Spleen-kidney supplementing formula alleviates insulin resistance via regulating AKT/glycogen synthase kinase 3β pathway in rats with type 2 diabetic induced by high-fat diet

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Abstract

OBJECTIVE: To explore the molecular mechanism underpinning the action by investigating its effect on glycogen content and AKT (also known as protein kinase B)/glycogen synthase kinase 3β (GSK-3β) pathway in the liver of rats with type 2 diabetic induced by high-fat diet.

METHODS: The rat model of type 2 diabetes was induced by high-fat diet and multiple low-dose streptozotocin injection. Diabetic rats were divided into five groups: the model control group, the Metformin group, spleen-kidney supplementing formula groups of low, medium and high doses. Fasting blood glucose (FBG) levels were measured before treatment and every two weeks during treatment. After the treatment, oral glucose tolerance test was performed, and hemoglobin A1c (HbA1c) and C-peptide were measured to assess the formula's effect on glucose metabolism and insulin resistance. The protein expression levels of AKT, GSK-3β and their phosphorylated forms in the liver were also measured to study the formula's role in insulin signaling pathway.

RESULTS: Spleen-kidney supplementing formula significantly relieved the symptoms of polydipsia, polyuria and weight loss in type 2 diabetic rats, reduced FBG and HbA1c levels, increased glycogen content, and improved insulin sensitivity. The anti-diabetic effects of spleen-kidney supplementing formula are dose dependent. It also increased the total AKT protein level and the GSK-3β phosphorylation in the liver of type 2 diabetic rats.

CONCLUSION: Spleen-kidney supplementing formula has hypoglycemic effect and relieves insulin resistance by enhancing AKT/GSK-3β signaling pathway in the liver of type 2 diabetic rats.

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Keywords: Spleen-kidney supplementing formula; Insulin resistance; Proto-oncogene proteins c-akt; Glycogen; Phosphorylation

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a long-term metabolic disorder characterized by hyperglycemia, insulin resistance of target tissues and relative insufficiency of insulin secretion. About 425 million people worldwide are estimated to have diabetes and some 629 million people are projected to develop diabetes by 2045. China has topped the world with the largest number of people diagnosed with diabetes mellitus.
As an important part of complementary and alternative therapies, Traditional Chinese Medicine (TCM) is promising in treating diabetes. Generations of medical practitioners have contributed a lot of valuable experiences and effective prescriptions for treating diabetes. Based on symptom pattern identification, we consider spleen-kidney deficiency as the fundamental pathogenesis mechanism of diabetes. Excessive intake of fatty and sweet foods injures the spleen-Qi and results in the spleen-Qi deficiency which weakens spleen’s transformation of food. The excessive food leads to the dampness-phlegm accumulation. It is proved that spleen-Qi deficiency and dampness-phlegm were strongly associated with diabetes. Middle-aged and senior adults are at the highest risk for type 2 diabetes, and they are also at the age stage of kidney-Qi deficiency. Huang Di Nei Jing linked diabetes to kidney-Qi and reported that "diabetes progresses after its first onset which was caused by kidney deficiency with sweet urine as its manifestation". In addition, it has been reported that kidney-Yin deficiency may be manifested throughout all stages of diabetes. The secondary cause of diabetes is dryness-heat, especially the dryness of lung and the hyperactivity of stomach fire. Lungs serve as a source of body fluids, and the dryness of the lung results in shortage of body fluid, causing thirst and polydipsia. Spleen-kidney supplementing formula was proposed based on the above pathogenesis mechanism and the therapeutic principle of supplementing spleen-kidney and clearing the extra heat of stomach-lung. In spleen-kidney supplementing formula, atractylus strengthens spleen-Qi and asiatric cornelian cherry fruit tonifies kidney. Chinese goldthread clears stomach fire and white mulberry root-bark clears lung heat. Kudzu root clears heat and promotes body fluid production. Fortune eupatorium herb expels dampness. We observed that spleen-kidney supplementing formula could obviously improve the sugar metabolism in type 2 diabetic patients. Our previous pharmacological experiments showed that berberine and puerarin are the main effective components in spleen-kidney supplementing formula, since studies have proven that berberine and puerarin have hypoglycemic effects. However, the molecular mechanisms of spleen-kidney supplementing formula are not clear. Liver helps maintain normal blood glucose level by storing excess glucose as glycogen. Glycogen synthase (GS) is the rate-limiting enzyme of glycogen synthesis. Insulin promotes activation of GS via inhibition of glycogen synthase kinase 3 (GSK-3) through phosphorylation. The signaling cascade is insulin receptor → IRS1/2 → PI3K → Akt → GSK-3. GSK-3 phosphorylates the C-terminal serine residues of glycogen synthase and reduces its activity. The activity of GSK-3 is inhibited by phosphorylation of a regulatory serine in either of the two isoforms of GSK-3, serine 21 in GSK-3α and serine 9 in GSK-3β. Strong evidence suggests that GSK-3β is involved in the pathology of type 2 diabetes mellitus, and the over-expression or over-activation of GSK-3β could induce T2DM, making it a novel target for treating diabetes. AKT (also known as protein kinase B), a major upstream kinase, when activated, directly inhibits GSK-3 activity by phosphorylating the serine residues of GSK-3. To understand the mechanism underlying the effect of Spleen-kidney supplementing formula, this study was aimed to explored its regulatory role on Akt/GSK-3β pathway in the liver of rats with type 2 diabetic induced by high-fat diet.

MATERIALS AND METHODS

Animal
Specific pathogen free Wistar male rats (290 ± 10) g were provided by Beijing Huafukang Bioscience Co., Inc. (Beijing China), permit No. SCXK (Jing) 2014-0002. The rats were caged in a temperature (21 ± 2 °C) and humidity (50% ± 10%) controlled environment with a 12 h dark/light cycle in Laboratory Animal Center of North China University of Science and Technology (MY10DXK07, Tangshan, China). All experiments were conducted according to the guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Drugs
Huangqi (Radix Astragalus Mongolici), Shanzhuyu (Fructus Comi), Huanglian (Rhizoma Codonopsis), Gegen (Radix Puerariae Lobatae), Sangbaipi (Cortex Mori Albae Radici), Peilan (Eupatorii Herba) from Beijing Tongrentang Chinese Medicine Co., Ltd., (Tangshan, China). After identification confirmed by Tian Chunyu (Department of Traditional Chinese Medicine, North China University of Science and Technology), these herbs were mixed in the ratio of 5:5:4:4:4:3:2. High-performance liquid chromatography method was conducted to determine the berberine content for quality control of Spleen-kidney supplementing formula. The decoction was concentrated and dehydrated in vacuum (70 °C), and ground into powder. The prepared powder was kept at 0-4 °C, and dissolved with purified water into suspension of different concentrations. Metformin hydrochloride tablet (500 mg/tablet) were manufactured by Sino-American Shanghai Squib Pharmaceutical Ltd. (#1407117).

Reagents
Rat C-peptide Elisa kit was purchased from Kainuo Bio Ltd. (Beijing, China); Rat/Mouse insulin Elisa kit was purchased from the Merck Millipore Co., Ltd. (Bedford, MA, USA); Glycogen Assay Kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China); Primary antibodies of AKT and GSK-3β was purchased from Waneliebio Co., Ltd. (Shenyang, China); Primary antibodies of AKT (phospho-S473), GSK-3β (phospho-S9) and GAPDH were...
purchased from Bioworld Technology Inc. (Nanjing, China); Western and IP cell lysis buffer, PMSF (100 mM), BCA kit (enhanced), SDS-PAGE Gel Parpation Kit, Prestained Protein Molecular Weight Marker, Horseradish Peroxidase (HRP) Conjugated Goat Anti-Mouse IgG (H + L), HRP- Conjugated Goat Anti-Rabbit IgG (H + L). were purchased from Bi Yuntian biological company (Shanghai, China).

Main instruments
Glucose meter (Sinocare Inc., Changsha, China); Nycocard Reader II (Alere/Axis-Shield, Oslo, Norway); IKA-T10 basic homogenizer (IKA, Staufen, Germany); Low-temperature high-speed centrifuge R134A (Eppendorf, Hamburg, Germany); Infinite F50 microplate reader (Tecan group Ltd., Männedorf, Switzerland); Multi-function electrophoresis apparatus (Liuyi instrument factory, Beijing, China).

Induction of type 2 diabetic rat model
Eighty male Wistar rats were fed for one week to adapt to the environment. Ten rats were randomly selected as the normal control group (Group N) and fed with standard diet. The remaining rats were fed with high-fat diet (kcal% fat, containing 60% fat, 20% protein, 20% carbohydrates from Beijing HuaFuKang Bioscience et al. (2019)). The rats in Group N were injected with streptozotocin (STZ) solution (25 mg/kg, twice at weekly intervals). The rats in Group N were injected with the same dose of buffer solution. All rats were fasted for 12 h before each injection of STZ. On day 7 after the final injection of STZ, all rats were tested for the levels of fasting blood glucose (FBG) and random blood glucose (RBG) (8:00 AM). Fifty-nine rats were considered to be diabetic with FBG ≥ 11.1 mmol/L or RBG ≥ 16.7 mmol/L.

Grouping and drug administration
After exclusion of 9 rats with FBG > 30.0 mmol/L, the remaining 50 diabetic rats were randomly divided into: the model control group (Group M), the metformin group (Group Met), and spleen-kidney supplementation formula groups of low, medium and high dose (Group SKSF1, SKSFm and SKSFh respectively), with 10 rats in each group. All rats were treated with corresponding drugs intragastrically (ig). According to the drug dosage conversion formula between rats and human, the rat dosage is 6.3 times of human dosage. Rats in Group N and Group M were given purified water; rats in Group Met were administrated with metformin hydrochloride suspension 85 mg·kg⁻¹·d⁻¹; rats in Group SKSF1, SKSFm and SKSFh were treated with alcohol extract of SKSF of 2000, 1000, 500 mg/kg respectively. Rats were weighed once a week for adjusting drug dosage in the 8 weeks’ intervention period.

FBG, HbA1c and glycogen
Before treatment and on days 14, 28 and 56 after treatment (after 16 h fasting), the blood from rat tail vein was collected for FBG measurement with glucose meter. Hemoglobin A1c (HbA1c) and glycogen levels were measured using the HbA1c ELISA Kit and Glycogen Assay Kit following manufacturer’s instructions respectively.

OGTT and AUC calculation
The rats of normal and diabetic groups were orally treated with 2 g/kg of glucose. The blood glucose levels in blood samples collected from tail vein were measured at 0, 30, 60, 120 min after glucose loading. The area under curve (AUC) was calculated by the formula: AUC (mmol/L × min) = 1/2 × (BG 0 min + BG 30 min) × 30 min + 1/2 × (BG 30 min + BG 60 min) ×30 min + 1/2 × (BG 60 min + BG 120 min) × 60 min.

Assessment of insulin sensitivity/resistance
The blood from the abdominal aorta was centrifuged to collect serum. FBG, fasting C-peptide and insulin were measured by ELISA with Tecan microplate reader. Insulin sensitivity and resistance indexes were calculated according to the formulae below:

\[
\text{Homa-IR (CP)} = \frac{\text{FBG} \times \text{fasting CP}}{\text{1} + \text{FBG}}
\]

Western Blot for protein contents of p-AKT(s473), AKT, p-GSK-3β(s9) and GSK-3β in the liver
The liver (120 mg) was mashed with homogenizer and cells were lysed in lysis buffer. After centrifugation, the supernatant was collected, and the concentration of each group was measured with bicinchoninic acid (BCA) protein assay kit. After mixing with SDS-sample buffer, cell lysates were separated by SDS-PAGE and transferred onto PVDF membrane (#ISEQ00010, Merck Millipore (Bedford, Ma, USA)), and were incubated with antibodies against p-AKT(s473), AKT, p-GSK-3β(s9), GSK-3β and GAPDH. After incubation with appropriate peroxidase-conjugated secondary antibodies, the immunoreactive bands were visualized by ECL reagents and quantified with Pro Plus 6.0 medical image analysis system.

Statistical analysis
All data were expressed as mean ± standard deviation ( x ± s). Statistical analysis were performed with SPSS 17.0 (SPSS Inc., Released 2008. SPSS Statistics for Windows, Version 17.0. Chicago, IL, USA). One-way analysis of variance (ANOVA) was conducted to test the differences different groups and a P < 0.05 (two-tailed) was the significant level.

RESULTS

Symptoms of diabetic rats
The rats in the normal control group (Group N) showed healthy signs, including normal daily food
and water intake, normal excretion of urine and stool, smooth fur, good mental state, and swift action. The rats in the untreated diabetic group (Group M) showed symptoms of diabetes, such as polydipsia, polyuria, loose stools, rough and dull fur, mental fatigue, slowness in reacting, back arched, and weight loss. Compared with those in Group M, the rats treated with Metformin or Spleen-kidney supplementing formula showed glossy fur and swift response, and the symptoms of polydipsia, polyuria and weight loss were significantly relieved (Figure 1).

During the experiment, the diabetic rats in treatment groups, especially in Groups Met and Group SKSFm, gained more weight than the untreated diabetic rats (Figure 1A). Food-intake in Group M kept high throughout the experiment. After treatment for 8 weeks, food-intake of treatment groups was remarkably reduced, with the exception of SKSFI, which did not show significant differences compared to Group M (Figure 1B). The water-intake remained relatively stable in Group N. However, it was higher in Group M and had an upward trend. After treatment, the water-intake of diabetic rat was lowered and maintained within a relatively normal range in Group Met, SKSFh and SKSFm (Figure 1C). Untreated diabetic rats had increased urine volume compared to the normal control group, but the urine volume of diabetic rats in Groups Met, SKSFh and SKSFm was decreased (Figure 1D).

**FBG, HbA1c levels, AUC of OGTT and hepatic glycogen levels in diabetic rats**

FBG levels in Group M remained significantly high throughout the experiment. On days 14, 28 and 56 of drug administration period, FBG levels descended significantly and gradually in all treatment groups ($P < 0.05$). In particular, the hypoglycemic effects were more prominent in Groups Met and Group SKSFh, suggesting that the effects of spleen-kidney supplementing formula were dose-dependent (Figure 2A). The HbA1C levels, another indicator of blood glucose concentration, were also significantly lower in the Spleen-kidney supplementing formula treatment groups than that of untreated diabetic group (Figure 2B). According to OGTT, the blood glucose level in the Group M was higher than those of Group N at each time point before and after glucose loading. The glucose tolerance in each SKSF treated group was improved and the effects were in dose dependent manner. The blood glucose levels in Groups Met and SKSFh were similar and close to the normal level in group M (Figure 2C). The AUCs of Groups Met and SKSFh were smaller than that of Group M (Figure 3). Glycogen contents were significantly increased in the SKSF treatment groups than that of Group M (Figure 4F) and the effects were in dose dependent manner.

![Figure 1 Effects of spleen-kidney supplementing formula on body weight gain, water/food intake, and urine excretion of diabetic rats A: body weight gain; B: food-intake; C: water-intake; D: urine volume. N: normal control group; M: model control group; Met: metformin group (metformin hydrochloride 85 mg/kg, 8 weeks); SKSFl: spleen-kidney supplementing formula group of low dose, extract of SKSF of 500 mg/kg, 8 weeks); SKSFm: spleen-kidney supplementing formula group of medium dose, extract of SKSF of 1000 mg/kg, 8 weeks; SKSFh: spleen-kidney supplementing formula group of high dose, extract of SKSF of 2000 mg/kg, 8 weeks. Compared with Group N, $^aP < 0.05$; compared with Group M, $^bP < 0.05$.](attachment:image.png)
Figure 2 Effects of spleen-kidney supplementing formula on FBG, HbA1c levels, OGTT in diabetic rats
A: fasting blood glucose; B: HbA1c (HbA1c); C: the changes in blood glucose concentration during OGTT in diabetic rats. N: normal control group; M: model control group; Met: metformin group (metformin hydrochloride 85 mg/kg, 8 weeks); SKSF: spleen-kidney supplementing formula group of low dose, extract of SKSF of 500 mg/kg, 8 weeks; SKSFm: spleen-kidney supplementing formula group of medium dose, extract of SKSF of 1000 mg/kg, 8 weeks; SKSFh: spleen-kidney supplementing formula group of high dose, extract of SKSF of 2000 mg/kg, 8 weeks. Compared with Group N, \( P < 0.05 \); compared with Group M, \( P < 0.05 \).

C-peptide, insulin and insulin sensitivity/resistance indexes in diabetic rats
Figure 4 shows the effect of SKSF on the levels of fasting blood glucose (Figure 4C), fasting c-peptide (Figure 4B) and insulin (Figure 4A), and the extent of insulin resistance in type 2 diabetic rats (Figure 4C), ISI (Figure 4D) and Homa-IR (Figure 4E), glycogen contents (Figure 4F). Compared with Group N, a lower level of fasting serum insulin and lower fasting c-peptide content was observed in diabetic rats of Group M (\( P < 0.05 \)). The C-peptide and insulin levels were significantly increased in each treatment group on day 56 after treatment, especially in Group SKSFh (\( P < 0.05 \)).

Protein levels of p-AKT and p-GSK-3β in the liver of T2DM rats
The proportion of p-AKT in diabetic rats (Group M) were significantly lower than that of normal rats (\( P < 0.05 \); Figure 5A, 5B). With Metformin and Spleen-kidney supplementing formula treatment, the p-AKT level increased compared to group M Elevated level of p-GSK-3β was observed in the liver of diabetic rats from Group M. After treatment, the phosphorylation of GSK-3β was significantly enhanced (\( P < 0.05 \)) (Figure 5A, 5C).

DIscussion
Insulin resistance (IR) is a major defect in type 2 diabetes mellitus (T2DM). In this study, high-fat diet combined with multiple low-dose STZ injections was used for developing stable animal model of type 2 diabetes mellitus. Although there was no weight loss observed in diabetic rats, the slower weight gain suggested the existence of metabolism disorder. These symptoms and abnormal biochemical parameter demonstrated that the rat’s model of T2DM was induced successfully. We observed total GSK-3β protein expression was increased significantly and its phosphorylation was significantly decreased in the liver of type 2 diabetic rats, which demonstrates the defects in signal pathway for
stimulation of glycogen synthesis by insulin. AKT is a major upstream kinase and activated AKT directly inhibits GSK-3 activity by phosphorylating serine residues of GSK-3 in response to insulin. 

The results suggested that the total AKT protein and its phosphorylation level were reduced in the liver of diabetic rats, which means there was lower content of AKT in the activated state and contribute to the over-activation of GSK-3β. After treatment with spleen-kidney supplementing formula for eight weeks, the FBG level was reduced, the glucose tolerance was improved, and insulin resistance index was lowered in type 2 diabetic model rats. We also observed that total GSK-3β was reduced, but its phosphorylation at serine increased, which means the content of GSK-3β in activated state was much lower. The total AKT protein was elevated after treatment, although there was no significant difference on its phosphorylation. In other words, the content of AKT in activated state was much higher. These observations suggested that spleen-kidney supplementing formula relieves insulin resistance by enhancing AKT/GSK-3β signaling pathway. Interestingly, although the enhancing effect on AKT/GSK-3β signal pathway was most remarkable in Group SKSFm, the glycogen content was lower than that in Group SKSFh. This result implies that other signaling/metabolic regulatory pathways may also be involved, which requires additional studies. Furthermore, we noticed that there were higher c-peptide and insulin levels in Group SKSFh. The level of c-peptide in the blood indicates the amount of insulin made by the pancreas, which typically releases c-peptide and insulin in equivalent amounts. According to literature analysis, berberine and puerarin, the important chemical compositions of SKSF, were reported to increase insulin secretion and β cell regeneration in STZ and high carbohydrate/fat diet induced diabetic rats.

Figure 4 Fasting C-peptide, insulin and fasting blood glucose in type 2 diabetic rats
A: INS; B: C-P; C: FBG; D: ISI; E: Homa-IR; F: Glycogen; N: normal control group; M: model control group; Met: metformin group (metformin hydrochloride 85 mg/kg, 8 weeks); SKSFh: spleen-kidney supplementing formula group of high dose, extract of SKSF of 2000 mg/kg, 8 weeks; SKSFm: spleen-kidney supplementing formula group of medium dose, extract of SKSF of 1000 mg/kg, 8 weeks; SKSFl: spleen-kidney supplementing formula group of low dose, extract of SKSF of 500 mg/kg, 8 weeks. INS: insulin; C-P: C-Peptide; FBG: fasting blood glucose; ISI: insulin sensitive index; Homa-IR: homeostasis model assessment-insulin resistance. Compared with Group N, *P < 0.05; compared with Group M, †P < 0.05.
We hypothesized that the increased endogenous insulin production and β cells function improvement also contribute to the stronger hypoglycemic effect of SKSFh than SKSFm.

In conclusion, Spleen-kidney supplementing formula has hypoglycemic effect and relieves insulin resistance by enhancing AKT/GSK-3β signaling pathway in the liver of type 2 diabetic rats, which is related to the up-regulation of total AKT expression and the inactivation of GSK-3β.

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