Original Article
Total alkaloids of *tripterygium hypoglaucum* (levl.) Hutch (THHta) attenuates high glucose-induced podocyte injury via mediating miR-29a/PTEN axis

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Abstract: Diabetic nephropathy (DN) is one of the chronic complications of diabetes and is also an important cause of end-stage renal disease (ESRD). Podocyte injury plays a key role in increased microalbuminuria in DN. Recent studies reported that the total alkaloids of *tripterygium hypoglaucum* (levl.) Hutch (THHta) plays an anti-apoptotic role in a number of cultured cells. The purpose of this study was to investigate the protective effects and its underlying mechanisms of THHta on podocyte injury induced by high glucose (HG).

The protective effects of THHta in HG-treated conditionally immortalized mouse podocytes were evaluated using MTT assay, 2',7'-DCF diacetate, flow cytometry and Western blot. To investigate whether THHta regulates the expression of miRNAs involved in podocyte injury, miRNA microarray was applied and the result was verified by quantitative reverse transcription PCR (qRT-PCR). Then, the involvement of relevant pathway in THHta-mediated protective effect in podocytes injury was explored. Our study showed that THHta displayed a dose-dependently protective effect against podocytes injury, as indicated by significantly reduced apoptosis, generation of ROS and enhanced cell viability. THHta also exerted anti-inflammatory effects as indicated by the decreased TNF-α and IL-1β levels in the podocytes. Moreover, we found that THHta upregulated the expression level of miR-29a in podocytes injury model. Gain- and loss-of-function studies showed that the protective effects of THHta were abolished by inhibition of miR-29, whereas enhanced by overexpression of miR-29a. More importantly, our data showed that THHta upregulated the expression level of miR-29a in podocytes injury model. Gain- and loss-of-function studies showed that the protective effects of THHta were abolished by inhibition of miR-29, whereas enhanced by overexpression of miR-29a. In HG-treated podocytes. Taken together, this study highlights that the protective effects of THHta depend on miR-29a/PTEN axis may be an important therapeutic target for podocytes injury.

Keywords: Diabetic nephropathy, podocyte injury, THHta, microRNA-29a, PTEN

Introduction
Diabetic nephropathy (DN), as one of the most chronic complication in patients with diabetes mellitus (DM), is the major cause of end-stage renal disease (ESRD) [1]. Podocytes are terminally differentiated visceral epithelial cells that are involved in the maintenance of the structure and function of the glomerular filtration barrier [2, 3], and numerous evidence has found that podocyte injury plays a pivotal role in the pathogenesis of DN and concluded that such injury can lead to proteinuria and glomerulosclerosis [4-6]. Therefore, attenuating podocyte injury has become a principal therapeutic target for DN.

Increasing evidence demonstrated that podocyte injury was associated with apoptosis, the excessive generation of reactive oxygen species (ROS) and inflammatory response [7-9]. For example, Zhang et al. found that hepatocyte growth factor (HGF) protected podocytes from HG-mediated injury through suppressing oxidative stress and apoptosis [10]. Therefore, various strategies targeting podocyte injury such as antioxidant, anti-apoptosis and anti-inflammatory have been proposed for DN treatment. A large body of studies supports that natural agents open up a novel avenue for treatment of DN [11, 12]. Huang et al. showed that notoginsenoside R1 (NR1) exerted protective effects in the podocytes of rats with DN by regulating the
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secretion of inflammatory cytokines as well as an anti-inflammatory cytokine [11]. Recently, total alkaloids of *Tripterygium Hypoglaucum* (levl.) Hutch (THHta) have been used as a traditional medicine with the pharmacological activities of anti-inflammation [13] and employed for the treatment of auto-immune diseases, such as rheumatoid arthritis, systemic lupus erythematosi and skin problems [14]. However, limited studies have pay attention on the therapeutic effects and underlying mechanisms of THHta on DN.

MicroRNA (miRNA)s are endogenous, single stranded, small noncoding RNA molecules with approximately 18 to 24 nucleotides, extensively exists for regulation of gene expression [15]. Recent studies have reported that miRNAs play an important role in podocytes. For example, Yang et al. found that miR-218 was upregulated in high glucose-induced podocytes, which accelerated cell apoptosis by suppressing its direct target gene, HO-1 [16]. Moreover, a recent study from Wang et al. showed that fructose induces miR-377 overexpression, which in turn decreases SOD1 and SOD2 levels and activates the \( \cdot O_2^-/p38 \) MAPK/TXNIP/NLRP3 inflamasome pathway to promote oxidative stress and inflammation, suggesting inhibition of miR-377 may be a new therapeutic strategy for glomerular podocyte injury in fructose-associated metabolic syndrome [17]. These studies suggest that miRNAs may be involved in the development and occurrence of DN by modulating podocytes functions.

In this study, we established a model of HG-induced podocytes *in vitro*, with an aim to explore the protective effects of THHta on HG-induced podocytes and find the possible mechanisms.

**Materials and methods**

*Conditionally immortalized mouse podocytes in culture*

Conditionally immortalized mouse podocytes purchased from the Cell Culture Center (PUMC, CAMS, Beijing, China) were cultured as previously described [18]. Podocytes were exposed to normal glucose (5.5 mM) and high glucose (10, 20, 30 and 40 mM), respectively. The normal glucose (5.5 mM) treatment was used as control.

**MTT assay**

Cells were seeded at a density of \( 1 \times 10^4 \) cells/well in 96-well culture plates. After treating with various concentrations of THHta (1.25, 2.5 and 5.0 \( \mu \)g/ml) for 24 h, MTT assay (Amresco, Solon, USA) was performed. Briefly, 20 \( \mu \)l of MTT solution (5 mg/ml) was added to each well for 4 h. After removal of MTT solution, dimethyl sulfoxide (DMSO) was added, and the absorbance at 490 nm was measured with Microplate Reader (Bio-Tek Instruments, Inc.). All experiments were done in triplicate.

**Cell apoptosis detection by flow cytometry**

After the indicated treatments, podocytes were resuspended in 500 \( \mu \)l binding buffer and then mixed with Annexin V-FITC (1 \( \mu \)g/ml; Invitrogen, Carlsbad, CA) in the dark for 15 min at 4°C. Five minutes after propidium iodide (PI) solution was added, the percentages of dead cells and cells undergoing apoptosis were evaluated by flow cytometry (Becton-Dickinson, San Jose, CA, USA).

**Detecting the ROS generation**

The generation of mouse podocytes ROS was assessed using 2',7'-DCF diacetate (DCF-DA; Sigma-Aldrich) as previously reported [19]. Briefly, podocytes were resuspended in PBS and the density was adjusted to \( 5 \times 10^5 \) cells/ml and the cells were incubated with 20 \( \mu \)mol/L DCFH-DA for 30 min at room temperature. Then, cells were observed under fluorescent microscope. The fluorescence intensity was recorded and used for statistical analysis.

**Enzyme-linked immunosorbent assay (ELISA)**

The concentrations of tumor necrosis factor (TNF)-\( \alpha \) and interleukin (IL)-1\( \beta \) in the culture media were determined using commercially available murine-specific sandwich ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA) according to manufacturer’s instruction.

**miRNA array**

Total RNA was isolated with the TRIzol reagent (Invitrogen) according to manufacturer’s instructions. The miRNA fraction was further purified using the mirVana™ miRNA Isolation Kit (Ambion). MicroRNA expression analysis was performed by the mirCURRY™ LNA miRNA Array
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Microarray images were acquired using the GenePix 4000B scanner (Axon Instruments, Foster City, CA, USA) and analyzed with GenePix Pro 6.0 software (Axon Instruments), which performed median normalization. After normalization, differentially expressed miRNAs were identified through Fold Change filtering. Hierarchical clustering was performed using cluster3.0 and treeview1.14.

Quantitative real-time PCR analysis

For microRNA expression analysis, 1 μg of total RNA were extracted from podocytes by TRIZol Reagent (Invitrogen, USA) following the manufacturer’s protocol. MiR-29a was reverse transcribed using the PrimeScript RT reagent Kit (TaKaRa, Tokyo, Japan) and quantified by real-time PCR with the TaqMan MicroRNA assay kit (Applied Biosystems). qRT-PCR analyses for PTEN and the normalization control gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were performed using SYBR Premix Ex Taq (TaKaRa) on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems). The relative expression of each gene was calculated and normalized using the 2^ΔΔCt method relative to RNU6B or GAPDH. All reactions were conducted in triplicate. The following primers were used: miR-29a forward: 5'-ACCTGTC- ACTGTCCTTGTACCCCTTG-3'; reverse: 5'-CGGGCG- TTTGAGTGCTGAGA-3'; U6 SnRNA, forward: 5'-CTGGGCAGCACGCA-3', Reverse: 5'-AACGCTACAGATTTCGC-3'; PTEN mRNA forward: 5'-AAGCTGGAAAAGGACGA-3', reverse: 5'-AACACATGCGCCTCTGACTG-3' and GAPDH mRNA forward: 5'-CCCTCTCTTATCAGCCCTC- TG-3', reverse: 5'-AGAAGGCTGGGGGCTCATTTG-3'.

Cell transfection

Podocytes were seeded at a density of 10 × 10^4/well in six-well plates and transfected with miR-29a mimics, miR-29a inhibitor or the respective negative controls obtained from RiboBio (Guangzhou, China) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The PTEN sequence was subcloned into the pcDNA3.1 vector (Invitrogen, USA). PTEN ectopic expression was achieved through pcDNA-PTEN transfection using Lipofectamine 2000 (Invitrogen, USA). Cell viability, apoptosis and ROS levels were analyzed 48 h after transfection.

Western blot

For western blot analysis, cells were lysed with RIPA lysis buffer (Beyotime, Jiangsu, China). 50 μg proteins was separated by SDS-PAGE and transferred onto nitrocellulose membranes. After blocking with 5% non-fat dry milk, the membranes were blotted with antibodies directed against cleaved-Caspase-3, cleaved-PARP, Bcl-2 and PTEN (1:1000, Santa Cruz Biotechnology, USA) and then with horseradish peroxidase-conjugated secondary antibodies. Anti-β-actin antibody was used as an internal control. Signals were visualized by ECL chemiluminescence. Equal protein loading was assessed by the expression of β-actin. Semi-quantitative evaluation of the bands was performed by densitometric analysis with the ImageJ software.

Luciferase reporter assays

A whole fragment of 3'UTR PTEN mRNA and a mutant form were cloned into pGL-3-Luc. The HEK 293T cells were seeded in 12-well plates and co-transfected with pGL-3-PTEN wild-type or mutant portion and TK100 Renilla combined with miR-29a mimics, miR-29a inhibitor or NC control using Lipofectamine 2000 (Invitrogen). After 48 h of incubation, cells were collected for application in the Dual-Luciferase Reporter System (Promega, Madison, WI) following the manufacturer’s recommendations. All of the dual-luciferase reporter assays were done in triplicate within each experiment, and three independent experiments were conducted.

Statistical analysis

Data are reported as mean ± standard deviation. Statistical significance among different groups was determined by Two-way ANOVA test of variance using the GraphPad Prism software (version 5.0). A p value of less than 0.05 was considered to be significant.

Results

Assessment of the HG induced podocyte injury model

Culturing podocytes in high glucose has been widely used as a model to simulate the podocyte injury of DN in vitro [6, 20]. To investigate the effect of glucose on the growth of podocytes, podocytes were treated with different doses of glucose from 10 to 40 mM for 72 h,
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and then the cells were analyzed by MTT Assay. As shown in Figure 1A, glucose inhibited the proliferation of podocytes in a dose-dependent manner. Next, levels of ROS and apoptosis were measured in podocytes under different doses of glucose. As shown in Figure 1B and 1C, glucose significantly increased levels of ROS and apoptosis in a dose-dependent manner. In the present study, the promoting effect of glucose on podocytes apoptosis was further confirmed by Western Blot. Glucose at the concentration of 10 and 30 mM increased the expression of the pro-apoptotic protein cleaved Caspase-3 and cleaved PARP, and decreased the expression of the anti-apoptotic gene Bcl-2 (Figure 1D). Likewise, the expressions of total caspase-3 and total-PARP did not change significantly in glucose-treated podocytes. Based on these results, treatment with 30 mM glucose was utilized in subsequent experiments.

THHta protected podocytes from HG-induced injury

To evaluate the effect of THHta on HG-induced podocyte injury, podocytes were treated with 30 mM glucose, and then incubated with 1.25, 2.5, or 5 μg/mL THHta for 48 h, after which cell viability, levels of ROS and apoptosis rate were measured. Compared with untreated controls, HG significantly suppressed cell viability, promoted levels of ROS and cell apoptosis, whereas THHta significantly increased cell viability and reduced levels of ROS and apoptosis of HG-treated podocytes in a dose dependent manner (Figure 2A-C). We also detected the expression of cleaved caspase-3 protein and found that THHta significantly decreased the expression of cleaved caspase-3 induced by HG (Figure 2D) and this effect was dose-dependent. Next, the levels of TNF-α and IL-1β were measured by ELISA in the media of podocytes. As shown in Figure 2E, 2F, compared with untreated controls, HG significantly increased the levels of TNF-α and IL-1β, whereas THHta significantly decreased the levels of TNF-α and IL-1β in a dose dependent manner. These data suggested that HG induced podocyte injury, and that THHta protected podocytes from these effects.

THHta promotes expression of miR-29a in HG treated podocytes

To determine the potential involvement of miRNAs in podocye injury, we used microarray
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A

B

C

D

E

F

Figure 2. THHta attenuates podocyte injury induced by HG. Podocytes were treated with 30 mM glucose, and then incubated with 1.25, 2.5, or 5 μg/mL THHta for 48 h. A. Cell viability was analyzed by MTT assay after THHta treatment. B. Levels of ROS were examined using 2',7'-DCF diacetate assay. C. Cell apoptosis was measured by flow cytometry. D. The expression of cleaved caspase-3 was determined by Western Blot. E and F. Levels of TNF-α and IL-1β were measured by ELISA in the media of podocytes. The data are expressed as the mean ± SD of three independent experiments. *P<0.05, **P<0.01 vs. control, ###P<0.01 vs. Glu alone group.

analysis to determine miRNA levels in HG treated podocytes after THHta treatment. A miRNA array revealed that a panel of 40 miRNAs was significantly changed, in which 19 miRNAs were upregulated, and 21 miRNAs were downregulated (Figure 3A). Among the aberrantly expressed miRNAs, miR-29a was one of the differentially expressed miRNAs after THHta treatment in HG treated podocytes. Moreover, miR-29a was found to be downregulated in various diabetic animal models and could ameliorate hyperglycemia-induced podocyte dysfunction.
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Therefore, miR-29a was chosen as the candidate for further study.

To further validate the expression of miR-29a in the HG-treated podocytes, miR-29a levels from HG-induced podocytes after treatment with different concentrations of THHta were determined by quantitative real-time PCR (qRT-PCR). The results showed that miR-29a expression was significantly upregulated in HG-treated podocytes after treatment with THHta in a dose-dependent manner (Figure 3B). These results indicated that THHta can increase the expression of miR-29a in HG-treated podocytes.

**Involvement of miR-29a in the protective effects of THHta in HG-induced podocytes**

Given the up-regulation of miR-29a mediated by THHta in HG-treated podocytes, we predicted that miR-29a may be involved in the protective effects of THHta on HG-induced podocytes. To verify our hypothesis, miR-29a inhibitor and miR-29a mimics were transfected into podocytes, and the expression level of miR-29a was significantly decreased or increased after transfection (Figure 4A, 4B). Then, we examined the effects of miR-29a on the cell viability, levels of ROS and apoptosis in podocytes following THHta and HG treatment. As shown in Figure 4C-E, inhibition of miR-29a significantly abrogated the protective effects of THHta on HG-treated podocytes, accompany with the reduction of cell viability, the enhancement of generation of ROS and apoptosis, whereas overexpression of miR-29a enhanced THHta-induced cell viability and suppressed the generation of ROS and apoptosis in HG-treated podocytes (Figure 4F-H). These results indicated that THHta protected podocytes from HG-induced cellular injury through upregulating the expression of miR-29a.

**PTEN is a direct target of miR-29a**

To explore the molecular mechanism by which miR-29a functions in the protective effects of THHta in HG-induced podocytes, the PicTar and TargetScan in silico miRNA target databases
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were used. We noticed that phosphatase and tensin homolog deleted on chromosome ten (PTEN) was commonly predicted by both algorithms. In addition, PTEN was found to be downregulated in podocytes and closely related to glomerular function and the progression of DN [23]. We found that PTEN harbored a common putative binding site for miR-29a in its 3'-UTR (Figure 5A). To confirm whether PTEN is a target of miR-29a, the THHta 3'-UTR containing miR-29a binding sites and mutant constructs were cloned into the pGL-3-Luc vector. Dual luciferase activity assay demonstrated that miR-29a mimics reduced PTEN 3'-UTR luciferase activity, whereas miR-29a inhibitor increased luciferase activity (Figure 5B). However, no obvious change in luciferase activity was significantly observed when PTEN 3'-UTR was replaced with a mutated PTEN 3'-UTR in which the miR-29a binding sites were disrupted (Figure 5B). In addition, qRT-PCR and Western Blot analysis showed that miR-29a overexpression decreased the levels of PTEN mRNA and protein expression, whereas inhibition of miR-29a increased the expressions (Figure 5C, 5D). Taken together, these results suggest that miR-29a/PTEN axis may play an important role in the protection of THHta in HG-induced podocyte injury.

Overexpression of PTEN reverses miR-29a mimics synergically mediated protective effects of THHta in HG-treated podocytes

The above mentioned results suggested that miR-29a mimics enhanced the protective ef-
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Figure 5. PTEN is a direct target of miR-29a. A. Schema of the firefly luciferase reporter constructs for the PTEN, indicating the interaction sites between miR-29a and the 3'UTRs of the PTEN. B. Luciferase assay of HEK293 cells co-transfected with firefly luciferase constructs containing the PTEN wild-type or mutated 3'UTRs and miR-29a mimics, mimics NC, miR-29a inhibitor or inhibitor NC, as indicated (n = 3). C and D. The expressions of PTEN mRNA and protein after treatment with miR-29a mimic or miR-29a inhibitor were measured by qRT-PCR and Western Blot (n = 3). Data represent the means ± SD, **P<0.01 vs. mimics NC, ###P<0.01 vs. inhibitor NC.
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**Discussion**

In this present study, we found that THHta could protect podocytes from HG-induced cellular injury by inhibiting levels of ROS, apoptosis and inflammation response. Moreover, we demonstrated that THHta attenuated HG-induced podocyte injury via mediating miR-29a by targeting PTEN. These results suggest that targeting the miR-29a/PTEN pathway by THHta could serve as a potential therapeutic strategy for DN.

Recent studies have demonstrated that podocyte injury represents one of the early events in diabetic nephropathy and podocyte dropout has been believed to contribute to the pathogenesis and development of DN [24-26]. Various risk factors such as hyperglycemia, oxidative stress and inflammation can induce podocyte apoptosis, leading to podocyte injury [27, 28]. Therefore, various strategies targeting podocyte injury such as anti-oxidant, anti-inflammation and anti-apoptosis have been proposed for treating patients with DN. Here, we investigated the influence of THHta, a natural compound with beneficial effects in various human autoimmune diseases and cancers [29, 30]. Our study reveals that THHta protects podocytes from HG-mediated injury through suppressing oxidative stress and apoptosis. Furthermore, our finding that THHta reduces the levels of TNF-α and IL-1β in HG-treated podocytes provides a novel insight into the mechanisms underlying the protective effects of THHta.

Dysregulations of miRNA have been implicated in the development and progression of DN [31-33]. For example, Putta et al. found that miR-192 was upregulated in cultured glomerular mesangial cells and in glomeruli from diabetic mice and inhibition of miR-192 can ameliorate renal fibrosis in diabetic nephropathy [34]. Another study conducted by Long et al. showed that miR-29c was identified as a signature miRNA in the diabetic environment and up-regulation of miR-29c could promote the progression of DN through a Spry1/Rho kinase pathway [35]. It has reported that some Chinese herbal medicines (CHM) have therapeutic effects on diabetes with fewer side effects [36-38]. And the underlying mechanism may be associated with the regulation of miRNAs [17]. However, it is unknown whether miRNAs mediated the protective effect of THHta on HG-induced podocyte injury. In the present study, using a miRNA array, we found that THHta treatment upregulated the expression of miR-29a in HG-treated podocytes, indicating that miR-29a may play a crucial role in the protective effects of THHta on HG-induced podocyte injury.

There is increasing evidence emerging to suggest that miR-29 family members (miRNA-29a/b/c) play important roles in the progression of DN. For example, all three members of the miR-29 family were suppressed by TGF-β1 in proximal tubular cells (NRK-52E), primary mouse mesangial cells, and human podocytes [39]. Du et al. found that high glucose/TGF-β1 induced significant down-regulation of miR-29a in HK-2 cells (human proximal tubule cell line), which mediated the pathogenesis of DN through directly targeting the 3'UTRs of collagen IV transcripts [21]. Recently, Lin et al. demonstrated that hyperglycemia-induced podo-
Podocyte dysfunction was ameliorated by miR-29a promotion of nephrin acetylation [22]. Our data showed that overexpression of miR-29a significantly enhanced the protective effect of THHta in HG-treated podocytes, whereas inhibition of miR-29a had an opposite result. Furthermore, we found that miR-29a contains several target binding sites for PTEN, a negative regulator of PI3K/AKT signaling pathway. A luciferase activity assay confirmed the direct binding relationship between miR-29a and PTEN. This information prompted us to investigate whether the protective effects of THHta were exerted through miR-29a-mediated PTEN expression. Our results showed that miR-29a mediated the expressions of PTEN were involved in the protective role of THHta in HG-induced podocytes. Overall, our findings have not only revealed the important role of THHta in the development of DN, but have also implicated the key role of miR-29a/PTEN axis in the treatment of THHta for DN.

In conclusion, our study has revealed that THHta can attenuate podocyte injury by suppressing oxidative stress, inflammation and apoptosis via miR-29a/PTEN pathway. On this basis, it is proposed that targeting the miR-29a/PTEN pathway by THHta might be a novel therapeutic strategy for DN.

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Disclosure of conflict of interest

None.

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