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Icariin treatment reduces blood glucose levels in type 2 diabetic rats and protects pancreatic function

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Abstract. Icariin, a flavonoid isolated from traditional oriental herbal medicines, has been demonstrated to exhibit several health benefits in animal models and in humans. The aim of the present study was to investigate the effect of Icariin on hyperglycemia in type 2 diabetes mellitus (T2DM) in rats. A model of diabetes was established in 50 Sprague Dawley rats using a high-sugar and high-fat diet and peritoneal injection of streptozotocin. Diabetic rats were divided into five groups: Diabetic control; metformin; and rats treated with three different doses of Icariin, 5, 10 and 20 mg/kg. Body weight and blood glucose levels were measured, and serum adiponectin levels, expression of phospho-AMP mediated protein kinase (p-AMPK) and glucose transporter isoform 4 (GLUT-4) were measured using ELISA, Realtime PCR and western blotting, respectively. Diabetic rats without drug treatment exhibited reduced body weight, increased blood glucose levels and decreased the number of islets. In T2DM rats treated with 10 or 20 mg/kg Icariin, the blood glucose levels were reduced, whereas serum adiponectin levels were not affected. Additionally, the mRNA and protein expression levels of p-AMPK and GLUT-4 protein were increased in the T2DM rats treated with Icariin. In conclusion, in the diabetes rat model, Icariin alleviated the severity of diabetes, and the effects may be associated with reduction of hyperglycemia by activating an AMPK/GLUT-4 pathway.

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Key words: Icariin, type 2 diabetes mellitus, AMP-mediated protein kinase, glucose transporter isoform 4

Introduction

Diabetes is a worldwide health problem with a prevalence of ~6% in adults (1). Type 2 diabetes mellitus (T2DM) accounts for 90-95% of all diabetic cases and is characterized by insulin resistance and impaired glucose and lipid metabolism (2). Treatment of diabetes and its complications are primarily dependent on chemical and biological agents, which are associated with certain side effects, including gastrointestinal problems and hypoglycemia. Natural medicines have exhibited anti-diabetic activity (3,4). Icariin ($C_{30}H_{40}O_{15}$; molecular weight, 676.67), the molecular structure of which is shown in Fig. 1 (5), is a flavonoid isolated from the traditional oriental herbal medicine, *Epimedium koreanum Nakai*. Icariin exhibits a variety of beneficial biological activities, including immunological functions (6), sexual function (7), cardiovascular diseases (8), and anti-cancer (9) and anti-Alzheimer's disease effects (10). In rats, Icariin was also found to alleviate renal damage (11), enhance neurite growth in retinal ganglion cells (12), ameliorate signs of impotence (13) and lower lipid levels (14). However, there is no direct evidence demonstrating how Icariin regulates glucose homeostasis.

Adiponectin is a biologically active polypeptide produced by adipocytes (15). Adiponectin shows anti-diabetic potential by improving insulin sensitivity (16,17). AMP-mediated protein kinase (AMPK) is a key molecule involved in regulation of energy metabolism, by increasing the ratio of intracellular AMP/ATP (18-20). Additionally, LKB1, an upstream kinase of the AMPK pathway, activates AMPK, promoting the phosphorylation of Thr172. Accordingly, LKB1, regulates glucose absorption during contractions of muscles (21). Drugs which regulate adiponectin levels or the AMPK-mediated pathway exhibit hyperglycemic actions which may be used for the treatment of diabetes (22,23).

Defects in skeletal muscle function have been associated with insulin resistance in diabetes (24). Glucose transporter isoform 4 (GLUT-4) expression is upregulated in skeletal muscle and adipose tissues (25). Insulin promotes intracellular GLUT-4 translocation to the cytoplasmic membrane,

increasing glucose uptake in skeletal muscle (26). Exercise increases GLUT-4 expression and AMPK activation in skeletal muscles (27,28). Overexpression of GLUT-4 improves glucose homeostasis (29). Flavonoids function as an antidiabetic, primarily by increasing the expression of and promoting translocation of GLUT-4 via the AMPK signaling pathway (4). The results of the present study suggest that regulation of the AMPK/GLUT-4 pathway in skeletal muscles may be an effective potential therapy for treatment of hyperglycemia.

The primary aim of the present study was to investigate the effects of Icariin on the levels of glucose in a rat model of diabetes. Additionally, the role of AMPK/GLUT-4 signaling pathway in the antidiabetic effects of Icariin were examined.

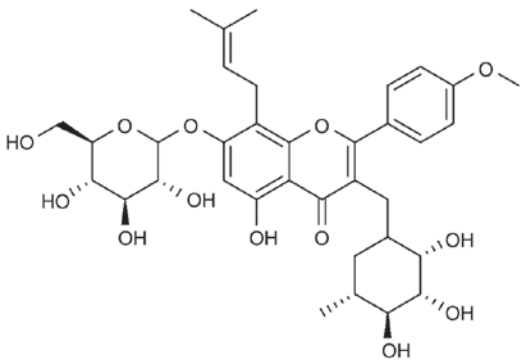

Figure 1. Chemical structure of Icariin.

Materials and methods

Animal models. Animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (publication no. 85-23, revised 1996). The present study was approved by the Animal Ethics Committee of Qingdao University. Sixty five-week-old male Sprague-Dawley rats, (100-120 g) provided by the Institute of Qingdao Platford Breeding Co., were maintained in a pathogen-free environment with a 12 h light/dark cycle with free access to food and water. The diabetic group (n=50) was fed with high-sugar and high-fat diet (kcal%: 45% fat, 20% protein, and 35% 100 carbohydrate; 4.73 kcal/gm, Research Diet, New Brunswick, NJ, USA) for 4 weeks (30), whereas the control group was fed with a normal diet for 4 weeks. Diabetes was induced by intraperitoneal injection of 40 mg/kg streptozotocin (STZ; S0130, Sigma).

Three days after STZ injection, T2DM was confirmed, as blood glucose levels were increased. A total of 50 rats with diabetes were randomly divided into five groups (n=10 per group): Diabetic control; metformin (400 mg/kg dissolved in water, administered by gavage) (31); and rats treated with either 5, 10 or 20 mg/kg Icariin (32)(489-32-7, Sigma) dissolved in carboxymethylcellulose sodium administered by intraperitoneal injection, once a day. 10 normal rats served as the control group. After a total of 3 weeks of drugs treatment, the body weight and fasting blood glucose levels were recorded. All the experimental animals survived.

Blood sample collection and tissue extraction. First of all, rats were anesthetized with 30 mg/kg sodium pentobarbital. Then, blood samples were collected from tail veins. An oral glucose tolerance test, in which 20% glucose was fed with a syringe at a dose of 2 g/kg, was performed after the rats were fasted for 10 h (33). Blood samples were collected from the caudal vein by means of a small incision at the end of the tail at 0, 15, 30, 60 and 120 min after the glucose administration. Subsequently, the level of blood glucose was measured.

After OGTT test, rats were euthanized using 150 mg/kg sodium pentobarbital. Pancreatic tissues were dissected, processed as paraffin blocks, then stained with hematoxylin eosin. Pancreatic tissues were rehydrated, incubated, washed, rapidly dehydrated and subsequently mounted on cover slips. Tissues were imaged using a microscope (DM750M, Leica) at x200 magnification.

Serum adiponectin measurement. Serum adiponectin concentrations were determined using a specific ELISA kit (ab108786, Abcam).

RNA extraction and gene microarray hybridization. Total RNA was extracted from bisected soleus muscle tissue using an RNA isolation kit (AM1912, Invitrogen, America). RNA concentrations were measured using spectrophotometric analysis by measuring the A260/280 ratio. The instrument for detecting RNA concentration is spectrophotometer (E300, Thermo, America). Reverse transcription-quantitative PCR was performed and analyzed on a Rotor-Gene 6000 system (Corbett Research). PCR was performed using a SYBR® Premix Ex Taq™ (Tli RNaseH Plus) kit (RR420A, Takara). The thermocycling conditions were: Initial denaturation, 9˚C for 30 sec; followed by 40 cycles of denaturation at 60˚C for 30 sec, primer annealing at 9˚C for 5 sec, and extension at 64˚C for 1 min. Fluorescence was measured at 72˚C in each cycle. To determine the specificity of PCR reactions, melt curve analysis was performed following amplification by slowly ramping the heat from 72˚C to 9˚C, with fluorescence acquisition at 1˚C intervals and a 5-sec hold at each increment. The forward and reverse primer sequences were as follows: GLUT-4 forward, 5'-TCATTCCTGTGAAAGTGATGACGA-3' and reverse, 5'-CTGCCACAGTGTCATATCATCCAA-3'; and β-actin forward, 5'-CCGTAAAGACCTCTATGCCAACA-3' and reverse 5'-GCTAGGAGCCAGGGCAGTAATC-3'. Expression of the target gene was normalized to β-actin. Primer 5 software was used for the primer design.

Western blotting. Homogenized skeletal muscle (0.1 g) at 4˚C in 1 ml of lysis buffer containing 50 mM Tris.HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 5 mM Na_3VO_4, 20 mM NaF, 10 mM sodium pyrophosphate and 50 μl protease inhibitor cocktail (B14001, Bimake). Equal quantities (50 μg) of total protein were resolved on a 12% gel using SDS-PAGE and transferred to a PVDF membrane (FFP32, Beyotime). After transfer, membranes were blocked using 5% skimmed milk containing 0.1% Tween-20. Subsequently the membranes were incubated with anti-GLUT-4 (2213S, Cell Signaling Technology, America), anti-phospho (p)-AMPK (2535S, Cell Signaling Technology), total-AMPK (2532S, Cell Signaling Technology) or anti-β-actin (ab8227, Abcam). Membranes were then incubated with horse radish peroxidase (HRP)-conjugated secondary antibodies, including

Table I. The effect of Icariin on body weight in T2DM rats.

Groups	Basal body weight (g)	Body weight (g) 21 days after drug treatment
Control	201.8±9.8	223.8±12.1
Diabetic	198.8±11.4	187.8±11.4[a]
Metformin	201.4±7.2	219.3±9.1[b]
Icariin 5 mg/kg	203.3±10.9	195.3±9.7
Icariin 10 mg/kg	202.3±11.2	208.7±6.9[b]
Icariin 20 mg/kg	200.9±8.2	215.3±12.1[b]

Values are means ± standard deviation. [a]P<0.05 vs. the control group; [b]P<0.05 vs. the diabetic group; n=10; T2DM, type 2 diabetes mellitus.

Table II. The effect of Icariin on blood glucose in T2DM rats.

Groups	Blood glucose (mmol/l) before STZ injection	Blood glucose (mmol/l) 21 days after drug treatment
Control	2.21±0.4	2.88±0.7
Diabetic	2.29±0.5	5.89±1.1[a]
Metformin	2.25±0.5	3.16±0.9[b]
Icariin 5 mg/kg	2.25±0.6	5.48±1.3
Icariin 10 mg/kg	2.35±0.4	4.02±1.2[b]
Icariin 20 mg/kg	2.27±0.7	3.27±0.7[b]

Values are means ± standard deviation. [a]P<0.05 vs. the control group; [b]P<0.05 vs. the diabetic group; n=10; T2DM, type 2 diabetes mellitus.

HRP-conjugated goat anti-rabbit immunoglobulin G (IgG) (AP132P, Merck Millipore), or HRP-conjugated goat anti-mouse IgG (ab6789, Abcam). Enhanced chemiluminescence plus kit (PE0010, Solarbio) was used to visualize the signals. Relative protein expression levels were quantified using densitometry analysis and normalized to β-actin expression levels.

Statistical analysis. GraphPad Prism version 4.0 (GraphPad Software, Inc.) was used to analyze the data. Statistical analysis was performed using a one-way ANOVA with a Tukey's post hoc test. All data are presented as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Results

The effect of Icariin on body weights, blood glucose levels and serum fasting blood glucose levels in diabetic rats. After 3 weeks of treatment with drugs, the body weight of rats decreased significantly (*P<0.05; Table I) compared with the non-diabetic control. There was no significant difference in the body weight of rats treated with 5 mg/kg Icariin compared with the diabetic control (P>0.05; Table I). However, the body weights of rats were significantly increased when treated with 10 or 20 mg/kg Icariin compared with the diabetic control (^P<0.05, Table I). There was no significant difference in body weight between the Icariin (10 and 20 mg/kg) group and the metformin group (P>0.05, vs. the metformin group, Table I).

The diabetic rats treated with 10 or 20 mg/kg Icariin exhibited reduced blood glucose levels compared with the diabetic control rats (^P<0.05; Table II). Meanwhile, there was no significant difference in blood glucose level between the Icariin (10 and 20 mg/kg) group and the metformin group (P>0.05, vs. the metformin group, Table II). However, the change in blood glucose levels were not considered significant in the rats treated with 5 mg/kg Icariin compared with the diabetic control rats (P>0.05, Table II).

In the oral glucose tolerance test, the blood glucose levels reached peak levels after 30 min, and returned to resting levels after ~120 min, except in rats treated with 5 mg/kg Icariin, where peak blood glucose levels were reached after 60 min. Glucose levels were significantly lower in the rats treated with 10 or 20 mg/kg Icariin compared with the diabetic control (^P<0.05; Table III). There was no significant difference in oral glucose tolerance between the Icariin (10 and 20 mg/kg) group and the metformin group (P>0.05, vs. the metformin group, Table III).

The effect of Icariin on pancreatic tissues in diabetic rats. The morphology of islets in the pancreatic tissues from the different groups are shown in Fig. 2. STZ treatment resulted in impaired pancreatic tissues, with fewer islets compared with the normal control (Fig. 2A and B, respectively). Icariin treatment reduced the loss in the number of islets, compared with the diabetic control rats, irrespective of the dose used, thus serving a protective role during the diabetic process (Fig. 2D-F). Metformin treatment also reduced the loss in the number of islets, compared with the diabetic control rats (Fig. 2C).

Table III. The effect of Icariin on blood glucose level during the oral glucose tolerance test in T2DM rats.

	Blood glucose (mmol/l)				
Groups	0 min	15 min	30 min	60 min	120 min
Control	2.88±0.7	6.45±1.3	7.09±1.2	6.24±1.0	3.87±0.7
Diabetic	5.89±1.1	12.3±1.8	19.3±1.0	20.51±1.1	3.83±1.8[a]
Metformin	3.16±0.9	8.23±1.7	9.63±1.4	7.98±1.3	3.82±1.1[b]
Icariin 5 mg/kg	5.48±1.3	8.70±1.3	12.88±1.8	13.86±2.1	9.83±2.3
Icariin 10 mg/kg	4.02±1.2	10.31±1.9	11.41±0.9	10.87±1.5	5.31±1.3[b]
Icariin 20 mg/kg	3.27±0.7	8.29±1.7	9.81±1.6	8.68±1.8	4.03±1.8[b]

Values are means ± standard deviation. [a]P<0.05 vs. the control group; [b]P<0.05 vs. the diabetic group; n=10; T2DM, type 2 diabetes mellitus.

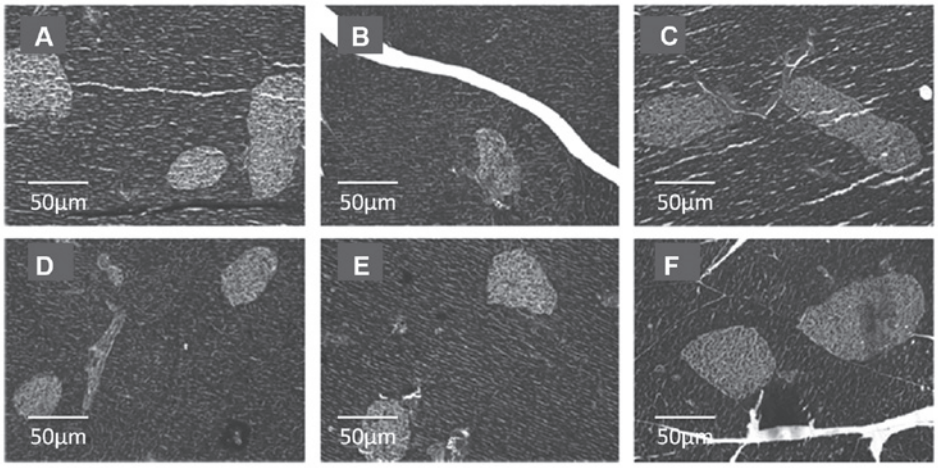

Figure 2. Images of pancreatic tissues stained with hematoxylin and eosin. Islets are colored lighter compared with the rest of the pancreatic tissue. Representative images of pancreatic tissues from (A) normal control, (B) diabetic control, (C) metformin control, and type 2 diabetes mellitus rats treated with (D) 5, (E) 10 or (F) 20 mg/kg *Icariin*. Magnification, x200.

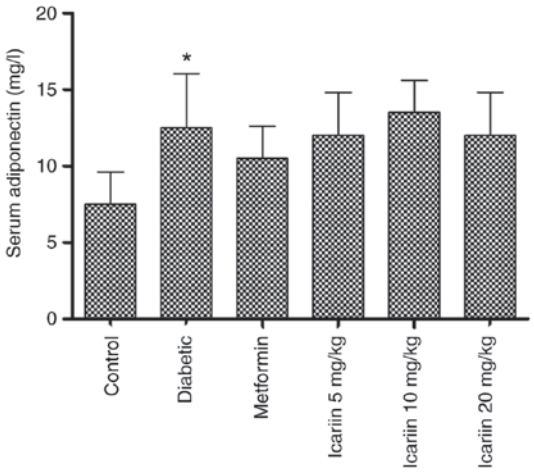

Figure 3. Effect of *Icariin* on serum adiponectin levels in type 2 diabetes mellitus rats. Data are presented as the mean ± standard deviation. *P<0.05 vs. control group. n=10.

The effect of Icariin on serum adiponectin levels in diabetic rats. STZ treatment increased the serum adiponectin levels compared with the non-diabetic control (Fig. 3; *P<0.05); However, no significant changes were observed in the serum adiponectin levels in the rats treated with Icariin (P>0.05, vs. the diabetic group).

The effect of Icariin on the mRNA and protein expression levels of GLUT-4 in diabetic rats. As shown in Fig. 4, the GLUT-4 mRNA expression levels in skeletal muscles were significantly lower in the diabetic rats compared with the control (P<0.05). In the rats treated with 10 and 20 mg/kg Icariin, GLUT-4 mRNA expression levels were significantly increased compared with the diabetic control (Fig. 4A; ^P<0.05). Similarly, GLUT-4 protein expression levels were also increased in Icariin treated mice compared with the diabetic control (Fig. 4B and C; P<0.05). There was no significant difference in expression of GLUT-4 between the Icariin (10 and 20 mg/kg) group and the metformin group (P>0.05, vs. the metformin group).

The effect of Icariin on phosphorylation of AMPK in diabetic rats. The phosphorylation of AMPK was decreased in the diabetic rats compared with the control (*P<0.05; Fig. 5). Phosphorylation of AMPK in the rats treated with 10 or 20 mg/kg Icariin was significantly increased compared with

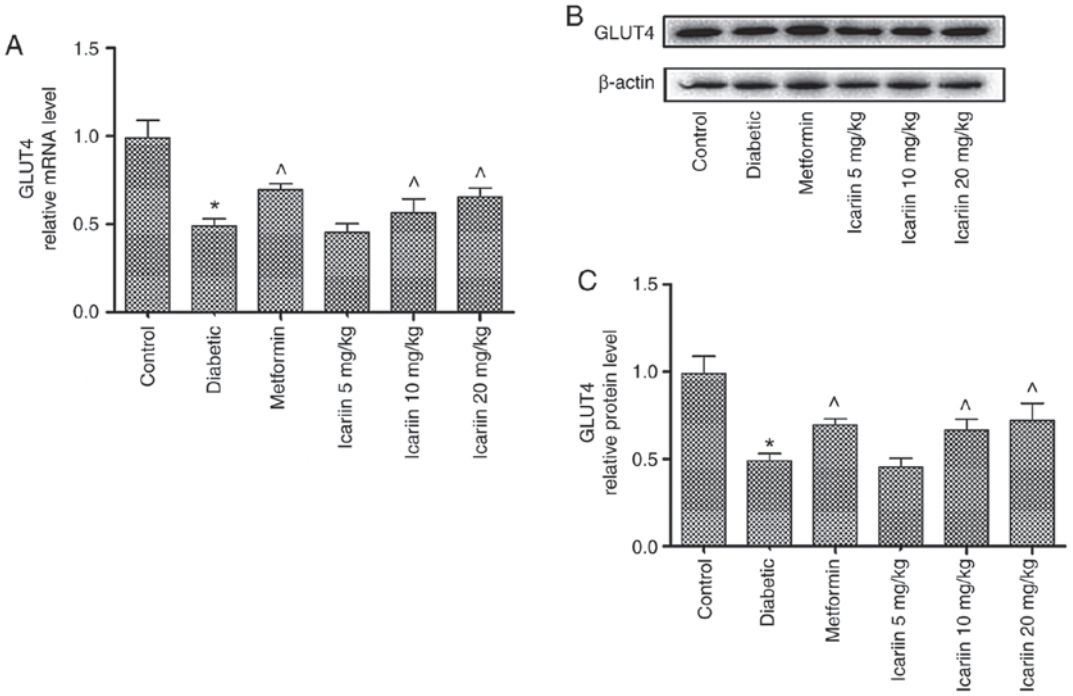

Figure 4. Effect of Icariin on the mRNA and protein expression levels of GLUT-4 in diabetic rats. (A) mRNA and (B) protein expression levels of GLUT-4 in type 2 diabetes mellitus rats in the normal control, diabetic control, metformin control, and type 2 diabetes mellitus rats treated with 5, 10 or 20 mg/kg *Icariin*. (C) Statistical analysis. Data were presented as the ratio of GLUT-4 to β-actin. Data are presented as the mean ± standard deviation. n=10 per group. ^P<0.05 vs. diabetic group. *P<0.05 vs. control group. GLUT-4, glucose transporter isoform 4.

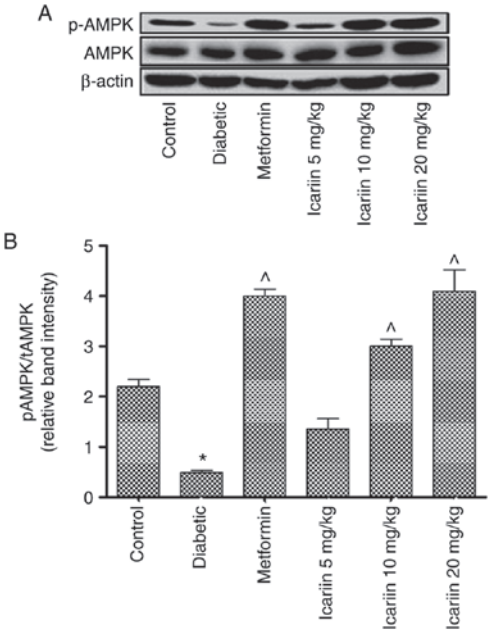

Figure 5. Effect of Icariin on phosphorylation of AMPK in diabetic rats. (A) Protein expression levels of phosphorylated of AMPK in type 2 diabetes mellitus rats in the normal control, diabetic control, metformin control, and type 2 diabetes mellitus rats treated with 5, 10 or 20 mg/kg *Icariin*. (B) Statistical analysis. Data were presented as the ratio of phosphorylated AMPK to total AMPK. Data are presented as the mean ± standard deviation. n=10 per group. *P<0.05 vs. control group; ^P<0.05 vs. the diabetic group.

the diabetic control (P<0.05; Fig. 5). There was no significant difference in phosphorylation of AMPK between the Icariin (10 and 20 mg/kg) group and the metformin group (P>0.05, vs. the metformin group, Fig. 5).

Discussion

The primary findings of the present study were that treatment with 10 or 20 mg/kg Icariin for 3 weeks reduced the blood glucose levels in diabetic rats. This treatment also reduced the peak glucose levels in an oral glucose tolerance test. Furthermore, treatment with Icariin resulted in reducing the loss in the number of islets in the pancreatic tissues and treatment with Icariin was associated with upregulated mRNA expression of GLUT-4 and increased phosphorylation of AMPK in the skeletal muscles. These results suggest that the beneficial effects of Icariin on T2DM may be associated with an AMPK/GLUT-4 signaling pathway.

Recent studies have suggested that polyphenolic compounds prevent the development of long-term diabetes and its complications, including cardiovascular disease, neuropathy, nephropathy and retinopathy (34,35). The therapeutic properties of *Epimedium koreanum* have been attributed to the flavonoid component of Icariin, which has been reported to exhibit a broad range of pharmacological effects, including anti-diabetic, anti-Alzheimer's disease, anti-tumor and hepatoprotective properties (36). To the best of our knowledge, the present study is the first to demonstrate the dose-dependent antidiabetic effect of Icariin, with hypoglycemic effects observed with 10 and 20 mg/kg. Treatment with Icariin for 3 weeks reduced the blood glucose levels as well as the peak glucose levels following a bolus dose of glucose.

Metformin is medically considered as the only biguanide which is used and recommended as oral anti-diabetic agent, which is crucial for decreasing the levels of plasma glucose. As known, metformin has been found to exert an

increasing effect on inhibiting hepatic gluconeogenesis, decreasing hyperinsulinemia, reducing protein synthesis, improving insulin sensitivity and enhancing glucose use in the muscle. In clinical practice, previous evidence has reported that metformin is widely accepted as an effective treatment for DM, and notably to T2DM by serving as the first-line therapy. Therefore, metformin was chosen as a positive control drug in this study.

Adipose tissue has been demonstrated to serve an endocrine role in recent years. Adiponectin, secreted by adipocytes, is an insulin-sensitizing hormone (15), improving insulin resistance in mice (37). And in studies on humans, adiponectin has the potential to be a biomarker for predicting metabolic diseases such as diabetes mellitus (38). In clinical trials, adiponectin was demonstrated to exhibit anti-diabetic (39), anti-atherosclerotic (40) and anti-cancer potential (41). Adiponectin improves insulin sensitivity by increasing insulin receptor expression and signal transduction, thereby alleviating insulin resistance (16,17,42). In the present study, there was no statistically significant difference in the adiponectin levels between the Icariin treated and diabetes control group, suggesting that the anti-diabetes effect of Icariin was likely not associated with the expression of adiponectin in the rat model diabetes used.

AMPK, a crucial component of cellular metabolism, has been demonstrated to inhibit many metabolic diseases including T2DM. Metformin lowers blood glucose levels by inhibiting hepatic glucose production, which is mediated by an AMPK-dependent mechanism (43). Increased glucose uptake following AMPK activation by AICA-riboside in perfused rat hindlimb muscles is attributed to an increase in translocation of GLUT-4 to the cell membrane (44). These results suggest that increasing GLUT-4 expression in skeletal muscles may be an effective therapy for treating diabetes. In the present study, Icariin treatment resulted in increased expression of AMPK and GLUT-4, suggesting that the anti-hyperglycemic effects of Icariin may be associated with the AMPK/GLUT-4 signaling pathway.

The limitation of this study is that there is no measurement of insulin levels under Icariin intervention. Thus, it is impossible to accurately assess the islet function. This study does not investigate whether Icariin has side effects in the treatment of T2DM. In this study, we did not observe the effect of Icariin on healthy rats. The side effects of Icariin were not elucidated by literature search. However, Icariin belongs to flavonoids which have common side effects (45), such as allergic reaction and pyrogen reaction. Studies have shown that the toxic side effects may be caused by the charge complexes formed by a class of lipoproteins and flavonoids. To clarify the common impurities and physicochemical properties of these flavonoids, effective separation methods should be adopted to provide safe and effective drugs for clinical use. Finally, there was no data on diabetic patients. This is also what needs to be done in future research.

In summary, the present study demonstrated that Icariin is an effective therapy for treating diabetes in a rat model of T2DM. The pharmacological effects of Icariin is related to preserve pancreatic islet number or function and increased expression levels of AMPK and GLUT-4 in the skeletal muscles.

Acknowledgements

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

YC and LW made substantial contributions to conception, design of the experiments, as well as the writing and revision of this manuscript. XL was responsible for acquisition and interpretation of data. XL, YW, PS, YL, TL and SL performed the experiments and participated in the writing of the manuscript. CW analyzed the data. The manuscript has been read and approved by each author, and all agree to this submission.

Ethics approval and consent to participate

The current study was approved by the Medical Ethics Committee of Affiliated Hospital of Qingdao University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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