Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org
C M B Association Publisher

Original Research

Ameliorative effect of polydatin on hyperglycemia and renal injury in streptozotocin-induced diabetic rats

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Received April 15, 2019; **Accepted** September 24, 2019; **Published** September 30, 2019

Doi: http://dx.doi.org/10.14715/cmb/2019.65.7.10

Abstract: To investigate the effect of polydatin on glucose transporter, blood glucose homeostasis and renal injury in streptozotocin (STZ)-induced diabetic rats. The *in vitro* inhibitory effect of polydatin on sodium-glucose cotransporter-1 (SGLT1) and 2 (SGLT2) was determined using HEK293 cells. The inhibitory effect of polydatin on GLUT1 and GLUT4 was evaluated using 3T3-L1 adipocytes. Streptozotocin-induced diabetic rats were used for this study. Fasting blood glucose (FBG), glycosylated hemoglobin (HbA1c), urea nitrogen, serum creatinine and urinary protein were determined using biochemical analyzer. Histopathological examination was performed on renal tissue. Serum levels of interleukin 1β (IL-1β), tumor necrosis factor α (TNF-α), monocyte chemoattractant protein 1 (MCP-1) and C-reactive protein (CRP) were also determined. Polydatin significantly inhibited SGLT1/2 and exhibited high selectivity for both GLUT1 and GLUT4. It significantly and dose-dependently decreased hyperglycemia, enhanced urine glucose excretion in the diabetic rats. The polydatin treatment significantly ameliorated symptoms of DN such as polyuria, polydipsia and hyperphagia. The hypoglycemic effect of polydatin was maintained throughout the treatment period. In addition,the levels of IL-1β, TNF-α, MCP-1 and CRP were significantly reduced in treated group. Treatment with polydatin significantly ameliorated most of the structural and morphological changes induced by STZ. Moreover, the levels of urinary protein, serum creatinine and urea nitrogen were significantly reduced after treatment with polydatin. As a potential dual inhibitor of SGLT1/2, polydatin has high selectivity for GLUT1 and GLUT4. Its long-term administration delays the development of DN, protects renal function and ameliorates renal tissue injury.

Key words: Polydatin; Diabetic nephropathy; Inflammatory factors; SGLT; Streptozotocin.

Introduction

Diabetic nephropathy (DN) is one of the microvascular complications of diabetes mellitus (DM), in which a patient's renal function deteriorates progressively until it develops into end-stage renal disease. According to World Health Organization (WHO), there are approximately 420 million diabetic patients worldwide, and the incidence of DN is 20 to 30 % (1). Hyperglycemia alters renal hemodynamics, and over-activates growth factors and cytokines. Increased concentration of urinary protein and decreased concentration of plasma albumin due to glomerular injury results in whole body edema in patients with DN (2). At present, maintenance of blood glucose concentration and blood pressure, and diet control are the major strategies for treating DN. Some patients still progress to end-stage renal disease even when they control their blood glucose and blood pressure. Therefore, the pathogenesis of DN has attracted much attention in recent times.

Sodium-dependent glucose transporters (SGLTs) are a family of glucose transporters found in intestinal mucosa of the small intestine and the proximal tubule of the nephron. They contribute to renal glucose reabsorption, and are targets of most antidiabetic drugs (4-6). Sodium-glucose cotransporter 2 (SGLT2) is expressed mainly in kidney, while SGLT1 is widely distributed in intestine, brain and kidney (7). The SGLTI in intestinal tract is responsible for glucose absorption (8). Inhibition of SGLT1 in the intestine promotes the release of glucagon-like peptide-1 (GLP-1) from intestinal endocrine cells, thus enhancing hypoglycemic activity. Inhibition of GLUT1 and GLUT4 leads to energy metabolism-related disorders because of their crucial physiological functions. The development of SGLT1/2 dual inhibitors has contributed immensely to the successes recorded so far in the treatment of DN (9). In 3T3-L1 adipocytes, GLUT1 primarily mediates non-insulin-dependent glucose uptake, while GLUT4 mediates mainly insulin-dependent glucose uptake (10-11).

Polydatin is the active principle in *Polygonum cuspidatum* Sieb.et Zucc., a traditional Chinese herb used

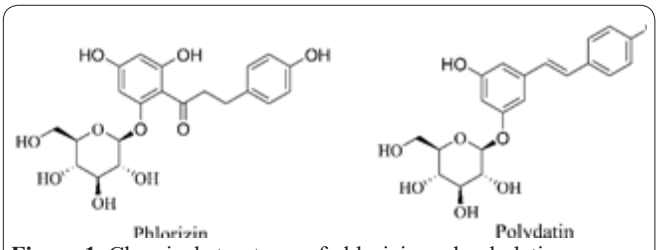

Figure 1. Chemical structures of phlorizin and polydatin.

to treat DM. As a structural analog of phlorizin, it has been speculated that polydatin may have SGLT-inhibitory activity. The aim of this study was to investigate the effect of polydatin on glucose transporter, blood glucose homeostasis and renal injury in STZ-induced diabetic rats.

Materials and Methods

Materials and chemicals

Thermo plate reader, ultra-pure water machine, and Thermo 50 µl 8-channel pipette were products of Thermo Fisher Ltd. (USA). Precision electronic balance (AUY120) was purchased from Shimadzu Co., Ltd. (Japan); HH-4 type constant temperature digital display water bath was obtained from Guohua Electric Co., Ltd., while blood glucose meter and blood glucose test strips were products of Changsha Sannuo Biosensing Co., Ltd. High-fat diet (45 % calories from fat) was obtained from Jiangsu Medison Biomedical Co., Ltd., while polydatin and phloridzin were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. Streptozotocin was a product of Sigma (USA). Interleukin-1β (IL-1β), TNF-α, MCP-1 and CRP ELISA kits were purchased from Nanjing Jiancheng Biotechnology Co., Ltd. Top count microplate scintillation counter was purchased from Packard Instrument Co., (USA).

Assessment of in vitro inhibitory activity of polydatin on SGLT1 and SGLT2

Human embryonic kidney (HEK) 293 cells with stably expressed SGLT2 or SGLT1 were seeded into 96-well plates and washed with buffer (pH 7.2 - 7.4). The uptake buffer consisted of 0.14 mol/L NaCl, 2 mmol/L KCl, 1 mmol/L $CaCl_2$), 1 mmol/L $MgCl_2$, 10 mmol/L HEPES, and 5 mmol/L Tris. Assay compounds of different concentrations were added, followed by addition of 14C labeled (4 µmol/L) and isotopically-labeled (10 µmol/L) α-methyl-D-glucose (α-MG). Finally, the solution was incubated at 37 °C for 2 h. The plates were washed twice with buffer, and digested with 200 mmol/L sodium hydroxide solution. The radioactivity of each well was determined using a top count microplate scintillation counter and the corresponding IC_{50} values were calculated.

Assessment of 2-Deoxy-D-glucose (2-DG) uptake in T3-L1 adipocytes

The 3T3-L1 fibroblasts were cultured in Dulbecco's Modified Eagle medium (DMEM) containing 10 % fetal bovine serum (FBS) in 5 % CO_2 incubator at 37 °C, and then seeded into 24-well collagen-coated plates (1 x 10^5 cells/well), and cultured for 5 days. The plates were cultured in 10 % FBS-supplemented DMEM containing 0.5 mmol/L 3-isobutyl-1-methylxanthine, 1 mmol/L dexamethasone and 10 mg/mL insulin for 2 days to stimulate 3T3-L1 cell differentiation into adipocytes, and further incubated in growth medium for another 8 - 9 days after washing. The differentiated 3T3-L1 adipocytes were cultured in buffer (pH 7.5) composed of 0.14 mol/L NaCl, 5 mmol/L KCl, 1 mmol/L $CaCl_2$, 2.5 mmol/L $MgCl_2$, and 20 mmol/L HEPES, at 37 °C for 4 h, and further incubated for 30 min in fresh DMEM supplemented with or without 100 nmol/L insulin. The 2-DG substrate (50 mmol/L) containing 37.4 KBq/mL [14C] 2-DG and test compound or dimethyl sulfoxide (DMSO) were added, incubated at 37 ° C for another 30 min, washed thrice with ice-cold phosphate-buffered saline (PBS), and then lysed with 0.5 mol/L NaOH solution. The radioactivity signal was measured using liquid scintillation counter, and the percent inhibition of 2-DG with or without insulin was determined.

Establishment of rat model of DN

Male Sprague-Dawley rats weighing 200 - 230 g, and aged 6 weeks were purchased from Yangzhou University Medical Laboratory Animal Center (license number: SCXK (Su) 2017-0001). They were fed high-fat diet for 8 weeks to induce insulin resistance, and then administered STZ intraperitoneally (35 mg/kg bwt) to establish diabetic nephropathy rat model. Fasting blood glucose (FBG) > 16.7 mmol/L and 24 h urine protein > 20 mg, were used as indicators of a successfully established rat model of DN.

Dose response study on polydatin

Rats with DN were randomly assigned to metabolic cages (6 rats per group), and the feed was removed immediately before the start of experiment. Blood was collected from the rats through their tails for determination of FBG (0 min), and then they were given vehicle (0.25 % sodium carboxymethyl cellulose solution) or polydatin (20 - 120 mg/kg bwt) using gavage, followed by the determination of blood glucose levels at intervals of 30, 60, 120 and 180 min after treatment. The rats were fed immediately after termination of blood glucose measurement. In addition, the urine of each rat was collected within 24 h of treatment and used for the determination of urinary glucose level.

Assessment of effect of long-term administration of polydatin

The rats (n = 24) were randomly assigned to four groups of six rats each: normal control group, DN control group, positive control group (phlorizin at a dose of 120 mg/kg bwt) and treatment group (polydatin at a dose of 120 mg/kg bwt). The rats were given drugs twice a day using gavage for 6 weeks. Fasting blood glucose was determined at intervals of day 0, day 10, day 20, day 30, and day 40 after 12 h of fasting, and random blood glucose levels were also monitored. Urine samples were collected at 5-day intervals from day 0 to day 35 within 24 h in the metabolic cages and used for determination of urinary glucose level. Feed and water intake were monitored on regular basis. On day 42 of treatment, 24 h urine was collected and centrifuged at 5000 rpm for 10 min, and the resultant supernatant was used for determination of protein content using biochemical automatic analyzer.

Determination of markers of inflammation and renal function

Whole blood collected in heparin containers were centrifuged at 3000 rpm for 10 min and the resultant plasma was used to determine the level of HbA1c using biochemical automatic analyzer. Serum levels of IL-1β, TNF-α, MCP-1 and CRP were determined using their respective ELISA kits. Serum levels of creatinine and

urea nitrogen were also determined using biochemical automatic analyzer.

Histological examination

Kidney tissues were excised after sacrifice, fixed with 10 % neutral formaldehyde for 24 h, dehydrated with gradient ethanol, embedded in paraffin, and then sliced using a microtome. The slices (5 µm) were baked in an oven at 60 °C for 2 h, dewaxed with xylene, and treated with absolute ethanol for 5 min, and gradient ethanol for 2 min. After washing thrice with running water, the slices were stained with hematoxylin within 5 min, rinsed with running water for 2 min, differentiated with 0.5 % hydrochloric acid alcohol, and rinsed again with running water. Subsequently, the slices were stained with 0.5 % eosin for 2 min, washed with running water within 1 min, dehydrated with ethanol step by step, cleared in xylene, and sealed with neutral gum. The treated tissue slides were observed under a light microscope at a magnification of 200.

Statistical analysis

Data are expressed as mean ± SEM, and the statistical analysis was performed using SPSS (20.0). Groups were compared using Duncan's multiple range test. Values of $p < 0.05$ were considered statistically significant.

Results

Inhibitory activity of polydatin on SGLT1 and SGLT2

Polydatin exerted moderate inhibitory effect on SGLT2 (IC_{50} = 878.5 nM), and was 5 times more selective for SGLT1 (Figure 2).

Inhibitory activity of polydatin on GLUT1 and GLUT4

As shown in Figure 3, in the presence of insulin, 1 µM polydatin only slightly inhibited the uptake of 2-DG, while 10 µM polydatin produced 32 % inhibition. On the other hand, in the presence of insulin, the inhibitory activity of cytochalasin B on GLUT4 was significantly increased up to 96 %. However, in the absence of insulin, 10 µM polydatin produced only 27 % inhibitory activity. The inhibitory activity of 10 µM cytochalasin B on GLUT1 was increased up to 90 %.

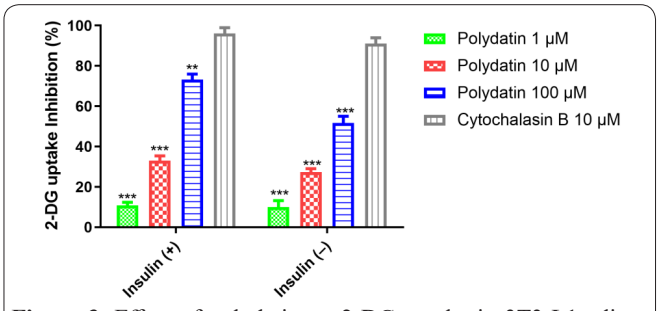

Figure 3. Effect of polydatin on 2-DG uptake in 3T3-L1 adipocytes, three independent experiments were performed for each group. $^{**}p < 0.01$ and $^{***}p < 0.001$, when compared with cytochalasin B group.

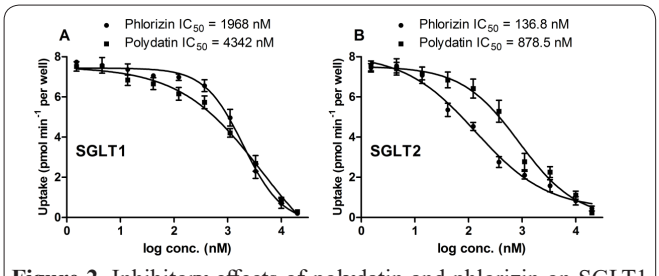

Figure 2. Inhibitory effects of polydatin and phlorizin on SGLT1 and SGLT2.

Effect of polydatin on levels of blood glucose in the rats

Polydatin significantly and dose-dependently improved the hyperglycemic state of diabetic rats ($p < 0.05$). A dose of 60 mg/kg bwt produced the best hypoglycemic effect. Polydatin also significantly and dose-dependently promoted urine glucose excretion ($p < 0.05$; Figure 4).

Long-term pharmacological activity of polydatin

On day 10 of treatment, the levels of random blood glucose and FBG were significantly reduced in the treatment group, when compared with DN control group, and the glucose-lowering effect was maintained throughout the treatment period ($p < 0.05$). The inhibitory effect of polydatin on SGLTs was significantly lower than that of phlorizin ($p < 0.05$). However, there was no significant difference in their *in vivo* hypoglycemic effects ($p > 0.05$). In addition, feed and water intake were significantly reduced in the treatment group relative to DN control group ($p < 0.05$). On day 1, urinary glucose level was significantly higher in the treatment group than in DN control group and was maintained throu-

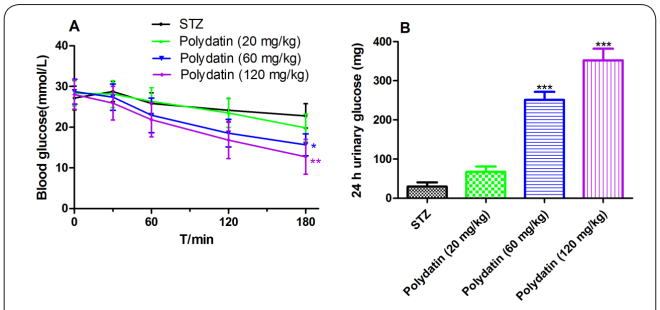

Figure 4. Dose-dependent effect of polydatin on blood glucose and urinary glucose excretion. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$, when compared with DN control group.

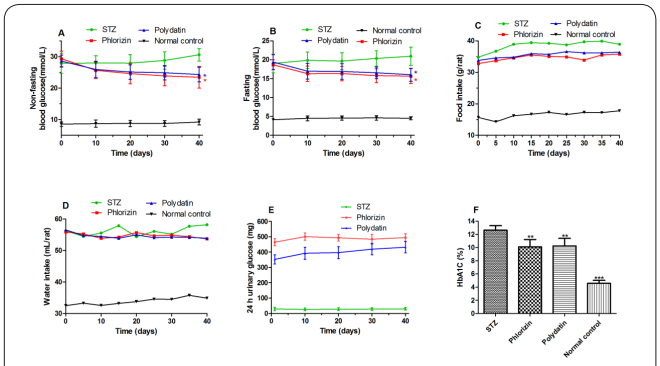

Figure 5. Charts showing long-term pharmacological effect of polydatin treatment. (A): Effect of polydatin on random blood glucose level; (B): Effect of polydatin on FBG level; (C): Effect of polydatin on feed intake; (C): Effect of polydatin on water intake; (D): Effect of polydatin on urinary glucose excretion; (E): Effect of polydatin on HbA1c level; and (F): Effect of polydatin in STZ-induced diabetic rats. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$, when compared with DN control group.

ghout the period of treatment ($p < 0.05$). After 6 weeks of treatment, HbA1c level was significantly reduced in the treatment group, when compared with DN control group ($p < 0.05$). These results are shown in Figure 5.

Effect of polydatin treatment on inflammatory responses

The levels of IL-1β, TNF-α, MCP-1 and CRP were significantly higher in DN control group than in normal control group, but were significantly reduced after treatment with polydatin over a long period ($p < 0.05$; Figure 6).

Effect of polydatin on kidney function in STZ-induced diabetic rats

The results of histopathological examination showed that renal cells were regularly organized, with intact tubular and glomerular structures, clear edges, and free from inflammatory cells in normal control group. However, there was marked deterioration of tubular and glomerular structures as well as numerous inflammatory cells infiltrate in DN control rats, relative to normal control group. Notably, the renal cells were arranged regularly, the glomerular and tubular structures were relatively intact, and the inflammatory cells were significantly reduced after treatment with polydatin or phlorizin, indicating that polydatin significantly ameliorated most of the structural and morphological lesions.. In addition, the levels of urinary protein, serum creatinine and urea nitrogen were significantly increased in DN control group, when compared with normal control group, but were significantly reduced after treatment with polydatin ($p < 0.05$). These results are shown in Figure 7.

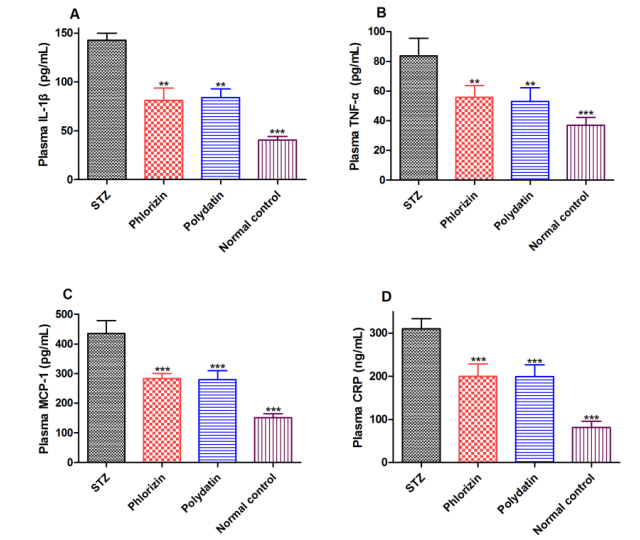

Figure 6. Effect of repeated administration of polydatin on plasma levels of IL-1β, TNF-α, MCP-1, and CRP. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$, when compared with DN control group.

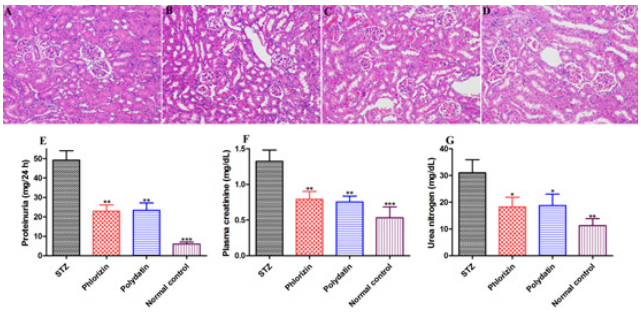

Figure 7. The effect of repeated administration of polydatin on renal tissue lesions (A) normal control, (B) STZ control, (C) Phlorizin group, (D) Polydatin and renal function (E-G).

Discussion

According to WHO, there are approximately 420 million diabetics worldwide, with type-2 DM accounting for more than 90 % of all diagnosed cases. Diabetic nephropathy is one of the major complications of DM and the leading cause of end-stage renal disease. Diabetic nephropathy is the result of multi-factor interactions and it is characterized by increased glomerular filtration, thickening of basement membrane and cumulative damage to extracellular matrix (12, 13). Polydatin, a glycoside extracted from the rhizome of *Polygonum cuspidatum* L. has a wide range of pharmacological activities, and it is a structural analog of the SGLTs inhibitor, phlorizin.

Sodium-glucose cotransporter-2 (SGLT2) which is mainly expressed in kidney, is responsible for over 90 % of glucose reabsorption in renal tubules. Sodium-glucose cotransporter-1 (SGLT1) is responsible for the absorption of glucose in the intestinal tract. Therefore, it is believed that selective inhibitors of SGLT2 reduce gastrointestinal side effects synonymous with most anti-diabetic drugs. At present, a number of SGLT2 selective inhibitors are available for the treatment of type-2 DM. These include dapagliflozin and canagliflozin (14-18). It has been reported that inhibiting SGLT1 in intestinal tract promotes the release of GLP-1 from intestinal endocrine cells, which in turn promotes hypoglycemic activity.

The results obtained in the present study showed that polydatin has moderate SGLT2 inhibitory activity, which was 5 times more selective for SGLT1, an indication that it may be a potential SGLT1/2 dual inhibitor. Due to the ligand of GLUT1 and GLUT4 is glucose, many glycosides are act as inhibitors of GLUT1 and GLUT4.(9, 17) It is likely that as a glycoside, polydatin might be interacted with GLUT1 and GLUT4 , which are responsible for most of the glucose transport in the body.(10-11) But actually, Polydatin revealed high selectivity for GLUT1 and GLUT4.

In this study, polydatin significantly and dose-dependently improved the hyperglycemic state of diabetic rats. These results suggest that the glucose lowering ability of this drug may be positively related to the amount of urinary glucose excreted. It is also possible that the glucose-lowering ability of polydatin is exerted via inhibition of SGLT1 and SGLT2. Treatment of diabetic rats with polydatin over a long period significantly ameliorated symptoms of DN such as polyuria, polydipsia and hyperphagia. Polydatin and phloridzi exerted similar hypoglycemic effect which was maintained throughout the treatment period. In addition, polydatin significantly reduced HbA1c level of DN control rats, an indication that it may reduce hyperglycemia-induced toxicity and delay the development of diabetes-related complications.

Metabolic disorders in DM enhance the reactivity of a number of vasoactive factors, which in turn dilate the

glomerular afferent arteriole, and increase the glomerular capillary blood flow. Glomerular internal hypertension reduces filtration, thereby altering glomerular structure and function. The progression of DN reduces glomerular filtration capacity and increases albumin content in urine. Proteinuria, and elevated serum creatinine and urea nitrogen are commonly used indicators for monitoring renal function (19). Proteinuria reflects the severity of glomerular injury, while serum creatinine reflects the glomerular filtration capacity.

After long-term administration of polydatin, the levels of urinary protein, serum creatinine and urea nitrogen were significantly reduced in DN control group, an indication that polydatin may significantly improve renal function in rats with DN. Hyperglycemia increases terminal glycosylation products, promotes endothelial cell apoptosis, and inhibits endothelial cell proliferation. The progression of DN results in glomerular endothelial cells apoptosis and necrosis, which in turn reduce the number of endothelial cells, thereby decreasing the integrity of the endothelium. The results of histopathological examination revealed the presence of massive inflammatory cells in the kidney of rats with DN. Inflammatory cells secrete cytokines which damage renal tissue and accelerate glomerular sclerosis (20). Long-term treatment with polydatin significantly decreased the serum levels of inflammatory factors such as IL-1β, TNF-α, MCP-1 and CRP in DN control rats. It also significantly ameliorated renal injury induced by STZ.
As a potential dual inhibitor of SGLT1/2, polydatin has high selectivity for GLUT1 and GLUT4. Its long-term administration delays the development of DN, protects renal function and ameliorates renal tissue injury.

Acknowledgements
This study was supported by Tranditional Chinese Medicine Bureau of Jiangsu Province (FY201506).

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

Author's contribution
All work was done by the authors named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Jiangyi Yu; Lijuan Wang, Liji Huang, Nan Li, Junjun Miao, Wei Liu, Jiangyi Yu collected and analysed the data; Lijuan Wang wrote the text and all authors have read and approved the text prior to publication.

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