Experimental Study on the Effect of Tibetan Medicine Triphala on the Proliferation and Apoptosis of Pancreatic Islet β Cells through Incretin–cAMP Signaling Pathway

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According to the data, there are 387 million people with diabetes in the world, and the number of people with diabetes is expected to reach 600 million by 2035 (Nature Reviews Endocrinology, 14, 2018, Zheng et al.). At present, there are nearly 110 million diabetic patients in China, the incidence of which is increasing (Diabetologia, 61, 2018, Ma). Islet β cell apoptosis and proliferation is an important basis for the occurrence and development of diabetes. It has been reported that enhancing the activity of incretin–cAMP signaling pathway can also promote islet β cell proliferation, reduce β cell apoptosis and promote insulin secretion (Diabetologia, 59, 2016, Iida et al.). Tibetan medicine Triphala (THL) is a traditional national medicine, it plays a good role in anti-fatigue, antioxidation, prevention and treatment of polycythemia at high altitude. Research have shown that it can reduce blood glucose in patients with diabetes and inhibit the activity of glucosidase in the intestines (The Journal of Alternative and Complementary Medicine, 23, 2017, Peterson et al.). After the diabetic Wistar rat model induced by Streptozocin (STZ) was successfully duplicated, the positive drug sitagliptin tablet and THL were given and the changes of body weight and blood glucose were measured. After 6 weeks, the expression of related factors in serum and pancreas was observed. Compared with the model group, in the treatment group, blood glucose decreased, body weight increased, incretin–cAMP signaling pathway related factors glucose-dependent insulin-promoting polypeptide (GIP), glucagon-like peptide-1 (GLP-1), GLP-IR, cAMP, P-protein kinase A (PKA), AKT were up-regulated, insulin secretion was increased, liporing protein interaction protein (TXNIP) expression was down-regulated. In addition, in the treatment group, the degree of islet atrophy was alleviated and the number of islet β cells increased. This study shows that THL may enhance the activity of incretin–cAMP signal pathway and affect the proliferation and apoptosis of islet β cells, so as to achieve the effect of anti-diabetes.

Key words Triphala; liporing protein interaction protein; incretin–cAMP signal pathway; islet β cell proliferation; islet β cell apoptosis

INTRODUCTION

Diabetes is a chronic metabolic disease with hyperglycemia as the main symptom caused by insufficient insulin secretion or islet dysfunction. Traditional Tibetan medicine believes that diabetes is caused by a serious imbalance in the “three causes” of the human body, which is characterized by the prosperity of “red bar” and “long” and the weakness of “bacon,” resulting in the normal decomposition, excretion and transportation of the seven fine and micro substances in the human body. Make blood filth gather too much, cause the human body. Make blood filth gather too much, cause the normal decomposition, excretion and transportation of the seven fine and micro substances in the human body. Make blood filth gather too much, cause the normal decomposition, excretion and transportation of the seven fine and micro substances in the human body. Make blood filth gather too much, cause the normal decomposition, excretion and transportation of the seven fine and micro substances in the human body. Make blood filth gather too much, cause the normal decomposition, excretion and transportation of the seven fine and micro substances in the human body. Make blood filth gather too much, cause the normal decomposition, excretion and transportation of the seven fine and micro substances in the human body. Make blood filth gather too much, cause the normal decomposition, excretion and transportation of the seven fine and micro substances in the human body. Make blood filth gather too much, cause the normal decomposition, excretion and transportation of the seven fine and micro substances

Enteropancreatin (incretin) is a kind of hormone synthesized and secreted by intestinal endocrine cells stimulated by food nutrients. It mainly includes glucose-dependent insulin-promoting polypeptide (GIP) and glucagon-like peptide-1 (GLP-1). It has many physiological effects, such as inhibiting feeding, inhibiting islet β cell apoptosis, promoting insulin secretion and so on.2 GLP-1, GIP and cAMP constitute incretin–cAMP signaling pathway. Recent studies have shown that incretin–cAMP signaling pathway can promote insulin secretion through protein kinase A (PKA) and activated exchange protein (Epac2A).3 At the same time, GLP-1 could activate protein kinase B (AKT), and enhance its expression, which could promote islet β cell proliferation and inhibit islet β cell apoptosis.4,5 The increased activity of incretin–cAMP signaling pathway can also promote the proliferation of islet β cells and reduce the apoptosis of β cells. It can also promote glucose-dependent insulin secretion.6,7 GLP-1 was released from L cells of intestinal mucosa and combined with glucagon-like peptide-1 receptor (GLP-1R). Glucose concentration-dependent hypoglycemic effect was played after GLP-1 was released into the blood. It will be rapidly degraded by dipeptidyl peptidase-4 (DPP 4) and lose its biological activity.8 The positive control drug (Sitagliptin tablets) which DPP-4 inhibitor, was used in this study.

TXNIP binds to thioredoxin (TRX) to inhibit its activity, resulting in the accumulation of intracellular active oxide (ROS), thus regulating the redox state of cells and affecting a variety of physiological processes of cells.9 It was found that,9 TXNIP is the key factor of islet β cell apoptosis in-
duced by high glucose toxicity. High glucose can induce the production and increase the expression of apoptosis-promoting factor TXNIP and promote islet β-cell apoptosis, while the deletion of TXNIP can reduce the occurrence of diabetes mellitus. The decrease of TXNIP expression can enhance the expression of GLP-IR signal and activate the downstream pathway. The results suggest that THL may improve the hyperglycemia state of the body by down-regulating the expression of TXNIP and enhancing the activity of incretin-cAMP signal pathway, reducing the apoptosis and promoting the proliferation of islet β cells. It is expected to be widely used in the field of diabetes treatment.

MATERIALS AND METHODS

Materials THL 60 g were purchased from Anguo Xufang Traditional Chinese Medicine Management Co., Ltd. (China). Sitagliptin tablets were purchased from Merck Sharp & Dohme Ltd. (U.K.). TXNIP, p-PKA, β-actin antibodies were purchased from Abcam Company (U.S.A.). GLP-IR was purchased from Shanghai Aibixin Company (China). Insulin was purchased from Shanghai Affinity Company (China). Rat GIP, GLP-1, TXNIP and cAMP enzyme-linked immunosorbent assay (ELISA) kits (Specifications are all 48T) were purchased from Jiangsu Green Leaf Biotechnology Co., Ltd. (China). Streptozocin (STZ) was purchased from Sigma Company (U.S.A.). Protein lysate RIPA (ordinary type) was purchased from Beijing Solarbio Company (China), BCA protein quantitative kit was purchased from Kaiji Biological (China). BD TM Skim milk powder was purchased from BD Company (U.S.A.). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) protein sample buffer (5X) was purchased from Beijing Baiaolaibo Technology Co., Ltd. (China).

Preparation and Dose Determination of THL THL preparation: THL (Phyllanthus emblica L., Terminalia chebula Retz. and Terminalia billerica Gaertn.) Roxb. were 1:1:1, break and enucleate, THL: water (1:40), soak 30 min and decoct 3 times, each time 30 min, combined with filtrate, filtered. The water extract of THL containing the original medicinal material 0.5 g/mL was concentrated and condensed on a rotary evaporator, lyophilized and set aside. The effective components can be extracted to a great extent by water decoction, and they are economical, easy to obtain and safe, so they are decocted in water.

Dose determination: The standard issued by the Ministry of Tibetan Medicine stipulates that the human dosage of THL Powder is 3 g/4 g twice a day. In this study, it is determined that the daily dose of THL adults (in terms of 70 kg body weight) is 6 g. The low and high doses of 0.43 and 1.72 g/kg were converted in rats, which were 5 and 20 times of the daily dose in adults, respectively, in order to increase the rationality of the experiment. It was decided to add the middle dose group with the dose of 0.86 g/kg, which was equivalent to 10 times of the daily dose of adults, and dissolved and diluted with physiological saline into three doses: high, medium and low.

Animals and Treatment Seventy Wistar rats, SPF grade, 150 g/220 g, were purchased from Sichuan Jianyang Dashuo Experimental Animal Co., Ltd. (R-082018062, China). Wistar rats were fed adaptively for 1 week. Ten rats were randomly selected as normal group and the other 60 rats were used as model group. The diabetic model was induced by intraperitoneal injection of 50 mg/kg STZ (72 h after injection, the tail vein blood glucose was measured by empty stomach blood glucose meter > 16.7 mmol/L as a successful diabetic model). A total of 45 models were successfully made, which were randomly divided into model group, positive group, high dose group, middle dose group and low dose group. There were 9 rats in each group. The rats in the normal group were given the same amount of normal saline, the model group was given the same amount of normal saline, the positive group were treated with the dose of 10.5 mg/kg of sitagliptin and the treatment group, THL, was given at a dose of 2.2 for 6 weeks. Every three days, weight and fasting blood glucose were measured. All animals were approved by the Ethics Committee of Yunnan University of Chinese Medicine.

Detection of the Expression of Related Factors The levels of GIP, GLP-1 and TXNIP in rats peripheral serum and cAMP in pancreatic tissues were detected by four ELISA kits. The expression of TXNIP, GLP-IR, PKA, P-PKA, AKT and insulin in rats pancreas were detected by Western blot method. HE staining and orange G staining were used to observe the sections of rats pancreas.

Statistical Analysis SPSS19.0 software was used for repeated measurement ANOVA. One-way ANOVA was used for inter-group comparison. LSD method was used for variance analysis, and image J was used for gray analysis. Results are expressed as mean ± standard deviation (S.D.) and p < 0.05 or p < 0.01 are the standard used for statistical significance.

RESULTS

THL Can Reduce Blood Glucose and Increase Body Weight in Diabetic Wistar Rats The male wistar rats were successfully established by intraperitoneal injection of STZ. The changes of body weight and blood glucose were observed after the THL was given for 6 weeks. As shown in Fig. 2 (a, b), the body weight of rats in the normal group increased
gradually, while the body weight of rats in other groups showed an overall downward trend. However, compared with the model group, the body weight of the positive group, the low, middle and high dose groups showed an upward trend at the third week of administration. The body weight of the low dose group was significantly higher than that of the model group ($p<0.05$). In Fig. 2 (c, d), the blood glucose in the normal group was maintained at a normal level. Compared with

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Fig. 2. Changes in Body Weight and Blood Glucose of Wistar Rats after 6 Weeks of Administration

Before indicates that before the model was successfully established, the drug was not given. After refers to 6 weeks after administration. The rats in the model group lost more weight and were thinner. After administration, the weight loss was slow, and after 3 weeks, the weight began to slowly increase (a, b); The blood glucose in the model group was higher, and the blood glucose began to decrease slowly after administration, but the effect was better than that in the non-positive group (c, d). (Color figure can be accessed in the online version.)

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Fig. 3. ELISA Kits Were Used to Detect the Changes of cAMP in Rats Pancreatic Tissue and Serum GIP, GLP-1, TXNIP and Other Factors

(a) Compared with the model group, the expression of cAMP in the pancreatic tissues of the THL group were enhanced ($p<0.01$). (b, c) Compared with the model group, the THL were administered. The levels of GIP and GLP-1 in the serum of the rats in the group were increased ($p<0.01$), but compared with GIP, the effect of the low-dose group of THL did not reach the middle-high dose group. (d) Compared with the model group, the level of TXNIP in the serum of rats in the THL group was significantly lower ($p<0.01$).
the model group, the blood glucose in the low dose group of THL did not decrease significantly, and increased in the later stage. The middle and high dose groups of THL had good hypoglycemic effect (significant difference, \( p < 0.05 \)), but the data showed that the hypoglycemic effect of middle and high dose groups was weaker than that of positive group. THL affects the expression of related factors of incretin–cAMP pathway.

**THL Affects the Expression of Related Factors of Incretin–cAMP Pathway** THL could decrease the level of TXNIP in peripheral serum of diabetic Wistar rats \( (p < 0.01) \). Fig. 3d), six weeks after administration of THL, it was found

**Fig. 4. Western Blot Was Used to Detect the Expression of Related Proteins in Rat Pancreas**

(a) Compared with the model group, the high-dose group of THL can reduce the expression of TXNIP in pancreatic tissue of diabetic Wistar rats by STZ modeling \( (p < 0.01) \). (b, d, e, f) Compared with the model group, THL can up-regulate the expression of GLP-1R, P-PKA, AKT and insulin in rat pancreatic tissues \( (p < 0.01) \). (c) For PKA, compared with the model group, there was significant difference between the low dose THL group \( (p < 0.05) \).
that the expression of cAMP in pancreatic tissue of was increased ($p < 0.01$. Fig. 3a), the levels of GIP and GLP-1 were increased, the TXNIP was decreased in peripheral serum of diabetic Wistar rats ($p < 0.05$, $p < 0.01$, $p < 0.01$. Figs. 3b–d).

Western results display that the expression of TXNIP in pancreatic tissue of diabetic Wistar rats was decreased in the middle and high dose group of THL ($p < 0.01$. Fig. 4a), compared to the model group. THL could up-regulate the expression of GLP-1R, P-PKA, AKT and insulin in rats pancreatic tissue ($p < 0.01$. Figs. 4b, d–f). There was a significant difference in the effect of on the low dose group of PKA ($p < 0.05$. Fig. 4c). THL could reduce the degree of pancreatic tissue atrophy and increase the number of islet $\beta$ cells in diabetic Wistar rats.

In the normal group, the pancreatic islets in the pancreatic tissues were larger, the shape of the islets was regular, oval, located in the center of the pancreas, and the boundary between the islets and the surrounding cells was clear, and the islets in the model group were atrophied. The size of the islet became smaller, and the shape of the islet was irregular and the boundary between the islet and the surrounding cells was blurred. Compared with the model group, the islet volume in the positive group and the THL group was larger, and the islet tissue morphology was significantly improved in the positive group and the THL group compared with the model group (Figs. 5a–f). After staining with aldehyde fuchsin-orange G, the granules in islet $\beta$ cytoplasm were purple and the background was yellow. In the normal group, the number of islet $\beta$ cells was more, and the particles in the islet $\beta$ cytoplasm were darker. In the model group, the number of islet $\beta$ cells decreased significantly. After 6 weeks of administration, compared with the model group, the number of islet $\beta$ cells in the high dose of THL was higher than that in the model group (Figs. 6a–f).

DISCUSSION

THL is a common drug in traditional Tibetan medicine. In recent years, a large number of people have studied its compound composition and pharmacological effects, and found that it has good pharmacological activities, such as antioxidation, anticancer, anti-inflammation, antibacterial and so on.\cite{14}
In addition, it also has great potential in anti-diabetes. Modern studies have shown that it has a good hypoglycemic effect and can be used in the treatment of diabetes. Ganeshpurkar et al. found that the aqueous extract of 250 μg/mL THL could play a hypoglycemic role by inhibiting the activity of glycogenase. However, THL effect on anti-diabetes by affecting the apoptosis and proliferation of islet β cells has not been reported. Therefore, this study shows that THL can inhibit the apoptosis of islet β cells and increase the proliferation of islet β cells to achieve the effect of anti-diabetes, which plays a key role in clinical drug use.

Incretin–cAMP signaling pathway is closely related to the occurrence and development of diabetes. GIP is the key factor to maintain glucose balance in incretin signaling system. cAMP activates PKA to play many physiological roles, PKA activation can enhance glucose-induced insulin release, and PKA phosphorylation can regulate the function of downstream target proteins, including ion channels, enzymes and transcription factors. cAMP/PKA signaling pathway regulates glucose homeostasis at multiple levels, including insulin secretion, glucose uptake, glycogen synthesis, decomposition and glycosylation. It is reported in the literature that GLP-1 can stimulate the release of somatostatin (SST) that an inhibitor of glucagon, and then inhibit the secretion of glucagon after acting on and binding to GLP-1R. TXNIP has a certain effect on the activity of incretin signaling pathway, the down-regulation of TXNIP expression in islet β cells can enhance the expression of GLP-1R that a key factor in incretin signaling pathway. As shown in Fig. 7.

The results of animal experiments in vivo showed that Tibetan medicine THL could increase the expression of GIP, GLP-1, GLP-1R, cAMP, P-PKA, AKT and insulin in pancreatic tissue, decrease the degree of pancreatic tissue atrophy and increase the number of islet β cells. It may enhance insulin secretion by inducing islet β cell proliferation and inhibiting islet β cell apoptosis through incretin–cAMP signaling pathway. In the course of this experiment, it was found that the expression of PKA had no significant change, but the expression of cAMP and P-PKA was significantly different \( p < 0.01 \), which suggested that THL could induce the proliferation of islet β cells. The inhibition of islet β cell apoptosis may be achieved through cAMP/PKA signaling pathway. THL can down-regulate the expression of TXNIP, suggesting that THL may have the effect of reducing islet β cell apoptosis induced by high glucose, and TXNIP may have synergistic anti-islet β cell apoptosis effect with incretin–cAMP signal pathway. Therefore, this study further clarified that THL could reduce the blood glucose of diabetic Wistar rats induced by STZ, increase the level of incretin in peripheral blood, and enhance the expression of related factors in pancreatic tissue and GLP-1R recep-
tor on islets. GLP-1 is the key factor of incretin–cAMP signal pathway, suggesting that it may inhibit islet β cell apoptosis and enhance islet β cell proliferation by regulating incretin–cAMP signal pathway, and the effect of high dose of THL is more obvious. In the future, it is necessary to further verify the objective necessity of THL in the process of anti-diabetes from the point of view of molecular level and in vitro experiments, so as to provide experimental basis for revealing its mechanism and exploring and developing new drugs for the treatment of diabetes.

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Conflict of Interest The authors declare no conflict of interest.

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