysaccharide (RBPP) was obtained from Rosa rugosa Thunb. (Rosaceae) by hot water extraction and ethanol precipitation. It was then separated by DEAE-Cellulose column and eluted with 0.4 M NaCl into an acidic fraction (RBPP-P), contains 93.6% of total carbohydrate (Figure 1A). Monosaccharide composition analysis by HPLC showed that RBPP-P is mainly composed of Ara (arabinose, 50.6%), Gal (galactose, 22.6%), GalA (galacturonic acid, 12.8%) and Rha (rhamnose, 5.6%), Glc (glucose, 4.4%), indicating that the polysaccharides we extracted are consistent with before (Figure 1B). Structural features of RBPP-P were characterized by analyzing 13C-NMR and FT-IR spectrum (Figures 1C,D), indicated that the polysaccharides we extracted are consistent with before (Figure 1B). Structural features of RBPP-P were characterized by analyzing 13C-NMR and FT-IR spectrum (Figures 1C,D), indicated that RBPP-P contained more proportion of arabinogalactan (AG) fragments and small amounts of rhamnogalacturonan I (RG-I) and homogalacturonan (HG) domains.

RBPP-P Activated β-Cell Proliferation and Insulin Synthesis in vitro

To investigate the polysaccharide caused proliferative effect, MIN6 cells were treated with increasing concentration of bee pollen polysaccharides for 24 h. Cell viabilities were assessed by MTT assay. Concentration-effect relationship indicated that the enhancement of cell proliferation by RBPP-P was 20.8, 45 and 30.3% at the concentration of 0.01, 0.1 and 1.0 mg/ml (Figure 2A). Followed, carried out Ki-67 immunostaining which cells stained with Ki-67 (in red) and DAPI (in blue) under epi-fluorescence microscope. Ki-67 only stained the proliferating cells in the active cell cycle, but not the resting cells. When compared with control cells, RBPP-P appeared more cells in active cell cycle (Figure 2B). The results were consistent with that of MTT assay that RBPP-P might play a role on stimulation cell proliferation.

We further investigated the potential role of RBPP-P in regulation of insulin expression. When compared with control cells, 0.1 mg/ml of RBPP-P activated the Ins2 mRNA in Min6 cells, but not Ins1 (Figure 2C). Because MafA is a master regulator of the insulin gene, we determined the effect of RBPP-P on its expression and found that MafA was strongly increased in potassium chloride (KCl) and RBPP-P cells (Figure 2D). Pdx1 (pancreas-duodenum homeobox1) is a key transcription factor which activated insulin synthesis and stimulated pancreatic beta cell proliferation. As expected, the expression of Pdx1 was stimulated with RBPP-P treatment (Figure 2D).

Next, we performed glucose stimulated insulin secretion. KCl treated at 30 mM induced strong insulin secretion in cells. Compared to control cells, RBPP-P also exhibited insulin release at basal glucose (Figure 2E). Collectively, these data

FIGURE 1 | Preparation and characterization of bee pollen polysaccharide. (A) The procedure used to prepare RBPP-P from Rosa rugosa Thunb. (Rosaceae). (B) Analysis of the monosaccharide composition of RBPP-P using high performance liquid chromatography (HPLC). (C) Analysis of chemical structure of RBPP-P using 13C-nuclear magnetic resonance (13C-NMR). (D) Analysis of the chemical structure of RBPP-P using fourier transform-infrared spectroscopy (FT-IR).
supported that RBPP-P modulates MafA and Pdx1 protein and activates Ins2 gene expression, showed its ability to activate β-cell proliferation and insulin synthesis.

**RBPP-P Reduced Blood Glucose and Alleviated the Symptoms of Diabetes in ALX-Induced Diabetic Mice**

We additionally investigated whether RBPP-P activation in β-cells reverses the hyperglycemia induced by alloxan (ALX). Intraperitoneal injection of alloxan monohydrate (150 mg/kg) caused over 2-fold elevation of blood glucose level, which was maintained over a period of 4 weeks. Daily oral treatment with 100 mg/kg of RBPP-P for 4 weeks, led to a significant decrease in fasting blood glucose (FBG) levels (Figures 3A,B). The results were in accordance with the previous results that RBPP-P had an anti-diabetes potential in HFD-mice.

Weight loss, polyphagia, polyuria and polydipsia are the main features of T1DM. Although administration of RBPP-P had the potential to block the ALX effect on body weight (Figure 3C), RBPP-P failed to reverse the increase in food intake after ALX damaged (Figure 3D). However, after treatment for 4 weeks, RBPP-P mice showed significantly decrease in water consumption (Figure 3E), urinary volume (Figure 3F) and ketone bodies level (Figure 3G) compared with ALX mice. This suggested that RBPP-P has shown a significant antidiabetic effect in alloxan-induced diabetic mice.

**RBPP-P Restored Islet Function of ALX-Mice**

Islet β-cell dysfunction leads to insulin insufficiency, a contributor to the onset of hyperglycemia in T1DM. We performed immunofluorescent staining of pancreatic sections using specific antibodies against insulin and glucagon. The C57BL/6J group showed islets of normal size, however, the ALX group observed reduction in the size of the insulin-expressing β-cells, enlarged the area of glucagon-expressing α-cells, and the disappearance of islet borders. This damage was considerably reduced after the administration of the RBPP-P, which was highly expressed of β-cells, and decreased α-cells located in the outer rim of the pancreatic islets (Figure 4A). It was demonstrated that RBPP-P displayed significantly increased the β-cell area/pancreatic area ratio with ALX group (Figure 4B). In addition, the insulin level was significantly increased of the RBPP-P treatment compared to the ALX mice (Figure 4C). These results showed RBPP-P
preserved structural integrity of pancreatic and restored islet function.

**RBPP-P Stimulates MAPK and AKT Signaling Pathways**

MAPK and AKT pathways have been shown to play key roles in regulation of the cell proliferation in mammalian cells. To elucidate if these classic pathways involved in RBPP-P reversed hyperglycemia in ALX-induced mice, we tested the expression levels focusing on pancreatic islet. Results showed that RBPP-P treated mice exhibited increased the phosphorylated levels of p38, ERK1/2 and Akt (S473) compared to C57BL/6J mice (Figures 5A,B). Taken together with other results in mice, suggested that AKT and MAPK signaling induction by RBPP-P plays a critical role in regulation of hypoglycemia.

**DISCUSSION**

The relevance between diabetes and β-cell dysfunction is a fundamental research topic for the development of new therapies increasing insulin secretion and optimizing metabolism of T1DM. Here, we prepared the active bee pollen polysaccharide RBPP-P from *Rosa rugose*, attempted to examine the effect of anti-T1DM and possible molecular mechanism of RBPP-P improving β-cell function.

Deregulation of a subset of transcriptional regulators in pancreatic β-cell is underlined a fundamental cause of β-cell dysfunction leading to T1DM (Harmon et al., 2005). The results that RBPP-P activated Ins2 gene expression by modulating MafA1 and Pdx1, which are critical transcription factors for insulin gene expression and secretion. Thus, RBPP-P promotes insulin production and induces expression of transcription regulators maintaining β-cell maturity.

T1DM is a metabolic disease characterized by impaired insulin secretion and resultant hyperglycemia (Lebovita, 2010). Weight loss, polyphagia, polyuria and polydipsia are the main features of T1DM (Bibak et al., 2014). Numerous studies have shown that alloxan destroys insulin-producing β-cells, a chemical commonly used in laboratories to make diabetic models (Katahira et al., 2020). Administration of RBPP-P (100 mg/kg) for 4 weeks showed marked hypoglycemic effect in ALX-induced diabetic mice. RBPP-P significantly reduced the signs of polydipsia and polyuria seen in diabetic mice, which could be the result of better control of hyperglycemia in T1DM. However, body weight and food intake were not significantly different between RBPP-P and ALX animals. Studies have reported that polysaccharides could restore damaged islets (Yang et al., 2019), histological examination in our studies showed RBPP-P significantly reversed the damage in islet induced by alloxan, increased the β-cell area/pancreatic area ratio. These results are
consistent with our previous work of RBPP-P carried out on the model of diabetes induced by HFD (Gong et al., 2017).

Cell proliferation, growth and survival are mediated by complex intracellular signaling network including several major pathways: PI3K/Akt and MAPK (p38, Erk and JNK) (Zhang and Liu, 2002; Song et al., 2019; Xie et al., 2021). We have observed that RBPP-P treatment increases the phosphorylation of p38, Erk1/2 and AKT in pancreas. Increasing evidence showed polysaccharides regulated cell proliferation via one of these signaling pathways or even several pathways synchronously. Lycium barbarum polysaccharide induced IGF-1-stimulated proliferation of MCF-7 cell through the ERK pathway (Huang et al., 2012). Capsosiphon fulvescens polysaccharides regulated effector β-catenin and Erk1/2 activation induced rat small intestinal epithelial cell proliferation (Go et al., 2011). IGF stimulated INS-1 cell proliferation via its downstream MAPK, which could phosphorylate ERK1/2 and p38 (Trumper et al., 2001). Thus, we speculated RBPP-P could stimulate growth factor to indirectly act on pancreatic beta cell, perhaps it may active multiple growth factors synchronously.

Based on these findings, we have explained how RBPP-P modulates β-cell production and β-cell function. RBPP-P activated key transcription factor MafA and Pdx1 in β-cells, increasing insulin expression and insulin secretion. RBPP-P also upregulates the phosphorylation levels of p38, ERK and AKT, promotes β-cell proliferation. Oral administration of RBPP-P showed potential hypoglycemic activities, improving the symptoms of diabetes, protecting the pancreas in T1DM mice, which maintains β-cell function and glucose homeostasis. Thus, RBPP-P could be used as a potentially natural functional food for the prevention and treatment of T1DM. Further pharmacological and biochemical studies are underway to elucidate the specific mechanism of RBPP-P for β-cell insulin secretion.

**FIGURE 4** | Effects of the RBPP-P on islet histology. (A) Immunofluorescence (IF) staining for insulin (green) and glucagon (red). Scale bar, 100 µm. (B) β-cell area/pancreatic area ratio, measured by immunofluorescence staining (n = 5). (C) Insulin levels in serum (n = 10). All data are expressed as mean ± s. d. *p < 0.05, vs. C57BL/6J mice; **p < 0.05, vs. ALX mice.
DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care and Use Committee of Northeast Normal University (SYXK 2018-0015).

AUTHOR CONTRIBUTIONS

All authors participated in the design of the study. SY were responsible for most experiments in vivo and in vitro. YQ prepared the polysaccharide fraction. JC detected mice pancreatic islets morphologies. SC examined insulin secretion of RBPP-P. LS revised the manuscript. YZ contributed to suggest in revised manuscript. YF contributed to data interpretation and the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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