Carbohydrate Polymers 117 (2015) 63–69

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Anti-diabetic effect mediated by Ramulus mori polysaccharides

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ARTICLE INFO

Article history:
Received 29 June 2014
Received in revised form
16 September 2014
Accepted 18 September 2014
Available online 28 September 2014

Keywords:
Ramulus mori polysaccharides
Hyperglycemia
Apoptosis
Metabolism

ABSTRACT

Diabetes mellitus is a complicated metabolic disease, whose pathogenesis is related to apoptosis within pancreatic tissue. In this study, the potential therapeutic benefits of Ramulus mori polysaccharides (RMP) on streptozotocin (STZ)-induced diabetic mice were evaluated. Our experiments indicated that RMP lowered hyperglycemia and increased insulin levels in diabetic mice. Histopathological examination revealed that RMP contributed to the reduction of STZ-lesioned pancreatic cells. In addition, the serum level of HbA1c was decreased. RMP treatment also showed increased Bcl-2 expression and reduced Bax protein level in pancreatic tissue. Furthermore, intrapancreatic expressions of p-JNK, p-p38 and cleaved-caspase-3 were down-regulated by RMP treatment. Collectively, the findings demonstrate that RMP exerts the pronounced hypoglycemic effect via regulation of the intrapancreatic JNK/p38 pathway to protect against STZ-induced apoptosis in pancreatic tissue, eventually ameliorating metabolic function in the pancreas.

1. Introduction

Diabetes mellitus (DM) is a chronic disease that is associated with metabolic dysfunction and is usually accompanied by severe complications (Tendon, Ali, & Narayan, 2012). Sustained hyperglycemia in patient can appear in classical symptoms, such as polyuria, polydipsia and polyphagia (Umpierrez et al., 2012). Due to pancreatic hypofunction characterized by insulin insufficiency, patients with DM may suffer from life-threatening comorbidities over time (Gerber et al., 2008). Thus, correcting hyperglycemia or hypoinsulinemia is an effective strategy for DM prophylaxis and treatment. At present, pharmacotherapy that is clinically utilized for diabetic therapy has achieved some successes. In particular, metformin (MF), an oral hypoglycemic medicine, is a first-line drug used in diabetes management (Moon, Bascombe, & Holt, 2007; Nakatani et al., 2012). Streptozotocin (STZ), a poisonous DNA alkylating reagent, performs targeted injury to pancreatic beta cells, presenting diabetes-like symptoms (Lenzen, 2008). The uncontrolled apoptosis (excessive cell death) can result in metabolic disorders, cancerous diseases, and tissue impairment (Elmore, 2007). Notably, type 1 diabetes treatment primarily acts on the reduction of pancreatic cell lesions via inhibition of apoptosis (Johnson & Luciani, 2010). Numerous scientific studies have demonstrated that the application of Traditional Chinese medicine (TCM) to diabetic control and clinical amelioration of syndromes has been achieved (Tong, Dong, Chen, & Zhen, 2012). Generally, the prescription of TCM refers to multicomponent recipes that are designed to produce the potent therapeutic effect with minimal toxicity (Yang, Chen, Wang, Lee, & Lai, 2009). Ramulus mori (Sangzhi named in Chinese) is the dried slender branch of *Morus alba* L. (Moraceae). Some reports have demonstrated that Ramulus mori extracts play the beneficial role in reduction of blood glucose and the amelioration of renal function in a manner that is similar to orthodox medication (Ye, Shen, & Xie, 2002; Shi et al., 2012). In addition, our preliminary research showed that Ramulus mori polysaccharides (RMP) exert the marked hypoglycemic effect in STZ-diabetic mice (Guo et al., 2013). However, the molecular mechanism of RMP-mediated anti-apoptotic effect in pancreatic cells remains relatively unexplored. Thus, this study was designed to further investigate the relationship between RMP-mediated hypoglycemic activity and apoptosis-dependent signaling pathway in STZ-diabetic mice.

2. Materials and methods

2.1. Chemicals

STZ (Sigma, USA) was dissolved in fresh sodium citrate buffer (pH 4.5, 0.1 mol/L), and the solution was stored in the dark to avert

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http://dx.doi.org/10.1016/j.carbpol.2014.09.052
0144-8617/

degradation. Metformin (MF, Beijing Jingfeng Pharmaceutical Co., Ltd., China) was stored as the solution in distilled water until use.

2.2. Preparation process and structural characterization of RMP

RMP preparation was detailed in our previous study (Guo et al., 2013). In brief, pulverized Ramulus mori (1 kg) was marinated in 10 L petroleum ether to degrease through a reflux condenser for 1.5 h. Used petroleum ether was volatilized prior to crude extracts being boiled twice for 1 h each time (herb:water = 1:5, w/v). Collected filtrate was separated via centrifugation at $1500 \times g$ for 10 min, and then aqueous extracts were prepared as 100 mL concentrated solution, followed by mixing with chloroform-*n* butanol (5:1 ratio, v/v, six times) to remove the unwanted protein in according to canonical Sevag method. After being stored at 12 h, the extracts were subjected to edulcoration with 92% ethanol (ratio 1:5, v/v), and partition with ethyl ether (1:3 ratio, v/v), dehydration via alcohol (1:4 ratio, v/v) and acetone (1:5 ratio, v/v, six times), then harvesting crude polysaccharides for further use. These primary polysaccharides were purified using a chromatography system, then RMP yielding ratio showing 89.6% (w/w) via determination analyses. The molecular weight was $4.6 \times 10^5 Mw$ (Da) through conducting Gel permeation chromatography method.

RMP was subjected to hydrolyzation prior to gas chromatography testing. 20 mg RMP was precisely weighed and placed into a test tube, followed by mixing with 2 mL trifluoroacetic acid solution (5 mol/L) at a water-bath with 100 °C water for further hydrolysis. After 10 h reaction, tube-loaded mixture was dried via nitrogen dioxide gas as solids. Subsequently, 20 mg monosaccharide standards of rhamnose, arabinose, xylose, mannose, glucose, galactose were accurately weighed and placed into different test-tubes. Hydrochloric acid amine (20 mg) and pyridine (0.5 mL) were added in the tubes, and then put into 90 °C water for 1 h. The mixture was removed for cooling at room temperature, and added in 1 mL acetic anhydride at 90 °C water for 1 h. After acetylation, reaction products was dried via nitrogen dioxide gas, and re-dissolved with 2 mL chloroform. 1 μL standard derivant mixture and hydrolyzed monosaccharides were detected by GC-201 series chromatographic system (Shimadzu, Japan), respectively. A DM-5 column (0.25 μm, 30 m × 0.25 mm i.d.) was used and the flow rate was controlled at 30–300 mL/min. The mobile phase was consisted of nitrogen dioxide (A) and hydrogen (B). The data analysis indicated that RMP mainly contains glucose (62.1%), and other galactose (13.8%), mannose (6.7%), rhamnose (11.6%) and arabinose (5.8%), respectively.

2.3. Animals and drug intervention

Sixty male mice (specific pathogen free), weighing approximately 18–20 g, were purchased from the Medical Laboratory Animal Center of Guangxi Medical University, China (No. SCXKG 2009-0001). The mice were quarantined and acclimated in controlled conditions with temperature of 25 ± 2 °C, relative humidity of 60 ± 10%, room air changes 12–18 times/h, and a 12 h light/dark cycle.

After adaptive feeding for seven days, the mice were intraperitoneally injected with STZ buffer solution (150 mg/kg). Caudal vein blood samples (100 μL) were taken from treated mice at 72 h. The mice presented fasting blood- glucose (FBG) values above 11.1 mmol/L were used to categorize as diabetic animals. STZ-diabetic mice were assigned to different groups (15 mice/group): the diabetic control group, the RMP-treated group (600 mg/kg/d), and the MF-treated group (600 mg/kg/d). Another fifteen healthy mice were selected as normal control group. The mice received dose of RMP or MF intragastrically daily for two weeks. In parallel, the mice from the normal and diabetic control groups were given equal volume of normal saline. The study was conducted in accordance with US guidelines (NIH publication no. 85-23, revised in 1985) for laboratory animal use and care.

2.4. Measurement of FBG

Procedures were conducted using a commercial glucose-reagent kit (Maker Science and Technology Co., Ltd., Sichuan, China) according to the manufacturer's protocols. The results (15 mice/group) were calculated and reported in mmol/L.

2.5. Measurement of hemoglobin A1c (HbA1c) concentration in serum

At the end of the experiment, the blood samples were collected and prepared by removal of precarious substances through the cation exchange resin. Absorption at 415 nm was measured using a spectrophotometer (BioSystems Ltd., Spain). The results (15 mice/group) were obtained according to the manufacturer's instructions.

2.6. Measurement of fasting serum insulin (FNS)

Testing was conducted using a commercial insulin radioimmunoassay kit (Maker Science and Technology Co., Ltd, Sichuan, China) according to the supplier's instructions. The results (15 mice/group) were calculated and reported in μU/ml.

2.7. Pathologic observation

Sectioned pancreatic samples (5 mice/group) were stained using hematoxylin and eosin (H&E) and were then examined for histophysiological changes under a light microscope (DMR + 550, Leica, Wetzlar, Germany).

2.8. Transmission electron microscopy examination

The pancreatic samples (5 mice/group) were fixed with 2.5% glutaraldehyde and 4% paraformaldehyde solution in PBS buffer for 3 h at 4 °C. After washing with PBS solution three times, specimens were re-fixed for 2 h with 1% osmium tetroxide. After stepwise dehydration with ethanol, the resin-embedded slices were stained with 3% urinal acetate/lead citrate. Each channel plate was scanned through an electron microscope at 80 kV (Hitachi, H-7650, Japan).

2.9. Western blot analysis for target proteins

The pancreatic tissues (100 mg) collected from each group were homogenized in ice lysis buffer at $8000 \times g$ for 10 min. The suspensions were subjected to ultrasonics using a cell disintegrator, and harvested protein contents were measured using a bicinchoninic acid procedure. The proteins were loaded onto a 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and separated under 90 V for 3 h. Then, transferred membranes were blocked for 1 h in 5% defatted milk powder and incubated with primary monoclonal antibodies against Bcl-2, Bax, p-JNK, p-p38, cleaved-caspase-3 (Santa Cruz, USA; 1:500), and beta-actin (Santa Cruz, USA; 1:800) overnight at 4 °C. Following three washes with TBST, the transmembranes were incubated with anti-mouse IgG antibodies conjugated with horseradish peroxidase for 1 h at 37 °C (Santa Cruz, USA; 1:800). The signals were detected using the ECL detection system (Pierce, USA), and the expression level was determined by Quantity One software (Bio-Rad, USA).

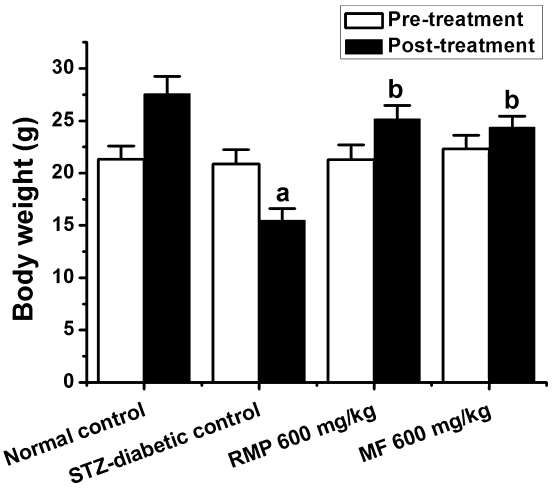

Fig. 1. RMP inhibited the body weight loss in diabetic mice.
The results were analyzed by one-way ANOVA followed by Tukey's post hoc test, and data were expressed as the Mean ± S.E. ($n = 15$). Notes: vs. normal control group, [a] $P < 0.01$; vs. STZ-diabetic control group, [b] $P < 0.01$.

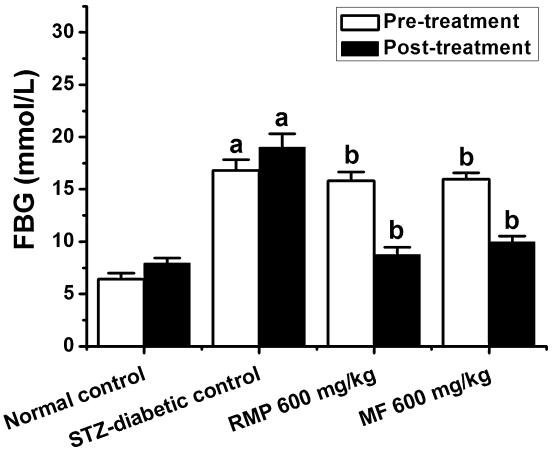

Fig. 2. RMP reduced the FBG in diabetic mice.
The results were analyzed by one-way ANOVA followed by Tukey's post hoc test, and data were expressed as the Mean ± S.E. ($n = 15$). Notes: vs. normal control group, [a] $P < 0.01$; vs. STZ-diabetic control group, [b] $P < 0.01$.

2.10. Statistical analysis

Data were expressed as the mean ± S.E. and analyzed by one-way ANOVA followed by Tukey's post hoc test using SPSS 13.0 software (SPSS, Chicago, IL, USA). P values less than 0.05 were considered statistically significant.

3. Results

3.1. RMP increased the body weight in diabetic mice

The potential protective role of RMP in diabetic mice was investigated by monitoring vital signs. STZ-diabetic mice exhibited significant emaciation, as evidenced by their reduced body weight compared with normal mice ($P < 0.01$, $n = 15$). After the RMP and MF treatments, the weight loss of STZ-diabetic mice was noticeably attenuated, which was reflected in elevated body weight in the tested mice ($P < 0.01$, $n = 15$) (Fig. 1).

3.2. RMP lowered the FBG level in diabetic mice

To investigate changes in blood glucose, the FBG was determined for all treated mice. The results suggested that serum FBG level in STZ-diabetic mice were markedly elevated compared with normal mice ($P < 0.01$, $n = 15$). Accordingly, the increased glycemia of the diabetic mice was effectively attenuated by RMP and MF treatments, as shown in the reduction of FBG when compared with that of untreated diabetic mice ($P < 0.01$, $n = 15$) (Fig. 2).

3.3. RMP decreased the serum HbA1c concentration in diabetic mice

To characterize the metabolic capability of RMP-treated mice, serum HbA1c levels were assessed. We found that the HbA1c content in STZ-diabetic mice was obviously increased relative to normal mice ($P < 0.01$, $n = 15$). Strikingly, both the RMP and MF-treatment contributed to the reduction of HbA1c concentration in serum ($P < 0.01$, $n = 15$) (Fig. 3).

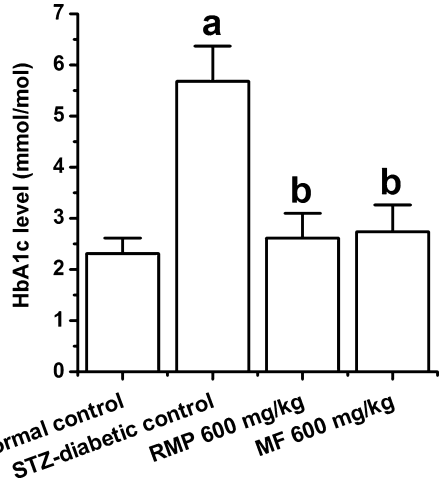

Fig. 3. RMP decreased the serum HbA1c concentration in diabetic mice.
The results were analyzed by one-way ANOVA followed by Tukey's post hoc test, and data were expressed as the Mean ± S.E. ($n = 15$). Notes: vs. normal control group, [a] $P < 0.01$; vs. STZ-diabetic control group, [b] $P < 0.01$.

3.4. RMP raised the FNS in diabetic mice

We sought to determine whether the RMP-mediated hpyerglycemic effect involved FNS changes. The results showed that the plasmatic insulin concentration in STZ-induced mice was significantly reduced when compared with that of normal mice ($P < 0.01$, $n = 15$). Following the RMP and MF treatments, the reduced FNS level in diabetic mice was blocked, as revealed by an increase in FNS when compared with the diabetic control group ($P < 0.01$, $n = 15$) (Fig. 4).

3.5. Histopathologic examination

To characterize the cytoarchitectural changes in the pancreatic tissue, the pathological sections were examined using H&E staining. In pancreatic samples of normal mice, a complete islet structure exhibiting regularly distributed and abundant pancreatic beta cells was observed. On the contrary, islet cytoarchitecture in STZ-lesioned mice showed pronounced endogenous destruction, such as cell rupture, cytolysis and apoptosis. Furthermore, the pancreatic beta cell number was reduced and was accompanied by inflammatory stress and necrosis. After treatment with RMP and MF, the abnormal changes in islet structure were rescued in the STZ lesions, contributing to architectural amelioration of islet structure and a gradual increase in beta cell counts (Fig. 5).

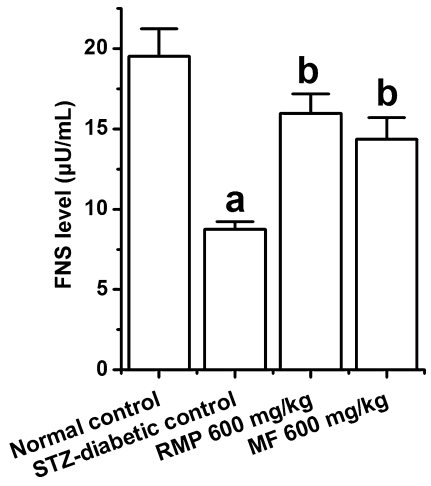

Fig. 4. RMP increased the FNS in diabetic mice.
The results were analyzed by one-way ANOVA followed by Tukey's post hoc test, and data were expressed as the Mean ± S.E. ($n = 15$). Notes: vs. normal control group, [a] $P < 0.01$; vs. STZ-diabetic control group, [b] $P < 0.01$.

Ultrastructural changes in pancreatic tissue of the STZ-diabetic mice were observed using a transmission electron microscope. In normal mice, the majority of ultrastructures in the pancreatic cells were integrated with an oval nucleolus that was accompanied by different patterns of secretory granules in the cytoplasm and the morphologically normal endoplasmic reticulum. Visually, islet beta cells in STZ-lesioned mice showed morphological irregularities and reduction or absence of secretory granules in the cytoplasm, as well as abnormalities of mitochondria and pronounced inflammation of the endoplasmic reticulum. Nucleus volume shrinkage and nuclear chromatin marginalization along with early symptoms of apoptosis were observed. Following the RMP and MF treatments, morphological amelioration in pancreatic cells was accompanied by the increase of secretory granules and cellular organs in the cytoplasm, indicating gradual attenuation of the apoptotic process (Fig. 6).

3.6. RMP up-regulated the Bcl-2 level and down-regulated Bax expression in diabetic mice

The findings from the western blotting assay indicated that the intrapancreatic Bcl-2 protein level in STZ-lesioned mice was down-regulated, while Bax expression was increased when compared to normal mice ($P < 0.01$, $n = 5$). Instead, the RMP and MF treated diabetic mice showed increased endogenous Bcl-2 expression and reduced Bax expression ($P < 0.01$, $n = 5$) (Fig. 7).

3.7. RMP inhibited the intrapancreatic p-JNK, p-p38, cleaved-caspase-3 expressions in diabetic mice

To further explore the apoptotic pathway, key regulators (JNK, p38 and caspase-3) were investigated. The results from western blotting showed that STZ-lesioned mice displayed elevated phosphorylation of JNK, p38 levels and activation of cleaved-caspase-3 in pancreatic tissue ($P < 0.01$, $n = 5$). Following the RMP and MF treatments, these changes were effectively blocked, as shown in the down-regulation of intrapancreatic p-JNK, p-p38 and cleaved-caspase-3 expressions ($P < 0.01$, $n = 5$) (Fig. 8).

4. Discussion

As revealed in structure characterization by assays, RMP represents a natural carbohydrate molecule that contains long chains of monosaccharide constituents binding together via glycosidic linkages. RMP may have diverse properties from the monosaccharide shaping in structure from linearity to branching. As a functional biological polymer, therapeutic application of RMP in diabetes management should be discussed as follows.

Hyperglycemia refers to high level of glycemia that circulates in blood stream (Shimizu et al., 2012). If hyperglycemia is untreated, the fatal complication termed ketoacidosis will develop over time (Paksu, Kalka, Asilioglu, Paksu, & Dinler, 2011). Evidence indicates that pancreatic beta-cell-mediated principal function is involved in generation and release of insulin. Accordingly, beta-cells function in reduction of blood glucose concentration through regulation of endogenous insulin (Domínguez-Bendala, Inverardi, & Ricordi,

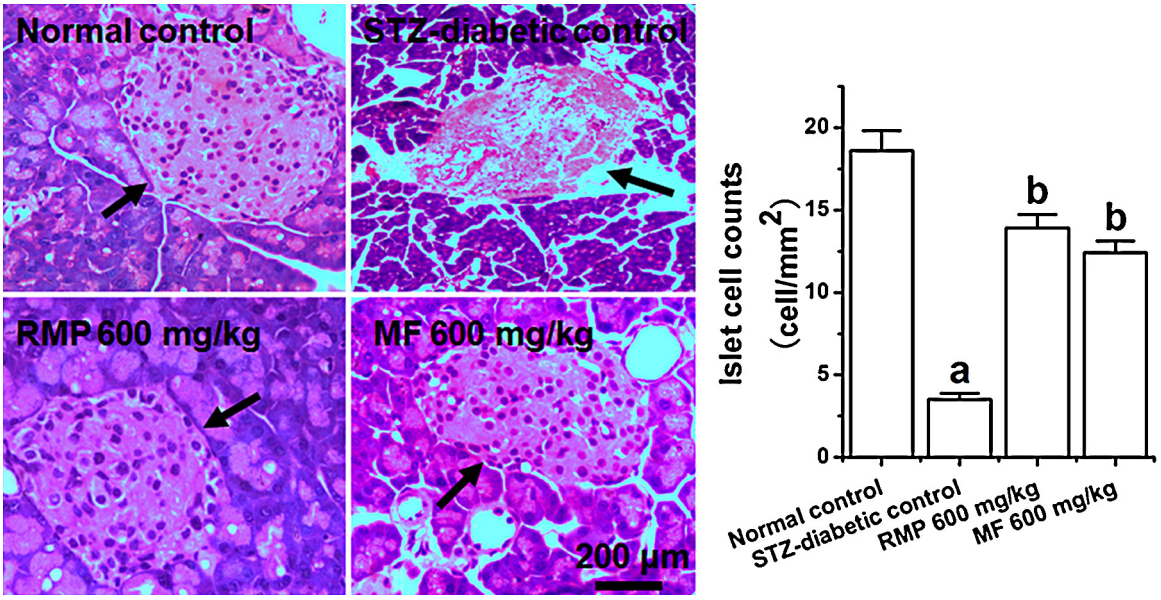

Fig. 5. Histopathologic examination of pancreas in STZ-diabetic mice (HE staining, scale bar: 200 μm).
Arrows represent the islet with pancreatic beta cells. The results were analyzed by one-way ANOVA followed by Tukey's post hoc test, and data were expressed as the Mean ± S.E. ($n = 5$). Notes: vs. normal control group, [a] $P < 0.01$; vs. STZ-diabetic control group, [b] $P < 0.01$.

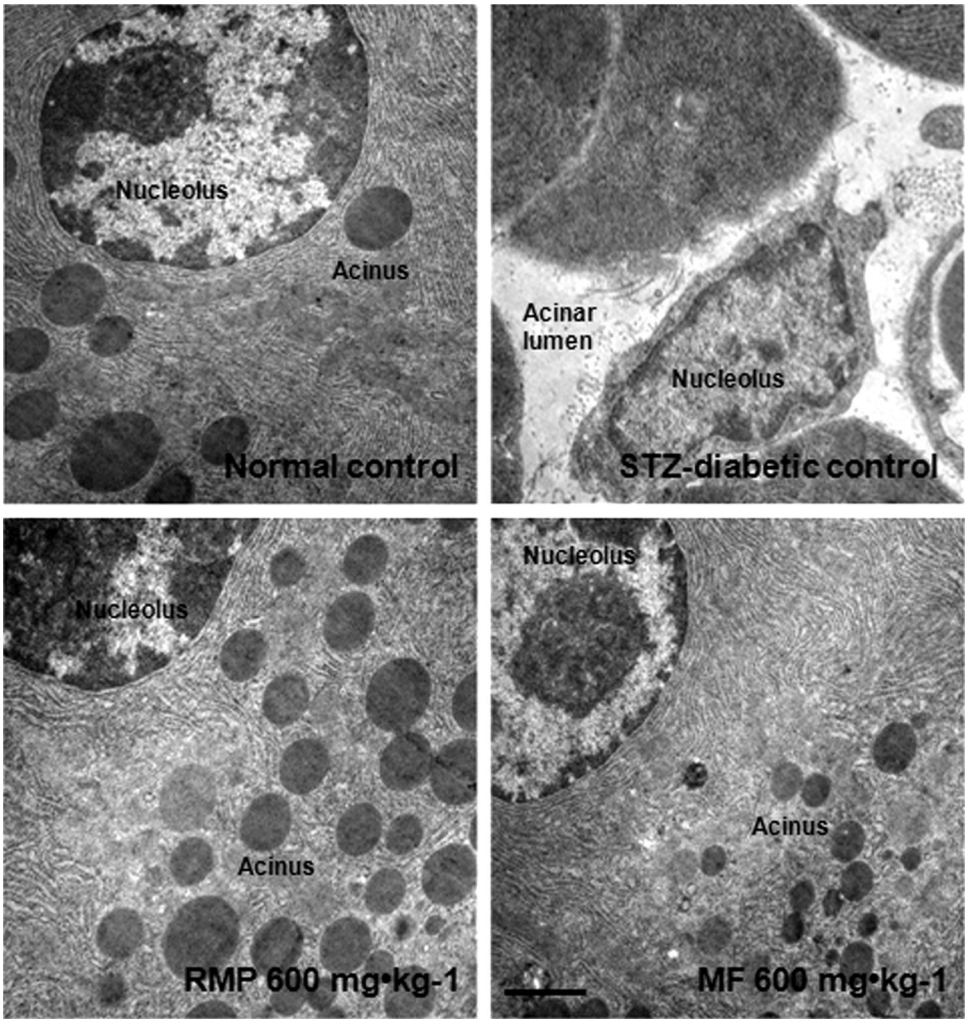

Fig. 6. Ultrastructural organization of pancreas in STZ-diabetic mice (Transmission electron microscopy assay, scale bar: 2 μm).

2012; Fu, Gilbert, & Liu, 2013). In addition, streptozotocin (STZ) is a naturally derived substance that can selectively destroy insulin-producing pancreatic beta-cells in mammals (Takeda et al., 2012). Therefore, effectively controlling the hyperglycemia contributed to suppression of the diabetes development. In this study, STZ-lesioned mice suffered pronounced marasmus and hyperglycemia, which outcome was consistent with beta-cell loss as shown by the H&E staining. However, our results suggested that RMP-treated diabetic mice showed the glycemia-lowering effect, due to water-soluble polysaccharides characterized by the potential benefits. Accordingly, the impact between hyperglycemia and insulin mediated by RMP was investigated.

Insulin, a secreted hormone that generates from pancreatic beta cells, regulates carbohydrate and lipid metabolism in organisms (Beardsall, Diderholm, & Danger, 2008). Insufficient insulin levels can result in hyperglycemia and further develop diabetes mellitus. Clinically, exogenous insulin is utilized to medically treat some phenotypes of diabetes (Aschner et al., 2012; Wilding et al., 2013). Thus, maintenance of insulin homeostasis is a promising strategy for managing diabetes. The results of this study suggested that RMP intervention simultaneously blocked diabetes-associated hypoinsulinemia and hyperglycemia in STZ-diabetic mice. These improvements were consistent with the cyto-architectural repairment of pancreatic cells as shown in pathological examination. However, the molecular mechanisms involved RMP-mediated anti-diabetic effect required further discussion.

Bcl-2 (B-cell lymphoma 2) is one of the most important apoptosis-regulated proteins and possesses potent anti-apoptotic characteristics (Luo & Rubinstein, 2010). Thus, the result indicates that resistance to apoptosis may suppress disease development. Bax is a pro-apoptotic protein that modulates programmed cell death. Intracellular Bax activation leads to increased permeabilization of mitochondrial outer membrane, triggering apoptotic cascade events (Bowen, Gibson, Keefe, & Cummins, 2005; Park et al., 2012). In this study, the Bcl-2 protein was significantly decreased and Bax level was elevated in STZ-diabetic mice, indicating that STZ-lesioned pancreatic cells underwent apoptosis. After treatment with RMP, these changes were effectively reversed and accompanied by an elevation of the Bcl-2/Bax ratio. The results suggested that the anti-apoptotic effect of RMP on pancreatic cells was mediated by regulation of Bax and Bcl-2 expressions, which distinct monosaccharide units might function as the coordinative effect to help reduce beta-cell loss in pancreas tissue.

Mitogen-activated protein kinases (MAPKs), a family of serine/threonine-specific protein kinases, mediate multifaceted physiological functions, such as cell proliferation/differentiation, mitosis, cellular survival and apoptosis (Wada & Penninger, 2004). Overactivation of MAPKs is induced by oxidative stress or toxin exposure, in which MAPK dysregulation leads to insulin-producing insufficiency associated with apoptosis of pancreatic beta cells (Runchel, Matsuzawa, & Ichijo, 2011). Recently, c-Jun N-terminal kinase (JNK) and p38 MAPK phosphorylation have been shown to

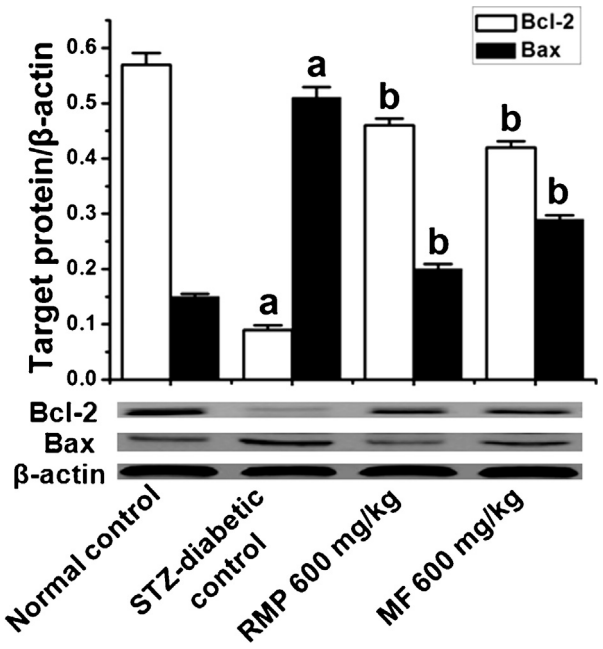

Fig. 7. RMP up-regulated the Bcl-2 expression and down-regulated Bax protein level in diabetic mice.
The results were analyzed by one-way ANOVA followed by Tukey's post hoc test, and data were expressed as the Mean ± S.E. ($n = 5$). Notes: vs. normal control group, [a] $P < 0.01$; vs. STZ-diabetic control group, [b] $P < 0.01$.

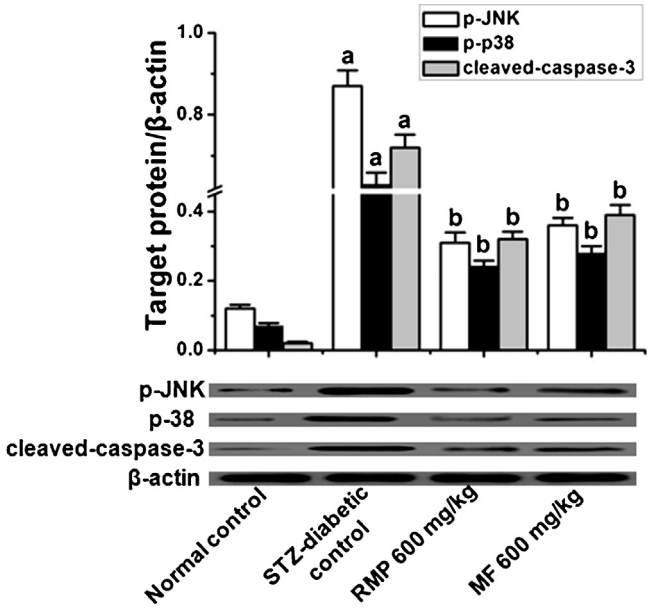

Fig. 8. RMP blocked the intrapancreatic p-JNK, p-p38, cleaved-caspase-3 expressions in diabetic mice.
The results were analyzed by one-way ANOVA followed by Tukey's post hoc test, and data were expressed as the Mean ± S.E. ($n = 5$). Notes: vs. normal control group, [a] $P < 0.01$; vs. STZ-diabetic control group, [b] $P < 0.01$.

regulate numerous activities of proteins located in mitochondria or nucleus. Activation of endogenous JNK and p38 MAPK alters cellular functions, including cell differentiation and apoptosis (Wagner & Nebreda, 2009; Ki, Park, Lee, Shin, & Kohl, 2013). In addition, overexpression of intrapancreatic JNK and p38 kinases induced by oxidative stress has been reported to be associated with pancreatic beta cell apoptosis/necrosis (Hou, Torii, Saito, Hosaka, & Takeuchi, 2008). Activation of caspase-3, the downstream inductor of the MAPK pathway, plays the vital role in execution phase of cellular apoptosis, in which uncontrolled caspase-3 expression is responsible for beta cell loss in diabetes-related conditions (Choi & Woo, 2010). Caspase-3 zymogen, an executioner caspase, is physiologically inactive until it is cleaved by an inducer caspase upon activation of the apoptotic signaling events (Walters et al., 2009). Our present findings showed that the expressions of p-JNK, p-38 and cleaved-caspase-3 proteins in the pancreatic tissue of STZ-lesioned mice were significantly increased, implying that abnormal protein expression was linked to severe apoptotic events. Impressively, intrapancreatic expressions of p-JNK, p-38 and cleaved-caspase-3 in diabetic mice were effectively down-regulated by RMP treatment. As mentioned above, we extrapolated that the pathogenesis of diabetes, especially type I diabetes, was involved in activation of the JNK/p38/caspase-3 axis pathways in pancreatic tissue, thus resulting in apoptosis-related pancreatic cell loss. Furthermore, the intrapancreatic cell loss might be optimized by RMP treatment via inhibition of the apoptosis-related pathway.

Furthermore, the reasoning behind RMP potential bioactivity showed that RMP with structural polysaccharides had the healthy benefits of well-balanced gut symbiont microbial community in contribution of beta-cell nutrition, metabolism, or regeneration. Given the limitation in current study, further investigation for relationship between structure units and RMP anti-diabetic activity needs to be conducted.

5. Conclusions

Overall, our present results demonstrate that RMP can serve as a promising strategy for managing diabetes. Additionally, RMP-mediated cytoprotection in the pancreas is implemented through inactivation of apoptosis-related components, such as Bcl-2, Bax, JNK, p38 and caspase-3. Furthermore, these findings provide insight into the pathogenesis of STZ-diabetes indicating an association with apoptosis-related cell loss in pancreatic tissue.

Acknowledgments

This study was partly supported by Foundation of Traditional Chinese Medicine Department of Guangxi Province, China (Nos. GZKZ1157, GZZC1042). Furthermore, we were grateful to the reviewers' helpful suggestions to improve current manuscript.

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