Anti-diabetic Activity of Zhenqing Recipe and *Ligustri Lucidi Fructus* in Type 2 Diabetic Rats

WEN Xiu-ying[1], XU Wen-guang[1], XIONG Ling[1], XU Ming-wang[1], LIU Hao[1], ZHANG Hong[1], LUO Qiong[2*], NIAO Qiu-hong[1], LIU Li-fang[3]

1. Liyuan Hospital of Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430077, China
2. General Hospital of the Yangtze River shipping, Wuhan 430015, China
3. The Central Hospital of Ezhou City, Ezhou 436000, China

Abstract: **Objective** To investigate the influence of Zhenqing Recipe (ZQR) and *Ligustri Lucidi Fructus* (LLF) on diabetic rats and its possible mechanism. **Methods** The model of type 2 diabetic rats was established by feeding a high-sucrose-high-fat diet and injecting a low dose of Streptozotocin in Wistar rats. The model rats were randomly divided into three groups: diabetic model, ZQR-treated, and LLF-treated groups for 8-weeks treatment. The normal Wistar rats were as a normal control group. **Results** The level of fasting blood glucose in ZQR and LLF groups was decreased compared with model group ($P < 0.01$, 0.05, respectively). Both ZQR and LLF markedly reduced serum triglycerides ($P < 0.01$, 0.05, respectively), and increased the insulin sensitivity index ($P < 0.05$). Histopathology revealed that ZQR and LLF reduced pancreatic damage. Immunohistochemistry evaluation showed that the percentage of insulin positive cells in pancreatic island was higher than model group ($P < 0.01$, 0.05, respectively).The mRNA and protein expression of SREBP-1c in pancreas were significantly decreased in ZQR and FLL group ($P < 0.01$). **Conclusion** ZQR has therapeutic effect on type 2 diabetes, it ameliorates the histopathological changes of pancreas, protects β cells, improves insulin resistance, and attenuates the expression of SREBP-1c. This study also provides the anti-diabetic evidence of FLL even its effects are weaker than ZQR.

Key words: insulin-producing cells; *Ligustri Lucidi Fructus*; SREBP-1c; type 2 diabetes rats; Zhenqing Recipe

DOI: 10.3969/j.issn.1674-6384.2011.01.010

Introduction

Type 2 diabetes comprises a group of metabolic disorders that results in hyperglycemia. Currently it is viewed that chronic insulin resistance (IR) and impairment of insulin secretion from pancreatic β-cell have been identified as the two fundamental features in the pathogenesis of type 2 diabetes (Song *et al*, 2007; Bouwens and Rooman, 2005; He *et al*, 2009). IR is defined as a reduced insulin action, leading to hyperinsulinemia (Bouwens and Rooman, 2005), which is a common phenomenon of the metabolic syndrome associated with type 2 diabetes, obesity, and non-alcoholic steatohepatitis. In normal circumstances, insulin stimulates glucose uptake into skeletal muscle, inhibits hepatic gluconeogenesis, and decreases adipose-tissue lipolysis. In contrast, IR led to the release of free fatty acids from adipose tissue, decreased skeletal muscle glucose uptake, and increased hepatic gluconeogenesis and β-cell dysfunction (DeFronzo and Ferrannini, 1991). Growing evidence suggested the progressive failure of β-cell function could be an important factor in causing elevated blood glucose (Kakuma *et al*, 2000). Based on data that a loss of β-cell function will be observed at the early stage of diabetes and that IR may make an additional contribution (Kahn, 2003).

Sterol regulatory element binding proton (SREBP), membrane-bound transcription factor, also includes SREBP-1a, SREBP-1c, and SREBP-2, *etc* (Shimomura *et al*, 1997). Each SREBP isoform is a basic helix-loop-helix leucine zipper factor and is synthesized as an endoplasmic-reticulum-bound precursor which is cleaved

* Corresponding author: Luo Q Address: Department of Tongji Medical College, Huazhong University of Science and Technology, Xueziyuan 505-322, Wuhan 430077, China Tel: +86-159 9749 3686 E-mail: xuwenguang2009@sina.com
Received: November 13, 2010; Revised: December 14, 2010; Accepted: January 21, 2011
Fund: National Natural Science Foundation of China (30672730); Developing Research Program of Science and Technology Bureau of Wuhan (20046001047)

to liberate an active nuclear fragment (Brown and Goldstein, 1999). SREBP-1c is highly expressed in metabolically active tissues, such as liver, pancreas, and adipose tissue, with important functions in the regulations of lipid metabolism, adipogenesis, and insulin action (Sewter *et al*, 2002). Evidence indicated that the fatty liver of IR was caused by SREBP-1c which was elevated in response to the high insulin level (Horton *et al*, 2002). Several studies have shown that SREBP-1c had been implicated in the regulation of glucose-responsive genes in pancreatic β-cells (Osborne, 2000; Wang, Maechler, and Antinozzi, 2003). Moreover, over-expression of the active nuclear fragment of SREBP-1c led to a dramatic lipid deposition in primary rat islets, and a profound decrease in glucose oxidation and glucose- stimulated insulin secretion (Diraison *et al*, 2004). Therefore, the active SREBP-1c by elevated glucose has been implicated in the development of pancreatic β-cells dysfunction and IR.

Zhenqing Recipe (ZQR), a Chinese herbal medicine prescription, contains *Coptidis Rhizoma*, *Rehmanniae Radix*, *Corni Fructus*, and *Ligustri Lucidi Fructus* (LLF). Previous studies have demonstrated that the administration of ZQR could reduce serum glucose (Wen *et al*, 2006). Moreover, we have reported that ZQR showed favorable ameliorative effects on metabolic syndrome, such as hyperglycemia and hyperlipidemia in diabetic rats (Zheng *et al*, 2006). However, the possible mechanism of ZQR on type 2 diabetes has not yet been determined. In the present study, ZQR was orally administered to high energy diet and STZ-induced diabetic rats for eight consecutive weeks to assess whether the consumption of ZQR might improve IR in the diabetic rats and to investigate the mechanisms involved in SREBP-1c expression.

Materials and methods

Biochemical reagents

Streptozotocin (STZ) was purchased from Sigma (Saint Louis, Missouri, USA). Trizol, MMLV reverse transcriptase, RNAase Inhibitor, dNTP, and Oligo (dT) Taq DNA polymerase were purchased from Toyobo (Tokyo, Japan). The primers were purchased from Invitrogen (Carlsbad, California, USA). DNA Marker I was purchased from Tiangen Biotech Co. Ltd. (Beijing, China). Rabbit anti-rat SREBP-1 polyclonal antibody and rabbit anti-rat β-actin polyclonal antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, California, USA).

Preparation of ZQR extract and LLF extract

LLF, *Corni Fructus*, *Coptidis Rhizoma*, and *Rehmanniae Radix* contained in ZQR were purchased from Wuhan Ginkgo Traditional Chinese Herbal Company (Wuhan, China). Both this prescription and LLF were extracted with boiled water (1:10) for 2 h. After filtration, the water extract was evaporated to 100% solution under reduced pressure. Then ethanol extract of the solution to meet the ratio of 1.5 g dry herbs/mL extract in the solution of ZQR and LLF, stored at 4 ℃ until use.

Treatment of animals

Male Wistar rats (clean grade), weighing (150－170) g, aged (8－10) weeks, were obtained from Hubei Experimental Animal Center of Academy of Medical Sciences, and kept in a plastic cage under a 12 h light/dark cycle. The room temperature was maintained automatically at (20 ± 2) ℃ with a humidity of (58 ± 5)%. The rats were allowed free access to a high energy diet comprising with 20% sucrose, 10% lard, 6% bean oil, 2% cholesterol, 1.5% yolk powder, and 60.5% regular rat chow, and water was given *ad libitum* for four weeks to induce IR. Then, STZ-diabetic rats were prepared by iv injecting 30 mg/kg STZ in 20% citrate buffer (pH 4.5), and normal group rats were given a Sham injection of citrate buffer without STZ. And then animals with non-fasting serum glucose concentration > 16.67 mmol/L were considered to be diabetic after 72 h of STZ injection. The diabetic rats were randomly divided into three experimental groups: diabetic mellitus (DM), ZQR-treated, and LLF groups, each consisted of eight rats, respectively. The DM group was given distilled water, while the ZQR group was ig administered ZQR extract with indicated dosage 12 g/(kg·d) (Zheng *et al*, 2006), and the LLF group was given LLF 2.6 mg/(kg·d) for eight weeks. The rats were weighed once a week, the dosage of drug was adjusted according to body weight changes. Another ten rats as normal control (Ctr) group were received the equivalent volume of distilled water. A week after STZ injection and eight weeks after the treatment, blood was sampled from tail vein between 8 and 10 am, and blood glucose was determined. Rats were sacrificed at the end of the

8-week treatment by exsanguinations from the carotid artery at 24 h after last administration. The whole pancreas was dissected and fixed in 4% neutralized Formalin for histology and immunohistochemistry. All animal procedures were approved by Ethic Committee of Tongji Medical School of Huazhong University of Science and carried out in accordance with the *Institutional Guidelines of China*.

Blood sampling and analysis

Blood samples of rats were centrifuged at 2000 × *g* for 10 min at 4 ℃, and serum was removed and aliquot for the respective analytical determinations. The fasting blood glucose (FBG) was determined by glucose oxidase method, serum triglycerides (TG) and total cholesterol (TC) were measured by enzyme coupled colorimetric analysis, and fasting insulin (FINS) was determined by radioimmunossay method. Insulin sensitivity index (ISI) was calculated as (Ju *et al*, 2010):

$$ISI = In\ [1/(FBG \times FINS)]$$

All of the biochemical parameters above were measured on AU—400 automatic biochemistry analyzer (Olympus, Japan).

Pancreatic histological analysis

Samples from the pancreas were removed immediately and embedded in paraffin to prepare 4-μm tissue slice. The tissue slices were mounted on glass slides and with HE stained for histological observation.

Immunohistochemistry staining of islet β-cell

Other sections were used for immunostaining using the streptavidin-peroxidase (SP) method. The pancreas was dewaxed, rehydrated, and incubated with methanol containing 3% H_2O_2 for 15 min to block endogenous peroxidase activity. After washing in phosphate-buffered saline (PBS), sections were incubated with mouse anti-human insulin monoclonal antibody for 1 h at 37 ℃, while PBS replaced primary antibodies as negative control. After washing with PBS, sections were incubated with secondary antibodies (goat anti-rabbit IgG or goat anti-mouse IgG) for 20 min at 37 ℃, then washed in PBS buffer, and finally incubated with SP complex for 20 min at 37 ℃. After washing, reactions were developed with diaminobenzidine (DAB) in PBS-H_2O_2. Sections were then counterstained with hematoxylin, dehydrated, and mounted. The insulin expression was brown or yellow granulae in cytoplasm of pancreas tissue sections. Insulin-positive cells were determined on HPIAS—2000 medical colored image analytic system (The Wuhan Championimage Co., Ltd., China).

Semiquantitative RT-PCR

Total RNA was isolated from islets in Trizol Reagent according to the manufacture's protocol (Invitrogen). Then the first strand cDNA was synthesized using the oligo (dT) primer and the Moloney murine leukemia virus (MMLV) retroviridase. PCRs were performed in a total volume of 25 μL comprising 1 μL cDNA product, 2 μL nucleotide triphosphate, 1 μL of each primer, and 0.5 μL Taq polymerase. Oligo-nucleotide primer sequences for SREBP-1c used in PCR amplification as follows: forward, 5'-GGAGCCATGGA TTGCACATT-3'; reverse, 5'-AGGAAGGCTTCCAGA GAGGA-3 (191 bp fragment); Oligonucleotide primers for β-actin mRNA were used as described: forward, 5'-CCAAGGCCAACCGCGAGAAGATGAC-3', reverse, 5'-AGGGTACATGGTGGT GCCGCCAGAC-3' (589 bp fragment). PCR was performed at 95 ℃ for 2 min, followed by 35 (SREBP-1c) or 30 cycles (β-actin) at 94 ℃ for 45 s, 58 ℃ for 45 s, and 72 ℃ for 1 min. The last cycle was followed by a final extension step at 72 ℃ for 10 min. PCR products were separated on 2% agarose gels containing ethidium bromide (0.5 μg/mL) and quantitated by JS—300 gel analytical system.

Western blotting analysis

Total islet protein was extracted from lysis buffer and protein concentration was determined by BCA protein assay kit. Proteins were separated by SDS-PAGE (8% acrylamide) and transferred onto nitrocellulose membranes. Membranes were blocked in PBS supplemented with 5% milk powder (PBS-milk) for 2 h at room temperature, followed by incubation with SREBP-1c antibody (1:200) at 4 ℃ overnight. Then, blots were probed with horseradish-peroxidase-conjugated anti-mouse IgG (1:5000) and visualized using the enhanced chemoluminescence (ECL) kit.

Statistical analysis

Data are expressed as the $\overline{x} \pm s$. The Student's *t* test or analysis of variance (ANOVA) was used to compare the means of different groups with SPSS 13.0 statistical software. Dunnett's test was used to compare the results between two groups. $P < 0.05$ was considered statistically significant.

Results

Biochemical estimations

A week after STZ injection, the FBG was more significantly increased while the FINS was significantly decreased in diabetic group than that in control group (Table1). After eight weeks of treatment, FBG and TG were significantly increased in diabetic group, and these changes were more obvious at the week 8 following diabetes induction (Table 2). ZQR effectively decreased FBG and TG after 8-week treatment. However, ZQR and LLF made little change on TC throughout the study period (Table 2). ZQR and LLF made significant influence on FINS in STZ-diabetic rats during entire experiments (Table 2). The level of ISI was markedly decreased compared to that of control group, the reduced ISI was significantly elevated by the oral administration of ZQR, whereas LLF made less effect (Table 2). These results implied that ZQR could prevent diabetic pathological conditions induced by hyperglycemia and hyperlipidemia in diabetes.

Table 1 Changes of FBG and FINS one week after STZ injection ($\bar{X} \pm s$)

Group	*n*	FBG / (mmol·L^{-1})	FINS / (mU·L^{-1})
Ctr	10	4.47 ± 0.48	28.85 ± 4.03
DM	30	15.34 ± 1.0^{b}	18.74 ± 5.01

$^{b}P < 0.01$ *vs* Ctr group

Table 2 Changes of FBG, TG, TC, FINS, and ISI after 8-week treatment ($\bar{X} \pm s$)

Groups	*n*	FBG / (mmol·L^{-1})	TG / (mmol·L^{-1})	TC / (mmol·L^{-1})	FINs / (mU·L^{-1})	ISI
Ctr	10	5.21 ± 0.53	0.66 ± 0.17	2.13 ± 0.19	28.25 ± 9.39	–4.03 ± 0.44
DM	8	16.67 ± 4.33^{b}	1.33 ± 0.33^{b}	4.35 ± 1.91^{b}	51.44 ± 7.15^{b}	–6.75 ± 0.57^{b}
ZQR	8	10.94 ± 3.33^{bd}	0.79 ± 0.27^{bd}	4.44 ± 0.87^{bd}	40.12 ± 6.13^{bd}	–5.33 ± 0.77^{bd}
LLF	8	12.86 ± 3.57^{bc}	1.02 ± 0.31^{bc}	4.37 ± 0.64^{bc}	36.29 ± 7.35^{bc}	–5.96 ± 0.69^{bc}

$^{b}P < 0.01$ *vs* Ctr group; $^{c}P < 0.05$ $^{d}P < 0.01$ *vs* DM group

Histopathological changes of pancreas

In the Ctr group, relatively well documented pancreatic islets and tightly arranged islet cells were observed in the non-diabetic rats. Severe islet necrosis and mild islet atrophy were detected in DM group. These abnormal histological signs were significantly improved after ZQR and FLL treatment, but ZQR showed more favorable effect than FLL (Fig. 1). At histomorphometrical analysis, a significant decrease of pancreatic islet numbers was detected in DM group compared to that of Ctr group. However, the size of islets was significantly increased, and the necrosis and atrophy of islets were significantly improved in the ZQR or LLF group compared to DM group.

Immunohistochemical evaluation

In Ctr group, the islets showed the normal structure with a large central core formed by insulin-secreting β-cells. The number of immunoreactive insulin-positive cells was reduced remarkably, and distributed in restricted pancreatic islets in DM group. However, these diabetic-induced changes of pancreas were partially reversed by ZQR administration (Fig. 2). At histomorphometrical analysis, a significant decrease ($P < 0.05$) in insulin-positive cells was detected in the DM group compared with Ctr group. Whereas, the percentage

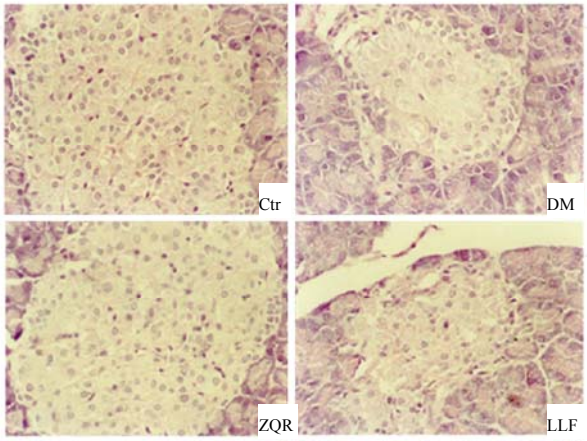

Fig. 1 Histological profiles of pancreatic islets in Ctr, DM, ZQR, and LLF groups

Relatively-well documented pancreatic islets and tightly arranged islet cells were observed in Ctr group. However, atrophy and vacuolation of islet cells according to destroy of β-cells were demonstrated in DM group. However, these abnormal changes were significantly reduced in ZQR and LLF groups compared to that in DM group

of insulin-positive cells significantly increased ($P < 0.05$) in ZQR and LLF group as compared to DM group, and more dramatic changes were detected in ZQR group than in LLF group.

RT-PCR and Western blotting analysis

The mRNA level of SREBP-1c slightly increased in diabetic rat pancreas. However, it significantly decreased

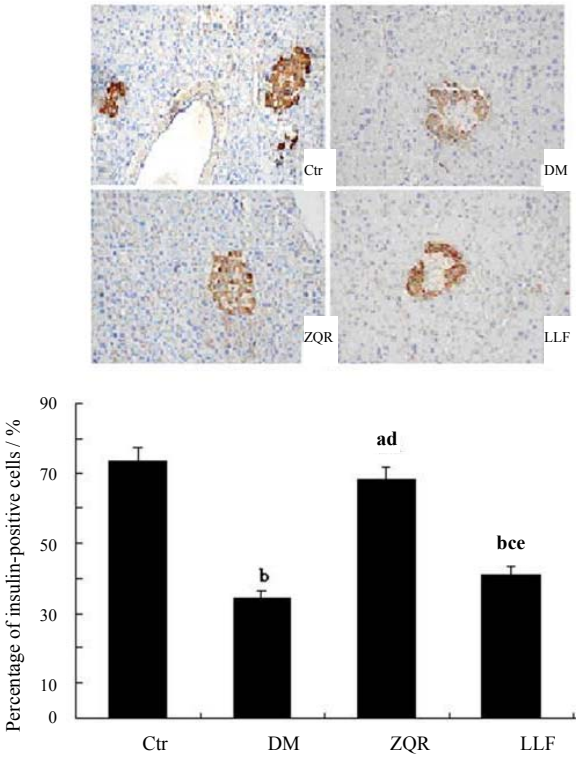

Fig. 2 Immunohistochemical staining for insulin of pancreas in Ctr, DM, ZQR, and LLF groups

Condense cords of insulin-positive cells were detected through all pancreatic islet of Ctr group. However, only a few insulin-positive cells were restrictively distributed in the central regions of pancreas in DM group. In the ZQR and LLF groups, restored density of insulin-positive cells in pancreatic islets was observed compared to that of DM group. The percentage of insulin positive cell was expressed as a ratio relative to the total number of each cell type within the same sections. Each value represents the $\bar{X} \pm s$ of three independent experiments

[a]$P < 0.05$ [b]$P < 0.01$ *vs* Ctr group; [c]$P < 0.05$ [d]$P < 0.01$ *vs* DM group; [e]$P < 0.01$ *vs* ZQR group, same as below

in the ZQR and LLF groups compared with the DM group ($P < 0.05$) (Fig. 3). The changes of mRNA level did not directly represent the functional changes. Therefore, we assessed the protein expression of SREBP-1c by Western blotting analysis with membrane protein samples extracted from pancreas of each group. When normalized to internal control with β-actin for protein sample input, the density of the SREBP-1c band was decreased dramatically in ZQR group compared with DM group, but was still increased compared with Ctr group ($P < 0.05$) (Fig. 4).

Discussion

STZs are drugs that selectively destroy β-cell, insulin-producing pancreatic cells, and thus induce experimental

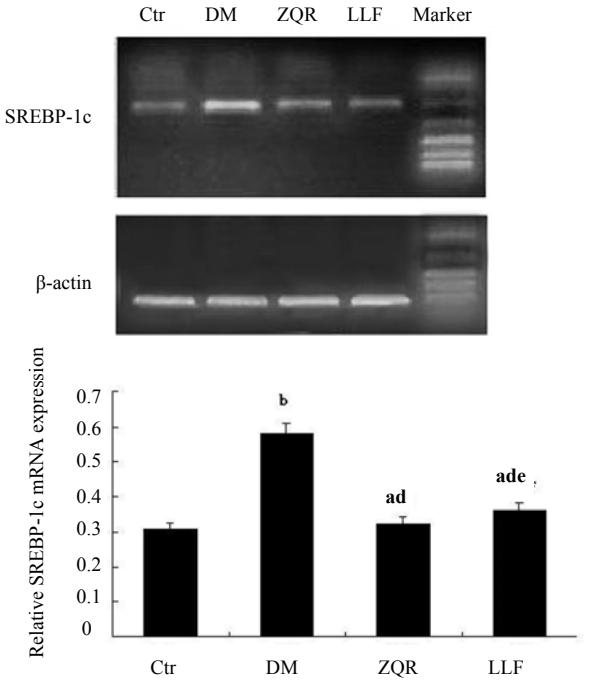

Fig. 3 Expression of SREBP-1c mRNA in pancreatic islet of rats

All results were expressed as the $\bar{X} \pm s$ and analyzed using One-way ANOVA. Dunnett's test was used to compare results between two groups. Each value represents the $\bar{X} \pm s$ of four independent experiments.

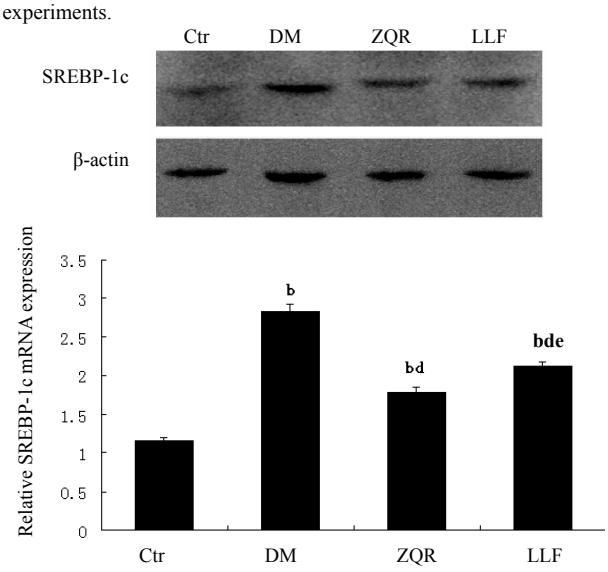

Fig. 4 Expression of SREBP-1c mRNA in pancreatic islet of rats

diabetes mellitus (Brenna *et al*, 2003). In our study, we first fed the rats with high energy diet for four weeks, then we used low-dose STZ (30 mg/kg) to induce insulin deficiency and chronic hyperglycemia in rats. This model provides a valuable pharmacological tool for antidiabetic drug research, because most of the diabetic patients are non-insulin dependent in nature. Our results showed that Wistar rats displayed

significant elevation of serum glucose, hyperlipidemia, IR, and islet rupture. In the present study, the effect of ZQR on dyslipidemia with IR condition in type 2 diabetes was investigated.

Glycemic control is the most important factor in the treatment of diabetes, therefore, the hypoglycemia effect have been treated as essential characteristics of anti-diabetic agents (Ojewole and Adewunmi, 2003). In order to ascertain whether ZQR is useful in glycemia control, its effects on blood glucose levels were investigated. Our results showed that continuous treatment with ZQR for a period of eight weeks, serum glucose levels were lower throughout the study period. In addition, FINS in DM group were significantly decreased than that in Ctr group a week after STZ injection, which suggested that the pancreatic islet underwent a major injury, and promoted further reduced insulin secretion when chronic hyperglycemia was present. However, ZQR showed a significant recovery effect on FINS during entire experiments. These results supported that the possible mechanism of ZQR brought about its hypoglycemic was by increasing either pancreatic secretion of insulin or insulin sensitivity for effective glucose disposal.

In the STZ-induced diabetic rats, inflammatory changes in the pancreatic islets led to the atrophy, decrease in numbers, and destruction of islet β-cells (Higdon *et al*, 2001). In the present study, severe necrosis and mild atrophy of islets according to the destruction of β-cells in the number and size were observed, all of which were improved after treatment with ZQR. In addition, by quantifying the insulin-positive cells, we found that the percentage of insulin-positive cells in ZQR group was much higher than that in DM group, but slightly lower than that in Ctr group. These results indicated that the number and function in the pancreatic islets of insulin-positive cells by ZQR were considered to be direct evidence that ZQR protected the pancreatic β-cells from the destruction induced by STZ, which corresponded to the results of serum insulin levels. This was considered to be a potential action mechanism that ZQR had relatively favorable antidiabetic effects.

Type 2 diabetes frequently has an abnormal blood lipid and other reactive metabolites caused by deficiency and resistance of insulin (Scott and King, 2004). Thus, the regulation of hyperlipidemia would play an important role in the etiology of diabetes and the complication of hyperglycemia. In this study, we investigated and observed a significant elevation of TG and TC. Our results showed that ZQR could effectively decrease the elevated TG level in diabetic rats, although TG level was still higher than that in control group, which was in accordance with the results of our previous studies (Wen *et al*, 2006). However, ZQR made slight changes on TC throughout the study period. These results indicated that ZQR might have some protective effects against diabetic lipid metabolism abnormality.

Chronic activation of hepatic SREBP-1c causes fatty liver, hypertriglyceridemia, and IR, leading to the development of metabolic syndrome (Shimano, 2009). While, SREBP-1c as the principal regulator was also involved in the accumulation of lipids in native islets due to β-cell destruction and impaired insulin secretion (Milburn *et al*, 1995; Kakuma *et al*, 2000). Additionally, in leptin deficient obese (ob/ob) mice, SREBP-1c levels were elevated with IR and hyperinsulinemia, which increased lipogenic gene expression, enhanced fatty acid synthesis, and accelerated triglyceride accumulation (Shimomura *et al*, 2000; 1999). While with the administration of leptin, these metabolic abnormalities were reversed that IR was correct and insulin level was lower (Shimomura *et al*, 1999). In this study, we found that SREBP-1c was less abundant in the islet of rats in ZQR group, whereas the opposite occurred in DM group. Our study has demonstrated that ZQR had a great effect on SREBP-1c expression, ZQR consumption prevented postprandial hyperinsulinemia and was associated with a decrease in SREBP-1 expression, and the changes of SREBP-1c expression were involved in the improvement of IR. These results suggested that ZQR had an ameliorating effect on dyslipidemia in type 2 diabetic rats through the regulation of SREBP-1c.

LLF is one of the common used Chinese herbs used to treat age-related diseases (Zhang *et al*, 2008). The present study was undertaken to examine the effect of LLF extracts on antidiabetic effects and to determine the underlying mechanism. Oleanolic acid, the principal chemical component of LLF, have hypoglycemic and hypolipidemic effects (Zhang and Shi, 2004). Our study

showed that after 8-week fed intervention of LLF extract, the inhibition of histological and immuno-histological changes in the pancreas as well as a decrease of the blood glucose level and an increase of the serum insulin concentration were significantly compared to DM group. But the percentage of insulin-positive cells did not increase obviously, indicating that the therapeutic effect of LLF extract in type 2 diabetes mellitus may be related to improvement of IR, and how LLF impacted on the islet β-cell has remained to be studied.

In summary, the present study indicates that ZQR treatment is effective in controlling hyperglycemia and hyperlipidemia in the STZ-induced diabetic rats. The lipid-lowering effect of ZQR is due to the regulation of SREBP-1 expression, which plays an important role in IR. Thus, ZQR may be useful in affecting the mRNA and protein expression of SREBP-1c and improve IR in type 2 diabetic rats.

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