

The Inhibitory Effect of Rhein on Proliferation of High Glucose-induced Mesangial Cell Through Cell Cycle Regulation and Induction of Cell Apoptosis

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Submitted: 09-07-2015

Revised: 17-08-2015

Published: 11-05-2016

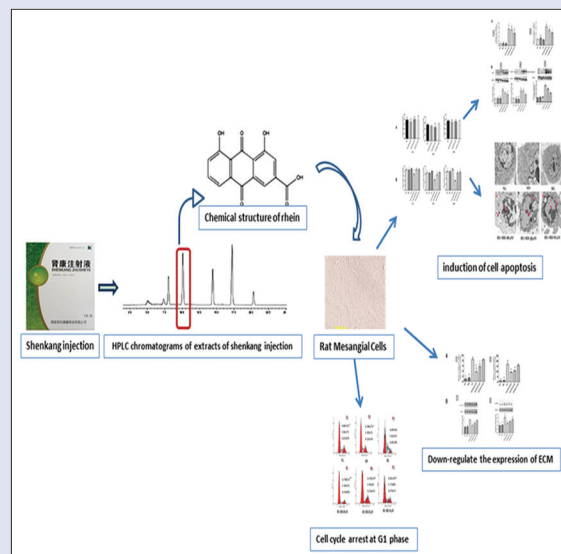
ABSTRACT

Objectives: Increased mesangial cell proliferation and accumulation of extracellular matrix (ECM) are the major pathological features of early-stage diabetic nephropathy. This study was sought to investigate the inhibitory effects of rhein (RH) on high glucose (HG)-cultured mesangial cells. Specially, we focus on the analysis of proliferation rate, cell cycle regulation, apoptosis, and the expression of collagen IV and laminin. **Materials and Methods:** The established rat renal mesangial cell (RMC) line was cultured in medium with different concentrations of glucose (5.6 mM or 25 mM) and RH (40 μ M, 20 μ M, and 10 μ M). Pro-treated cells were collected at 12 h, 24 h, and 48 h for cell proliferation analysis and after 24 h for the experiments of flow cytometry, transmission electron microscope, real-time polymerase chain reaction, and Western blotting. **Results:** Our data shows HG can promote the proliferation of RMCs and RH has an inhibitory effect on HG-induced RMC proliferation and expression of ECM. Based on our data, we hypothesize this inhibitory effect might be a result of cell cycle regulation and the induction of cellular apoptosis. **Conclusion:** RH can inhibit cellular proliferation and downregulate the expression of ECM under the circumstance of HG. The mechanism of growth suppression may be due to cell cycle arrest at G₁ phase, induction of cell apoptosis, and upregulation of apoptotic mediators bax and caspase-3.

Key words: Apoptosis, cell cycle, diabetic nephropathy, extracellular matrix, mesangial cell, rhein

SUMMARY

- Rhein (RH) has an inhibitory effect on high glucose-induced rat mesangial cells proliferation
- RH has an inhibitory effect on the expression of extracellular matrix
- RH has a growth-suppression effect
- RH can upregulate the expression of apoptotic mediators bax and caspase-3
- All above shows RH is one of the main active ingredient in Shengkang injection.



Abbreviations used: RH: Rhein, ECM: Extracellular matrix, DN: Diabetic nephropathy, RMC: Renal mesangial cell, SKI: Shengkang injection, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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DOI: 10.4103/0973-1296.182158

Access this article online

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INTRODUCTION

Diabetic nephropathy (DN) is one of the most severe microvascular complications of type 1 and type 2 diabetes, and also a major cause of end-stage renal disease.^[1] Increased renal mesangial cell (RMC) proliferation is one of the major pathologic features in the early stage of DN.^[2,3] Though the precise mechanisms of DN have not been elucidated, several *in vivo* studies have recently unraveled the significance of RMC in the early stages of DN. It seems that mesangial cell hypercellularity precedes an increase in the extracellular matrix (ECM) and glomerular sclerosis.^[4] RMC proliferation has been shown to be correlated with the degree of glycemic control, indicating that abnormally high blood glucose may be a crucial risk factor for DN.^[5] There are few data on the effects of pharmacological intervention on RMC proliferation in DN.

Therefore, a search for a drug potentially capable of inhibiting RMC proliferation could be of significant importance.

Shengkang injection (SKI) is a patented Chinese drug and is used to treat chronic renal failure. Several clinical and laboratory reports have shown

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Cite this article as: Xu S, Lv Y, Zhao J, Wang J, Wang G, Wang S. The inhibitory effect of rhein on proliferation of high glucose-induced mesangial cell through cell cycle regulation and induction of cell apoptosis. *Phcog Mag* 2016;12:S257-63.

SKI has a certain function to DN.^[6-10] In the prescribed composition of SKI, rhubarb as a monarch drug, while rhein (RH) (4,5-dihydroxy-9,10-dioxo-9,10-dihydroanthracene-2-carboxylic acid) is one of the main active ingredients (our previous study have identified the concentration of RH in SKI) [Figure 1].^[11] In studies performed *in vitro*, it can inhibit Integrin-linked kinase expression, regulate the ratio of matrix metalloproteinase-9/tissue inhibitor of metalloproteinase-1^[12] and inhibit renal tubular epithelial cell hypertrophy and ECM accumulation.^[13] Furthermore, some reports show it can potently interfere with organic anion transporter-mediated renal elimination^[14] and reverse the diabetic phenotype of mesangial cells over-expressing the glucose transporter-1.^[15] While, *in vivo*, it can improve renal lesion and ameliorate dyslipidemia in db/db mice with DN.^[16]

In this study, we explore the inhibitory effect of RH on the rate of cellular proliferation and expression of ECM cultured in condition of high glucose (HG).

MATERIALS AND METHODS

Cell culture and reagents

The established RMC line HBZY-1 was obtained from The Chinese Center for Type Culture Collection (Wuhan, China). The cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in an atmosphere containing 5% CO₂. RMC cells between passages 3 and 10 were used for experiments. After preincubation in DMEM (with glucose 5.6 mM) supplemented with 0.1% fetal calf serum for 24 h, cells were then treated with different concentrations of glucose or RH as indicated: Normal glucose group (NG, 5.6 mM glucose); HG group (25 mM glucose); HG with different concentrations of RH (RH - 40 µM, 25 mM glucose + 40 µM RH; RH - 20 µM, 25 mM glucose + 20 µM RH; RH - 10 µM, 25 mM glucose + 10 µM RH); mannitol group (MN, 5.6 mM glucose and 19.4 mM MN). DMEM (5.6 mM or 25 mM glucose) was purchased from Thermo Fisher Scientific Inc., (Beijing, China). MN was from Sigma (St. Louis, MO, USA). RH bought from National food and drug testing institute (Beijing, China).

Cell proliferation assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay was used as a qualitative index of cell viability. All cells were incubated with different conditions as described above and 20 µl MTT (5 mg/mL, Invitrogen Corp., Carlsbad, CA, USA) was added after 12 h, 24 h, or 48 h, and then cells were cultured for additional 4 h. Subsequently, cells were lysed using dimethyl sulfoxide (0.15 mL/well,

Sigma, St. Louis, MO, USA). When the formazan crystals were completely dissolved, the optical density was measured at 490 nm using a Microplate Reader Model M680-UV Spectrophotometer (Bio-Rad Laboratories, Marnes-La-Coquette, France).

Cell cycle analysis

The cell cycle distribution was analyzed using flow cytometry. After 24 h treatments with different conditions (0.5–1) × 10⁶ cells were harvested by trypsinization, washed twice with phosphate-buffered saline (PBS), and fixed in 70% ethanol at 20°C. The fixed cells were collected and resuspended in 500 µL of PBS with RNase A (TaKaRa, Japan) (100 µg/mL) at 37°C for 1 h. Subsequently, the cell samples were incubated with propidium iodide (TaKaRa, Japan) (50 µg/mL) for 30 min. DNA content of 2 × 10⁵ cells for each sample was analyzed by Flow Cytometer (Becton Dickinson, San Jose, CA, USA). The data were analyzed with Mod Fit LT 2.0 software (Verity Software, Topsham, ME, USA).

Transmission electron microscope

The transmission electron microscope (TEM) was used to observe the cell apoptosis. After 24 h incubation with different compounds as described above, cells were collected by centrifugation (1000 rpm, 3 min), washed twice with PBS solution, and fixed in freshly made 1% paraformaldehyde with 2% glutaraldehyde for 24 h. Samples were fixed for 2 h in 1% osmium tetroxide, and dehydrated in graded ethanol, and embedded in Araldite. Ultrathin sections were cut and stained with uranyl acetate and lead citrate, and then observed with TEM (JEM-101, Jeol Electron Inc., Japan).

Real-time polymerase chain reaction analysis

After being treated with different compounds for 24 h, cells were harvested and washed with ice-cold phosphate buffer. Total RNA was prepared using trizol reagent (TaKaRa, Japan) according to the manufacturer's instructions. The quantity and purity of the RNA were assessed by measuring the absorbance at 260 and 280 nm. The cDNA was synthesized from total RNA (2 µg) with oligo (dT) 20 primers using an M-MLV reverse transcriptase First-Strand Kit (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction (RT-PCR) was performed using SYBR Green master mix, and the detection of mRNA was analyzed using an ABI StepOne RT-PCR System (Applied Biosystems, Foster City, CA, USA). Primer sequences for the reference gene β-actin and the genes of interest were shown in Table 1. Typical profile times used were the initial step, 95°C for 10 min followed by a second step at 95°C for 15 s and 60°C for 30 s for 40 cycles with a melting curve analysis. The level of target mRNA was normalized to the level of the β-actin and compared with the control. Data were analyzed using the $\Delta\Delta C_T$ method.

Western blotting

After being treated with different compounds for 24 h, cells were harvested and washed with ice-cold phosphate buffer. The whole-cell extracts were obtained by lysing cells with radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, China). Protein concentrations were determined using the BCA method (Beyotime Institute of Biotechnology, China). Equal amounts of protein were loaded, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After being blocked with 5% skimmed milk in Tris-buffered saline (pH 7.6) at room temperature, the membranes were incubated overnight at 4°C with primary antibodies for bax (1:5000; ID: ab32503, Abcam, USA) and caspase-3 (1:3000; ID: p42574, Epitomic - An Abcam Company, China), laminin (1:1000; ID: ab11575, Abcam, USA), collagen IV (1:800; ID: ab6586, Abcam, USA), and β-actin (1:3000; Cat no.: AP0060, BioWorld, USA). After being

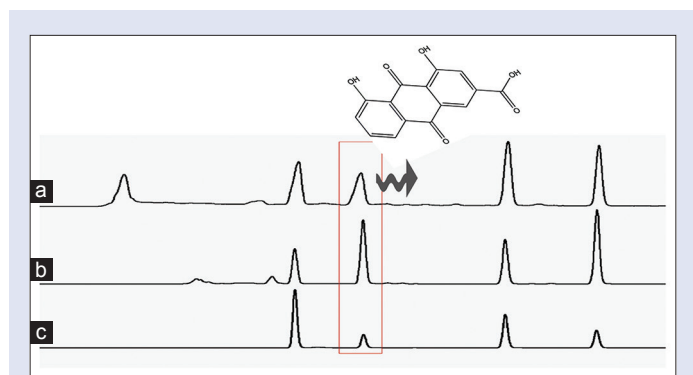


Figure 1: Chemical structure of rhein and HPLC chromatograms of extracts of Shenkang injection; (a) chromatograms of extracts of Shenkang injection; (b) chromatograms of extracts of Rhubarb; and (c) chromatograms of extracts of blended standard substances

incubated with the respective second antibody, immune complexes were detected using ECL Western blotting reagents (Thermo scientific, Thermo Fisher Scientific Inc. USA). Immunoreactive bands were quantified using the Gel Doc TM XR with Lab image 4.0.1 software (Bio-Rad Laboratories, Marnes-La-Coquette, France). Values were corrected with the absorbance of the internal control (β -actin).

Statistical analysis

The differences were tested using ANOVA. All values are expressed as mean \pm standard deviation, and statistical significance was defined as $P < 0.05$.

RESULTS

Effect of rhein on cell proliferation induced by high glucose

RMC proliferation was evaluated using MTT analysis. First, we performed MTT assay on cells cultured under NG and found no significant different [Figure 2a]. Second, we performed the same assay on cells which were exposed to HG, and the data show that compared with the NG group, 25 mM glucose (HG) alone increased RMC proliferation

after treatment for 12 h, 24 h, and 48 h, respectively. RH notably inhibited HG-induced RMC proliferation with the concentration of RH 40 μ M at 12 h and inhibited cell proliferation with the concentration of RH 40 μ M, 20 μ M, and 10 μ M after treatment for 12 h, 24 h, and 48 h and which was positively related to the concentration of RH [Figure 2b]. As expected, 25 mM MN, as an osmotic control, did not alter mesangial cell growth, suggesting that the HG-triggered RMC proliferation was not due to high osmolality [Figure 2b].

Effect of rhein on cell cycle distribution exposed to high glucose

To further evaluate the effect of RH treatment upon cell proliferation; and after exposed to various concentration of RH for 24 h, we then performed flow cytometry. The results show that compared with the HG group RH - 40 μ M, RH - 20 μ M, and RH - 10 μ M, significantly increased the proportion of cells in G_1 phase with the proportion of 78.8 \pm 3.1%, 67.8 \pm 2.1%, and 65.1 \pm 2.5%, respectively; which was related to the concentration of RH. Compared with the group of MN and NG group, HG is more likely to promote the cell cycle, with G_1 : 45.9 \pm 1.6%, S: 24.2 \pm 1.5%, G_2 : 30.1 \pm 2.8%. There was no notably difference between MN and NG group which suggested that the cell cycle change of HG-triggered was not due to high

Table 1: Primer sequences for the reference gene β -actin and the genes of interest

Name	Forward primer (5'-3')	Reverse primer (5'-3')
Laminin	GATCTCTAGACCGCAGCCTC	GCTCTGCCCGTAACTCACTT
Collagen IV	CAAAGGTGCTATCCCTCTGTAAG	ATAAGCTCTGGGTTGGTTCTGA
Caapase-3	GAGCTTGGAAACGCGAAGAAA	TAACCGGGTCCGGTAGAGTA
Bax	GTCCTCACTGCCTCACTCAC	TTCCCCGTTCCCCATTCATC
β -actin	CGTAAAGACCTCTATGCCAACA	CGGACTCATCGTACTCTGCT

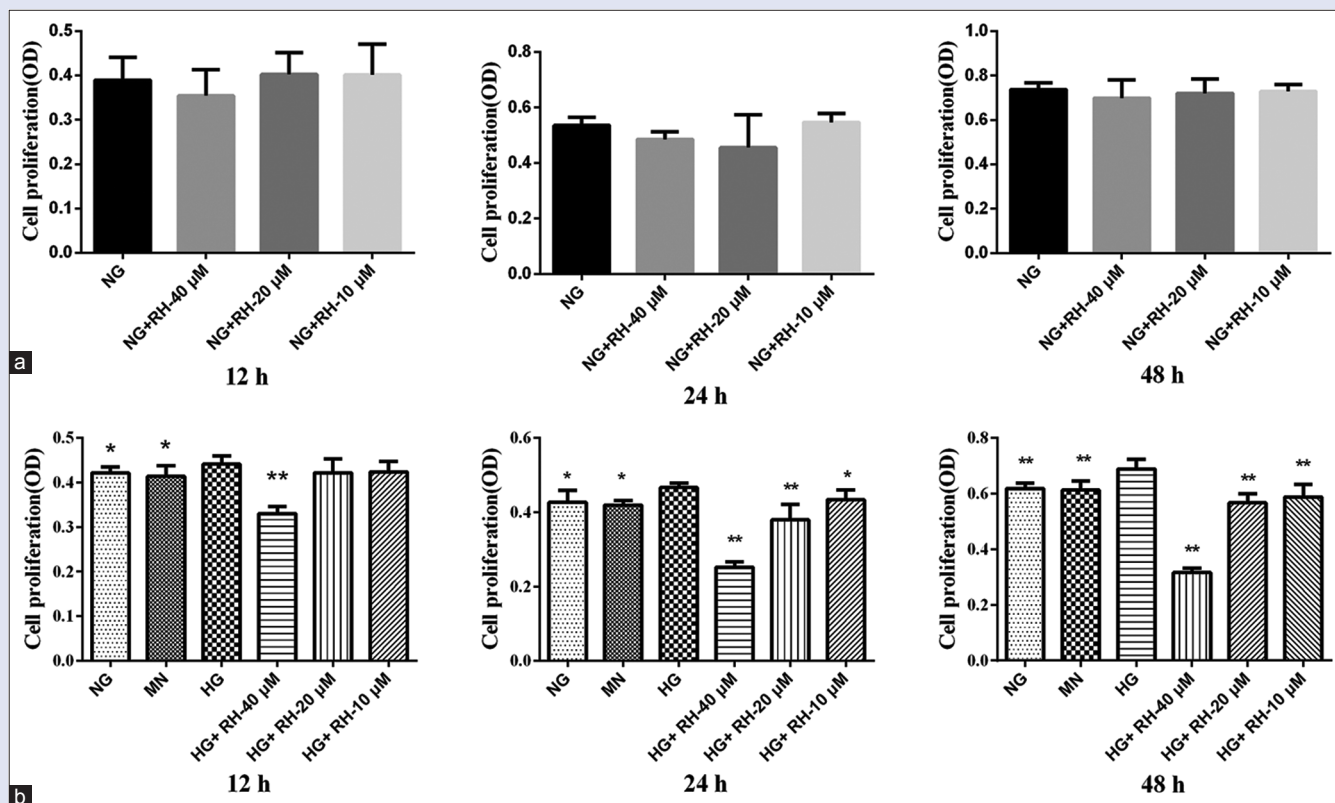


Figure 2: Effect of rhein on mesangial cell proliferation stimulated by high glucose using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay ($n = 8$). (a) Effect of rhein on mesangial cell proliferation under normal glucose for different time; (b) Effect of rhein on mesangial cell proliferation under high glucose for different time. Values are given as mean \pm standard deviation. * $P < 0.05$ versus high glucose group, ** $P < 0.01$ versus high glucose group

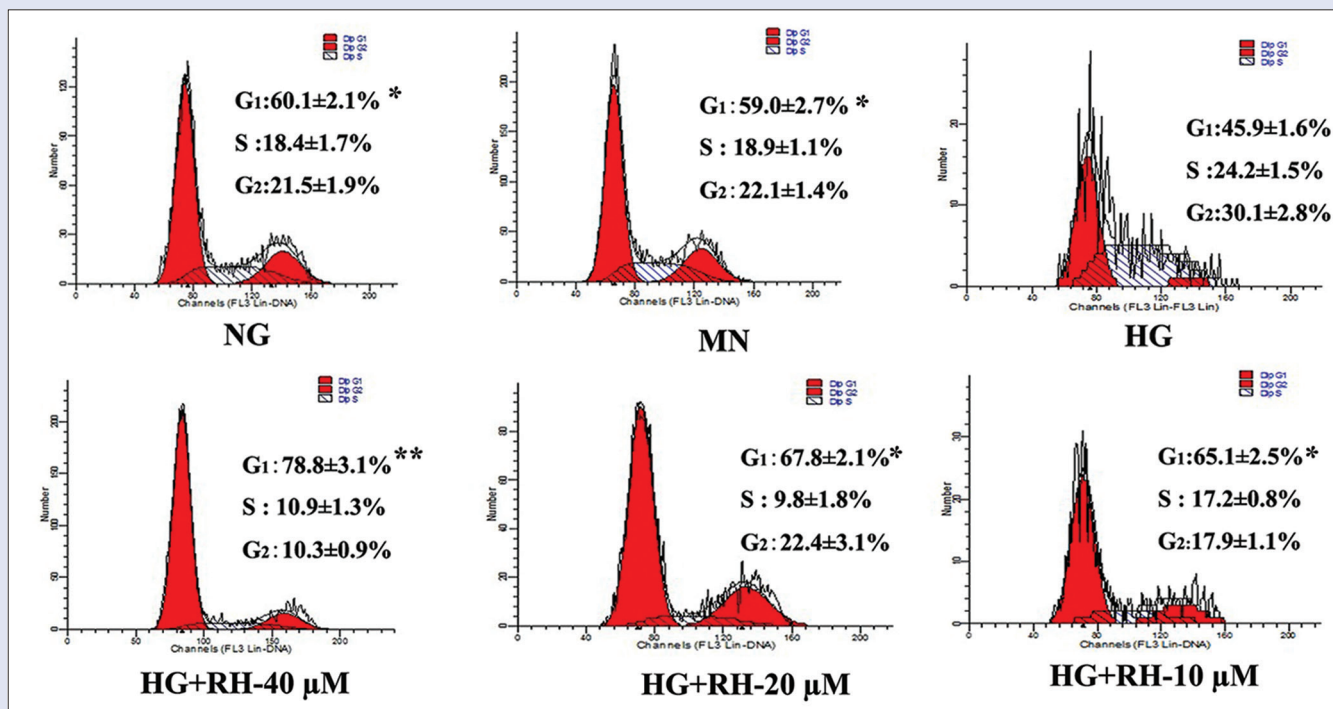


Figure 3: Effect of rhein on cell cycle distribution induced by high glucose in renal mesangial cell ($n = 3$). Values are given as mean \pm standard deviation. * $P < 0.05$ versus high glucose group, ** $P < 0.01$ versus high glucose group

osmolarity [Figure 3]. This experiment showed RH could inhibit RMC cell proliferation (cultured in HG) via increased the proportion of G1 phase; and HG could promote the cell cycle of RMC.

Effect of rhein on cell apoptosis exposed to high glucose

Effect of RH on cell apoptosis exposed to HG assayed by TEM.

TEM is a method for morphological observation through clearly differentiating nuclei and organelle and is used to confirm apoptosis in this research. As showed in Figure 4, there were no typical morphological changes in the group of MN, NG, and HG cells. However, when cells were exposed to different concentrations of RH for 24 h, obvious apoptotic morphological changes were observed in these cells. Typical morphological changes are chromatin condensation [Figure 4c], vacuolization in mitochondria [Figure 4a], and degranulation in endoplasmic reticulum [Figure 4b]. This result demonstrated that RH could induce RMC apoptosis.

Effect of rhein on cell apoptosis exposed to high glucose assayed by real-time polymerase chain reaction and Western blot

To determine the effects of RH upon apoptosis in RMC cells, changes in the mRNA of apoptosis-related proteins were evaluated using RT-PCR analysis. After exposed to different concentration of RH for 24 h, the mRNA of caspase-3 and bax were significantly increased in comparison with MN, NG, and HG; while there is no notable difference between MN and NG [Figure 5a]. To verify the results of RT-PCR, then we evaluated the expression of related proteins using Western blotting and after exposed to different concentration of RH for 24 h. RH can raise the expression of related proteins of bax, caspase-3, and cleaved-caspase-3 [Figure 5b] and which was related to concentration of RH.

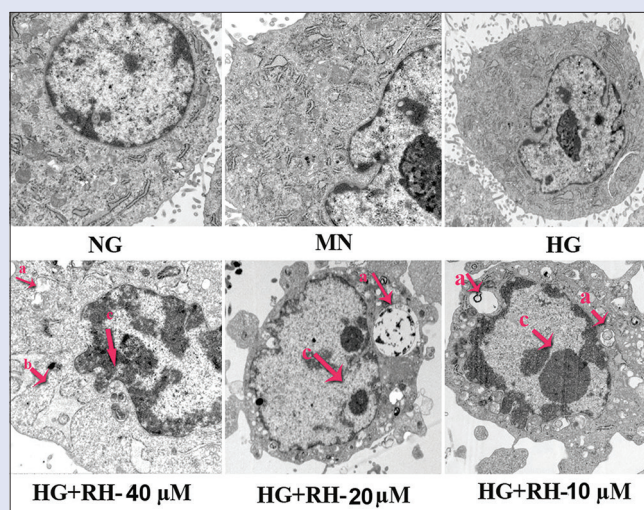


Figure 4: Effect of rhein on cell apoptosis exposed to high glucose assayed by transmission electron microscope ($\times 20,000$). Chromatin condensation (c), vacuolization in mitochondria (a) and degranulation in endoplasmic reticulum (b)

Effect of rhein on the expression of collagen IV and laminin exposed to high glucose

To explore the effect of different concentrations of RH on the expression of ECM exposed to HG. In comparison with NG group, cells were exposed to HG can significantly upregulate the protein of collagen IV and laminin as well as its related mRNA. While both RH 40 μM and 20 μM can significantly downregulate the protein of collagen IV and laminin as well as its related mRNA in contrast with HG group. However, there are

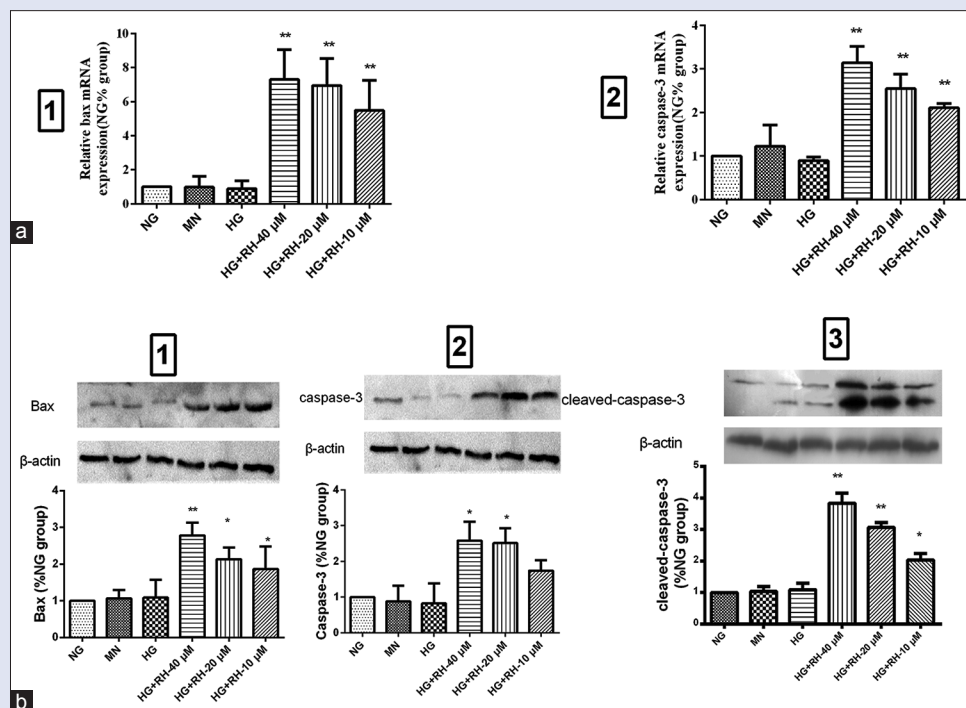


Figure 5: Effect of rhein on cell apoptosis induced by high glucose ($n = 3$). The mRNA expression assayed by real-time polymerase chain reaction (a) and the protein assayed by Western blot (b). Values are given as mean \pm standard deviation, * $P < 0.05$ versus high glucose group, ** $P < 0.01$ versus high glucose group

no notable differences between NG group and MN group, which shows high osmolarity cannot affect the expression of ECM [Figure 6].

DISCUSSION

In this study, we have found an inhibitory effect of RH on HG-induced proliferation of RMCs and downregulation of collagen IV and laminin. To future elucidate the underlying mechanism and demonstrated this function via inhibiting DNA synthesis that resulting in arrest of the cell-cycle transition from G_1 to S phase. We also find morphological changes of RMCs following exposure to a different concentration of RH, which is characterized by chromatin condensation, vacuolization in mitochondria, and degranulation in endoplasmic reticulum. Moreover, for future corroborated this data through flow cytometric assay of cell apoptosis in RMCs exposed to RH for 24 h. Finally, we demonstrate the underlying molecular mechanism by RT-PCR and Western blot analysis and found upregulation the mRNA of caspase-3, bax, and downregulation the mRNA of collagen IV and laminin as well as their related protein. Altogether, our data show RH can inhibit HG-triggered proliferation of RMCs via regulating the cell cycle and induction of cellular apoptosis.

Study shows RMCs proliferative responses to a variety of stimuli are associated with matrix accumulation and the development of glomerular sclerosis, which finally leads to progressive renal disease.^[17] An HG concentration has been shown to contribute mainly to uncontrolled cell proliferation and hypertrophy in RMC,^[18] distal tubular epithelial cells,^[19] and vascular smooth muscle cells^[20] in diabetes. In this research, rat RMCs were used as an *in vitro* model that has been widely used in the study of cell proliferation in the early stage of DN.^[21,22] As a consequence of clinical trials have demonstrated that HG is the principal cause of renal damage in both type 1 and type 2 diabetes, and HG culture condition was employed to stimulate RMC proliferation in this study.

In cell cycle progression, traversing the G_1 -S phase boundary is coupled to DNA synthesis and followed by entry into G_2 phase, and finally, mitosis

occurs in M phase. Here, compared with NG treatment, HG significantly promote the cell cycle while treating with different concentration of RH, the cell cycle was restrained at G_1 phase, and there was no difference between NG and MN groups. This phenomenon indicated HG could induce RMC proliferation by promoting the progression of RMCs cell cycle, and RH can inhibit RMC proliferation by restraining the progression of cell cycle at G_1 phase.

The process of apoptosis is characterized by specific biochemical and morphological changes. Many methods have been developed to test apoptosis including the morphological study, flow cytometric analysis, biochemical assay, and so on. For morphological observation, following exposed to different concentration of RH, typical morphological changes were observed (chromatin condensation, vacuolization in mitochondria, and degranulation in endoplasmic reticulum). However, there were no typical morphological changes in MN, NG, and HG. For further explore, the mechanism of RH on cell apoptosis, we performed flow cytometry to test cell apoptosis and found RH could obviously induce cell apoptosis in comparison with MN, NG, and HG.

Bax is a Proapoptotic protein allowing apoptosis to occur through the intrinsic, damage-induced pathway, and amplifying that one occurring *via* the extrinsic receptor-mediated pathway.^[23] Bax is present in viable cells and activated by Proapoptotic stimuli. Caspase-3 is a member of cysteine proteases originally discovered for its role in apoptosis (programed cell death). During apoptosis, caspases participate in cascades whereby the upstream (initiator) caspases activate the downstream (executioner) caspases, which cleave a specific group of cellular targets.^[24] In this paper, we demonstrated RH can up-express the level of the mRNA of bax, caspase-3, and its related protein.

SKI is a traditional Chinese medicine who has a special effect to treat DN. Although it is widely used in the clinic in the china, the precise molecular functions are unclear. Here, we show an inhibitory effect of RH on RMCs not only cellular proliferation but also expression of ECM

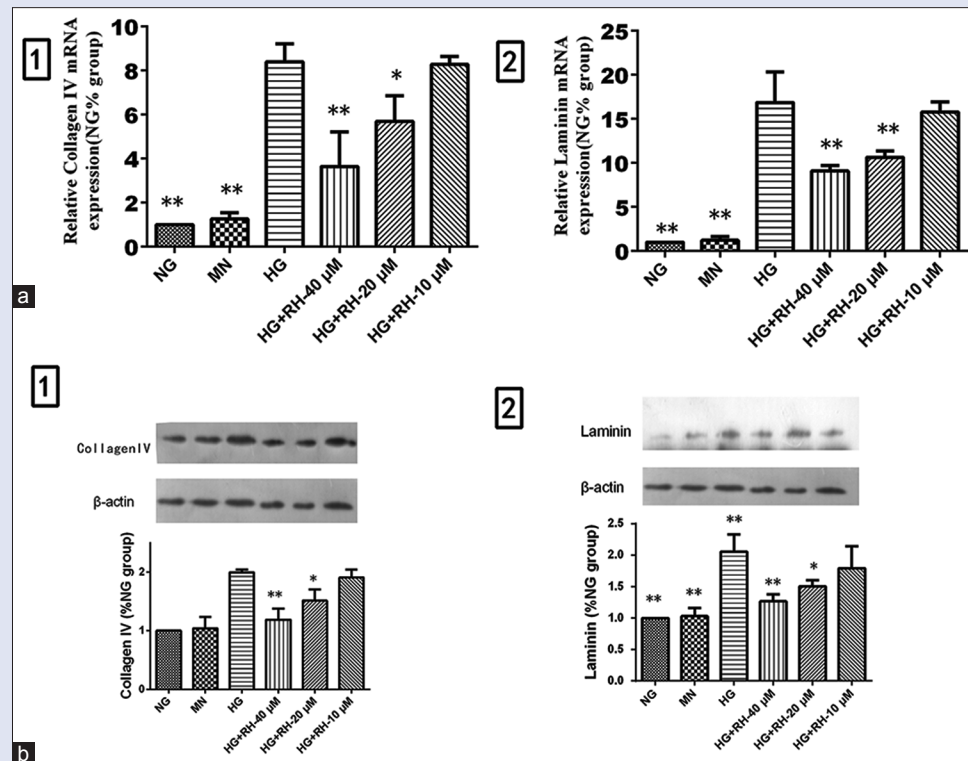


Figure 6: Effect of rhein on the expression of collagen IV and laminin exposed to high glucose ($n = 3$). The mRNA expression assayed by real-time polymerase chain reaction (a) and the protein assayed by Western blot (b). Values are given as mean \pm standard deviation, * $P < 0.05$ versus high glucose group, ** $P < 0.01$ versus high glucose group

and the underlying functions due to cell cycle arrest at G_1 phase and induction of cellular apoptosis.

CONCLUSION

In this study, we have demonstrated that RH can inhibit HG-induced RMCs proliferation and the expression of ECM. At the molecular level, we have shown that the mechanism of growth suppression due to cell cycle arrest at G_1 phase and induction of RMCs apoptosis which is through upregulating the gene of bax, caspase-3, and its related proteins. Our study provides evidence that RH is the main active ingredient of SKI in treating DN.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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