Original Research

Protective effect of Specnuezhenide on islet β cell of rats with gestational diabetes mellitus

Jing Yang[1#], Jian Jia[1#], Yongkang Yang[1, 2*], Yaning Zhao[1, 2], Qin Li[1, 2]

[1] The Second Affiliated Hospital of Shaanxi University of Chinese Medicine, Xianyang, PR China
[2] Shaanxi University of Chinese Medicine, Xianyang, PR China

Abstract: Gestational diabetes mellitus (GDM) refers to diabetes mellitus and impaired glucose tolerance first diagnosed during pregnancy without previous history of the disease. Usually, GDM has a high risk of inducing type 2 diabetes mellitus. The incidence of GDM is increasing year by year worldwide, and it seriously affects the quality of life of patients, and increases the risk to pregnancy. Specnuezhenide (SPZ) is a characteristic active component of *Ligustrum lucidum*, a plant which exerts a variety of pharmacological effects. In this study, the protective effect of SPZ on β cells in gestational diabetes mellitus rats was investigated in a rat model of gestational diabetes. Based on oral glucose tolerance test, ELISA, qRT-PCR and western blotting assays, It was found that SPZ effectively improved blood sugar control and glucose tolerance in gestational diabetic rats, inhibited inflammation in islet tissue, and reduced inflammation-mediated insulin resistance.

Key words: Specnuezhenide; Proliferation; Apoptosis; Inflammation; Chondrocytes.

Introduction

Gestational diabetes mellitus (GDM) is a complication of pregnancy caused by disorder in glucose metabolism during pregnancy. Its pathological characteristics are similar to those of type 2 diabetes mellitus. It is characterized by insulin resistance and compensatory enhancement of the secretory function of islet β cells. In severe cases, it results in islet β cell damage (1). Investigations have shown that the incidence of GDM increases year by year with changes in women's lifestyle. There are also increasing trends in the associated childbirth risk to pregnant women and incidence of neonatal hypoglycemia and macrosomia (2). At present, the pathogenesis of GDM is not completely clear. However, insulin resistance (IR) caused by abnormal secretion of related adipokines, and impairment of pancreatic β cell function are crucial in the development of GDM (3). The major treatment strategy for GDM is insulin injection which can effectively control blood sugar. However, this treatment has no obvious therapeutic value for insulin resistance and damage to the function of islet β cells.

Fructus Ligustri lucidi is the dry and mature fruit of *Ligustrum lucidum* Ait which is used in traditional Chinese medicine (TCM) for nourishing *yin, reinforcing* kidney, and *nourishing* the liver to improve visual acuity. Specnuezhenide (SPZ, Figure1) is a characteristic bioactive compound in *fructus ligustri lucidi* and one of the secoiridiod glycosides present in the plant. Studies have shown that iridoid glycosides exert protective effects on the liver and gallbladder, lower blood sugar and blood lipid, and are effective anti-spasmolytic, anti-

inflammatory, anti-tumor and immunoregulatory agents (4). Specnuezhenide is a bioactive compound extracted from *Ligustrum lucidum* fruits with 60% methanol (5). It has anti-inflammatory, immuno-regulatory and anti-aging properties. It has been reported that SPZ exhibited strong antioxidant effects on free radical-induced erythrocyte hemolysis, and protected brain cells from 6-hydroxydopamine-induced neurotoxicity (6).

Changes in the structure and function of islet β cells during pregnancy are important parts of the etiology and pathogenesis of GDM. However, the protective effect of SPD on islet β cells in gestational diabetic rats is rarely reported. Therefore, in the present study, the protective effect of SPZ on pancreatic islet β cells in gestational diabetes rats was determined.
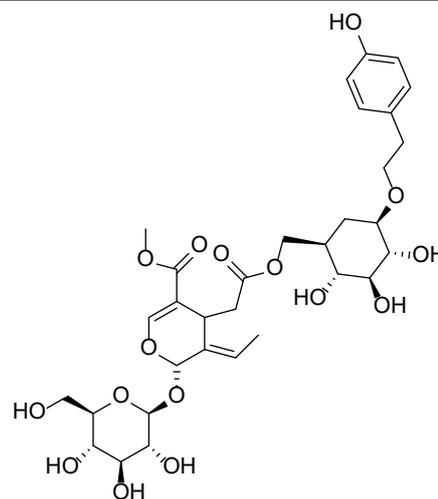

Figure 1. Chemical structure of specnuezhenide.

Materials and Methods

Drugs and major materials

Specnuezhenide (SPZ) was purchased from Chenguang Biotechnology Co. Ltd., Baoji City. High-fat feed was purchased from Research Diet Company of America. Sprague-Dawley (SD) rats and normal feed were obtained from Shanghai Slake Company, while ACCU-CHEK blood glucose meter and blood sugar test paper were products of Roche Company of Sweden. Insulin and resistin ELISA kits were purchased from Mercodia Company, Sweden; Bcl-2, Bax, Caspase-3 and GAPDH antibodies were purchased from Santa Cruz Biotechnology Company. Horseradish peroxidase-labeled secondary antibodies were bought from Wuhan Boshide Bioengineering Co., Ltd. Other reagents were provided by Sigma Company.

Animal grouping and modeling

The rat model of gestational diabetes mellitus was established according to the method reported in the literature (7). Female SD rats aged 8 weeks were fed according to the standard operating procedures. They were randomly divided into drug group (SPZ group), gestational diabetes mellitus group (GDM group) and control group, with 8 rats in each group. The rats in GDM group were fed with high-fat diet for 12 weeks, while rats in the drug group were fed with high-fat diet and STZ at a dose of 20 mg/kg/day, for 12 weeks. Rats in control group were fed with normal diet for 12 weeks. At the 12th week, all three groups of female rats were mated with male rats, and the occurrence of vaginal plug was recorded as day zero of pregnancy. The rats in GDM group were maintained on their high-fat diet, while the rats in drug group were maintained on their high-fat diet with SPZ (20 mg/kg daily).

Oral glucose tolerance test

Oral glucose tolerance was performed according to the method reported in the literature (8). Twenty-four hours before the experiment, rats in each group were fasted for 12 hours (overnight). After measuring blood sugar, each group was given glucose through gavage at a dose of 2g/kg body weight in aqueous solution. Tail vein blood samples were collected 15, 30, 60 and 120 minutes after gavage. The blood sugar concentration was measured with blood glucose meter and blood sugar test paper, and the results were used to draw oral glucose tolerance curve. The area under the curve was calculated and the differences among the groups were determined.

Determination of serum insulin and resistin concentrations

On the 18th day of pregnancy, the rats were fasted for 12 hours. Isoflurane gas anesthesia was administered on the next day, and abdominal aortic blood was collected after anesthesia. The blood was centrifuged for 3 minutes at 4 000 rpm, and the resultant supernatant was frozen in a refrigerator at -20ºC. The levels of insulin and resistin in the supernatant were assayed using ELISA method.

Determination of mRNA expressions of NF-κB and IκB in pancreatic tissues

On the 20th day of gestation, the rats were anesthetized and sacrificed, and the islet tissue was dissected. Total RNA was extracted with Trizol lysate reagent. The RNA extraction and cDNA preparation were done using appropriate kits according to the kit instructions. Then, PCR amplification was performed. The primer sequences used were:
NF-κB upstream primer: 5'-AGTTGGAGCAGCTG-CAGCGC-3'; downstream primer: 5'-GGAGGGCTC-CGCTGGTT-3';
IκB upstream primer: 5'-GCAACCCAGTGGTGGCC-CAG-3'; downstream primer: 5'-CTCCCGCTGGC-TGACCTGGA-3';
β-action upstream primer: 5'-AGAACATCATCCCTG-CATCC-3'; downstream primer: 5'-TGGATACATTGG-GGGTAGGA-3'.

Determination of expressions of islet tissue apoptotic proteins with Western blotting

On the 20th day of gestation, rat pancreatic tissue was harvested, frozen in liquid nitrogen and ground with a grinder. Total protein was extracted by adding RIPA lysate. Samples of pancreatic protein were taken and 10% SDS-PAGE gel electrophoresis was carried out at a constant current of 32 mA for 70 minutes. After transfer of the proteins to PVDF membrane at a constant pressure 100 mV for 75 minutes, 5% skimmed milk powder (TBST buffer formulation) was used to block non-specific binding for 1 hour. Then, the membrane was incubated overnight with antibodies for Bcl-2, Bax, Caspase-3 and GAPDH in shaking bed at 4ºC, after which it was washed thrice with TBST buffer (each wash for 5 minutes). Thereafter, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for 2 hours, and washed three times with TBST buffer (3 minutes for each wash). The bands were subjected to enhanced chemiluminescence imaging, and the intensity of the luminous signals and the gray values were determined.

Statistical analysis

GraphpadPrism7.0 statistical software was used for statistical analysis. Measurement data are expressed as $\overline{x} \pm SD$. Two-sample comparison was done with *t*-test, while one-way ANOVA was used to compare two groups. Values of $p<0.05$ were assumed as indicative of statistical significance.

Results

SPZ improved glucose tolerance in gestational diabetic rats

On the 18th of gestation, rats in each group were fasted for oral glucose tolerance test. Compared with the control group, the glucose tolerance of gestational diabetes mellitus model rats was significantly impaired. However, SPZ significantly improved the oral glucose tolerance of the gestational diabetes model rats. These results are shown in Figure 2.

Effect of SPZ on serum insulin and resistin concentrations

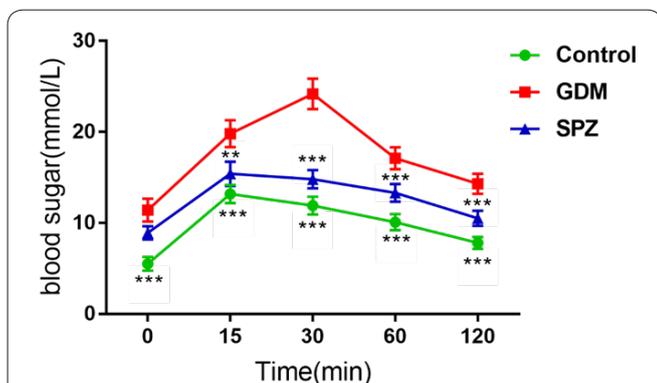The results showed that insulin and resistin levels

Figure 2. Effect of SPZ on glucose tolerance of gestational diabetic rats, showing oral glucose tolerance curve for each group of rats on day 18 of pregnancy. The data are presented as mean ± standard deviation (SD). *$p<0.01$, **$p<0.001$, compared with the GDM group.

Figure 3. Effect of SPZ on serum insulin (A) and resistin (B) concentrations. The data are presented as mean ± standard deviation (SD). *$p<0.01$, ***$p<0.001$, compared with the GDM group.

Figure 4. Effect of SPZ on the relative expressions of NF-κB and IκB mRNA in islet β cells of each group. The data are presented as mean ± standard deviation (SD). *$p<0.01$, ***$p<0.001$, compared with the GDM group.

Figure 5. Effect of SPZ on the expressions of Bcl-2, Bax and Caspase-3 proteins in islet β cells of each group. The data are presented as mean ± standard deviation (SD). *$p<0.05$, **$p<0.01$, ***$p<0.001$, compared with the GDM group.

in GDM group were significantly higher than those in pregnant rat control group ($p<0.001$), while those in SPZ group were significantly lower than in GDM group ($p<0.01$; Figure 3), suggesting that SPZ reduced hyperinsulinemia and insulin resistance in GDM rats.

Effect of SPZ on the expressions of NF-κB and IκB mRNA in pancreatic β cells

The results of RT-PCR showed that the expression level of NF-κB mRNA in GDM group was significantly higher than that in pregnant rat control group, while the expression of IκB showed the reverse trend ($p<0.001$). The SPZ treatment significantly reduced the expression of NF-κB mRNA and increased the expression of IκB ($p<0.01$; Figure 4), suggesting that the mechanism of action of STZ might involve regulation of the expressions of NF-κB and IκB.

Effect of SPZ on the expression levels of Bcl-2, Bax and Caspase-3 in pancreatic β cells of different groups

Compared with the control group, the expression level of Bcl-2 in islet β cells of GDM group was significantly lower ($p<0.001$). However, the expression level of Bcl-2 in islet β cells of SPZ group was significantly higher than that in GDM group ($p<0.01$). Moreover, the expression levels of Bax and Caspases-3 in islet β cells of GDM group were significantly higher than those of normal control group ($p<0.001$). Moreover, the protein expressions of Bax and Caspases-3 in pancreatic β cells of SPZ group were significantly lower than those in GDM group ($p<0.01$, Figure 5).

Discussion

Gestational diabetes mellitus (GDM) is a common complication of pregnancy. The basic pathological features of GDM are similar to those of type 2 diabetes mellitus, i.e. insulin resistance and compensatory enhancement of islet β cell secretion (9). Long-term hyperglycemia induces a series of cellular responses (including oxidative stress and inflammation), thereby forming a vicious cycle by exacerbating islet cell apoptosis and worsening the impairment of islet cell function (10, 11). Therefore, it is of great significance to find new drugs
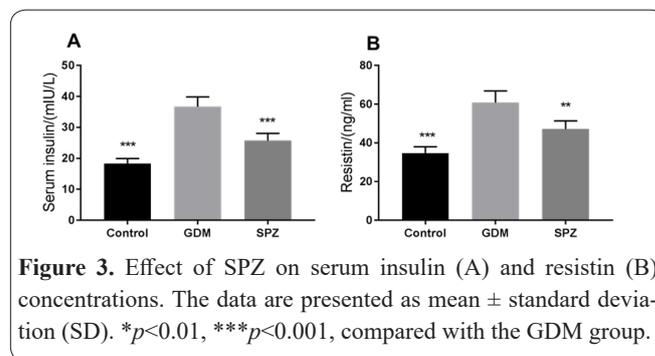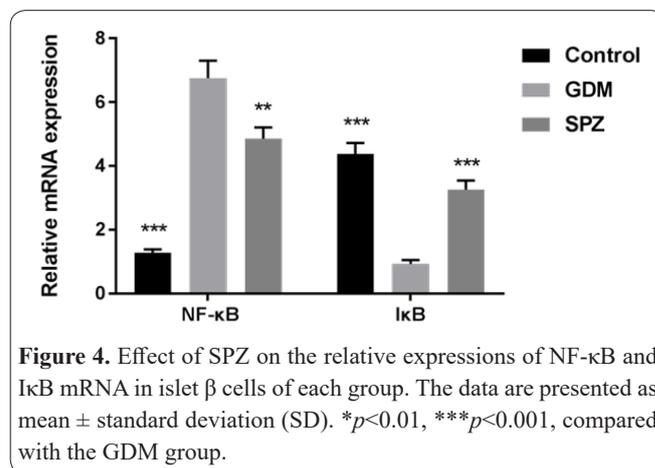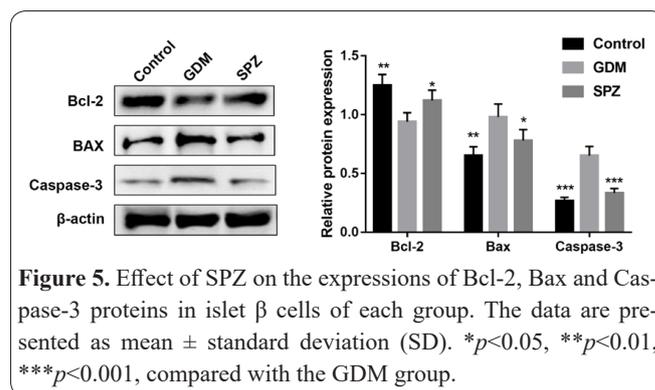
for diabetes mellitus to stop islet cell injury induced by high glucose.

Although insulin resistance during pregnancy is a physiological metabolic change, insulin sensitivity in gestational diabetes mellitus patients is lower than that in normal pregnant women (12). Therefore, islet β cells of gestational diabetes mellitus patients need to secrete more insulin to cope with insulin resistance. Long-term maintenance of compensatory high insulin levels in islet β cells often leads to islet dysfunction, resulting in insufficient insulin in gestational diabetes mellitus patients after childbirth. This results in more severe insulin resistance and islet β cell apoptosis, and induces reduction of glucose tolerance or even diabetes. The results obtained in this study show that SPZ effectively improved blood sugar control and glucose tolerance in gestational diabetic rats. Moreover, it reduced hyperinsulinemia and insulin resistance in these rats.

The NF-κB is a regulatory factor with multidirectional transcriptional activation function. It is an important inflammation pathway involved in mediation of insulin resistance and islet β cell dysfunction, and a key regulatory factor in inflammation. It causes inflammation through oxidative stress, leading to diabetes mellitus

and its complications (13). The nuclear localization of NF-κB is blocked by IκB which is located in the cytoplasm. This leads to inhibition of the activation of NF-κB. It is known that IκB is regulated by IκK through phosphorylation of its serine residue (14). This induces the rapid decomposition of IκB, thereby fully exposing the nuclear localization sequence of NF-κB. The activation of NF-κB leads to its rapid translocation to the nucleus where it binds to κB, thereby regulating target gene transcription and participating in inflammatory responses and cell differentiation, all of which lead to insulin resistance and islet β cell dysfunction. At the same time, the NF-κB complex induces peripheral blood mononuclear cells to migrate to the intima of the arteries to form foam cells, accelerate atherosclerosis, and cause diabetes-related complications (15). The results obtained in this study showed that SPZ decreased the expression of NF-κB mRNA, and increased the expression of IκB mRNA, indicating it inhibited the inflammatory responses in islet tissue and mitigated insulin resistance mediated by inflammatory response.

In the course of GDM, persistent insulin resistance and compensatory enhancement of islet β cell secretion function gradually lead to β cell damage (16, 17). The Bcl-2 protein family is located outside the mitochondrial membrane, and it regulates apoptosis with mitochondria as target. It plays an important role in the regulation of apoptosis. It comprises Bcl-2 and Bax proteins. Under normal conditions, these two proteins are mutually restricted. They participate in regulating apoptosis through the mitochondrial pathway, in a dynamic equilibrium (18). The pro-apoptotic protein Bax down-regulates mitochondrial transmembrane potential, enhances the release of cytochrome (Cyt) C which then enters the cytoplasm to bind with apoptotic protein activator 21 (Apaf). This binding triggers a cascade reaction, leading to activation of downstream factor Caspase-3, thereby initiating apoptosis. In contrast the anti-apoptotic protein Bcl-2 blocks the decrease in mitochondrial transmembrane potential, prevents CytC release and the associated caspase cascade reaction, ultimately blocking apoptosis (19-21). This study has demonstrated that the protective mechanism of SPZ on pancreatic β cells occurred through inhibition of pancreatic β cell apoptosis by increasing the expression of Bcl-2 and decreasing the expressions of Bax and Caspase-3.

In conclusion, SPZ not only effectively controls the rise in blood sugar in gestational diabetes mellitus, but also inhibits mitochondrial pathway apoptosis in islet tissue, and reduces islet β cell damage. It effectively protects islet β cells and prevents progression of diabetes mellitus. Thus, SPZ may be helpful in the targeted treatment of gestational diabetes mellitus. These results show that SPZ reduces damage to islet cells induced by high-sugar and high-fat, and protects islet function, thereby exerting a hypoglycemic role. Liraglutide improves insulin resistance by inhibiting the apoptosis of islet β cells, and increasing the number of islet β cells. Thus, liraglutide can effectively improve insulin resistance and inhibit apoptosis of pancreatic β cells.

Based on these results, some preliminary conclusions can be drawn i.e. corosolic acid improves insulin signaling pathway in placenta of gestational diabetes mellitus rats, inhibits the activation of inflammatory

reactions, and mitigates insulin resistance. It also inhibits mitochondrial pathway apoptosis in islet tissue and alleviates damage to islet β cells.

Acknowledgements

Conflict of Interest
There are no conflicts of interest in this study.

Author's contribution
All work was done by the author named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Yongkang Yang; Jing Yang, Jian Jia, Yongkang Yang, Yaning Zhao and Qin Li collected and analysed the data; Jing Yang and Jian Jia wrote the text and all authors have read and approved the text prior to publication.

Jing Yang and Jian Jia contributed equally to this work and should be considered as co-first authors.

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