Tohoku J. Exp. Med., 2023, **260**, 35-45

Polysaccharide H-1-2 Ameliorates High Glucose-Induced Podocyte Dysfunction by Suppressing Epithelial-to-Mesenchymal Transition via Restoration of SIRT1 *in Vivo* and *in Vitro*

Minzhou Li,[1] Zhong Wu,[1] Wei Gao,[1] Kaiying Zhao,[1] Xiaobo Yang,[1] Huiyan Zhang,[2] Bin Deng[2] and Yang Niu[2]

[1]Department of Traditional Chinese Medicine, the People's Hospital of Inner Mongolia Autonomous Region, Hohhot, Inner Mongolia, China
[2]College of Traditional Chinese Medicine, Inner Mongolia Medical University, Hohhot, Inner Mongolia, China

Renal interstitial fibrosis, a pathological feature of diabetic nephropathy, is closely related to endothelial-to-mesenchymal transition (EMT). This study aimed to explore the effect of H-1-2, a polysaccharide of *Pseudostellaria heterophylla*, on high glucose (HG) induced-podocyte EMT *in vivo* and *ex vivo*. DBA/2 mice were given five consecutive days of streptozotocin injection to induce the diabetic nephropathy model. H-1-2 treatment effectively attenuated general states (bodyweight and blood glucose level) and reduced oral glucose tolerance, insulin tolerance, kidney index, as well as the level of serum urine nitrogen, serum creatinine, and urinary albumin excretion rate in diabetic nephropathy mice. The injury and EMT of podocytes in diabetic nephropathy mice were restrained by H-1-2. After exposing podocytes to HG, the impaired cell viability, apoptosis, the downregulation of nephrin, synaptopodin, sirtuin 1 (SIRT1) and P-cadherin, and the upregulation of N-cadherin were observed in podocytes. H-1-2 treatment could reverse these effects induced by HG. To uncover the mechanism underlying H-1-2 suppressing EMT, small interference RNA for SIRT1 was transfected into podocytes. Mechanically, silencing SIRT1 largely restrained the protective effect of H-1-2 on HG-induced podocytes. In conclusion, H-1-2 exerts a potential role in alleviating HG-induced dysfunction and EMT of podocytes *in vivo* and *ex vivo* via SIRT1.

Keywords: diabetic nephropathy; endothelial-to-mesenchymal transition; fibrosis; H-1-2; SIRT1
Tohoku J. Exp. Med., 2023 May, **260** (1), 35-45.
doi: 10.1620/tjem.2023.J015

Introduction

Diabetic nephropathy, one of the fatal complications of diabetes mellitus (DM), develops in 30~40% of DM patients and is the leading cause of end-stage renal disease worldwide (Gross et al. 2005). Clinically, the stages of diabetic nephropathy can be classified according to the development of proteinuria and the degree of renal impairment. Before any noticeable clinical changes occurred, the function of nephrons changed at the level of the glomerulus, such as glomerular hyperfiltration and hyperperfusion. Afterward, a series of structural pathological changes take place in the kidney, including basement membrane thickening, mesangial expansion, glomerular hypertrophy and even sclerosis (Alicic et al. 2017). As a type of highly specialized epithelial cells, podocytes are the main component of glomeruli, which are responsible for preventing the leakage of protein into the urine. Increasing evidence supported that the progression of diabetic nephropathy is closely linked with abnormalities and depletion of podocytes (Barutta et al. 2022).

It is widely accepted that myofibroblasts contribute to glomerulosclerosis and interstitial fibrosis in diabetic nephropathy (Badid et al. 2001; Simonson 2007). As a source of myofibroblasts, epithelial-to-mesenchymal transition (EMT) is a process that epithelial cells lose their hallmark epithelial features and acquire the characteristics of mesenchymal cells, which has implicated in diabetic nephropathy (Loeffler and Wolf 2015). In the last decade, data from experimental and clinical studies demonstrated

Received November 19, 2022; revised and accepted February 15, 2023; J-STAGE Advance online publication March 2, 2023
Correspondence: Minzhou Li, Department of Traditional Chinese Medicine, the People's Hospital of Inner Mongolia Autonomous Region, No. 20 Zhaowuda Road, Saihan District, Hohhot, Inner Mongolia 010017, China.
e-mail: liminzhou521@163.com
©2023 Tohoku University Medical Press. This is an open-access article distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License (CC-BY-NC-ND 4.0). Anyone may download, reuse, copy, reprint, or distribute the article without modifications or adaptations for non-profit purposes if they cite the original authors and source properly.
https://creativecommons.org/licenses/by-nc-nd/4.0/

that podocyte depletion may be attributed to EMT (Yamaguchi et al. 2009; Tu et al. 2019); therefore, podocyte EMT could be a potential target in developing novel therapeutic strategies for diabetic nephropathy patients.

The promising clinical effect of Traditional Chinese Medicine (TCM) in diabetic nephropathy treatment has become an attractive point for researchers (Liu et al. 2014). A seminal study by Mou et al. (2020) indicated the efficacy and underlying mechanism of Shenxiao decoction on alleviating renal tubular epithelial cell EMT in diabetic nephropathy based on *in vivo* and *ex vivo* experiments. More recently, a multicenter and randomized clinical trial reported that Zicuiyin decoction is capable of improving and protecting kidney function with no severe adverse events for diabetic nephropathy patients (Liu et al. 2022). Tailing Pills is an original prescription for diabetic nephropathy with *Pseudostellaria Heterophylla* as monarch drug in our hospital. Our previous study showed that Tailing Pills (10 g/kg/day) can effectively attenuate proteinuria and renal pathological changes in diabetic nephropathy model rats (Li et al. 2018). A growing number of studies demonstrated that sirtuin 1 (SIRT1) is a target of diseases driven by EMT (Simic et al. 2013), including diabetic nephropathy (Du et al. 2021). In recent years, H-1-2, a novel polysaccharide isolated from *Pseudostellaria Heterophylla*, was proven to alleviate DM by regulating SIRT1 (Fang et al. 2018). However, the effects of H-1-2 on diabetic nephropathy and podocyte depletion are largely unknown.

Based the above-mentioned literature, the present study aimed to explore whether H-1-2 can alleviate diabetic nephropathy and podocyte depletion by EMT regulation via enhancing SIRT1 *in vivo* and *in vitro*. The mechanism underlying the effect of H-1-2 on EMT was also determined based on *in vitro* analysis.

Materials and Methods

Animal grouping and treatment

All animal experimental procedures were approved by the Ethics Committee of the People's Hospital of Inner Mongolia Autonomous Region. A total of twenty-five 6-week age DBA/2 mice (male, 18~22 g) purchased from Shanghai Laboratory Animal Co., Ltd (SLAC, Shanghai, China) were included in this study. After a week of adaptive feeding, 15 mice were randomly selected for consecutive five days of intraperitoneal injection of streptozotocin (STZ; 50 mg/kg) after 12 h fasting to establish the DM model, while the remaining 10 mice as the control were given the equal volume of the vehicle (0.1 M citrate buffer). The mice with consecutive morning blood glucose levels ≥ 300 mg/dL were chosen as DM models for the following study.

After the successful establishment of DM model, DM mice were divided into three groups: (1) diabetic nephriopathy (DNP) group, (2) DNP+H-1-2-L group, and (3) DNP+H-1-2-H group. Three groups of mice (n = 5/group) were subjected to either vehicle or H-1-2 (1.5 g/kg or 3 g/kg, dissolved in drinking water) administrations by oral gavage for eight weeks. Meanwhile, the control mice were divided into two groups: normal group (vehicle) and normal+H-1-2 group (1.5 g/kg). The body weight and fasting blood glucose (FBG) of each group of mice were recorded every other week from the day of the first administration.

Oral glucose tolerance test (GTT) and insulin tolerance test (ITT)

After eight weeks of administration with H-1-2, all mice were subjected to overnight fasting and the baseline (0 min) glucose levels were determined in blood sampled from the tail vein. After intraperitoneal injection of either glucose (2 g/kg) or insulin (1 U/kg), the blood samples were collected from the tail vein at time points of 15, 30, 60, 90, and 120 min for determining blood glucose levels.

Measurement of biochemical parameters and kidney index

The 24 h urine collection was performed using metabolic cages on the day before the end of the experiment. On the last day, mice were anesthetized with pentobarbital for blood collection, followed by excising the kidney. After weighting, the collected kidneys were stored at −80°C for the subsequent experiment.

The kidney index (kidney/body weight ratio), blood urine nitrogen (BUN), serum creatinine (Scr), and urinary albumin excretion (UAE) were detected to assess renal function.

Cell culture, treatment, and transfection

The mouse podocytes (MPC-5) were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific Inc., Waltham, MA, USA) and 1% penicillin-streptomycin (Gibco) solution at 37°C and 5% CO_2. The *ex vivo* hyperglycemic condition for podocytes was induced by 33 mM glucose, which is defined as high glucose (HG) group. Meanwhile, podocytes cultured in media containing 5.5 mM glucose were defined as normal glucose (NG) group.

SIRT1 small interference (si) RNA (si-SIRT1) and a negative control scramble siRNA (si-NC) were supplied by Shanghai Genechem Co., Ltd. (Shanghai, China). In brief, when the cell density reached approximately 70%, podocytes were subjected to the transfection with the above-indicated plasmids using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific Inc.) in line with the instructions provided by the manufacturer. After 48 h transfection, transfection effectiveness was verified by both RT-PCR and western blot.

CCK-8 assay

CCK-8 assay was exploited to evaluate cell viability. After incubation of 24 h, the CCK-8 reagent (Dojindo Laboratories Inc., Kumamoto, Japan) (10%, v/v, dissolved in RPMI-1640) was added to podocytes from different

groups for another 1.5 h cultivation, the absorbance was examined with a microplate reader at 450 nm to determine the cell viability.

Annexin-V/propidium iodide (PI) double staining assay

The apoptosis rate of podocytes was detected by Annexin-V/PI staining and subsequent flow cytometry analysis. The podocytes from three groups were harvested using Trypsin/EDTA and the concentration was adjusted to 1.5×10^5 cells/mL with phosphate-buffered saline (PBS). After washing thrice, the collected podocytes were stained with annexin V-FITC and PI (Solarbio, Beijing, China) in the dark for 15 min. Finally, the percentage of Annexin-V-FITC+ was determined on flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) to evaluate the percentage of apoptotic podocytes.

Immunofluorescence

After finishing diverse treatments, podocytes were fixed with 2% formaldehyde, followed by permeabilized with 0.3% Triton-X 100 and blocked with PBSB solution. Then, podocytes were incubated with anti-Nephrin (#PA5-106921, Thermo Fisher Scientific Inc.) or anti-Synaptopodin (#PA5-21062, Thermo Fisher Scientific Inc.) antibodies for 1 h. Next, cells were washed thrice with cold PBS and then incubated with Cy3 conjugated anti-rabbit secondary antibodies (#A0516, Beyotime, Jiangsu, China) for 45 min. Coverslips were washed and mounted on DAPI Fluoromount-G. Finally, staining podocytes were observed and imaged under the Zeiss confocal microscope.

RT-PCR

The extraction of total RNA from tissues or podocytes and the following reverse transcription was conducted as per standard protocols. Afterward, RT-PCR analysis was performed with SYBR green reagent (Thermo Fisher Scientific Inc.) on the 7500 Real-Time PCR System. Our study selected GAPDH (Forward: 5′-AGTTAATGCCGCCCCTTACC-3′; Reverse: 5′-CAGGGCTGACTACAAACCCA-3′) as an internal reference gene to quantitate mRNA expression of SIRT1 (Forward: 5′-GTGGTGAGCGACTCAAGGAT-3′; Reverse: 5′-GAGCCGCAGCCTTTTGATCT-3′) based on the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Western blot

The collected kidney tissues and podocytes from diverse groups were lysed with RIPA Buffer on ice for 20 min at 4°C to obtain the total protein. After determining the concentration of total protein, equivalent amounts of protein were separated on 10% SDS-PAGE gels and subsequently transferred onto PVDF membranes. Membranes were blocked with skimmed milk and then incubated with primary antibodies as follows: anti-SIRT1 (#AF0282, 1:1,000; Beyotime), anti-β-actin (#AF0003, 1:1,000; Beyotime), anti-Desmin (#AF1414, 1:2,000; Beyotime), anti-Nephrin (#PA5-106921, 1:2,000; Thermo Fisher Scientific Inc.), anti-Synaptopodin (#PA5-21062, 1:2,000; Thermo Fisher Scientific Inc.), anti-P-cadherin (#13-2000Z, 1:1,000; Thermo Fisher Scientific Inc.), and anti-N-cadherin (#AF0243, 1:800; Beyotime). Then, the membranes were rinsed thrice before incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies. Finally, by using an ECL kit (Beyotime), the protein bands were visualized, of which intensities were measured by Image J 6.0 software.

Statistical analysis

The data were analyzed by GraphPad Prism 8.0.1 software. The quantitative data were represented as the mean ± standard deviation (SD) of triplicate determinations. The comparisons between the two groups or among more than two groups were performed with Student's t-test or one-way ANOVA with Tukey's *post hoc* tests, respectively. $P < 0.05$ was considered a statistically significant difference.

Results

H-1-2 exerted anti-hyperglycemic effect and improved renal function in DM mice

The chemical structure of H-1-2 was shown in Fig. 1A. Before the onset of administration, mice in both the DNP and DNP+H-1-2 groups exhibited a significantly higher FBG than those in the normal mice (Table 1), confirming the successful establishment of the DM mice model. As for body weight monitor results, there was no obvious difference among the three groups of mice at the beginning of treatment. From week 6 to the end of the experiment, the body weight in the normal group was continuously and significantly increased the compared with those in the DNP group (Table 1). After the administration of either a low or high dosage of H-1-2, the body weight of the diabetic nephropathy mice increased slowly, and their FBS significantly decreased from week 4 (Table 1). In GTT and ITT, diabetic nephropathy mice showed oral glucose tolerance and insulin tolerance, which were significantly improved after treatment with H-1-2, especially at the high dosage (Fig. 1B, C). These data revealed that H-1-2 could improve body weight, FBG, as well as insulin resistance in the DM mice model. In the meantime, H-1-2 possessed no effect on the control mice (normal+H-1-2 group).

Thus, we further explore the role of H-1-2 in protecting kidneys in the DM mice model. Compared with those of the normal mice, both the kidney weight and kidney index of the mice in the DNP group were significantly increased (Fig. 1D, E). H-1-2 treatment significantly reduced these increases (Fig. 1D, E). At the same time, the DNP group exhibited significantly greater Scr (Fig. 1F), BUN (Fig. 1G), and UAE (Fig. 1H) than the normal group, while these differences were obviously suppressed by 8-week H-1-2 treatment (Fig. 1F-H). Collectively, H-1-2 has anti-hyperglycemic effect and renal protective function on DM mice, while exerts no effect on normal mice.

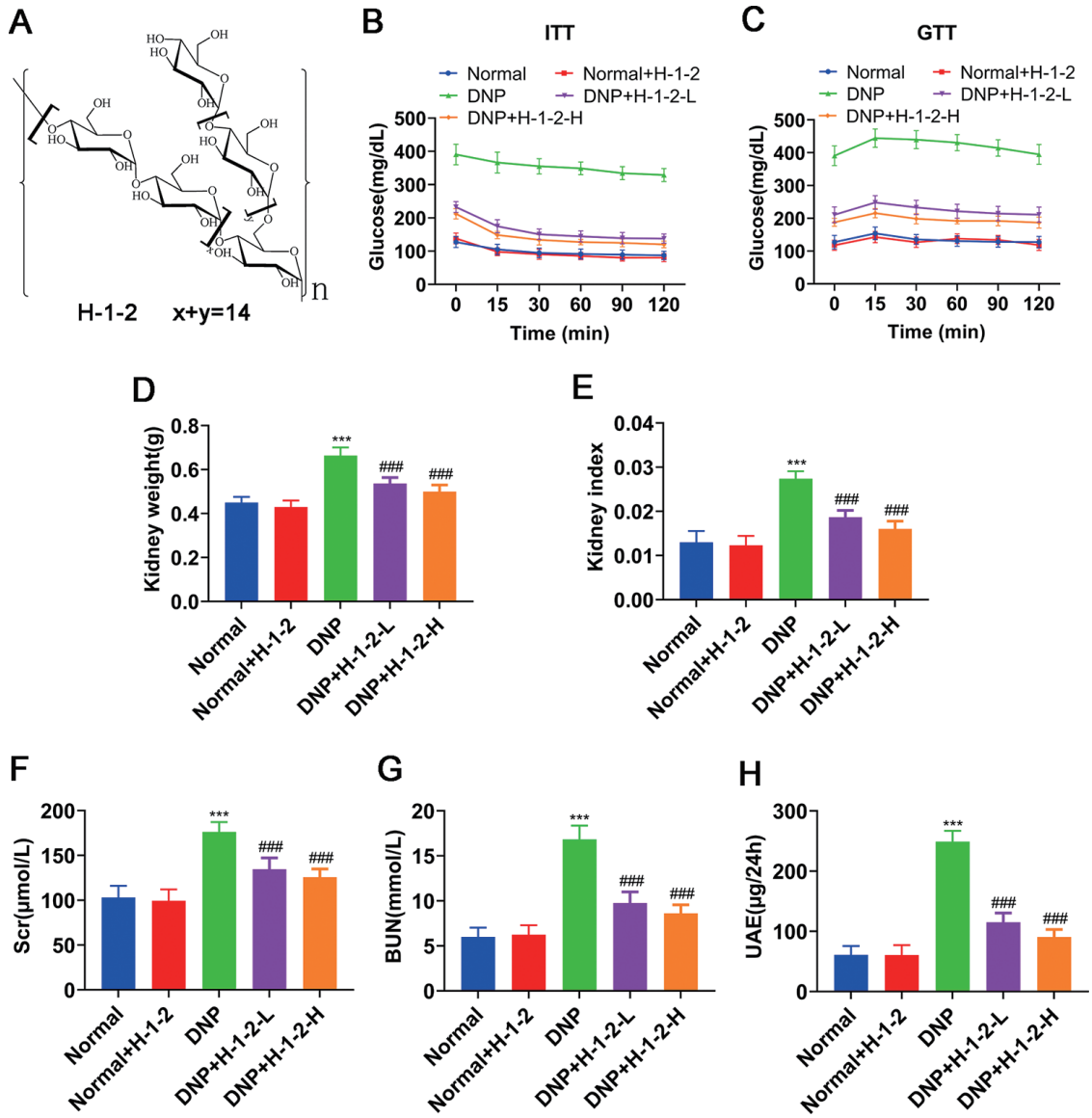

Fig. 1. H-1-2 improved biochemical indexes and renal function in DM mice.
(A) H-1-2 chemical structure. H-1-2 treatment was started 2 weeks after streptozocin (STZ) injection and was administered once daily by the oral garage for eight weeks. The following analyses were performed after the completion of the H-1-2 treatment. (B) Insulin tolerance test (ITT). (C) Oral glucose tolerance test (GTT). (D) Kidney weight. (E) Kidney index (kidney weight/body weight). (F) Serum creatinine (Scr). (G) Blood urea nitrogen (BUN). (H) Urine albumin excretion (UAE). Normal, control mice; Normal+H-1-2, control mice treated with 1.5 g/kg/day of H-1-2; diabetic nephropathy (DNP), STZ-induced DM mice; DNP+H-1-2-L, DM mice treated with 1.5 g/kg/day of H-1-2; DNP+H-1-2-H, DM mice treated with 3 g/kg/day of H-1-2. $^{***}P < 0.001$, vs. normal group; $^{###}P < 0.001$, vs. DNP group; n = 5.

H-1-2 ameliorated podocyte injury and suppressed EMT in the kidney of DM mice

Given that podocytes are crucial in supporting the integrity of the filtration barrier, we detected the expression of the key podocyte markers in kidneys from three groups using western blot to investigate whether H-1-2 protects podocytes against DM-induced injury. The podocyte injury was observed in the kidney of DM mice, which was evidenced by a significant decrease in the expression of synaptopodin and nephrin (key podocyte markers) and a significant increase in the expression of desmin (podocyte injury marker) (Fig. 2A). After intervention with H-1-2, these abnormalities were partially restored (Fig. 2A). These results suggested that H-1-2 protected the kidney by attenuating podocyte injury. EMT and SIRT1 have been proven to be closely related to the pathogenesis of diabetic nephropathy. Western blot showed a significant downregulation of P-cadherin and a significant upregulation of N-cadherin in the DNP group relative to the normal group (Fig. 2B), suggesting that EMT occurred in the kidney of mice from the DNP group. Intriguingly, this phenomenon was restrained by the 8-week H-1-2 administration (Fig. 2B). Moreover, compared with the normal group, the expression of SIRT1 in kidney tissue of the DNP group sig-

Table 1. Body weight and fasting blood glucose of different groups of mice during 8 weeks.

Index	Time	Normal	Normal+H-1-2	DNP	DNP+H-1-2-L	DNP+H-1-2-H
Body weight (g)	0W	24.48 ± 3.12	24.16 ± 3.54	22.94 ± 3.85	23.27 ± 3.59	23.41 ± 3.44
	1W	26.15 ± 3.64	25.87 ± 3.75	23.21 ± 4.07	23.88 ± 3.92	24.06 ± 3.82
	2W	28.09 ± 4.16	27.91 ± 4.02	23.68 ± 4.51	25.17 ± 4.25	25.51 ± 4.05
	3W	29.89 ± 3.95	29.42 ± 4.11	24.17 ± 4.37	26.04 ± 5.10	26.77 ± 4.83
	4W	32.06 ± 4.28	32.26 ± 4.38	23.96 ± 4.65	28.23 ± 5.48	29.01 ± 5.14
	5W	33.53 ± 4.49	33.40 ± 4.25	24.59 ± 3.86	29.36 ± 4.79	30.01 ± 5.37
	6W	35.27 ± 3.92	34.73 ± 4.07	24.82 ± 4.18*	31.14 ± 5.13	31.89 ± 5.04$^{\#}$
	7W	36.44 ± 4.38	36.09 ± 3.95	24.14 ± 3.73*	33.16 ± 4.12$^{\#}$	34.21 ± 4.83$^{\#\#}$
	8W	37.21 ± 3.60	36.84 ± 3.79	23.65 ± 3.91*	33.85 ± 4.39$^{\#}$	35.06 ± 4.95$^{\#\#}$
Fasting blood glucose (mg/dL)	0W	125.34 ± 18.47	121.69 ± 20.35	351.72 ± 32.16***	348.94 ± 36.25	352.47 ± 35.11
	1W	119.62 ± 20.35	115.73 ± 21.66	365.15 ± 35.80***	341.57 ± 38.21	337.28 ± 40.07
	2W	128.17 ± 21.71	123.92 ± 22.87	372.23 ± 39.22***	335.14 ± 35.82	328.16 ± 38.75
	3W	130.44 ± 23.04	125.33 ± 20.84	380.79 ± 36.14***	318.45 ± 36.90	306.94 ± 40.33$^{\#}$
	4W	126.63 ± 20.95	128.42 ± 21.77	384.81 ± 35.29***	302.83 ± 33.75$^{\#}$	291.56 ± 36.80$^{\#\#}$
	5W	122.82 ± 17.79	124.39 ± 19.60	391.54 ± 36.10***	286.47 ± 35.62$^{\#}$	269.32 ± 37.61$^{\#\#\#}$
	6W	120.47 ± 22.68	126.01 ± 20.54	405.73 ± 38.46***	251.38 ± 32.15$^{\#\#}$	240.35 ± 35.27$^{\#\#\#}$
	7W	125.60 ± 24.56	124.35 ± 21.36	397.20 ± 35.84***	238.41 ± 30.25$^{\#\#}$	223.66 ± 32.89$^{\#\#\#}$
	8W	127.41 ± 21.87	122.69 ± 23.82	390.85 ± 37.62***	210.33 ± 29.49$^{\#\#}$	204.50 ± 33.45$^{\#\#\#}$

Data are shown as mean ± SD (n = 5). $^{*}P < 0.05$, $^{***}P < 0.001$ vs. Normal; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. DNP.
Normal, control mice; Normal+H-1-2, control mice treated with 1.5 g/kg/day of H-1-2; diabetic nephropathy (DNP), STZ-induced DM mice; DNP+H-1-2-L, DM mice treated with 1.5 g/kg/day of H-1-2; DNP+H-1-2-H, DM mice treated with 3 g/kg/day of H-1-2; W, week(s).

nificantly decreased at both mRNA and protein levels (Fig. 2C). In the DNP+H-1-2 group, the suppressed SIRT1 mRNA expression was significantly reversed (Fig. 2C), as was the case for the SIRT1 protein expression (Fig. 2D).

H-1-2 restrained injury and EMT in HG-induced podocytes ex vivo

To verify the protective role of H-1-2 in podocyte injury, *ex vivo* studies were performed in podocytes exposed to HG. Firstly, the cytotoxicity of H-1-2 in podocytes was observed under normal conditions. CCK8 assay demonstrated that H-1-2 treatment did not cause any conspicuous cytotoxicity in podocytes when its concentration is lower than 200 mg/L (Fig. 3A). Next, we examined cell viability under a series of concentrations (5, 10, 50, 100, and 200 mg/L) of H-1-2 to evaluate the protective function of H-1-2 in podocytes under HG conditions. HG caused a significant reduction in cell viability, while H-1-2 could improve the cell viability impaired by HG in a concentration-dependent way (Fig. 3B). Ameliorative effects of H-1-2 on HG-induced podocyte injury did not differ significantly between 100 and 200 mg/L (Fig. 3B). Thus, 100 mg/L of H-1-2 was selected to carry out the following experiments.

Annexin-V/PI double staining assay was conducted to identify podocyte apoptosis. The result showed that the apoptosis rate of podocytes was significantly higher in the HG group than in the NG group, while H-1-2 effectively alleviated podocyte apoptosis (Fig. 3C). In immunofluorescence analysis, exposing podocytes to HG led to a significant decrease in the expression of nephrin (Fig. 4A) and synaptopodin (Fig. 4B). Meanwhile, H-1-2 reversed these changes induced by HG in podocytes (Fig. 4A, B). Western blot analysis for nephrin and synaptopodin showed a trend similar to that observed with immunofluorescence analysis (Fig. 4C), which corroborated the protective effect of H-1-2 on podocyte injury.

In addition, we perceived that H-1-2 repressed HG-induced podocyte EMT, as evidenced by the increased levels of P-cadherin and the decreased levels of N-cadherin (Fig. 4D). Consistent with the findings of *in vivo* experiment, H-1-2 could block the suppressive effect of HG on the expression of SIRT1 in podocytes (Fig. 4D). These results revealed that H-1-2 can ameliorate podocyte injury, EMT, as well as SIRT1 downregulation induced by HG.

Effect of H-1-2 on HG-induced podocyte injury and EMT is mediated by SIRT1

To investigate whether H-1-2 is involved in regulating SIRT1 under the HG condition, the expression levels of SIRT1 were detected in HG-induced podocytes after the intervention of H-1-2 with diverse concentrations. The results showed that SIRT1 expression levels were elevated with increasing H-1-2 concentrations (Fig. 5A), which let us wondering the involvement of SIRT1 in the effect of H-1-2 on HG-induced podocytes. Thus, si-SIRT1 transfection was applied to HG-induced podocytes before H-1-2 treatment. Both RT-PCR and western blot analysis showed that si-SIRT1 effectively blocked the SIRT1 upregulation in

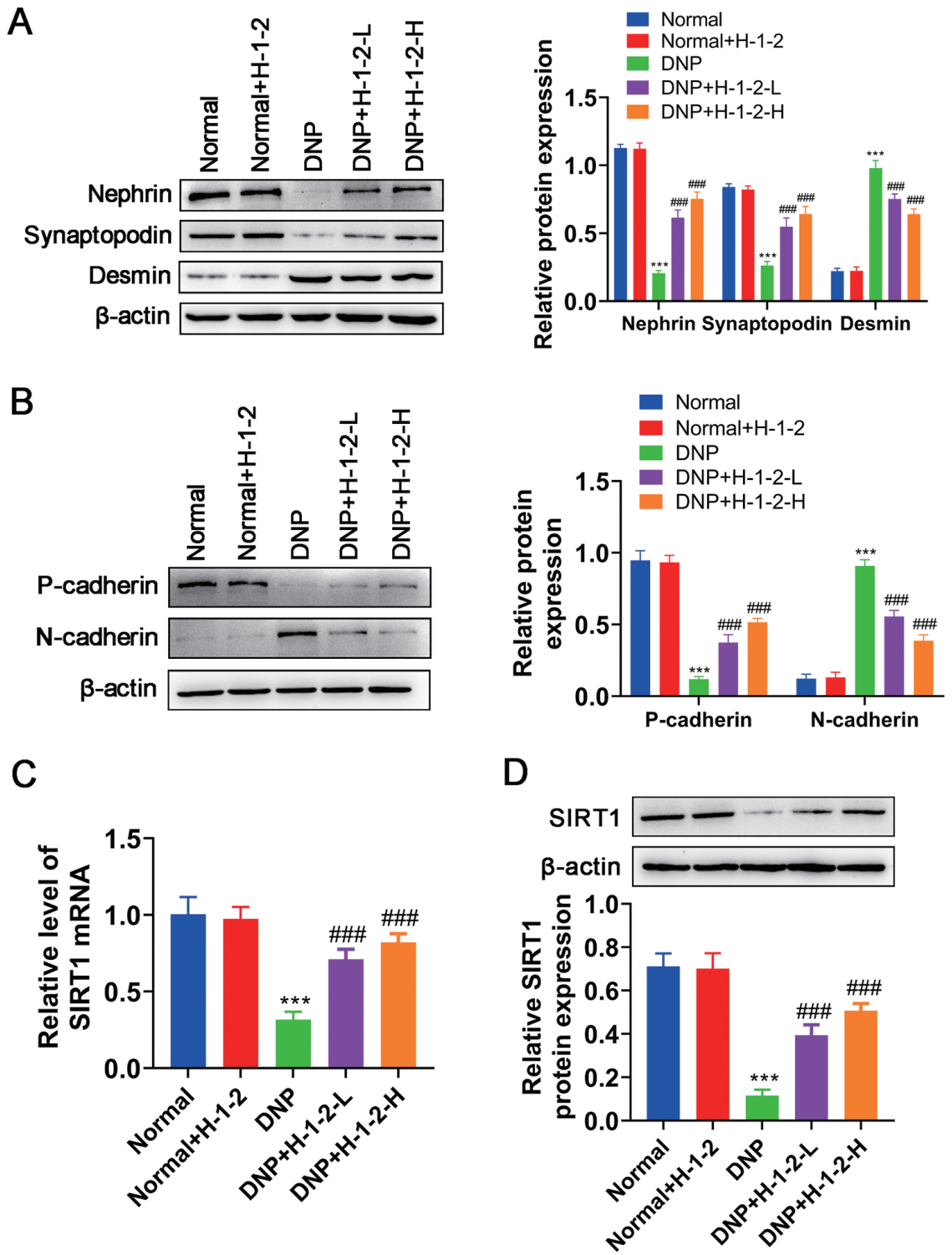

Fig. 2. H-1-2 suppressed podocyte depletion and EMT in renal tissues of DM mice.
(A) The protein expressions of nephrin, synaptopodin, and desmin were detected to evaluate the podocyte depletion in renal tissues by western blot. (B) The protein expressions of EMT-related markers (P-cadherin and E-cadherin) in renal tissues were detected by western blot. The expression of SIRT1 in renal tissues was examined at (C) mRNA and (D) protein levels using RT-PCR and western blot, respectively. Normal, control mice; Normal+H-1-2, control mice treated with 1.5 g/kg/day of H-1-2; diabetic nephropathy (DNP), STZ-induced DM mice; DNP+H-1-2-L, DM mice treated with 1.5 g/kg/day of H-1-2; DNP+H-1-2-H, DM mice treated with 3 g/kg/day of H-1-2. $^{***}P < 0.001$, vs. normal group; $^{\#\#\#}P < 0.001$, vs. DNP group; n = 3.

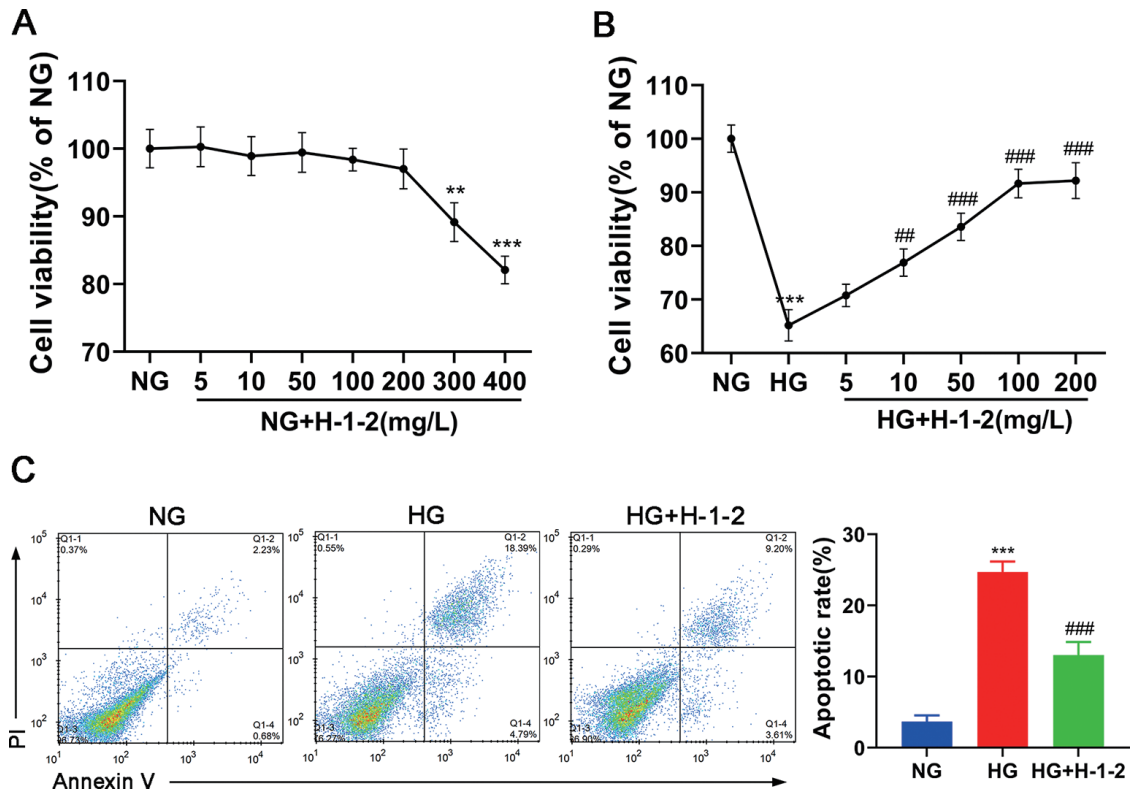

Fig. 3. H-1-2 alleviated podocyte depletion induced by high glucose (HG).
(A) The cell viability of podocytes was evaluated by CCK-8 after treatment with a series of concentrations of H-1-2. (B) The cell viability of HG-induced podocytes was evaluated by CCK-8 after treatment with a series of concentrations of H-1-2. (C) The cell apoptosis of podocytes was analyzed by flow cytometry with Annexin-V/PI double staining. Normal glucose (NG) group and HG group were cultured in the media containing 5.5 mM glucose and 33 mM glucose, respectively. $^{**}P < 0.01$ and $^{***}P < 0.001$, vs. NG group; $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$, vs. HG group; n = 3.

HG-induced podocytes with H-1-2 treatment (Fig. 5B, C), indirectly revealing the successful transfection of si-SIRT1.

It was observed that no significant difference in the cell viability and apoptosis between the HG+H-1-2 and HG+si-NC+H-1-2 groups, while the effect of H-1-2 on the cell viability and apoptosis of HG-induced podocytes was markedly abrogated by silencing SIRT1 (Fig. 5D, E). The restoration by H-1-2 in the downregulation of nephrin and synaptopodin induced by HG was also evidently blocked by the transfection of si-SIRT1 in podocytes (Fig. 5F, G). Moreover, in the HG+H-1-2 group, the protein levels of P-cadherin were dramatically increased compared with those of the HG group, while the protein levels of E-cadherin were evidently decreased, and si-SIRT1 transfection remarkably blocked this effect (Fig. 5H), indicating that SIRT1 plays a crucial role in the protective effect of H-1-2 on HG-induced EMT in podocytes. Besides, silencing SIRT1 has a slight effect on the HG-induced injury in podocytes (Fig. 5D-H). We speculated it may be because HG exposure remarkedly downregulated the activity of SIRT1 at a deficient level that knocking down SIRT1 could not further injure podocytes. Besides, silencing SIRT1 has slight effect on the HG-induced injury in podocytes (Fig. 5D-H). Taken together, the protective effect of H-1-2 on podocyte injury and EMT in HG conditions was associated with the upregulation of SIRT1.

Discussion

The incidence of diabetic nephropathy has been increasing dramatically because of the rapid economic growth and urbanization, and changes in lifestyle in China (Zhang et al. 2020). Diabetic nephropathy has more complicated metabolic disorders relative to general kidney diseases; hence, there is no completely effective prevention and treatment for it at present. Considerable evidence indicates that podocyte depletion plays a decisive role in the progression of diabetic nephropathy (Li et al. 2007; Dai et al. 2017). Increasing studies have highlighted the importance of podocyte EMT in the development of diabetic nephropathy. Dai et al. (2011) found the reduction of epithelial markers and the induction of mesenchymal proteins in podocytes in the early stages of diabetic nephropathy in STZ-induced rodents. Another *in vivo* study demonstrated that podocyte EMT may be a potential factor causing proteinuria in diabetic nephropathy (Li et al. 2008). Hence, targeting inhibition of podocyte EMT is recognized as a new therapeutic strategy for diabetic nephropathy. H-1-2 is a novel polysaccharide of *Pseudostellaria heterophylla*, which has been reported to exert anti-hyperglycemic and anti-cancer effects in recent studies (Fang et al. 2018; Sun

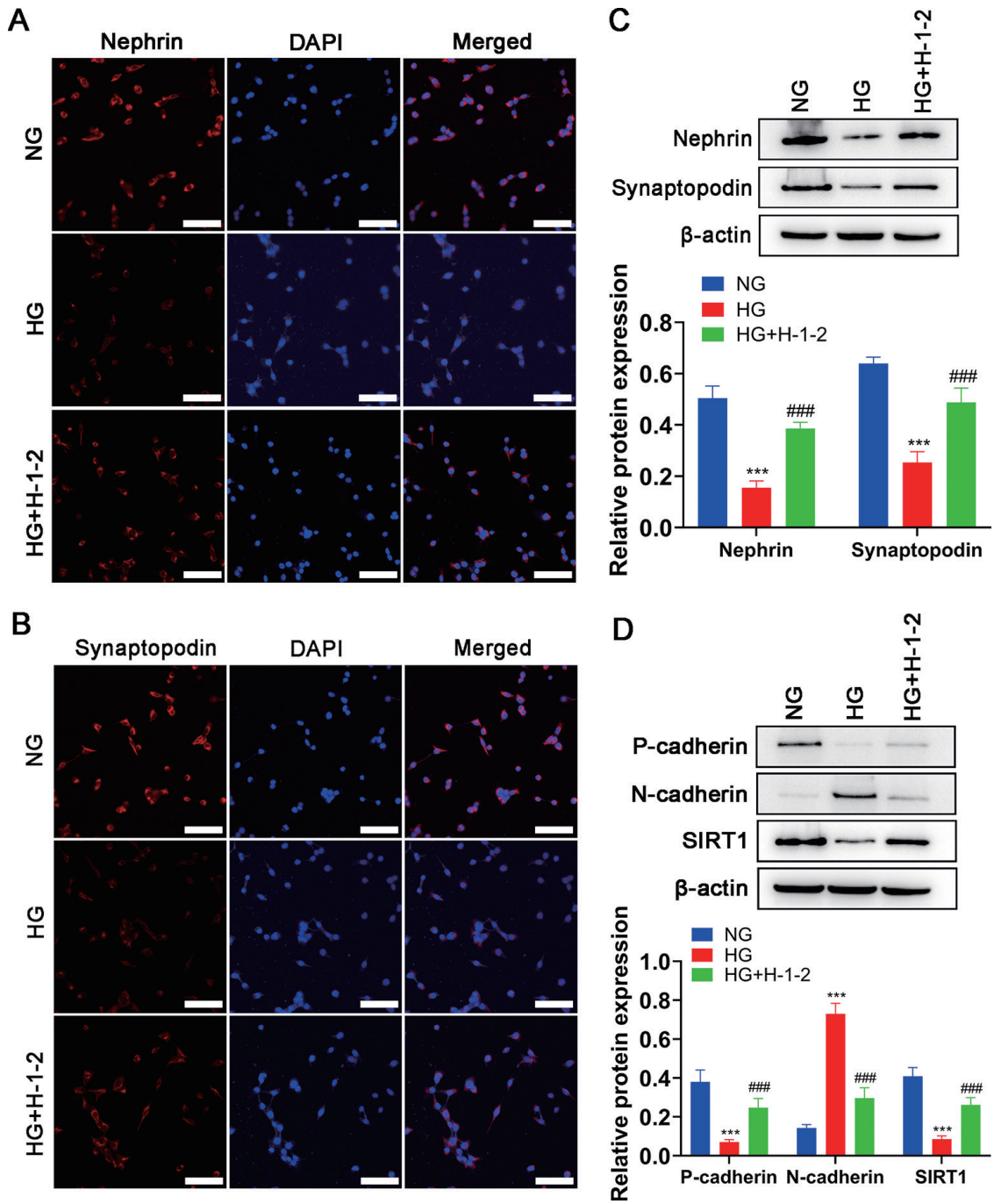

Fig. 4. H-1-2 reversed HG-induced podocyte depletion and EMT *ex vivo*. Podocytes were exposed to normal glucose (NG, 5.5 mM) or high glucose (HG, 33 mM) conditions, and were subsequently treated with or without H-1-2 (100 mg/L). Immunofluorescence analysis of (A) nephrin (scale bar, 50 μm) and (B) synaptopodin (scale bar, 50 μm). (C) The protein expressions of nephrin and synaptopodin were detected by western blot. (D) The protein expressions of EMT related markers (P-cadherin and E-cadherin) and SIRT1 were detected by western blot. $^{***}P < 0.001$, vs. NG group; $^{###}P < 0.001$, vs. HG group; n = 3.

et al. 2020). Herein, we investigated whether H-1-2 ameliorates HG-induced-podocyte depletion and EMT *in vivo* and *ex vivo*, and preliminarily uncovered the underlying mechanism.

Considering that DBA/2 mice are more susceptible to renal injury during STZ-induced DM compared with other strains of mice (Qi et al. 2005), the present study used DBA/2 mice to induce diabetic nephropathy. H-1-2 treatment effectively attenuated diabetic symptoms of DM mice, such as increased FBG and body weight loss, which was identical to a previous study conducted by Fang et al. (2018). In addition, DM mice exhibited renal dysfunction characterized by the increased kidney index, and elevated levels of Scr, BUN, and UAE, which means that DM has

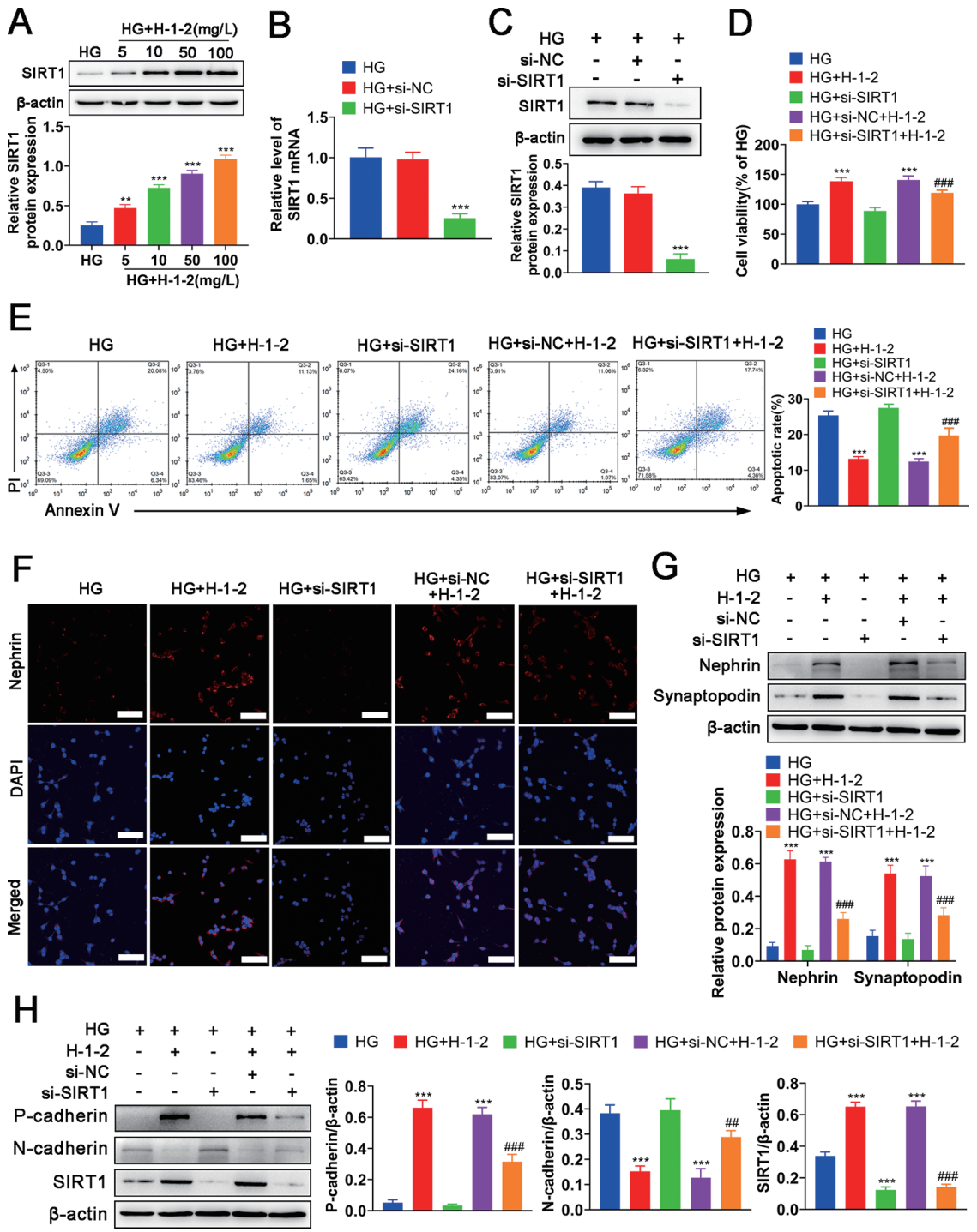

Fig. 5. Effect of H-1-2 on HG-induced podocyte depletion and EMT depending on SIRT1.
(A) The expression changes of SIRT1 in HG-induced podocytes with a series of concentrations of H-1-2 were evaluated by western blot. After being transfected with si-NC or si-SIRT1, the (B) mRNA and (C) protein expression levels of SIRT1 in HG-induced podocytes were analyzed by RT-PCR and western blot, respectively. (D-G) Podocytes were transfected with si-NC or si-SIRT1 before hyperglycemic exposure, followed by incubation with H-1-2 (100 mg/L). (D) The cell viability was evaluated by CCK-8. (E) The cell apoptosis was analyzed by flow cytometry with Annexin-V/PI double staining. (F) Nephrin expression in HG-induced podocytes was investigated by immunofluorescence staining (scale bar, 50μm). (G) The protein expressions of nephrin and synaptopodin were detected by western blot. (H) The protein expressions of EMT related markers (P-cadherin and E-cadherin) were detected by western blot. $^{**}P < 0.01$ and $^{***}P < 0.001$, vs. HG group; $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$, vs. HG+H-1-2 group; n = 3.

developed diabetic nephropathy in mice. Our study firstly indicated the renoprotection of H-1-2 in DM-induced kidney injury. Besides, our *ex vivo* experiments revealed that H-1-2 effectively enhanced the ability of podocytes against HG, as shown by the restoration of cell viability and reduced apoptosis in HG-induced podocytes. As one of the epithelial markers, nephrin is an important protein that constitutes the key functional unit of podocytes (Ruotsalainen et al. 1999); thus, reducing nephrin expression may contribute to the increasing albuminuria in early DPN (Kim et al. 2007). Synaptopodin has long been suggested as an important marker protein of podocyte maturation, of which expression was decreased when podocytes were damaged (Susztak et al. 2006). In our study, HG decreased nephrin and synaptopodin expression *in vivo* and *ex vivo*, which was consistent with the above literature. However, H-1-2 administration for eight weeks preserved both renal nephrin and synaptopodin expression in DM mice. These *in vivo* observations were corroborated by *ex vivo*. H-1-2 significantly restored nephrin and synaptopodin expression in HG-induced podocytes. Simultaneously, HG-induced EMT was observed *in vivo* and *ex vivo* in this study. Notably, treatment with H-1-2 effectively ameliorated the decreasing P-cadherin expression and increased N-cadherin expression in podocytes. These results collectively indicated that H-1-2 ameliorated HG-induced EMT and subsequent podocyte depletion, which might be a potential novel therapeutic option for diabetic nephropathy.

It is well established that SIRT1 plays a salutary role in multiple kidney diseases (Wakino et al. 2015), including diabetic nephropathy (Yacoub et al. 2014). Hasegawa et al. (2013) and Chuang et al. (2011) previously found that SIRT1 expression is significantly reduced in the kidney of patients with diabetic nephropathy. A growing number of studies revealed that the therapeutic effect of multiple potential strategies for diabetic nephropathy is related to their role in SIRT1 regulation (Kitada et al. 2011; Xu et al. 2012; Zhong et al. 2018). It has been reported that H-1-2 treatment caused SIRT1 upregulation to alleviate DM (Fang et al. 2018). To uncover the mechanisms underlying the effect of H-1-2 on HG-induced podocyte EMT, we investigated the effects of H-1-2 and si-SIRT1 transfection on podocyte depletion and EMT in HG-induced podocytes. Consistent with previous studies, our data showed that HG stimulated podocyte EMT and SIRT1 downregulation. SIRT1 has been shown to reduce TGF-β-induced EMT in diabetic nephropathy (Du et al. 2021). In this study, H-1-2 significantly restrained HG-induced podocyte depletion and EMT, and concentration-dependently enhanced SIRT1 expression in HG-induced podocytes. Furthermore, silencing SIRT1 significantly blocked these effects of H-1-2 in HG-induced podocytes. Therefore, the regulatory effect of H-1-2 on SIRT1 seems to be responsible for its protective effects against HG-induced podocyte injury and EMT.

In conclusion, our research has provided experimental evidence that H-1-2 can reduce the symptoms of renal dysfunction in DM mice. H-1-2 alleviates podocyte injury and EMT, at least in part by regulating the SIRT1 expression, thus exerting a protective effect for podocytes under a HG environment. Nevertheless, since the pathogenesis of diabetic nephropathy is complex, and may involve many pathways, the specific mechanism underlying H-1-2 in diabetic nephropathy still calls for further investigations.

Acknowledgments

This study was supported by *In vitro* study on intervention of Tai Ling Dan in podocyte transdifferentiation induced by high glucose by Natural Science Foundation of Inner Mongolia Autonomous Region (NO.2019MS08096); Inner Mongolia Medical University Joint Project (NO. YKD2021LH046). Project Name: Study on the Regulation Mechanism of Chinese Medicine Tailingdan on STZ-induced podocyte transdifferentiation in diabetic rats, and 2022 Inner Mongolia Autonomous Region Health Science and Technology Plan Project (NO.202201044): Tailingdan inhibits glucose induced epithelial-mesenchymal transition via the PI3K-AKT signaling pathway.

Conflict of Interest

The authors declare no conflict of interest.

References

Alicic, R.Z., Rooney, M.T. & Tuttle, K.R. (2017) Diabetic kidney disease: challenges, progress, and possibilities. *Clin. J. Am. Soc. Nephrol.*, **12**, 2032-2045.

Badid, C., Vincent, M., Fouque, D., Laville, M. & Desmouliere, A. (2001) Myofibroblast: a prognostic marker and target cell in progressive renal disease. *Ren. Fail.*, **23**, 543-549.

Barutta, F., Bellini, S. & Gruden, G. (2022) Mechanisms of podocyte injury and implications for diabetic nephropathy. *Clin. Sci.* (*Lond.*), **136**, 493-520.

Chuang, P.Y., Dai, Y., Liu, R., He, H., Kretzler, M., Jim, B., Cohen, C.D. & He, J.C. (2011) Alteration of forkhead box O (foxo4) acetylation mediates apoptosis of podocytes in diabetes mellitus. *PLoS One*, **6**, e23566.

Dai, H., Liu, Q. & Liu, B. (2017) Research progress on mechanism of podocyte depletion in diabetic nephropathy. *J. Diabetes Res.*, **2017**, 2615286.

Dai, H.Y., Zheng, M., Tang, R.N., Ni, J., Ma, K.L., Li, Q. & Liu, B.C. (2011) Effects of angiotensin receptor blocker on phenotypic alterations of podocytes in early diabetic nephropathy. *Am. J. Med. Sci.*, **341**, 207-214.

Du, L., Qian, X., Li, Y., Li, X.Z., He, L.L., Xu, L., Liu, Y.Q., Li, C.C., Ma, P., Shu, F.L., Lu, Q. & Yin, X.X. (2021) Sirt1 inhibits renal tubular cell epithelial-mesenchymal transition through YY1 deacetylation in diabetic nephropathy. *Acta Pharmacol. Sin.*, **42**, 242-251.

Fang, Z.H., Duan, X.C., Zhao, J.D., Wu, Y.J. & Liu, M.M. (2018) Novel polysaccharide H-1-2 from pseudostellaria heterophylla alleviates type 2 diabetes mellitus. *Cell. Physiol. Biochem.*, **49**, 996-1006.

Gross, J.L., de Azevedo, M.J., Silveiro, S.P., Canani, L.H., Caramori, M.L. & Zelmanovitz, T. (2005) Diabetic nephropathy: diagnosis, prevention, and treatment. *Diabetes Care*, **28**, 164-176.

Hasegawa, K., Wakino, S., Simic, P., Sakamaki, Y., Minakuchi, H., Fujimura, K., Hosoya, K., Komatsu, M., Kaneko, Y., Kanda,

T., Kubota, E., Tokuyama, H., Hayashi, K., Guarente, L. & Itoh, H. (2013) Renal tubular Sirt1 attenuates diabetic albuminuria by epigenetically suppressing Claudin-1 overexpression in podocytes. *Nat. Med.*, **19**, 1496-1504.

Kim, J.J., Li, J.J., Jung, D.S., Kwak, S.J., Ryu, D.R., Yoo, T.H., Han, S.H., Choi, H.Y., Kim, H.J., Han, D.S. & Kang, S.W. (2007) Differential expression of nephrin according to glomerular size in early diabetic kidney disease. *J. Am. Soc. Nephrol.*, **18**, 2303-2310.

Kitada, M., Takeda, A., Nagai, T., Ito, H., Kanasaki, K. & Koya, D. (2011) Dietary restriction ameliorates diabetic nephropathy through anti-inflammatory effects and regulation of the autophagy via restoration of Sirt1 in diabetic Wistar fatty (fa/fa) rats: a model of type 2 diabetes. *Exp. Diabetes Res.*, **2011**, 908185.

Li, J.J., Kwak, S.J., Jung, D.S., Kim, J.J., Yoo, T.H., Ryu, D.R., Han, S.H., Choi, H.Y., Lee, J.E., Moon, S.J., Kim, D.K., Han, D.S. & Kang, S.W. (2007) Podocyte biology in diabetic nephropathy. *Kidney Int. Suppl.*, S36-42.

Li, M.Z., Wu, Z., Zhang, R.X., Gao, W. & Zhao, K.Y. (2018) Effects of Tai Ling pills on renal protection of the rats with STZ-induced diabetic nephropathy. *Gansu Journal of Traditional Chinese Medicine*, **31**, 5-9.

Li, Y., Kang, Y.S., Dai, C., Kiss, L.P., Wen, X. & Liu, Y. (2008) Epithelial-to-mesenchymal transition is a potential pathway leading to podocyte dysfunction and proteinuria. *Am. J. Pathol.*, **172**, 299-308.

Liu, J., Gao, L.D., Fu, B., Yang, H.T., Zhang, L., Che, S.Q., Xu, Y., Du, X., Liu, Z.C., Xue, Y., Lv, C.X., Huang, Y.H., Wang, B.H., Gao, S.X., Xing, Y.F., et al. (2022) Efficacy and safety of Zicuiyin decoction on diabetic kidney disease: a multicenter, randomized controlled trial. *Phytomedicine*, **100**, 154079.

Liu, X., Liu, L., Chen, P., Zhou, L., Zhang, Y., Wu, Y., Jiang, L., Cheng, D., Huang, W. & Yi, D. (2014) Clinical trials of traditional Chinese medicine in the treatment of diabetic nephropathy--a systematic review based on a subgroup analysis. *J. Ethnopharmacol.*, **151**, 810-819.

Livak, K.J. & Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*, **25**, 402-408.

Loeffler, I. & Wolf, G. (2015) Epithelial-to-mesenchymal transition in diabetic nephropathy: fact or fiction? *Cells*, **4**, 631-652.

Mou, X., Zhou, D.Y., Zhou, D., Liu, K., Chen, L.J. & Liu, W.H. (2020) A bioinformatics and network pharmacology approach to the mechanisms of action of Shenxiao decoction for the treatment of diabetic nephropathy. *Phytomedicine*, **69**, 153192.

Qi, Z., Fujita, H., Jin, J., Davis, L.S., Wang, Y., Fogo, A.B. & Breyer, M.D. (2005) Characterization of susceptibility of inbred mouse strains to diabetic nephropathy. *Diabetes*, **54**, 2628-2637.

Ruotsalainen, V., Ljungberg, P., Wartiovaara, J., Lenkkeri, U., Kestila, M., Jalanko, H., Holmberg, C. & Tryggvason, K. (1999) Nephrin is specifically located at the slit diaphragm of glomerular podocytes. *Proc. Natl. Acad. Sci. U. S. A.*, **96**, 7962-7967.

Simic, P., Williams, E.O., Bell, E.L., Gong, J.J., Bonkowski, M. & Guarente, L. (2013) SIRT1 suppresses the epithelial-to-mesenchymal transition in cancer metastasis and organ fibrosis. *Cell Rep.*, **3**, 1175-1186.

Simonson, M.S. (2007) Phenotypic transitions and fibrosis in diabetic nephropathy. *Kidney Int.*, **71**, 846-854.

Sun, H., Shi, K., Qi, K., Kong, H., He, Q. & Zhou, M. (2020) Pseudostellaria heterophylla extract polysaccharide H-1-2 suppresses pancreatic cancer by inhibiting hypoxia-induced AG2. *Mol. Ther. Oncolytics*, **17**, 61-69.

Susztak, K., Raff, A.C., Schiffer, M. & Bottinger, E.P. (2006) Glucose-induced reactive oxygen species cause apoptosis of podocytes and podocyte depletion at the onset of diabetic nephropathy. *Diabetes*, **55**, 225-233.

Tu, Q., Li, Y., Jin, J., Jiang, X., Ren, Y. & He, Q. (2019) Curcumin alleviates diabetic nephropathy via inhibiting podocyte mesenchymal transdifferentiation and inducing autophagy in rats and MPC5 cells. *Pharm. Biol.*, **57**, 778-786.

Wakino, S., Hasegawa, K. & Itoh, H. (2015) Sirtuin and metabolic kidney disease. *Kidney Int.*, **88**, 691-698.

Xu, Y., Nie, L., Yin, Y.G., Tang, J.L., Zhou, J.Y., Li, D.D. & Zhou, S.W. (2012) Resveratrol protects against hyperglycemia-induced oxidative damage to mitochondria by activating SIRT1 in rat mesangial cells. *Toxicol. Appl. Pharmacol.*, **259**, 395-401.

Yacoub, R., Lee, K. & He, J.C. (2014) The role of SIRT1 in diabetic kidney disease. *Front. Endocrinol.* (*Lausanne*), **5**, 166.

Yamaguchi, Y., Iwano, M., Suzuki, D., Nakatani, K., Kimura, K., Harada, K., Kubo, A., Akai, Y., Toyoda, M., Kanauchi, M., Neilson, E.G. & Saito, Y. (2009) Epithelial-mesenchymal transition as a potential explanation for podocyte depletion in diabetic nephropathy. *Am. J. Kidney Dis.*, **54**, 653-664.

Zhang, X.X., Kong, J. & Yun, K. (2020) Prevalence of diabetic nephropathy among patients with type 2 diabetes mellitus in China: a meta-analysis of observational studies. *J. Diabetes Res.*, **2020**, 2315607.

Zhong, Y., Lee, K. & He, J.C. (2018) SIRT1 is a potential drug target for treatment of diabetic kidney disease. *Front. Endocrinol.* (*Lausanne*), **9**, 624.