Sangguayin preparation prevents palmitate-induced apoptosis by suppressing endoplasmic reticulum stress and autophagy in db/db mice and MIN6 pancreatic β-cells

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[ABSTRACT] Sangguayin preparation (SGY-P) is refined from the traditional Chinese medicinal compound Sangguayin, which “clears heat and promotes fluid” and “tonifies kidney and spleen” for “Xiaoke”, commonly known as ‘Diabetes mellitus’ in clinics. Previous studies have shown that SGY-P could reduce insulin resistance and repair damaged pancreas in db/db mice, but the underlying mechanisms were unclear. Here, we investigated whether treatment with SGY-P could protect pancreatic β-cells from apoptosis and uncovered the underlying mechanisms. db/db mice were used to observe the hypoglycemic and islet protective effect in vivo. Apoptosis was induced in mouse insulinoma 6 (MIN6) cells by palmitate, following which the cells were treated with SGY-P for elucidating the anti-apoptotic mechanism in vitro. Cell viability and nuclear morphology were detected by CCK-8 assay and Hoechst 33258 staining. The expression levels of apoptosis-, endoplasmic reticulum (ER) stress-, and autophagy-related proteins were measured by western blot. The results showed that SGY-P reduced fasting blood glucose, pancreatic pathological changes, and islet β-cell apoptosis in db/db mice. Palmitate-induced apoptosis in MIN6 cells was decreased by SGY-P treatment. Hence, SGY-P therapy exhibited a protective effect on pancreatic β-cells by decreasing the expression of cleaved caspase-3, cleaved PARP and Bax, and increasing Bcl-2 by suppressing ER stress (Bip/XBP1/IRE1α/CHOP/Caspase-12) and autophagy (LC3/p62/Atg5) pathways.

[KEY WORDS] Sangguayin preparation; MIN6 cells; Apoptosis; Endoplasmic reticulum (ER) stress; Autophagy; Lipotoxicity

Introduction

Diabetes mellitus (DM), associated with the highest morbidity and mortality in China, attracts extensive attention worldwide. Type 2 diabetes mellitus (T2DM) accounts for about 90% of DM cases globally [1]. Currently, clinical drugs for T2DM cannot reverse the disease course and drug tolerance always develops on long-term usage because they have a single target. Traditional Chinese medicine (TCM) has been used for the treatment of T2DM for a long time in China [2], which integrally regulated the organism via multi-components, multi-targets, and multi-action pathways. Developing a novel anti-diabetes TCM compound may provide a new strategy for the treatment of T2DM.

Despite the complex pathogenesis of T2DM, islet damage which contributes to insulin secretion impairment plays an important role in the progression of T2DM. Excess free fatty acid (FFA) is considered a pancreatic β-cell killer because of its lipotoxic effects during islet damage [3]. Lipotoxicity induced endoplasmic reticulum (ER) stress and activated unfolded protein response (UPR) [4-5]. In addition, autophagy is considered a lysosomal degradation system that can eliminate impaired organelles or aggregated proteins. It is usually triggered by ER stress and involved in the survival of pancreatic β-cells [6, 7].

In conclusion, relieving ER stress and autophagy in pancreatic islets may be an effective method for the prevention and therapy of T2DM. Sangguayin preparation (SGY-P) is refined from the traditional Chinese medicinal compound Sangguayin which significantly “clears heat and promotes fluid” and “tonifying kidney and spleen” for “Xiaoke” in clinics [9]. Previously, SGY-P was reported to have hypoglycemic activity in db/db mice [9-12]. However, little is known...
about its effects on islet β-cells. In this study, the protective effects and the possible mechanisms of SGY-P against palmitate-induced apoptosis in vitro and in vivo were evaluated.

Materials and Methods
SGY-P preparation and chemical composition analysis
SGY-P mainly includes four kinds of TCM: Mori Folium, Puerariae Lobatae Radix, Dioscorea Rhizoma, and Fructus Momordicae Charantiae. All four were procured from Hunan ZiranTang Chinese Herbal medicine Co., Ltd. (Hunan Province, China) and can be found in Pharmacopoeia of China (2015 edition, Chinese medical science and Technology Press) and Hunan province standard for TCM (2009 edition). The preparation and analysis of SGY-P were performed as per previous research [9]. Briefly, total alkaloids of Mori Folium, total flavonoids of Puerariae Lobatae Radix, and total saponins of Fructus Momordicae Charantiae were extracted by alcohol at different concentrations, and then purified by macroporous resin. Total polysaccharides of Dioscorea Rhizoma were extracted by water and purified by alcohol precipitation. The four extracted powders, in the ratio 1 : 5 : 6 : 6 were used to make SGY-P. Chromatographic separations were then carried out on an Agilent 1200 HPLC system using a TSK gel Amide-80 column (4.6 mm × 250 mm, 5 μm; TOSOH, Japan). The column oven temperature was set at 25 °C. The mobile phase consisted of deionized water with 6.5 mmol·L⁻¹ ammonium acetate solution (solvent B) and acetonitrile (solvent A). The separation flow-rate was 1.0 mL·min⁻¹ and gradient elution for solvent B was as follows: 0–15 min, 10%; 15–25 min, 10%–25%; 25–45 min, 25%; 45–55 min, 25%–50%. The HPLC instrument was coupled to micrOTOF-Q quadrupole-time of flight mass spectrometer from Bruker Daltonics Inc. (American) with electrospray ionization (ESI) source in the positive and negative mode. The detection was performed in the Auto Ms mode. The mass spectrometric condition was optimized as follows: capillary voltage, 5000 V; offset voltage, –500 V; and gradient elution for solvent B was as follows: 0–15 min, 10%; 15–25 min, 10%–25%; 25–45 min, 25%; 45–55 min, 25%–50%. The mobile phase consisted of deionized water with 6.5 mmol·L⁻¹ ammonium acetate solution (solvent B) and acetonitrile (solvent A). The separation flow-rate was 1.0 mL·min⁻¹ and gradient elution for solvent B was as follows: 0–15 min, 10%; 15–25 min, 10%–25%; 25–45 min, 25%; 45–55 min, 25%–50%. The HPLC instrument was coupled to micrOTOF-Q quadrupole-time of flight mass spectrometer from Bruker Daltonics Inc. (American) with electrospray ionization (ESI) source in the positive and negative mode. The detection was performed in the Auto Ms mode. The mass spectrometric condition was optimized as follows: capillary voltage, 5000 V; offset voltage, –500 V; RF value, 150 Vpp; temperature, 180 °C; gas flow, 5.0 L·min⁻¹; gas pressure, 3.0 Bar. Data acquisition and processing were performed using Data Analysis software (version 4.0). SGY-P samples were weighed and subjected to ultrasonic extraction with 50 % methanol for 10 min. After centrifugation, the supernatant was filtered through a 0.45 μm filter and 5 μL of the filtrate was injected into the HPLC column. Sixteen compounds were identified, (1) Daidzein; (2) Momordicoside K; (3) 6”-O-Acetylaidaidzin; (4) Pueroside D; (5) Genistin; (6) 3’-hydroxypuerarin; (7) Daidzin; (8) 3’-methoxy puerarin; (9) Puerarin; (10) 3’-hydrogenated puerarinxyloside; (11) Puerarin-7-xyloside; (12) Momordicoside A; (13) Puerarin-6”-O-xyloside; (14) Puerarin-7-O-glucoside; (15) Puerarin-4’-O-B-D-Glucoside; (16) 1-DNJ. The results are depicted in Fig. S1 and Table S1.

Animal experiments
Male wild-type C57BLKS/Lepr db (db/db) mice and C57BLKS/Lepr db (db/db) mice (6-week-old) were procured from Nanjing Biomedical Research Institute. All experimental procedures strictly adhered to the Guide of Care and Use of Laboratory and were approved by the Ethics Committee of Hubei University of Chinese Medicine. The mice were raised under specific-pathogen-free (SPF) environment with standard conditions of light (12-h light/dark), temperature (23 ± 2 °C), and diet (chow). After 1 week of adaptive feeding, the db/db mice were randomly divided into three groups based on blood glucose levels such that the average blood glucose level was similar in each group. The model control group (MC) was orally administered normal saline, while the SGY-P treated groups were orally administered SGY-P at low (175 mg kg⁻¹) and high (700 mg kg⁻¹) doses daily. The wild-type db/db mice were considered the normal control group (NC) and orally administered normal saline.

Fasting blood glucose (FBG), fasting insulin (FINS), homeostasis model assessment of insulin resistance (HOMA-IR), and pancreas viscera index measurement
At the 9th week, all mice were fasted for 12 h before blood sample collection. The collected blood samples were allowed to stand at 28 °C for 30 min and subsequently centrifuged (4 °C, 1500 × g, 10 min) to obtain serum for the analysis of FBG and FINS levels. The pancreas of each mouse was peeled off and weighed. The HOMA-IR and viscera index were calculated using the following formulas: HOMA-IR = FBG (mmol·L⁻¹) × FINS (mU·L⁻¹) / 22.5, viscera index = pancreatic weight (g) / body weight (g) × 100 %. FINS levels were evaluated using an enzyme-linked immunosorbent assay (ELISA) kit (Cusabio Biotech Co., Ltd, Wuhan, China).

Histology and TUNEL assay

The pancreas of each mouse was harvested, followed by a 24 h fixation with 4% paraformaldehyde, and then sectioned to a thickness of 3 µm. The sections were stained with hematoxylin and eosin (H&E). To detect apoptotic cell death in the tissues, the TUNEL assay was performed using a commercial kit (Roche, Cat#11684818625), according to the manufacturer’s instructions.

Cell culture and treatment
MIN6 cells were maintained in high-glucose Dulbecco’s Modified Eagle Medium (DMEM) (HyClone Laboratories, South Logan, Utah, USA) supplemented with 10% fetal bovine serum (FBS) and then incubated in a humidified atmosphere of 5% CO₂ at 37 °C. Palmitate (PA) was dissolved in 0.1 mol·L⁻¹ NaOH with 20% BSA in a 70 °C water-bath and stored at −20 °C as a stock solution (20 mmol·L⁻¹), and SGY-P was dissolved in double distilled water and stored at −20 °C as a stock solution (10 mg·mL⁻¹). Both stock solutions were filtered using 0.1 µm sterile filter for sterilization and diluted with complete DMEM before treatment.

Cell viability assay
MIN6 cells (8 × 10^3 cells mL⁻¹) were seeded in 96-well plates and treated with either PA (0, 0.05, 0.1, 0.2 and 0.4 mmol·L⁻¹) PA or SGY-P (0, 25, 50 and 100 μg·mL⁻¹) along with 0.2 mmol·L⁻¹ PA for 36 h. Then, 10 μL CCK-8 (En-
green Biosystem Ltd., Beijing, China), which can be reduced to a water-soluble formazan dye by intracellular dehydrogenase, was added in each well and incubated for 4 h, OD values were determined at 450 nm.

**Hoechst 33258 Staining**

MIN6 cells (2 × 10^5 cells·mL⁻¹) were seeded in 6-well plates and treated with 0.2 mmol·L⁻¹ PA with or without SGY-P (25–100 μg·mL⁻¹) for 36 h. The cells were fixed in Carnoy’s solution (methanol : acetate = 3 : 1) for 20 min at 4 °C. The cells were then washed with phosphate buffer saline (PBS) and incubated away from light for 20 min to allow for staining in 10 μg·mL⁻¹ Hoechst 33258 (Beyotime Institute of Biotechnology, Jiangsu, China). The cellular morphology was then observed under a fluorescence microscope (Olympus, Japan).

**Western blot analysis**

MIN6 cells (1 × 10^6 cells·mL⁻¹) were seeded in 6-well plates and treated with 0.2 mM PA and SGY-P as mentioned above for 36 h. The cells or tissue samples were lysed with Radio Immunoprecipitation Assay (RIPA) (Beyotime Institute of Biotechnology, Jiangsu, China) on ice. Protein concentrations of whole cell lysates were determined using BCA Protein Quantitation Kit (Takara, Kusatsu, Shiga, Japan). Then, equal amounts of protein samples were separated by 10% or 12% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes (Amersham Biosciences, UK). The nitrocellulose membranes were blocked in 5% non-fat milk in TBS with 0.05% Tween-20 (TBST) for 1 h and incubated in primary antibodies including GAPDH (#5174), XBP1s (#40435), Bax (#2772), Bcl-2 (#2870), PARP (#9532), cleaved PARP (#94885), Caspase-3 (#9665), cleaved Caspase-3 (#9662), Caspase-12 (#2202), CHOP (#2895), Bip (#3177), IRE1-α (#3294), Atg5 (#12994), Beclin-1 (#3495), LC3A/B (#12741) and p62 (#39749) (1 : 1000 dilution in TBS-T) (Cell Signaling Technology, USA). The membranes were then washed with PBS and incubated in HRP-conjugated secondary antibody (1 : 2000) (Cell Signaling Technology) for 1 h at room temperature. Blots were developed using ECL detection reagent (Bio-Rad) under FluorChem FC3 system (AlphaInnotech, San Leandro, CA, USA).

**Statistical analysis**

All quantitative values were depicted as means ± standard deviation (SD). Difference between groups was calculated using Student’s t-test with the statistical software SPSS 19.0. P-values < 0.05 were considered statistically significant.

**Results**

**SGY-P reduced the HOMA-IR of db/db mice**

At the start of the experiment, MC and SGY-P treated groups of mice exhibited similar average FBG and body weight. However, after 9 weeks of treatment with SGY-P (175 and 700 mg·kg⁻¹), FBG and HOMA-IR (P < 0.01) clearly decreased as shown in Figs. 1A and 1C. The FINS and the viscera index of the pancreas showed no significant

**Fig. 1** SGY-P reduced HOMA-IR of db/db mice. (A) FBG; (B) FINS; (C) HOMA-IR; (D) viscera index of pancreas. Values are expressed as means ± SD, n = 6. **P < 0.01, *P < 0.05 vs the MC group; #P < 0.01, ##P < 0.05 vs the NC group**
change after SGY-P treatment compared with the NC group and the MC group (Figs. 1B and 1D).

SGY-P alleviated the pathology and apoptosis in pancreatic islets of db/db mice

H&E staining showed the pathological changes, including frequently atrophied pancreatic islets in the MC group, and the lesions were alleviated after 9 weeks of SGY-P treatment (Fig. 2A). Western blot revealed that the expression of cleaved Caspase-3 and Bax significantly increased in the MC group compared to the NC group (P < 0.01), but decreased in the SGY-P treated groups compared to the MC group (P < 0.01 and P < 0.05) (Fig. 2B). The number of islet β-cells that were stained red (insulin-positive) decreased in the MC group (Fig. 2C), and the administration of SGY-P prevented the decrease of insulin-positive cells. Nuclei displaying DNA fragmentation (TUNEL-positive nuclei) were detected mainly in the pancreatic islet β-cells of the MC group. The SGY-P treated groups also showed improved inhibition of apoptosis of the pancreas islet β-cells in vivo.

SGY-P alleviated PA-induced mitochondrial apoptosis pathway in MIN6 cells

Whether SGY-P protects cells from PA-induced apoptosis was investigated. As shown in Fig. 3A, when MIN6 cells were exposed to PA (0–0.4 mmol·L⁻¹), the cell death decreased in proportion to the dose. PA (0.2 mmol·L⁻¹) was chosen in the following experiments. The results (Fig. 3B) indicate that 36 h of SGY-P (25–200 μg·mL⁻¹) treatment did not have a significant effect on MIN6 cells. However, SGY-P (25–100 μg·mL⁻¹) significantly alleviated PA-induced inhibition of cell proliferation (P < 0.01, as shown in Fig. 3C). The results of Hoechst 33258 staining showed that the control group cells exhibited uniform round or oval-shaped nuclei with evenly distributed chromatin and the PA-treated cells exhibited typical apoptotic morphological changes including shrunken cell body, brightly-dyed nuclear chromatin, nuclear fragmentation, and formation of apoptotic bodies. The apoptosis induced by PA was significantly ameliorated by SGY-P treatment (Fig. 3D). Consistent with Hoechst staining, western blot assays (Fig. 3E) indicated that the apoptotic markers, including cleaved caspase-3 and PARP, significantly increased in the PA group (P < 0.01) compared to the Control group, and significantly decreased in the SGY-P group compared to the PA group (P < 0.01). The Bcl-2 was increased and Bax decreased by SGY-P treatment compared to that in the PA-treated group (P < 0.01), which indicated that PA-induced apoptosis in MIN6 cells was suppressed by SGY-P treatment.

SGY-P reduced ER stress in MIN6 cells

To further explore the mechanism through which SGY-P exhibits a protective effect on MIN6 cells, the expression of ER stress pathway-related proteins was detected by western blot. The results showed that all the biomarkers including Bip, IRE1α, XBP1, CHOP and cleaved caspase-12 significantly increased in the PA group compared with Control group and decreased in SGY-P groups (Fig. 4, P < 0.01, P < 0.05).

SGY-P reduced autophagy in MIN6 cells

To determine whether autophagy is involved in the protective effects of SGY-P, the expression of LC3, p62, Atg5, and Beclin-1 was evaluated. Western blot analyses showed that SGY-P treatment effectively down-regulated the ratio of LC3-II/LC3-I and the expression of p62 and Atg5 without significant changes in Beclin-1 (Fig. 5, P < 0.01, P < 0.05). These findings suggested that autophagy is related to the mechanism of the effects of SGY-P.

Discussion

SGY-P is composed of the total alkaloids of Mori Foli um, flavonoids of Puerariae Lobatae Radix, sapo nins of Fructus Momordicae Charantiae, and polysaccharides of Dioscoreae Rhizoma. The main components include 1-DNJ, puerarin, and Momordicoside A, which have high alpha-glucosidase inhibitory, antioxidant, anti-inflammatory and anti-diabetic activity. Pre-meal intake of 1-DNJ in therapeutic concentration has resulted in the inhibition of postprandial hyperglycemia and hyperinsulinemia; Puerarin can attenuate streptozotocin induced pancreatic cell apoptosis by inhibiting the expression of caspase family proteins and AIF; Molecular docking studies showed that compounds Momordicoside A and K bound close to the active site of both enzymes by interacting with key amino acid residues [13-15]. In the present study, db/db mice were used as the model animal to study insulin activity. The mice are derived from the inbreeding of the heterozygous C57BL/LSJ [16]. After 9 weeks of treatment, SGY-P was shown to modulate FBG without any regulation of fasting blood insulin and pancreas index. This result indicates that SGY-P does not cause pancreatic hyperplasia or atrophy, and does not reduce blood glucose by promoting insulin secretion. Combined with the histology, immunohistochemistry, and TUNEL assays, it was speculated that SGY-P might play a role in the treatment of T2DM by improving the insulin sensitivity of peripheral tissues or by protecting the pancreatic islets from apoptosis.

The western blot and TUNEL assay indicated that SGY-P can protect pancreatic islets from apoptosis in db/db mice. However, the mechanism of islet protection effect of SGY-P needs to be explored further in vitro. It has been well documented that FFA-induced β-cell apoptosis is closely associated with the onset and progression of T2DM [17, 18]. During fasting, circulating FFA is critical for basal pancreatic β-cell function and effective insulin secretion. However, prolonged exposure to elevated saturated FFA levels induced β-cell dysfunction and apoptosis [19]. Apoptosis observed in MIN6 cells exposed to 0.2 mmol·L⁻¹ PA for 36 h in this study, was consistent with previous findings [20]. In addition, SGY-P also prevented the pancreatic islet β-cells from apoptosis in db/db mice. In vitro and in vivo research reveals the islet protection effect of SGY-P, which contributes to alleviation of the progression of T2DM.

ER stress and autophagy play an important role in the mechanisms underlying PA-induced pancreatic β-cells
Fig. 2  SGY-P improved the structure of pancreas islets, inhibited apoptosis of islets \( \beta \) cells in \( db/db \) mice. (A) Representative images of H&E staining at \( \times 200 \) magnification; (B) Expression of apoptosis-related proteins in pancreatic tissues of mice was determined by western blot analysis, values were expressed as means \( \pm \) SD, \( n = 3 \). *\( P < 0.05 \), **\( P < 0.01 \) vs the MC group; #\( P < 0.05 \), ##\( P < 0.01 \) vs the NC group; (C) Representative images of TUNEL staining at \( \times 200 \) magnification, FITC (TUNEL-positive) stains green, INS (insulin-positive) stain red, and DAPI stains blue.
In this study, PA induced the cleavage of the apoptotic markers caspase-3 and PARP and the ratio of Bax/Bcl-2 \[7, 21, 22\]. The Bcl-2 family plays a key role in the mitochondrial-mediated apoptosis pathway and initiate the caspase cascade, triggering the cleavage of executioner caspases-3 and PARP, resulting in \(\beta\)-cell apoptosis \[23\]. Here, SGY-P treatment decreased the expression of cleaved caspase-3 and cleaved PARP while up-regulating Bcl-2/Bax ratio, indicating that SGY-P may protect MIN6 cells from apoptosis induced bypassing the mitochondrial pathway.

It has been reported that ER stress potentially contributes to \(\beta\)-cell apoptosis response to lipotoxicity \[22-24\]. Here, PA was used to induce ER stress. The overexpression of ER chaperone Bip, which is a marker for ER stress, is increased.
by PA treatment. Bip is overexpressed to bind to unfolded proteins in the UPR, and once the unfolded proteins exceed the mitigation ability of intolerably high levels of Bip, the protective ER stress becomes pro-apoptotic. Thus, IRE1α (an important transmembrane protein), one of three key signaling proteins, is activated and functions as a rheostat capable of regulating cell fate [25]. The activated IRE1α splices XBP-1 mRNA to produce a spliced XBP-1 (XBP1s), which is an active transcriptional factor and regulates the genes related to proper functioning of ER and cellular stress response [26]. In this study, SGY-P reversed the up-regulation of Bip and IRE1α, which could reverse severe ER stress. In addition, an important mediator, CHOP is activated and switches cell fate from survival to death. The activation of CHOP increases the expression of Bim and DR5, while decreasing the expression of Bcl-2, which results in mitochondrial apoptosis [27-30]. Furthermore, IRE1α activation was reported to trigger the recruitment of caspase-12 which is translocated from ER to cytosol and activates subsequent effector caspase mediating apoptosis [31, 32]. Our results showed that SGY-P treatment blocked the ER stress induced apoptosis by down-regulating CHOP and cleaved caspase 12, which was consistent with the suppression of mitochondrial apoptosis, suggesting that perhaps SGY-P protects pancreatic β-cells against apoptosis via the Bip/XBP1/IRE1/CHOP/caspase-12 pathway.

Autophagy plays a key role in maintaining pancreatic β-cell homeostasis [33]. Maiuri et al. established a link between autophagy and apoptosis which were considered separate processes in controlling cell fate for a long time [34]. In subsequent years, autophagy was proved to delay or promote apoptosis and cell death under certain conditions [35, 36]. The anti-apoptotic protein, Bcl-2, interacts with the evolutionarily conserved autophagy protein, Beclin-1, which provides evidence of the crosstalk between cell apoptosis and autophagy [37]. Newly synthesized LC3 is primarily processed to a soluble form LC3-Ⅰ, but then modified to a lipidated and autophagosome-associated form LC3-Ⅱ under the action of Atg5/Atg7/Atg12, which has been recognized as a marker for autophagy [38]. In addition, p62 is considered as an autophagic flux marker, and interacts with LC3-Ⅱ to regulate protein aggregation and degradation in autolysosomes. An accumulation of p62, parallel to an increase in LC3-Ⅱ, reflects...
an impaired autophagic flux and results in cell death \cite{38, 40}. This study demonstrated that autophagy was involved in PA-induced cellular apoptosis and that SGY-P decreased the ratio of LC3-II/LC3-I and the expression of p62, Atg5 but without significantly changing Beclin-1 expression. This suggests that SGY-P may protect pancreatic β-cells against apoptosis associated with restoration of LC3, p62, and Atg5 proteins; however, further research is required for investigating the autophagy regulation by SGY-P.

In conclusion, this research verifies the efficacy and protective mechanism of SGY-P against pancreatic β-cell apoptosis induced by PA. However, further research is required to better understand the relationship between β-cell apoptosis and insulin secretion impairment, which may contribute to uncovering the mechanisms and provide stronger evidence for the use of SGY-P in the treatment of T2DM.

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Supplementary Materials

See Fig. S1 and Table S1 in the Supplementary Material for comprehensive image analysis.

References


