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ORIGINAL ARTICLE

Effect of icarisid II on diabetic rats with erectile dysfunction and its potential mechanism *via* assessment of AGEs, autophagy, mTOR and the NO–cGMP pathway

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Erectile dysfunction (ED) is a major complication of diabetes mellitus. Icariin has been shown to enhance erectile function through its bioactive form, icarisid II. This study investigates the effects of icarisid II on diabetic rats with ED and its potential mechanism *via* the assessment of advanced glycosylation end products (AGEs), autophagy, mTOR and the NO–cGMP pathway. Icarisid II was extracted from icariin by an enzymatic method. In the control and diabetic ED groups, rats were administered normal saline; in the icarisid II group, rats were administered icarisid II intragastrically. Erectile function was evaluated by measuring intracavernosal pressure/mean arterial pressure (ICP/MAP). AGE concentrations, nitric oxide synthase (NOS) activity and cGMP concentration were assessed by enzyme immunoassay. Cell proliferation was analysed using methyl thiazolyl tetrazolium assay and flow cytometry. Autophagosomes were observed by transmission electron microscopy, monodansylcadaverine staining and GFP-LC3 localisation. The expression of NOS isoforms and key proteins in autophagy were examined by western blot. Our results have shown that Icarisid II increased ICP/MAP values, the smooth muscle cell (SMC) growth curve, S phase and SMC/collagen fibril (SMC/CF) proportions and decreased Beclin 1 ($P<0.05$). Icarisid II significantly increased the proliferative index and p-p70S6K(Thr389) levels and decreased the numbers of autophagosomes and the levels of LC3-II ($P<0.01$). Icarisid II decreased AGE concentrations and increased cGMP concentration, NOS activity ($P<0.05$) and cNOS levels ($P<0.01$) in the diabetic ED group. Therefore, Icarisid II constitutes a promising compound for diabetic ED and might be involved in the upregulation of SMC proliferation and the NO–cGMP pathway and the downregulation of AGEs, autophagy and the mTOR pathway.
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Keywords: advanced glycosylation end products (AGEs); autophagy; cell proliferation; diabetes mellitus (DM); erectile dysfunction (ED); icariin; icarisid II; mTOR; NO–cGMP; NOS activity

INTRODUCTION

Currently, some phosphodiesterase type 5 (PDE5) inhibitors have been widely used in the treatment of erectile dysfunction (ED).[1–3] However, these drugs have many side effects, such as headaches and visual impairment, which show that it is impending for further research with highly selective PDE5 inhibitor and for the development of natural drugs.[4] Investigations have suggested that the most metabolically active extract of Epimedium is icariin, which has been shown to exert inhibitory effects against PDE5.[5] In addition to its erotogenic role, icariin has demonstrated testosterone-mimetic properties.[6] Icariin has been shown to increase the intracavernous pressure in rats, which could be abolished nitric oxide synthase (NOS) and guanylate cyclase inhibitors.[7] Furthermore, icariin has successfully ameliorated both castration-related and arteriogenic impairment of erectile function and has reduced penile neuron NOS concentration in a rodent model.[8]

However, the effect of icariin in inhibiting PDE5 is much weaker.[9] Therefore, chemical modifications of the native structure of icariin have been extensively conducted to achieve high PDE5 inhibitory activity and multiple effects in the NO–cGMP pathway.[10] In 2008, a modification of the native icariin compound with two hydroxyethyl moieties enhanced the inhibition of PDE5 80-fold.[9] Icarisid II has also been successfully isolated and assessed its PDE5 inhibitory effect.[11,12] This study investigates the effect of icarisid II on diabetic rats with ED and its potential mechanism *via* assessment of advanced glycosylation end products (AGEs), autophagy, mTOR and the NO–cGMP pathway.

MATERIALS AND METHODS

Animals and treatment

All experimental protocols were approved by the Institutional Animal Care and Use Committee at Peking University (Beijing, China). Male

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Wistar rats (Grade A, certificate no. scxk11-00-0006) were obtained from the Animal Breeding Center at the Peking University Health Science Center. Thirty rats with normal function with intracavernosal pressure/mean arterial pressure (ICP/MAP) values greater than 0.6 were chosen and classified as the control group. Sixty diabetic rats with erectile dysfunction and ICP/MAP values less than 0.45 were chosen from streptozotocin-induced diabetic male rats and were randomly divided into the diabetic ED group (n=30) and the icarisid II group (n=30). The rats were administered normal saline in the control and diabetic ED groups. In the icarisid II group, the rats were administered 10 mg/kg icarisid II intragastrically every day for 8 weeks. Body weights and glucometer tail-vein blood glucose levels (Bayer HealthCare, Tarrytown, NY, USA) were measured biweekly. Corpus cavernosum tissue specimens and blood samples were obtained from every rat in each group and were stored at −20 °C after 8 weeks.

ICP and MAP measurements

To exclude the impact of individual differences, we evaluated erection function using ICP/MAP. Before and after the eight-week treatment, surgery was performed under 2% isoflurane anaesthesia at 37 °C. The right corpora cavernosa and carotid arteries were isolated and cannulated with 23-G butterfly needles primed with 250 U/ml heparin-saline solution and were connected to a pressure transducer (Utah Medical Products, Midvale, UT, USA) for ICP and MAP measurements. The right cavernous nerve near the major pelvic ganglion was identified for electric stimulation (20 Hz, 5 V and 60 s).

Masson trichrome staining of cavernous tissue

To exclude the impact of individual differences, we evaluated the cavernous tissue *via* smooth muscle cell/collagen fibril (SMC/CF) staining. The cavernous tissue specimens from each group were fixed in 2% paraformaldehyde and embedded in paraffin. Sections (3 μm in thickness) were then stained with a Masson trichrome staining kit (Sigma-Aldrich, St Louis, MO, USA). The corpus cavernosum SMCs were red, and CFs were blue.

Glucose, AGEs, NOS activity and cGMP assay

Blood specimens were checked for blood glucose levels using the Roche Diagnostics Cobas Integra 400 Plus assay system (Roche Diagnostics, Indianapolis, IN, USA). Corpus cavernosum tissue specimens from each group were performed for AGEs (Cell Biolabs, Inc. San Diego, CA, USA), NOS activity and cGMP (Cayman Chemicals, Ann Arbor, MI, USA) with the corresponding ELISA kits according to the manufacturer's instructions.

Cell harvests, cell growth curve assay and cell cycle analysis

The primary SMCs were isolated from every rat of each group and were cultured in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum, 100 U ml^{-1} penicillin and 100 mg ml^{-1} streptomycin (Invitrogen, Carlsbad, CA, USA). These primary rat SMCs were used in the corresponding experiments.

Cell proliferation capacity was investigated *via* a cell growth curve drawn using the MTT method, and the cell cycle was analysed using a flow cytometer (FACS; Becton Dickinson, Franklin Lakes, NJ, USA). The optical density (o.d.) values were measured at 570 nm and 630 nm with a microplate reader (Bio-Rad, Hercules, CA, USA). The growth curve was constructed according to o.d. values. Cell cycle S phase (DNA synthesis period) analysis and proliferative index (PI) were evaluated, with $PI=(S+G_2M)/(G_0G_1+S+G_2M)$. For each sample, 2×10^5 cells were measured.

Autophagosome observations using transmission electron microscopy (TEM), monodansylcadaverine (MDC) staining or GFP-LC3 localisation

The SMCs were post-fixed in osmium tetroxide (OsO_4) and embedded in Epon. Sections were then stained with uranyl acetate/lead citrate (Sigma-Aldrich) and viewed with a JEM1230 transmission electron microscope (JEOL, Tokyo, Japan). The SMCs on cover slips were stained with MDC (Sigma-Aldrich) and were observed with an SP5 confocal system (Leica, Solms, German) with excitation and emission filters with wavelengths of 380 nm and 525 nm, respectively.

The SMCs were transfected with GFP-LC3 plasmid using LipofectamineTM^{2000} reagent (Invitrogen) and were observed for LC3 distribution using the SP5 confocal system. Fifty non-overlapping SMCs in each specimen were randomly selected for autophagosome distribution analysis *via* TEM, MDC and GFP-LC3 assays.

Protein isolation and Western blot analysis

Cell lysates containing 100 μg protein were electrophoresed using sodium dodecyl sulphate–polyacrylamide gel electrophoresis and were then transferred to a polyvinylidene fluoride membrane (Millipore Corp., Bedford, MA, USA). Detection of target proteins on the membranes was performed with an electrochemiluminescence kit (Amersham Life Sciences Inc., Arlington Heights, IL, USA) using primary antibodies for nNOS, iNOS, eNOS, LC3-I/II, Beclin 1, p70S6K, p-p70S6K(Thr389) and β-actin (1 : 1000, all antibodies were from Santa Cruz Biotech, Santa Cruz, CA, USA). After the hybridisation of the secondary antibodies, the resulting images were analysed using a BioRad GS-670 densitometer (BioRad) and UTHSCSA Image Tool for Windows (3.0) (University of Texas Medical School at San Antonio, San Antonion, TX, USA) to determine the integrated density value of each protein band.

Statistical analysis

All experiments were repeated three times, and the similar results were obtained. Data were expressed as mean±s.d. and were analysed using one-way ANOVA in the SPSS13.0 software package (SPSS Inc., Chicago, IL, USA). $P<0.05$ was considered statistically significant.

RESULTS

Effects of icarisid II on ICP/MAP and SMCs/CF measurement

Compared to the diabetic ED group, the values for the ICP/MAP and SMC/CF measurements were higher in the icarisid II ($P<0.05$)and control groups ($P<0.01$). The values were lower in the icarisid II group than in the control group ($P<0.05$) (**Figures 1 and 2**).

Effects of icarisid II on cell multiplication using cell growth curve and cycle analysis

Compared to the diabetic ED group, the SMCs grew faster and the o.d. values were greater in the icarisid II and control groups, especially at days 5, 6 and 7 ($P<0.05$) (**Figure 3a**). Compared to the diabetic ED group, the percentages of cells in S phase (DNA synthesis period) were higher in the icarisid II ($P<0.05$) and control groups ($P<0.01$), and the PIs were significantly higher in the icarisid II and control groups ($P<0.01$). However, there was no statistical difference between the icarisid II group and the control group (**Figure 3**).

Effects of icarisid II on autophagosomes using TEM, MDC and GFP-LC3 localisation assays

Compared to the diabetic ED group, autophagosome quantities were significantly lower in the icarisid II and control groups ($P<0.01$).

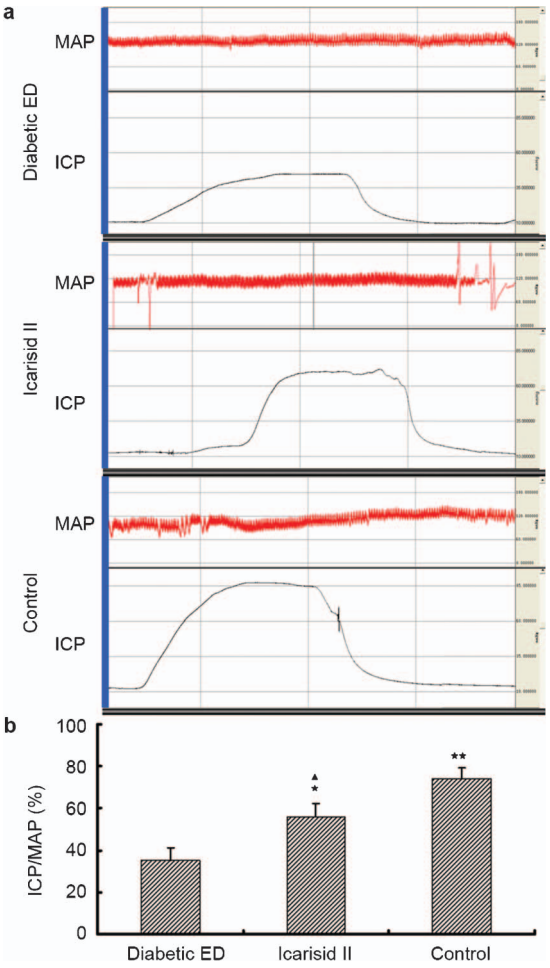

Figure 1 The ratio of intracavernosal pressure and mean arterial pressure (ICP/MAP) was investigated to evaluate erectile function in each group. (**a**) A Biopac physiograph displays the ICP (red curve) and MAP (black curve) values of representative rats in each group. (**b**) ICP/MAP levels were analysed with acqKnowledge software (BioPac Systems, Santa Barbara, CA,USA) and ANOVA. ★★$P<0.01$, ★$P<0.05$ *vs.* diabetic ED group. ▲$P<0.05$ *vs.* control group.

However, autophagosome quantities in the icarisid II group were still greater than those in the control group ($P<0.05$) (**Figure 4**).

Effects of icarisid II on the expression levels of key proteins in the mTOR pathway

Compared to the diabetic ED group, LC3-II and Beclin 1 expression levels were significantly lower in the icarisid II group ($P<0.01$ and $P<0.05$, respectively), and they were significantly lower in the control group ($P<0.01$). Compared to the diabetic ED group, p-p70S6K(Thr389) expression levels were significantly higher in the icarisid II and control groups ($P<0.01$). However, LC3-II and Beclin 1 expression levels were still higher in the icarisid II group than those in the control group ($P<0.05$ and $P<0.01$, respectively), and the p-p70S6K(Thr389) expression level was still lower in the icarisid II group than that in the control group ($P<0.01$). However, the p70S6K expression levels were not significantly different ($P>0.05$) (**Figure 5**).

Effects of icarisid II on glucose level, AGE concentrations, NOS activity and cGMP concentration

Blood glucose levels were lower in the control group than those in the diabetic ED and icarisid II groups ($P<0.01$). Concentrations of AGEs were higher in the diabetic ED group than those in the icarisid II and control groups ($P<0.05$ and $P<0.01$, respectively), and AGE concentrations were higher in the icarisid II group than those in the control group ($P<0.05$). NOS activity levels and cGMP concentrations were lower in the diabetic ED group than those in the icarisid II and control group ($P<0.05$ and $P<0.01$, respectively), but they were still lower in the icarisid II group compared to the control group ($P<0.05$) (**Table 1**).

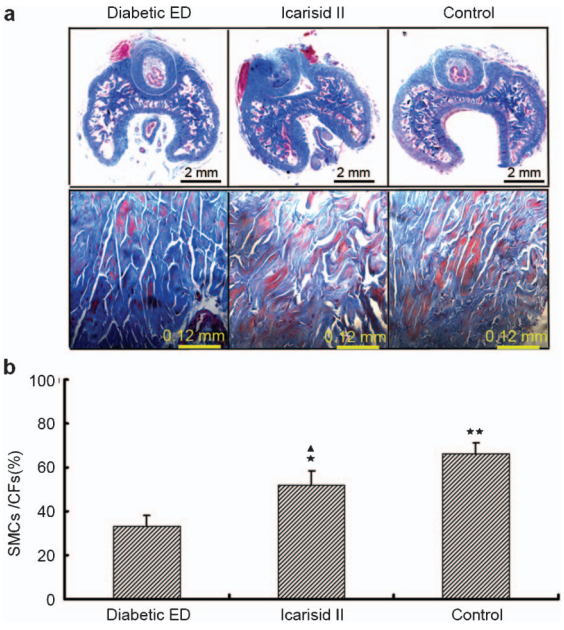

Figure 2 Morphological analysis of the ratio of smooth muscle cells/collagen fibrils (SMCs/CFs) in the corpus spongiosum from a representative of each group. (**a**) Masson's trichrome staining shows SMCs (red areas) and CFs (blue areas) of representative rats in each group. (**b**) The morphometric analysis of the area fraction (SMCs/CFs) was calculated using Leica QWin Pro V2.6 image analysis and processing software (Leica DMIRB, Leica, Wetzlar, Germany). Data are presented as the area fraction means from the 20 visual fields randomly selected from every specimen in each group. The differences were analysed by ANOVA. ★★$P<0.01$, ★$P<0.05$ *vs. diabetic ED group.* ▲$P<0.05$ vs. *control group.*

Effects of icarisid II on the expression of NOS isoforms

Compared to the diabetic ED group, nNOS and eNOS expression levels were higher in the icarisid II and control groups ($P<0.01$), and iNOS expression levels were lower in the icarisid II and control groups ($P<0.01$). However, the eNOS expression level was still lower in the icarisid II group compared with the control group ($P<0.01$) (**Figure 6**).

DISCUSSION

Icarisid II might enhance the proliferation of SMCs and attenuate excessive autophagy in diabetic ED rats by regulating the mTOR signalling pathway

It is well known that SMCs/CFs are significantly reduced in the erectile tissue of ED patients.[13–15] The renin-angiotensin system plays an important role in causing SMC fibrosis, and angiotensin II type I receptor blockers and angiotensin-sconverting enzyme inhibitors can ameliorate SMC fibrosis and extend the life span of SMCs in animal models.[16–19] Tankyrase 1 also has a similar effect.[14,15] This study determined that a decrease in erectile function coincided with the downregulation of SMC proliferation and SMC/CF proportions in diabetic ED rats and that icarisid II could ameliorate these effects.

The autophagy phenomenon was first recorded with the observation of autophagosomes by TEM in 1962, which is considered the

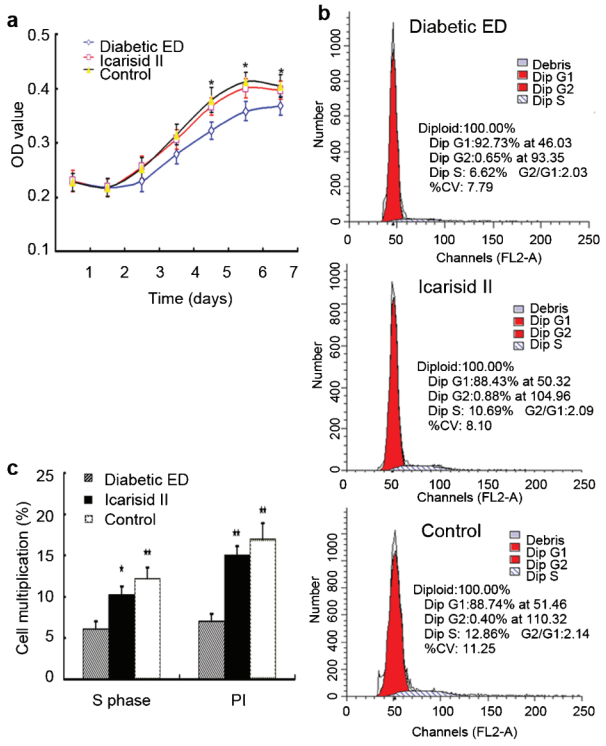

Figure 3 The cell growth curve and cell cycle analysis in the smooth muscle cells (SMCs) from each group. (**a**) The cell growth curve was assayed from the optical density (o.d.) values using the MTT method. (**b**) The cell cycle was analysed by flow cytometry. (**c**) The differences were then analysed by ANOVA. $^{\star\star}P<0.01$ and $^{\star}P<0.05$ *vs.* the diabetic ED group. PI, proliferative index.

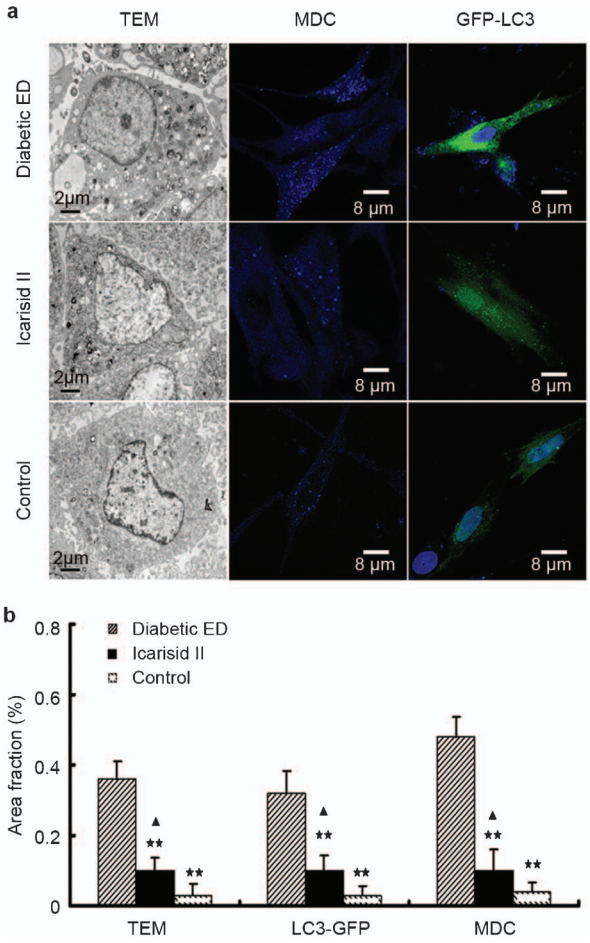

Figure 4 Morphological analysis of autophagy in the smooth muscle cells (SMCs) from each group. (**a**) Punctate autophagosomes were observed using transmission electron microscopy monodansylcadaverine staining and GFP-LC3 transfection localisation methods. (**b**) The area fractions (autophagosomes/cytoplasm) were calculated using Leica QWin Pro V2.6 image analysis and processing software. Data were presented as the means of the area fractions (autophagosomes/cytoplasm) in the 50 cells randomly selected from every specimen in each group. The differences were then analysed by ANOVA. $^{\star\star}P<0.01$ *vs.* the diabetic erectile dysfunction (ED) group. $^{\blacktriangle}P<0.05$ *vs.* the control group.

diagnostic gold standard of autophagy detection.[20] MDC staining, GFP-LC3 fluorescent localisation and key protein expression levels by Western blot are normally used for autophagy study.[21] LC3 is located on the membrane surfaces of preautophagic vacuoles and autophagic vacuoles. LC3-II reflects autophagy activity to some extent and is a common membrane marker for autophagic vacuoles.[22] This study found that excessive autophagy occurred in diabetic ED rats and was attenuated by icarisid II, which was confirmed by LC3-II expression by western blot and with autophagosome characterisation *via* TEM, MDC staining and GFP-LC3 localisation.

The signal transduction molecules in autophagy are complex and the mTOR pathway is widely studied in autophagy.[23] TOR kinase is a negative control element in autophagy and might play an important role in the regulation of cell growth.[24,25] Beclin 1 is a regulatory protein in autophagy.[26] Beclin 1 is involved in autophagosome formation and forms a complex with class III PI3K.[27,28] Beclin 1 participates in the formation of autophagosomes and plays an important role in cell growth by regulating autophagy activity;[29] studies have shown that autophagy activity is lower in Beclin 1 knockout mice[30] and that Beclin 1 is critical for autophagy activity.[31] P70S6K regulates 5′-TOP mRNA translation and biosynthesis, which plays a critical role in the growth of the cytoskeleton through the phosphorylation of the S6 protein.[32,33] This study determined that Beclin 1 levels were significantly increased in diabetic ED rats and that icarisid II decreased these levels. This study also found that the proliferation of SMCs and p-p70S6K(Thr389) levels were significantly decreased in diabetic ED rats and that icarisid II treatment resulted in increases in both.

This study found that the decreases in erectile function, SMC proliferation and SMC/CF proportions coincided with autophagy upregulation and mTOR pathway downregulation in diabetic ED rats.

Icarisid II might ameliorate erectile function in diabetic ED rats *via* a downregulation in AGE concentrations and an upregulation in the NO–cGMP pathway

Diabetes mellitus, the third serious chronic disease in humans, may induce diabetic ED.[34] Corpus cavernosum SMC fibrosis and cavernous nerve damage occurs in nearly 70% of diabetic patients.[35,36] In such patients, treatment with existing drugs is almost always ineffectual because drugs such as PDE5 inhibitors require the presence of intact and undamaged penile nerves and SMCs. Therefore, there is a great need for a new compound that has the capacity to help regenerate damaged nerves and SMCs.

We have reported that icariin preserved erectile function in castrated rats with nNOS preservation.[37] Further, icariin stimulated myocardial cell differentiation by the generation of reactive oxygen species, which increased p38MAPK levels.[38] Chung *et al.*[39] found that

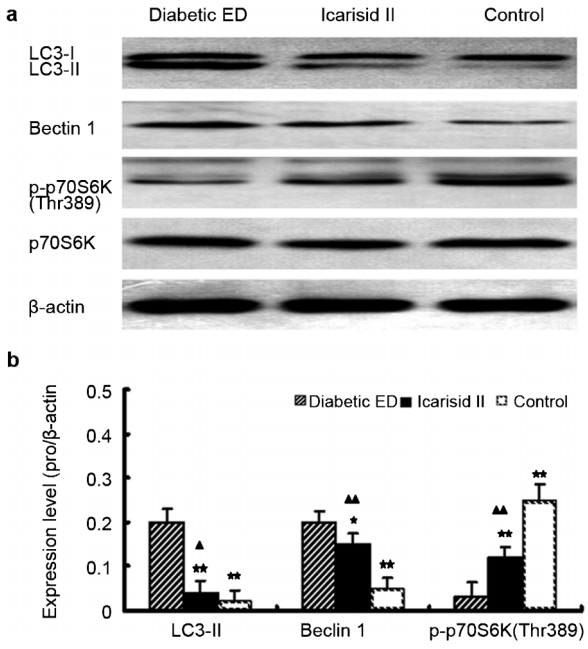

Figure 5 Expression of mTOR signalling pathway proteins in the corpus cavernosum tissue from each group. (**a**) Western blot of LC3-I/II, Beclin 1, p70S6K, p-p70S6K(Thr389) and β-actin. (**b**) The relative expression levels were calculated by normalising to β-actin. The differences were then analysed by ANOVA. ★★$P<0.01$ and ★$P<0.05$ *vs.* the diabetic erectile dysfunction (ED) group. ▲▲$P<0.01$ and ▲$P<0.05$ *vs.* the control group.

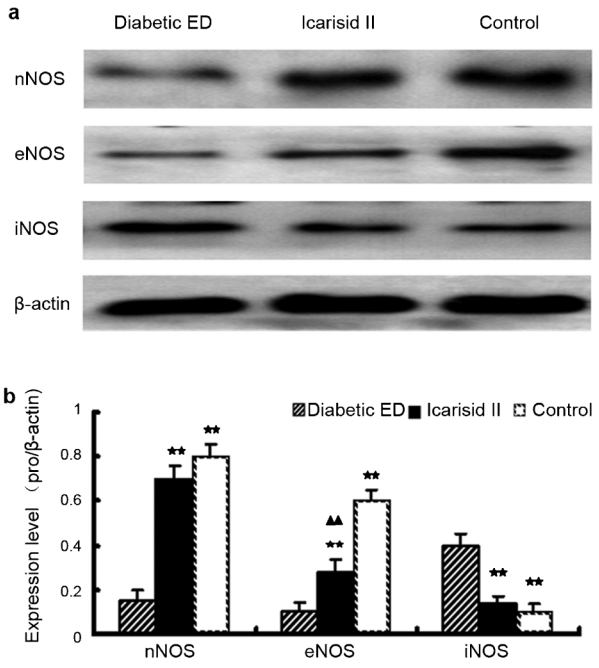

Figure 6 Expression of nitric oxide synthase (NOS) isoforms in the corpus cavernosum tissue from each group. (**a**) Western blot of nNOS, eNOS and iNOS expression. (**b**) The relative expression levels were calculated by normalising to β-actin. The differences were then analysed by ANOVA. ★★$P<0.01$ *vs.* the diabetic erectile dysfunction (ED) group. ▲▲$P<0.01$ *vs.* the control group.

icariin increased MEK/ERK and PI3K/Akt/eNOS path-dependent protein levels in human umbilical vein endothelial cells. Icariin has also been shown to enhance eNOS expression and NO production in human endothelial cells and to decrease caspase-3 expression and cellular apoptosis in response to hydrogen peroxide.[40] Icarisid II has been reported to be the main bioactive form of icariin *in vivo*.[41] We innovatively isolated and purified icarisid II and found that icarisid II increases intracellular cGMP levels through the enhancement of nNOS expression and NOS activity in rat corpus cavernosum tissue *in vitro*.[42]

AGEs form as the result of the nonenzymatic glycosylation of proteins through a process by which a reducing sugar attaches to an amino group of an amino acid residue and then undergoes rearrangement to form a ketoamine-linked sugar.[43,44] AGEs accumulate in diabetic tissue, and AGE concentrations are increased in the penile cavernosal smooth muscle tissues and the vascular beds of diabetic ED patients.[45] AGEs interfere with ion channels, gap junctions and receptors so that calcium ion release and blood flow both decrease; as a result, the SMC relaxation mechanism is impaired.[46] Seftel *et al.*[47] reported that AGEs quenched the production of epoxide and NO in endotheliocytes and speculated on a pathophysiologic mechanism for AGE-mediated ED *via* the upregulation of iNOS and the downregulation of eNOS. However, Chen *et al.*[48] reported that AGEs could attenuate the activity of cNOS (nNOS and eNOS) and could increase iNOS activity in rat cavernosum tissue, resulting in the impairment of penile erectile function. Ishibashi reported that vardenafil could block the AGE-induced upregulation of MCP-1 mRNA levels in HUVECs by suppressing AGE receptor expression levels and subsequent ROS generation *via* the elevation of cGMP levels.[49]

This study found that a decrease in erectile function coincided with an upregulation in AGE concentrations and a downregulation in the NO–cGMP pathway in diabetic ED rats and that icarisid II could ameliorate these effects. We also observed that icarisid II increased erectile function, SMC proliferation and SMC/CF proportions and decreased AGE concentrations in diabetic ED rats. These findings might be related to the upregulation of the NO–cGMP pathway and the downregulation of autophagy and the mTOR pathway. Further study is needed.

AUTHOR CONTRIBUTIONS

JZ carried out the design of the study, drafted the manuscript and participated in every parts of the experiment. AML participated in the design of the study, performed the statistical analysis, Protein isolation and Western blot analysis. BXL participated in the design of the study, Intracavernosal pressure (ICP) and mean arterial pres-

Table 1 Comparisons of glucose levels, AGE concentrations, NOS activity levels and cGMP concentrations among groups ($\bar{x}\pm$s.d.)

Group	*Icarisid*	*Control*	*Diabetic ED*
Blood sugar (mmol l^{-1})	23.71±3.92	6.87±0.83★★	29.06±4.75
AGEs (pg ml^{-1})	54.28±2.76★,▲	48.83±2.19★★	62.35±4.63
NOS activity (U mg^{-1})	0.36±0.04★,▲	0.49±0.06★★	0.21±0.04
cGMP content (pmol mg^{-1} min^{-1})	0.42±0.04★,▲	0.53±0.06★★	0.27±0.04

[a] $P<0.01$ and [b]$P<0.05$ *vs.* the diabetic ED group.
[c] $P<0.05$ *vs.* the control group.

sure (MAP) measurement. FH participated in cell harvest, cell growth curve and cell cycle analysis. FL participated in NOS activity. SZX participated in Masson trichrome staining of cavernous tissue. GQK participated in Masson trichrome staining of cavernous tissue. SJC participated in animals and treatment. CGM participated in cGMP assay. XL participated in Glucose and AGEs. SPS participated in Statistical Analysis. ZLJ participated in the design of the study, Autophagosome observation with transmission electron microscopy (TEM), Monodansylcadaverine (MDC) staining or GFP-LC3 localization. ZCX participated in the design of the study, conceived of the study, participated in its design and coordination, helped to draft the manuscript and have given final approval of the version to be published. All authors read and approved the final manuscript.

COMPETING FINANCIAL INTERESTS

The authors have no conflicting interests to disclose.

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