doi.org/10.36721/PJPS.2020.33.3.REG.1063-1072.1

Chlorogenic acid inhibits forming of diabetes mellitus in rats induced by high-fat high-sucrose and streptozotocin

Yan Wang[1,2], Shuguang Peng[1], Zihan Mei[2,3], Chenzhong Jin[2,3], Jinhe Kang[4], Minghui Xiang[5], Zheng Wang[1*] and Yihong Hu[2,3*]

[1]College of Bioscience and Biotechnology, Hunan Agricultural University, Changsha, China
[2]Department of Life Sciences, Hunan University of Humanities, Science and Technology, Loudi, China
[3]Innovation Center for Field Weeds Control of Hunan Province, Loudi, China
[4]Institute of Subtropical Agriculture, Chinese Academy of Science, Changsha, China
[5]College of Medicine, University of Florida, Florida, USA

Abstract: To evaluate the inhibitory effect of chlorogenic acid on the forming of type 2 diabetes mellitus (T2DM), using Sprague Dawley (SD) rats, a recognized T2DM model induced by high-fat high-sucrose diet (HFSD) and streptozotocin (STZ). Thirty female SD rats were assigned equally to three groups randomly: normal control with standard commercial (NC), chlorogenic acid treatment with HFSD and chlorogenic acid (90mg/kg, CA), and diabetes model with HFSD (DM). Upon treatment with chlorogenic acid, suppression of the onset of diabetes, reduced serum glucose and insulin concentrations, improved glucose tolerance and increased body weight and visceral fat weight were observed. Serum triglyceride, total cholesterol, low density lipoprotein levels, and kidney and pancreas morphology were significantly ameliorated. Chlorogenic acid also inhibited the mRNA levels of hepatic G-6-Pase and up-regulated the mRNA levels of skeletal muscle GLUT4. Our results indicated that before the onset of diabetes, chlorogenic acid had an inhibitory effect against the forming of T2DM induced by HFSD and STZ through regulating the glucose and lipid metabolism.

Keywords: Chlorogenic acid, Type 2 diabetes mellitus, high-fat high-sucrose diet, hepatic glucose-6-phosphatase, skeletal muscle glucose transporter 4.

INTRODUCTION

Diabetes mellitus, characterized by chronic hyperglycemia, polydipsia and polyuria, results in serious complications on eyes, kidneys and nerves. Diabetes mellitus is also a known cause for atherosclerosis and cardiovascular diseases that significantly increase the risks of human mortality (Cooper-DeHoff *et al.*, 2010). It is a main cause on morbidity or mortality in human populations. The overall prevalence of diabetes in China was 11.6% in 2010, i.e. 114 million Chinese adults (Chang *et al.*, 2017; Hu *et al.*, 2017). Based on the recent data, the International Diabetes Federation declared that there were 415 million people with diabetes in the world across 2015 and the number will reach 642 million in 2040 (Ogurtsova *et al.*, 2017).

Genetic and environmental factors or the complex gene-environmental interplays can induce diabetes mellitus, but all forms of diabetes mellitus (Type 1 and Type 2 diabetes) are either the results of the decrease of insulin concentration (insulin deficiency) or the lower responsiveness to insulin (insulin resistance). Type 1 diabetes is an autoimmune condition characterized by the destruction of pancreatic beta cells and absolute insulin deficiency (Farooq *et al.*, 2018; Turton *et al.*, 2018). Type 2 diabetes is a complicated disease that is primarily characterized by insulin resistance and relative insulin deficiency mediated by numerous organs (Chu and Leung., 2009; Turner *et al.*, 1992). Many factors including lifestyle, physical activity, adiposity and diet contribute to the forming of type 2 diabetes mellitus (T2DM), accounting for nearly 90% of all diabetes cases (Brito *et al.*, 2009). Therefore, proper diabetes management is essential to restore the carbohydrate metabolism to its normal state, typically with oral administration of anti-diabetic drugs, insulin intervention therapy, or a combination of both. However, those drugs have undesirable side effects. For example, glitazones, drugs to improve insulin sensitivity and reduce hepatic glucose production, show an increased risk of myocardial infarction (Thompson and Davis., 2017). Many anti-diabetic drugs may also increase the risk of cancer. In recent years, researchers have been searching for new drugs that can reduce blood glucose but cause no adverse effects. Some other researchers are beginning to investigate natural products from plants or employ dietary intervention. Early studies supported that dietary intervention can improve health conditions by reducing critical risk factors such as obesity, dyslipidemia, hyperglycemia, hypertension and insulin sensitivity (Bazzano *et al.*, 2005; Zarraga and Schwarz., 2006). Natural compounds including folic acid, oleanolic acid, and ursolic acid were isolated from plants which were effective on regulating insulin signaling pathway and cellular metabolism (Baumgartner *et al.*, 2010; Paoli *et*

**Corresponding author:* e-mail: wz8918@163.com; huyhongwangyi@163.com

al., 2015). Previous studies also revealed that *p*-coumaric acid, caffeic acid and their derivatives could prevent vascular complications, ameliorate hypercholesterolemia, and regulate antioxidant property in diabetic rats (Amalan *et al.*, 2016; Yin *et al.*, 2014).

Chlorogenic acid is a natural compound abundant in many plants such as coffee bean and green tea (Del Rio *et al.*, 2010). Known as a free radical scavenger, chlorogenic acid is an important active component in Chinese medicine that improves the plasma glucose tolerance and gastrointestinal hormone secretion in human (Johnston *et al.*, 2002). The intake of foods and drinks rich in chlorogenic acid is directly associated with lower risks of coronary disease and T2DM (Lin *et al.*, 2011). Chlorogenic acid was reported to inhibit the hepatic G-6-Pase activity (Bassoli *et al.*, 2008), increase mRNA and protein levels of hepatic peroxisome proliferator-activated receptor-α (PPAR-α) in lipid metabolism (Zhang *et al.*, 2011), increase the production of glucagon-like peptide-1 (McCarty, 2005) and promote the mRNA levels of skeletal muscle glucose transporter 4 (GLUT4) (Prabhakar and Doble, 2009). Chlorogenic acid also prevents the forming of the advanced glycation end products induced by hyperglycemia (Kim *et al.*, 2011).

However, no previous reported the modulating effects of chlorogenic acid on the forming of T2DM. Hence, the aims of the present study were to evaluate the inhibitory effect of chlorogenic acid during the forming of T2DM and to investigate the preventive role of chlorogenic acid in diabetes treatment, using a rat model induced by high-fat, high-sucrose diet (HFSD), and streptozotocin (STZ).

MATERIALS AND METHODS

Animals and diets

Thirty female SD rats (100-130 g) with certificate No. HNASLKJ20101790 were purchased from Hunan Sileike Jingda Co. (Changsha, China) and maintained under a 22 ± 2°C with 12h light-dark cycle. All animal procedures and the protocol were followed according to the Regulation on Management of Experimental Animals (Hunan Province, No. 259, 2012).

Commercial standard pellets and HFSD were purchased from the Hunan Sileike Jingda Co. (Changsha, China). HFSD was composed of 68.5% commercial standard pellets in 10% lard oil, 20% sucrose, 0.5% pig bile and 1% cholesterol (table 1).

Chemicals and drugs

Chlorogenic acid of 98% purity was purchased from Changsha Xiangzi Biotechnology Co. (Changsha, China). STZ and diethyl oxydiformate (DEPC) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Rat ELISA kit was purchased from R&D Systems (Oxon, UK). Reverse transcription kit was obtained from MBI Fermentas Co. (Waltham, MA, USA). Tryglyceride (TG), total cholesterol (TC), low density lipoprotein (LDL-C), high density lipoprotein (HDL-C) and free fatty acid (FFA) reagent kits were purchased from Shenzhen Mindray Co. (Shenzhen, China). Trizol reagent was obtained from Invitrogen Co. (Carlsbad, USA), and SYBR green premix was obtained from Takara Bio Co. (Shiga, Japan). All of the other chemicals were of analytical grade.

Experimental design

During the experimental period all animals were randomly assigned to three groups (n=10): (1) normal control with commercial standard pellets (NC); (2) diabetes model with HFSD (DM); (3) chlorogenic acid treatment with HFSD and chlorogenic acid (90 mg/kg, CA) continuously for eight weeks. Chlorogenic acid was dissolved in sterile saline, and the chlorogenic acid treatment group rats were individually treated with chlorogenic acid by gavage needle one time in a day, while the normal control and diabetes model groups were simply conducted with sterile saline. The rats can enjoy food and water with freedom, and their weight was determined once a week. After fed with HFSD for four weeks, chlorogenic acid treatment and diabetes model groups were induced by intraperitoneal injection of freshly prepared STZ (35 mg/kg b.w.) diluted in 0.1M citrate buffer (pH 4.5) according to Jeszka-Skowron *et al* (Jeszka-Skowron *et al.*, 2014). Normal control group rats were instilled with the same volume of citrate buffer. After 72 h of injection, dynamic fasting blood glucose concentration of diabetes model group and chlorogenic acid treatment group were monitored for two weeks with an Accu-Chek Advantage Meter glucometer (Roche Diagnostics GmbH, Germany) using the blood drawn from tail veins. T2DM was confirmed by glucose level which was higher than 250 mg/dl from tail veins (Karthikesan *et al.*, 2010). After treated with chlorogenic acid for eight weeks, each rat was used for oral glucose tolerance test (OGTT). One week later, all rats were anesthetized with pentobarbital sodium (35 mg/kg) and the blood was exsanguinated and collected from eyes for determination of the biochemical indexes. Liver and skeletal muscle (soleus) were excised immediately, washed with pre-cooled physiological saline, and stored at -80°C until use. Visceral fat was excised and weighted, and kidney and pancreas were removed to 10% of neutral formalin solution for pathological detection.

Evaluation of fasting blood glucose and oral glucose tolerance test

After STZ injection, fasting blood glucose (FBG) level was monitored weekly with the blood collected from tail veins. During the last week of the experimental period, the rats had been fasted overnight firstly, and then OGTT was conducted with the blood from tail veins collected at 0, 0.5, 1 and 2 h after the oral administration of glucose (2.5 g/kg b.w.) according to the procedure from de Sotillo and Hadley (Rodriguez de Sotillo and Hadley, 2002).

Determination of plasma insulin resistance and sensitivity levels

Plasma insulin levels were determined with enzyme linked immunosorbent assay (ELISA) method according to the instruction from the manufacturer. Homeostasis model assessment of insulin resistance index (HOMA-IR) and insulin sensitivity index (ISI) were calculated using FBG and fasting insulin levels (FINS) data according to Li *et al* (Li *et al.*, 2009) and Matthews *et al* (Matthews *et al.*, 1985) as the following formulae, respectively:

$$\text{HOMA - IR} = \text{FBG(mmol/L)} \times \text{FINS(mIU/L)}/22.5 \quad (1)$$

$$\text{ISI} = \ln(1/(\text{FBG(mmol/L)} \times \text{FINS(mIU/L)})) \quad (2)$$

Measurement of serum lipid and free fatty acid

To measure the levels of TG, TC, LDL-C, HDL-C, and FFA, a BS-200 full automatic biochemical analyzer (Shenzhen Mindray Co, Shenzhen, China) was applied using their corresponding reagent kits according to Siedel *et al* (Siedel *et al.*, 1983).

RNA isolation and quantitative real-time PCR

Total RNA from the liver and skeletal muscle was extracted with Trizol reagent in accordance with the manufacturer's instructions. To obtain cDNA, total RNA (1μg) was reversely transcribed using a Fermentas reverse transcription kit with Oligo dT as primer. The mRNA expression of G-6-Pase and GLUT4 were quantified by quantitative real-time PCR (qRT-PCR), which was performed with a Thermo Scientific PikoReal96 PCR (MA, USA) using SYBR green premix following the manufacturer's instructions. Relative expressions of G-6-Pase and GLUT4 were normalized with β-actin. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method. The sequences of the reverse and forward primers used in the study are listed below.

G-6-Pase forward primer: 5'-CACCTTGACACTACACC CTT-3'
G-6-Pase reverse primer: 5'-GTGGCTGTGAACACCT CT-3'
β-actin forward primer: 5' –GAAATCGTGCGTGACA TTA-3'
β-actin reverse primer: 5'-ACTCATCGTACTCCTGCT TG-3'
GLUT4 forward primer: 5'-GCCAGCCTACGCCACCA TA-3'
GLUT4 reverse primer: 5'-ATGCCAACGATGAAGTTA CAGG-3'
All primers were designed with Primer Premier 5.0 (Premier Biosoft, Palo Alto, USA), and synthesized by Huada Gene Co (Shenzheng, China).

Histological assessment and immunohistochemical analysis

Kidney and pancreas were fixed in 10% neutral formalin solution one week for hematoxylin and eosin (HE) staining and normal histological assessment. The section slices were stained according to Fischer *et al* (Fischer *et al.*, 2008) and Gu *et al* (2012) with modifications. In brief, the sections were dehydrated with a serial alcohol gradient, washed in xylene, and embedded in paraffin. Before immunostaining, paraffin-embedded sections (5μm thick each) were dewaxed in xylene, rehydrated through different concentrations of ethanol, and washed in 0.1M phosphoric acid buffer (PBS, pH 7.4). And then, the section slices were stained for 10 min with hematoxylin, rinsed in PBS for 5 min, and stained with 1% eosin Y solution for 5 min with agitation. Sections were dehydrated through increasing concentrations of ethanol and xylene after staining. Cytoplasm and extracellular were stained pink, nuclei was stained deep purple, and pancreatic islets was stained pale. Slices were observed by an Eclipse TE2000U inverted microscope with a twin CCD camera (Nikon, Japan). To evaluate the expression of insulin in pancreas, immunohistochemical staining was performed according to Shang *et al* (2013) with modification. After deparaffinization, the serial sections of the rat pancreas were washed in distilled water consisting of 0.1% bovine serum albumin (BSA). Then the endogenous peroxidase activity was quenched for 10 min by incubating the slides in 3% H_2O_2. After washed for three times in distilled water, the samples were incubated for 20 min at ambient temperature in 5% BSA, and then reacted with the primary antibody at ambient temperature for 3 h. Then the sections were cleaned in PBS for three times, biotinylated anti-mouse IgG (Birmingham, USA) conjugated secondary antibody was put in, incubated at 25°C for 20 min, and rinsed with PBS. The immunostaining was visualized using diaminobenzidine tetrahydrochloride (DAB), and the slides were counterstained with hematoxylin to facilitate nuclear identification.

STATISTICAL ANALYSIS

All data were analyzed with SPSS 21.0 (SPSS Inc., Chicago, IL, USA) and expressed as mean ± SE or mean + SE, and statistical significance was assessed by the Student's t-test. * and **indicated significances between diabetes model group (DM) and normal control group (NC) at 0.05 and 0.01 levels, respectively, whereas $^{\Delta}$ and $^{\Delta\Delta}$ indicated significances between diabetes model group (DM) and chlorogenic acid treatment group (CA) at 0.05 and 0.01 levels, respectively.

RESULTS

Effects of chlorogenic acid on body weight, visceral fat weight, and incidence of diabetes

During treated with chlorogenic acid for eight weeks, ten rats developed into diabetes in model group and no diabetes rat was found in the chlorogenic acid treatment group (table 2). No differences of body weight were observed during the initial three weeks (fig. 1A). On the 4th week and the 5th week, body weight of diabetes

model group was remarkably higher than that of normal control group (P<0.01). Body weights of diabetes model group and chlorogenic acid treatment group started to decrease on the 6th week probably due to the injection of STZ at the beginning of the 5th week. On the 8th week, body weight of chlorogenic acid treatment group showed an increasing trend, whereas the weight of diabetes model group continued to decline and the significant difference of body weight between diabetes model group and chlorogenic acid treatment group was observed (P<0.05). On the 9th week, the weight of the rats of chlorogenic acid treatment group was remarkably higher than weight of rats in diabetes model group (P<0.01). Body weight of normal control group showed a trend of increasing during the whole experimental period. And at the end of the experiment, visceral fat weight of chlorogenic acid treatment group was remarkably higher than weight of rats diabetes model group (P<0.01). Visceral fat weight of diabetes model group was remarkably lower than that of normal control group (P<0.01). Visceral fat weight of chlorogenic acid treatment group was 65.03% lower than that of normal control group (fig. 1B). These outcomes support that chlorogenic acid significantly ameliorate the weight that the rats lost both on body and visceral fat in the diabetic rat model.

Table 1: Composition of commercial standard pellets

Ingredients	Content (%)
Wheat starch	20
Rice starch	20
Corn starch	10
Soymeal	24
Fish meal	10
Wheat bran	10
Salt	1
Milk powder	2
Multiplex vitamin	1
Phosphorus and calcium (bone meal)	2

Effects of chlorogenic acid on blood glucose

FBG concentrations from diabetes model group showed an increasing trend upon STZ treatment, and ten days after the STZ injection, the increasing slowed down (fig. 2A). FBG concentrations from diabetic model group at three time points (day 3, day 10 and day 17) were all remarkably higher than that from normal control group (P<0.01) (fig. 2A). However, FBG concentrations from chlorogenic acid treatment group were remarkably decreased than those from diabetic model group (day 3 P<0.01, day 10 P<0.05, and day 17 P<0.01 after STZ injection).

The acute effect of chlorogenic acid was evaluated using oral glucose tolerance test with overnight-fasted SD rats (fig. 2B). The oral administration of glucose increased the blood glucose concentrations in diabetic model group. Although the blood glucose concentrations in chlorogenic acid treatment group also increased after STZ injection, its blood glucose concentrations were remarkably lower than those of diabetic model group at 0h and 2h (P<0.01). After 2 h of the oral administration of glucose, FBG concentration from chlorogenic acid treatment group was 37.63% lower than that from diabetic model group. These results showed that chlorogenic acid could improve glycaemic control.

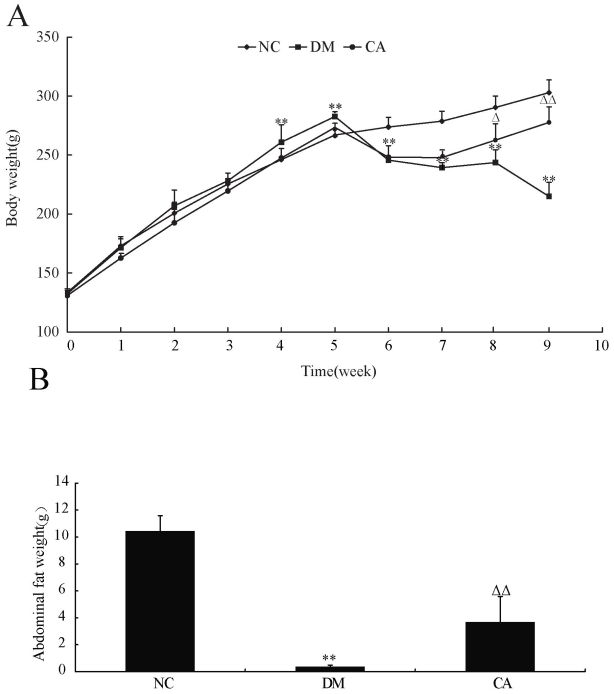

Fig. 1: Effects of chlorogenic acid treatment for 8 weeks on the body weight and visceral fat weight of HFSD and STZ-induced diabetic rats. (A) Body weight. (B) Visceral fat weight. Values were expressed as mean + SE, n=10. $^{**}p$<0.01 for DM versus NC. $^{\Delta}p$<0.05, $^{\Delta\Delta}p$<0.01, for CA versus DM.

Effects of chlorogenic acid on insulin sensitivity

FIN levels (P<0.01) and HOMA-IR levels (P<0.05) in diabetic model group was remarkably higher than those in normal control group (fig. 3). And ISI levels (P<0.01) in diabetic model group was remarkably lower than those in normal control group (fig. 3). Chlorogenic acid treatment reversed the effect that FIN levels in chlorogenic acid treatment group significantly decreased (P<0.01), and HOMA-IR levels in chlorogenic acid treatment group s remarkably decreased (P<0.05), whereas ISI levels in chlorogenic acid treatment group remarkably increased (P<0.05) (comparing to diabetes model group). These results indicated that chlorogenic acid could increase insulin sensitivity through decreasing FINS and HOMA-IR levels and increasing ISI levels.

Effects of chlorogenic acid on serum lipids and FFA levels

Examining chlorogenic acid treatment and diabetes model groups, we found that the concentrations of the serum TG,

TC and LDL-C in chlorogenic acid treatment group decreased (P<0.01), concentrations of the serum HDL-C increased (P<0.05) (fig. 4A) even though concentrations of serum FFA did not decrease (fig. 4B). Examining normal control and diabetes model groups, we found that concentrations of serum TG, TC, LDL-C, and HDL-C in diabetes model group increased (P<0.01), and concentrations of serum FFA also increased (P<0.01). These results supported that chlorogenic acid could protect against diabetic-induced hyperlipidemia.

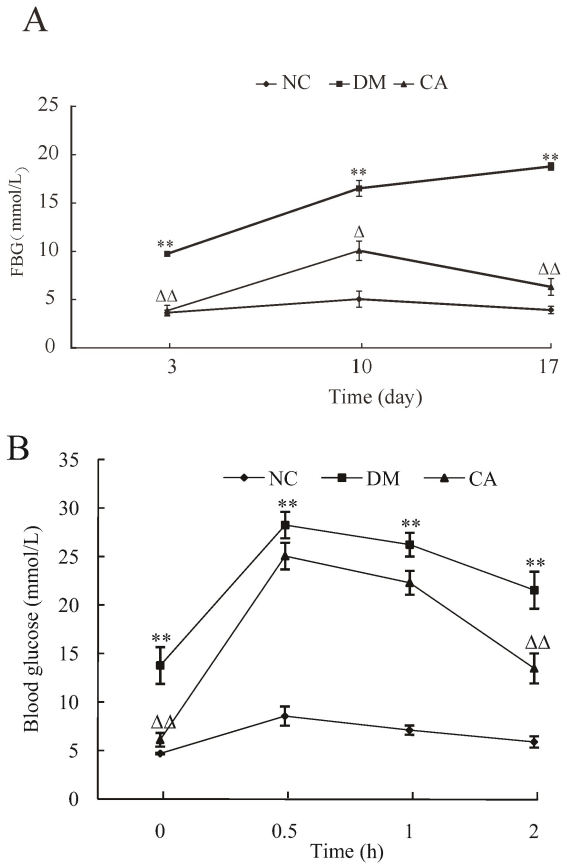

Fig. 2: Acute effect of chlorogenic acid on FBG concentrations and OGTT in HFSD and STZ-induced diabetic rats. (A) FBG levels. (B) OGTT. Blood samples were collected to determine FBG concentrationson day 3, 10 and 17 after the STZ injection. During the last week of the experimental period, blood samples were collected at 0 h, 0.5 h, 1 h and 2 h after imbuing 2.5g/kg glucose for OGTT. Values are expressed as mean ± SE, n=10. $^{**}P$<0.01 for DM versus NC. $^{\Delta}P$<0.05, $^{\Delta\Delta}P$<0.01 for CA versus DM.

Effects of chlorogenic acid on hepatic G-6-pase and skeletal muscle GLUT4 mRNA levels

While the expression of hepatic G-6-pase mRNA in chlorogenic acid treatment group decreased (P<0.05) (fig. 5A), the expression of skeletal muscle GLUT4 mRNA increased (P<0.05) (fig. 5B). In contrast, G-6-pase mRNA in diabetes model group increased (P<0.05) (fig. 5A), whereas the mRNA levels of hepatic GLUT4 in diabetes model group decreased (P<0.05) (fig. 5B). The results suggested that chlorogenic acid inhibits glucose production in liver and stimulate glucose uptake in skeletal muscles.

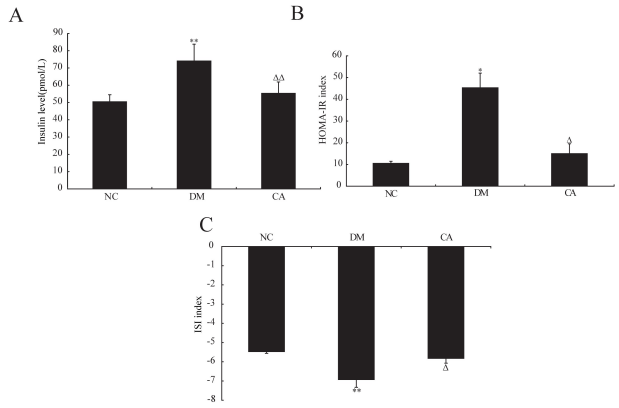

Fig. 3: Effects of chlorogenic acid treatment for 8 weeks on the insulin sensitivity of HFSD and STZ-induced diabetic rats. (A) Insulin concentrations. (B) HOMA-IR index. (C) ISI index. Values are mean ± SE, n=10. $^{*}P$<0.05, $^{**}P$<0.01 for DM versus NC. $^{\Delta}P$<0.05, $^{\Delta\Delta}P$<0.01 for CA versus DM.

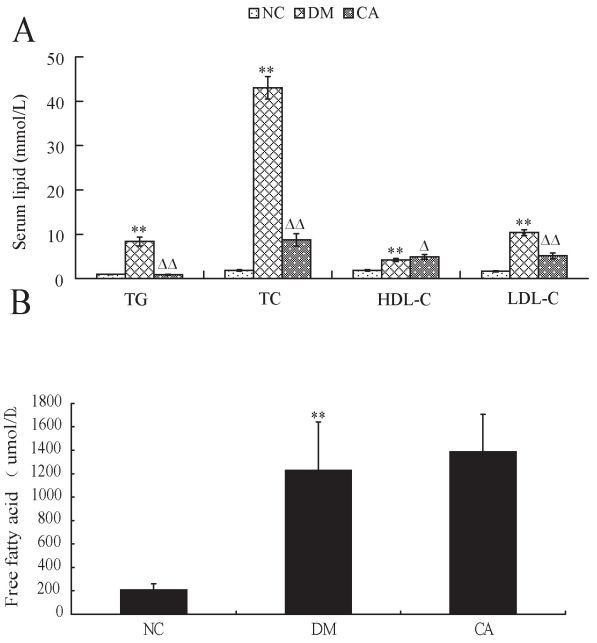

Fig. 4: Effects of chlorogenic acid treatment for 8 weeks on the serum lipids and FFA levels of HFSD and STZ-induced diabetic rats. (A) Serum lipid levels. (B) serum FFA levels. Values are mean ± SE, n=10. $^{**}P$<0.01 for DM versus NC. $^{\Delta}P$<0.05, $^{\Delta\Delta}P$<0.01 for CA versus DM.

Effects of chlorogenic acid on histopathologic changes on kidney and pancreas

The diameters of glomeruli in diabetes model group were increased (P<0.05), a sign of hypertrophy, and the

vacuolar degeneration appeared in its pipe wall of distal tubules and proximal tubules (fig. 6Aa, 6Ab, 6B). With chlorogenic acid treatment, the diameters of glomeruli were reduced (P<0.05), and the pipe wall of distal tubules showed only a slight vacuolar degeneration (fig. 6Ab, 6Ac). Histopathologic analysis of the pancreas in diabetes model group revealed a high frequency of degenerative changes: decrease of insulin-positive granules, islet atrophy, pancreas degeneration, and pancreas necrosis (fig. 6Ad, 6Ae). Chlorogenic acid treatment reversed the changes (fig. 6Af). Chlorogenic acid treatment also normalized glomeruli diameter (P<0.05) (fig. 6B). These findings suggested that chlorogenic acid had the potential to ameliorate diabetic-induced kidney failure and pancreas damage.

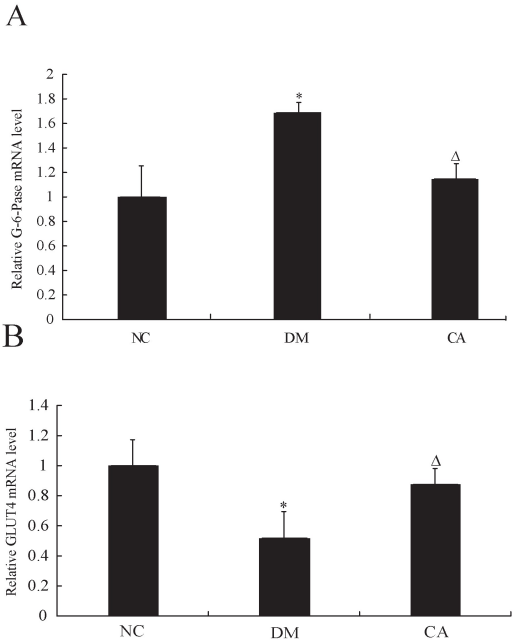

Fig. 5: Effects of chlorogenic acid treatment for 8 weeks on the hepatic G-6-pase and skeletal muscle GLUT4 expression of HFSD and STZ-induced diabetic rats. (A) G-6-pase mRNA levels. (B) GLUT4 mRNA levels. Values are mean ± SE, n=10. *P<0.05 for DM versus NC. $^{\Delta}P$<0.05 for CA versus DM.

Effects of chlorogenic acid on immunohistochemical expression of insulin in pancreas

The immune-histochemical results of pancreas insulin of SD rats were shown in fig 7. More β-cells were observed in insulin islet in normal control group, and less β-cells in insulin islet in diabetes model group (fig. 7a, 7b). When treated with chlorogenic acid, more β-cells in insulin islet were observed (CA vs DM, fig. 7b, 7c).

DISCUSSION

Diabetes mellitus and insulin resistance are results of metabolic disorders of multiple etiologies. Early studies revealed that the combination of controlled diet (with less fat and energy intake) and proper physical activity could decrease the risk of diabetes (Davis *et al.*, 2013). Chlorogenic acid, a special phenolic acid, has recently been found to have anti-diabetic and anti-hyperlipidemia effects in various animal models: db/db mice, high-fat diet-induced-obese mice and rats, and STZ-induced non-obese diabetic rats (Huang *et al.*, 2015; Jeszka-Skowron *et al.*, 2014; Kim *et al.*, 2011; Liu *et al.*, 2015). Nevertheless, the mechanism of the preventive effect of chlorogenic acid on T2DM remains elusive. In this study, a rat model was prepared by feeding animals with HFSD, and then injecting a low-dose STZ. This rat model closely mimics the metabolic features of T2DM in human (Rodriguez de Sotillo *et al.*, 2006). Chlorogenic acid along with HFSD was administrated to rats to assess the effects of chlorogenic acid on T2DM. We evaluated body weight, visceral fat weight, blood glucose and lipid metabolism, insulin sensitivity, mRNA levels of hepatic G-6-Pase and skeletal muscle GLUT4, histological staining of kidney and pancreas, and immunohistochemical test of insulin.

Our experiment showed that all of the rats of diabetes model group developed into diabetes at the end of experiment. On the contrary, when treated with chlorogenic acid for eight weeks, no diabetes rat was found in the chlorogenic acid treatment group. These findings indicated that chlorogenic acid could inhibit of the incidence of diabetes.

Early report supported that oral administration of chlorogenic acid could reduce mice body weight and visceral fat weight under normal fed conditions (Shimoda *et al.*, 2006) and chlorogenic acid failed to recover body weight of diabetic rats (Stefanello *et al.*, 2014). In our study, eight-week chlorogenic acid preventive treatment caused a notable body weight gain and visceral fat weight gain in HFSD and STZ influenced rats. This discrepancy may be due to higher doses of the chlorogenic acid and longer experiment period in our study, and the different treatments between prevention and treatment. The above results suggested that chlorogenic acid could ameliorate health conditions of the diabetic.

Blood glucose, serum lipids, and FFA concentrations are general indexes for diabetes. In this study, we showed that chlorogenic acid decreased the concentrations of blood glucose and serum lipids (serum TG, TC, and LDL-C). These results strongly supports that chlorogenic acid could effectively weaken the symptoms of hyperlipidemia and hyperglycemia induced by HFSD and STZ. The sugar-reducing effects and lipid-lowering effects were consistent with those of the early reports (Li *et al.*, 2009; Rodriguez de Sotillo and Hadley, 2002). A remarkable increase in HDL-C was observed in chlorogenic acid treatment group (fig. 4A). Consequently, the lower TC/HDL-C ratio in chlorogenic acid treatment group

could reduce atherosclerosis risk (Randy *et al.*, 2016). Besides, chlorogenic acid also ameliorated glucose tolerance and reduced plasma glucose concentrations after STZ injection (fig. 2), consistent with that of the early studies with db/db mice or Zucker rat (Jeszka-Skowron *et al.*, 2014; Ong *et al.*, 2012). Hyperglycemia in T2DM is characterized by enhanced hepatic glucose production. Inhibitors against hepatic glucose production can be used clinically in glycemic control in the diabetic patients as insulin sensitizers, for example, metformin, sitagliptin, and exenatide (He *et al.*, 2009; Tokajuk *et al.*, 2015). Our experiment supported that fasting hyperinsulinemia observed in diabetic rats induced by HFSD and STZ was associated with hyperglycemia and insulin resistance. The treatment with chlorogenic acid could ameliorate the symptom of hyperinsulinemia and increase insulin sensitivity. As insulin sensitivity increased, the response of insulin secretion in the pancreas was reduced, which could improve glucose tolerance and reduce plasma glucose rate. Furthermore, our observation of the trend of decreasing expression of G-6-Pase in liver also suggested that chlorogenic acid inhibited the hepatic glucose production. Our results are in agreement with early reports of the suppression of chlorogenic acid on G-6-Pase expression in lepr$^{db/db}$ mice (Ong *et al.*, 2013) and albino wistar rats (Bassoli *et al.*, 2008). We believe that chlorogenic acid effectively inhibit hepatic gluconeogenesis.

GLUT4, a glucose transporter mainly expressed in

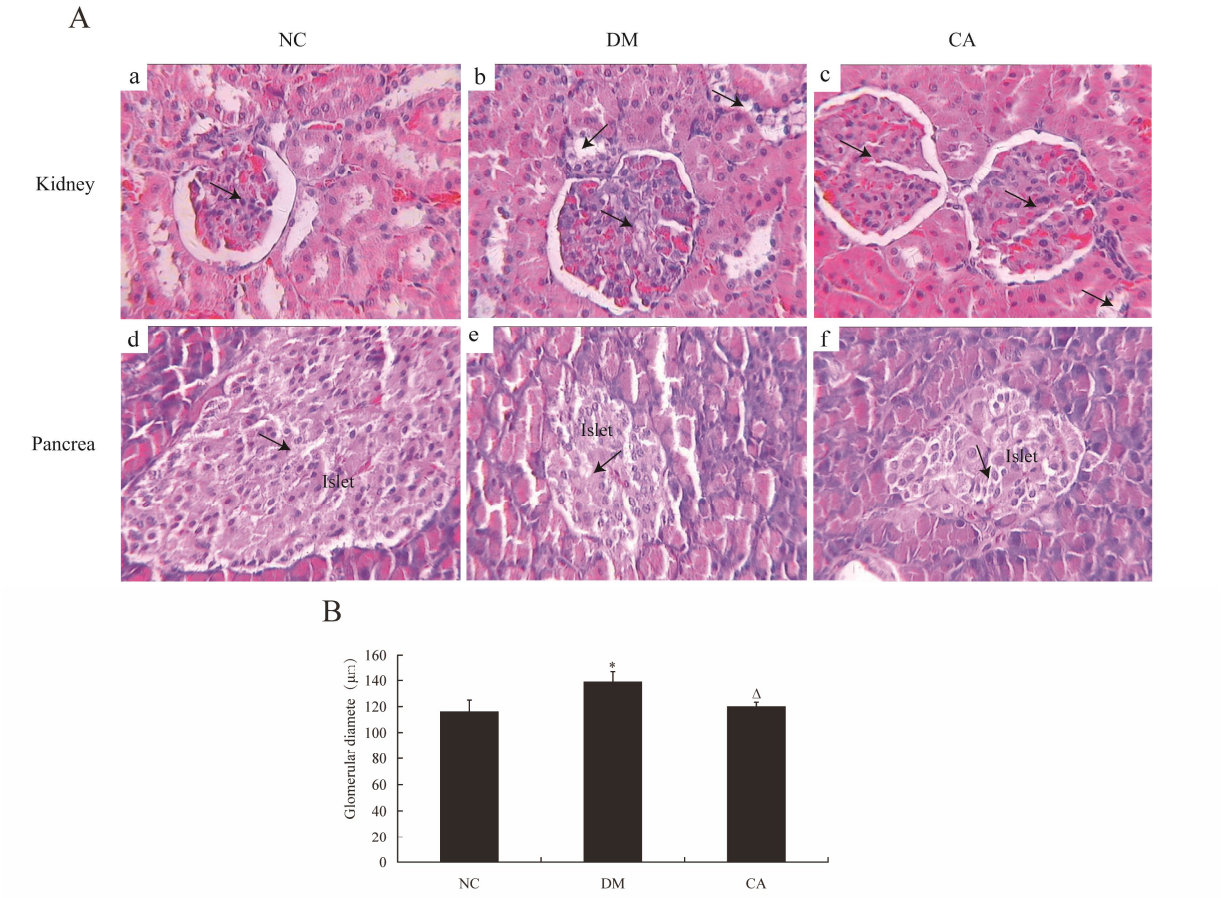

Fig. 6: Hematoxylin & Eosin staining of kidney and pancreas of HFSD and STZ-induced diabetic rats. (A) Hematoxylin and eosin staining of kidney of rats from NC (a), DM (b) and CA (c). Light microscope of pancreas sections from NC (d), DM (e) and CA (f). (B) The calculated glomeruli diameter size. Original magnification ×400.Values are mean ± SE, n=10. $^{*}P$<0.05 for DM versus NC.$^{\Delta}P$<0.05 for CA versus DM.

Table 2: Chlorogenic acid on incidence of diabetes in HFSD and STZ-induced diabetic rats

Groups	At 3 days after the STZ injection	At the end of experiment
Normal control group	0	0
Diabetes model group	8	10
Chlorogenic acid treatment group	1	0

skeletal muscles and adipocytes (Sivitz *et al.*, 1989) controls glucose uptake in those tissues. Chlorogenic acid stimulated glucose uptake acutely by increasing expression and translocation of GLUT4 in skeletal muscles (Ong *et al.*, 2013; Prabhakar and Doble., 2009; Shimoda *et al.*, 2006). Our study also showed that chlorogenic acid treatment increased GLUT4 mRNA expression. The result illustrated that chlorogenic acid was anti-hyperglycemic in the preventive treatment of diabetes mellitus.

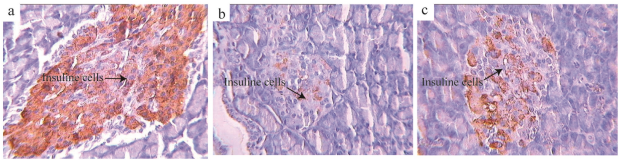

Fig. 7: Effects of chlorogenic acid on the expression of insulin in pancreas of HFSD and STZ-induced diabetic rats. Anti-insulin antibody immunostaining from NC (a), DM (b) and CA (c). Original magnification×400.

Metabolic syndromes accompanied by diabetes mellitus cause many pathological changes. Besides liver and skeletal muscle, diabetes mellitus also causes notable pathological changes inside pancreas and kidney. The early reports indicated that the islet β-cells were impaired in T2DM (Saisho, 2015). In the impaired islet β-cells the insulin-signaling pathway is blocked. Other studies showed that the insulin resistance was related to diabetic nephropathy in kidney: glomerular hypertrophy and the damages of renal tubules and interstitial tubules (Wu *et al.*, 2016). Diabetic nephropathy is characterized by glomerulosclerosis, tubular matrix deposition as well as kidney hypertrophy (Ahn *et al.*, 2014). Without proper treatment, T2DM patients with diabetic nephropathy will most likely lead to end-stage renal failure. Our study showed that chlorogenic acid protects from renal damages in HFSD and STZ induced diabetic rats by reducing the glomerular expansion and improving the vacuolar degeneration. Moreover, chlorogenic acid increased more intense insulin staining and fewer vacuoles emerged in insulin islets. These findings indicated that chlorogenic acid exhibited a good efficacy on the HFSD and STZ-induced beta cell destroy during the forming of diabetes mellitus that was related to a lower blood glucose and an higher insulin sensitivity. Previous studies in rodent islets showed that glucagon-like peptide-1(GLP-1) acted directly to regulate the insulin gene and enhanced insulin signaling as well as prevented degradation of IRS-1 and IRS-2 by suppressing ubiquitin-proteasome pathway (Drucker *et al.*, 1987; Wang *et al.*, 2014). We also believed that GLP-1 was probably an important aspect of the improved β-cell function in those animals.

CONCLUSION

The present study showed that chlorogenic acid has an inhibitory effect against the forming of T2DM induced by HFSD and STZ through regulating the glucose and lipid metabolism. Chlorogenic acid inhibited the hepatic mRNA expressions of G-6-Pase and up-regulated the skeletal muscle GLUT4 mRNA expression, to ameliorate glucose tolerance, insulin sensitivity, dyslipidemia, glomerular expansion and β-cell function. In conclusion, chlorogenic acid can have preventive effect on the forming of T2DM.

ACKNOWLEDGEMENTS

This study was supported by National Natural Science Foundation of China (31071531), the Scientific Research Fund of Hunan Provincial Education Department (14A071), and Youth Project of Hunan Provincial Education Department (16B138).

REFERENCES

Ahn JH, Yu JH, Ko SH, Kwon HS, Kim DJ, Kim JH, Kim CS, Song KH, Won JC, Lim S, Choi SH, Han K, Cha BY and Kim NH (2014). Taskforce Team of Diabetes Fact Sheet of the Korean Diabetes A. Prevalence and determinants of diabetic nephropathy in Korea: Korea national health and nutrition examination survey. *Diabetes Metab J.,* **38**(2): 109-119.

Amalan V, Vijayakumar N, Indumathi D, Ramakrishnan A (2016). Antidiabetic and antihyperlipidemic activity of p-coumaric acid in diabetic rats, role of pancreatic GLUT 2: In vivo approach. *Biomed Pharmacother.,* **84**: 230-236.

Bassoli BK, Cassolla P, Borba-Murad GR, Constantin J, Salgueiro-Pagadigorria CL, Bazotte RB, da Silva RS and de Souza HM (2008). Chlorogenic acid reduces the plasma glucose peak in the oral glucose tolerance test: effects on hepatic glucose release and glycaemia. *Cell Biochem Funct.,* **26**(3): 320-328.

Baumgartner RR, Steinmann D, Heiss EH, Atanasov AG, Ganzera M, Stuppner H and Dirsch VM (2010). Bioactivity-guided isolation of 1,2,3,4,6-Penta-O-galloyl-D-glucopyranose from Paeonia lactiflora roots as a PTP1B inhibitor. *J. Nat. Prod.,* **73**(9): 1578-1581.

Bazzano LA, Serdula M and Liu S (2005). Prevention of type 2 diabetes by diet and lifestyle modification. *J Am Coll Nutr.,* **24**(5): 310-319.

Brito EC, Lyssenko V, Renstrom F, Berglund G, Nilsson PM, Groop L and Franks PW (2009). Previously associated type 2 diabetes variants may interact with physical activity to modify the risk of impaired glucose regulation and type 2 diabetes: A study of 16,003 Swedish adults. *Diabetes.* **58**(6): 1411-1418.

Chang X, Cai H and Yin K (2017). The regulations and mechanisms of laparoscopic sleeve gastrectomy (LSG) for obesity and type 2 diabetes: A systematic review. *Surg Laparosc Endosc Percutan Tech.* **27**: e122-e126.

Cooper-DeHoff RM, Gong Y, Handberg EM, Bavry AA, Denardo SJ, Bakris GL and Pepine CJ (2010). Tight

blood pressure control and cardiovascular outcomes among hypertensive patients with diabetes and coronary artery disease. *JAMA.* **304**(1): 61-68.

Chu KY, Leung PS (2009). Angiotensin II in type 2 diabetes mellitus. *Curr. Protein Pept. Sci.,* **10**(1): 75-84.

Davis NJ, Ma Y, Delahanty LM, Hoffman HJ, Mayer-Davis E, Franks PW, Brown-Friday J, Isonaga M, Kriska AM, Venditti EM, Wylie-Rosett J and Diabetes Prevention Program Research G (2013). Predictors of sustained reduction in energy and fat intake in the Diabetes Prevention Program Outcomes Study intensive lifestyle intervention. *J. Acad Nutr. Diet.,* **113**(11): 1455-1464.

Del Rio D, Stalmach A, Calani L and Crozier A (2010). Bioavailability of coffee chlorogenic acids and green tea flavan-3-ols. *Nutrients.,* **2**(8): 820-833.

Drucker DJ, Philippe J, Mojsov S, Chick WL and Habener JF (1987). Glucagon-like peptide I stimulates insulin gene expression and increases cyclic AMP levels in a rat islet cell line. *Proc. Natl. Acad Sci. U S A.,* **84** (10): 3434-3438.

Farooq T, Rehman K, Hameed A and Akash MSH (2018). Stem cell therapy and type 1 diabetes mellitus: treatment strategies and future perspectives. *Adv. Exp. Med. Biol.,* **195**(1084): 95-107.

Fischer AH, Jacobson KA, Rose J and Zeller R (2008). Hematoxylin and eosin staining of tissue and cell sections. *CSH Protoc.* **2008**(6): pdb prot4986.

Gu X, Zhao HL, Sui Y, Guan J, Chan JC, Tong PC (2012). White rice vinegar improves pancreatic beta-cell function and fatty liver in streptozotocin-induced diabetic rats. *Acta Diabetol.,* **49**(3): 185-191.

He L, Sabet A, Djedjos S, Miller R, Sun X, Hussain MA, Radovick S and Wondisford FE (2009). Metformin and insulin suppress hepatic gluconeogenesis through phosphorylation of CREB binding protein. *Cell.* **137**(4): 635-646.

Hu M, Wan Y, Yu L, Yuan J, Ma Y, Hou B, Jiang X, Shang L (2017). Prevalence, Awareness and Associated Risk Factors of Diabetes among Adults in Xi'an, China. *Sci Rep.* **7**(1): 10472.

Huang K, Liang XC, Zhong YL, He WY and Wang Z (2015). 5-Caffeoylquinic acid decreases diet-induced obesity in rats by modulating PPARalpha and LXRalpha transcription. *J. Sci. Food Agric.,* **95**(9): 1903-1910.

Jeszka-Skowron M, Flaczyk E, Jeszka J, Krejpcio Z, Król E and Buchowski MS (2014). Mulberry leaf extract intake reduces hyperglycaemia in streptozotocin (STZ)-induced diabetic rats fed high-fat diet. *J. Funct. Foods,* **8**(1): 9-17.

Johnston KL, Clifford MN and Morgan LM (2002). Possible role for apple juice phenolic compounds in the acute modification of glucose tolerance and gastrointestinal hormone secretion in humans. *J. Sci. Food Agric.,* **82**(15): 1800-1805.

Karthikesan K, Pari L and Menon VP (2010). Antihyperlipidemic effect of chlorogenic acid and tetrahydrocurcumin in rats subjected to diabetogenic agents. *Chem. Biol. Interact.,* **188**(3): 643-650.

Kim J, Jeong IH, Kim CS, Lee YM, Kim JM and Kim JS (2011). Chlorogenic acid inhibits the formation of advanced glycation end products and associated protein cross-linking. *Arch. Pharm. Res.,* **34**(3): 495-500.

Li SY, Chang CQ, Ma FY and Yu CL (2009). Modulating effects of chlorogenic acid on lipids and glucose metabolism and expression of hepatic peroxisome proliferator-activated receptor-alpha in golden hamsters fed on high fat diet. *Biome. Environ. Sci.,* **22**(2): 122-129.

Lin WY, Xaiver Pi-Sunyer F, Chen CC, Davidson LE, Liu CS, Li TC, Wu MF, Li CI, Chen W and Lin CC (2011). Coffee consumption is inversely associated with type 2 diabetes in Chinese. *Eur. J. Clin. Invest,* **41**(6): 659-666.

Liu SL, Peng BJ, Zhong YL, Liu YL, Song Z and Wang Z (2015). Effect of 5-caffeoylquinic acid on the NF-kappaB signaling pathway, peroxisome proliferator-activated receptor gamma 2 and macrophage infiltration in high-fat diet-fed Sprague-Dawley rat adipose tissue. *Food Funct.,* **6**(8): 2779-2786.

Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF and Turner RC (1985). Homeostasis model assessment: Insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia,* **28**(7): 412-419.

McCarty MF (2005). A chlorogenic acid-induced increase in GLP-1 production may mediate the impact of heavy coffee consumption on diabetes risk. *Med. Hypotheses.,* **64**(4): 848-853.

Ogurtsova K, da Rocha Fernandes JD, Huang Y, Linnenkamp U, Guariguata L, Cho NH, Cavan D, Shaw JE and Makaroff LE (2017). IDF Diabetes Atlas: Global estimates for the prevalence of diabetes for 2015 and 2040. *Diabetes Res Clin Pract.* **128**: 40-50.

Ong KW, Hsu A and Tan BK (2013). Anti-diabetic and anti-lipidemic effects of chlorogenic acid are mediated by AMPK activation. *Biochem. Pharmacol.,* **85**(9): 1341-1351.

Ong KW, Hsu A and Tan BK (2012). Chlorogenic acid stimulates glucose transport in skeletal muscle via AMPK activation: A contributor to the beneficial effects of coffee on diabetes. *PLoS One.* **7**(3): e32718.

Paoli P, Caselli A, Camici G and Cirri P (2015). Effect of natural compounds on insulin signaling. *Curr. Med. Chem.,* **22**(1): 80-111.

Prabhakar PK and Doble M (2009). Synergistic effect of phytochemicals in combination with hypoglycemic drugs on glucose uptake in myotubes. *Phytomedicine.,* **16**(12): 1119-1126.

Randy A, Kim M and Nho CW (2016). Ligularia fischeri and its constituent 3,4-dicaffeoylquinic acid improve obesity-induced nonalcoholic fatty liver disease by

regulating lipid metabolism and activating AMPK. *J. Funct. Foods,* **27**(1): 1-16.

Ritz E (1999). Nephropathy in type 2 diabetes. *J. Interl. Med.,* **245**(2): 111-126.

Rodriguez de Sotillo DV and Hadley M (2002). Chlorogenic acid modifies plasma and liver concentrations of: Cholesterol, triacylglycerol, and minerals in (fa/fa) Zucker rats. *J. Nutr. Biochem.,* **13**(12): 717-726.

Rodriguez de Sotillo DV, Hadley M and Sotillo JE (2006). Insulin receptor exon 11+/- is expressed in Zucker (fa/fa) rats and chlorogenic acid modifies their plasma insulin and liver protein and DNA. *J. Nutr. Biochem.,* **17**(1): 63-71.

Saisho Y (2015). beta-cell dysfunction: Its critical role in prevention and management of type 2 diabetes. *World J. Diabetes,* **6**(1): 109-124.

Shang G, Gao P, Zhao Z, Chen Q, Jiang T, Zhang N and Li H (2013). 3,5-Diiodo-l-thyronine ameliorates diabetic nephropathy in streptozotocin-induced diabetic rats. *Biochim. Biophys. Acta.* **1832**(5): 674-684.

Shimoda H, Seki E and Aitani M (2006). Inhibitory effect of green coffee bean extract on fat accumulation and body weight gain in mice. *BMC Complement Altern. Med.,* **6**(1): 9.

Siedel J, Hagele EO, Ziegenhorn J and Wahlefeld AW (1983). Reagent for the enzymatic determination of serum total cholesterol with improved lipolytic efficiency. *Clin. Chem.,* **29**(6): 1075-1080.

Sivitz WI, DeSautel SL, Kayano T, Bell GI and Pessin JE (1989). Regulation of glucose transporter messenger RNA in insulin-deficient states. *Nature.* **340**(6228): 72-74.

Stefanello N, Schmatz R, Pereira LB, Rubin MA, da Rocha JB, Facco G, Pereira ME, Mazzanti CM, Passamonti S, Rodrigues MV, Carvalho FB, da Rosa MM, Gutierres JM, Cardoso AM, Morsch VM, Schetinger MR (2014). Effects of chlorogenic acid, caffeine, and coffee on behavioral and biochemical parameters of diabetic rats. *Mol. Cell Biochem.,* **388**(1-2): 277-286.

Thompson PL and Davis TME (2017). Cardiovascular effects of glucose-lowering therapies for type 2 diabetes: new drugs in perspective. *Clin Ther.,* **39**(5): 1012-1025.

Tokajuk A, Krzyzanowska-Grycel E, Tokajuk A, Grycel S, Sadowska A and Car H (2015). Antidiabetic drugs and risk of cancer. *Pharmacol. Rep.,* **67**(6): 1240-1250.

Turton JL, Raab R and Rooney KB (2018). Low-carbohydrate diets for type 1 diabetes mellitus: A systematic review. *PLoS One*. **13**(3): e0194987.

Turner R, Hattersley A and Cook J (1992). Type II diabetes: Search for primary defects. *Ann. Med.,* **24**(6): 511-516.

Wang ZQ, Yu Y, Zhang XH and Komorowski J (2014). Chromium-insulin reduces insulin clearance and enhances insulin signaling by suppressing hepatic insulin-degrading enzyme and proteasome protein expression in KKAy mice. *Front Endocrinol (Lausanne),* **5**: 99.

Wu CC, Hung CN, Shin YC, Wang CJ and Huang HP (2016). Myrciaria cauliflora extracts attenuate diabetic nephropathy involving the Ras signaling pathway in streptozotocin/nicotinamide mice on a high fat diet. *J Food Drug Anal.,* **24**(1): 136-146.

Yin Y, Qi F, Song Z, Zhang B and Teng J (2014). Ferulic acid combined with astragaloside IV protects against vascular endothelial dysfunction in diabetic rats. *Biosci Trends,* **8**(4): 217-226.

Zarraga IG and Schwarz ER (2006). Impact of dietary patterns and interventions on cardiovascular health. *Circulation,* **114**(9): 961-973.

Zhang LT, Chang CQ, Liu Y and Chen ZM (2011). Effect of chlorogenic acid on disordered glucose and lipid metabolism in db/db mice and its mechanism. *Zhongguo Yi Xue Ke Xue Yuan Xue Bao.,* **33**(3): 281-286.