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Original Article

Herbal compound of pueraria and hawthorn alleviates fibrotic renal injury in diabetic fatty rats

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Abstract: Objective: Pueraria and hawthorn (RH) contains anti-oxidative properties that improve insulin resistance and lower blood glucose and lipid levels. The purpose of this study was to investigate the protectional effect of RH in diabetic nephropathy model. Methods: Sprague-Dawley rats treated with streptozocin or not were randomly divided into four groups: normal, diabetes, Diabetes mellitus (DM) plus RH treatment, and DM plus RH preventative treatment. Renal damage indexes body weight, blood glucose, and blood lipids were measured. Meanwhile, we detected that PTEN, FN, α-SMA and collagen IV levels in kidney in each rats. Results: Our data identified that RH alleviated 24 h urinary protein excretion, blood glucose level and renal injury in diabetic rats, reduced the expression of α-SMA and collagen IV, and increased expressions of PTEN and FN. Conclusion: These findings highlight the potential of RH as a traditional Chinese medicine (TCM), by shedding lights on the mechanism of RH action in diabetes treatment.

Keywords: Diabetes, renal fibrosis, pueraria, hawthorn, PTEN

Introduction

Diabetic nephropathy (DN) is one of the most common microvascular complications of diabetes mellitus and a major cause of mortality among diabetic patients [1]. The pathological changes of DN mainly include basement membrane thickening, glomerular mesangial expansion, endothelial cell proliferation and seriously, glomerular sclerosis and extracellular matrix (ECM) accumulation that could ultimately lead to end stage renal disease (ESRD) [2]. However, current treatment options for DN are still limited.

Although the mechanism of DN has not yet been fully understood, previous studies suggest that disturbed lipid metabolism, oxidative stress and abnormally expressed cytokines play an important role [3, 4]. In addition, long-term high glucose environment can lead to abnormal glycometabolism which in turn can activate cytokines such as TGF-β and CTGF as well as type IV collagen, resulting in renal fibrosis [5]. Fibronectin (FN) is an important component of extracellular matrix and is distributed in glomerular basement membrane, mesangial and blood plasma. Previous research has shown that FN is a key factor involved in ECM accumulation and glomerular fibrosis [6]. Moreover, blocking FN can significantly alleviate the degree of pathological changes.

At present, traditional Chinese medicine (TCM) has been used for treating DN in China with promising potential [7]. However, the majority of studies about the therapeutic effect of TCM in treating DN and its mechanism have focused on TCM effective constituents such as puerarin. Furthermore, exiting studies have found that pueraria and hawthorn decoction can improve metabolic syndrome and hyperlipemia. Pueraria, known as 'Gegen' in Chinese, is the root of Pueraria Leguminosae family of plants

[8], which was shown recently to be beneficial for diabetic patients [9]. Puerarin, one of the main effective components of pueraria, can enhance the immunity, eliminate oxygen free radicals, reduce tumor sizes, and lower blood pressure, in both animal experiments and clinical trials [10]. Hawthorn is a Rosaceae family of plants widely grown in China [11], which has strong antioxidant activity in vitro [12].

Pueraria and hawthorn (RH) were widely used to make TCM decoction in China. However, whether RH compound is effective in regulating blood lipid and glucose, and the renoprotection mechanisms of RH in diabetic patients remain unclear. Here, we examined whether RH could rescue renal injury in a diabetic rat model, whose renal pathological damage was induced by high-fat diet and low-dose STZ. We found that RH decrease serum glucose and urinary protein effectively in the experimental diabetic rats. Pretreated and treated with RH alleviate glycogen, collagen and fats deposition in the renal as well as diabetic glomerulopathy, including glomerular hypertrophy, glomerular extracellular matrix accumulation. Additionally, our data illustrate that RH alleviate renal fibrosis obviously in diabetic rats, possibly by decreasing expressions of PTEN, α-SMA and collagen IV.

Materials and methods

Drugs and animals

The herbal compound RH was produced by Shanghai Second Military Medical University. The production process was as follows: two successive reflux extractions of one kilogram of each medicinal herb with 60% alcohol (5:1 solvent to herb volume ratio) for 90 min each [7]. The extract was reduced-pressure evaporated till the volume was 590 ml. Pueraria solution and hawthorn solution were mixed at a volume ratio of 1:1 and the mixture was used as herbal compound in this study.

Eight-week-old male Sprague-Dawley (SD) rats, purchased from the Laboratory Animal Center of the Academy of Zhejiang Medical Sciences (Zhejiang, Certificate No 0012371), weighed between 250 and 300 grams. All rats were housed at a SPF-grade laboratory animal room in the Animal Laboratory Center of Ningbo University. The room was kept at a temperature of 20-25°C, humidity of 50-60%, with a 12/12 h light/dark cycle. The experimental procedure lasted 15 weeks, during which rats were kept on the standard or high fat diet (HFD) according to their group assignments. The high fat diet was purchased from Pu Luteng Bio-Technique Co. Ltd. The high fat diet is the standard diet supplemented with 16.9% fat and 10.2% casein. All animal studies were approved by the Animal Ethics and Welfare Committee (AEWC) of Ningbo University (no: AEWC-2015-32).

The rats were then randomly divided into 4 groups (n=5 each): 1) Normal group, fed with the standard diet. 2) Diabetes mellitus (DM) group, fed with the high fat diet, gavaged with normal saline, and injected with STZ. 3) DM plus RH treatment group (gavage of RH solution 2 ml/day, which means 3/day). 4) DM plus RH pretreatment and treatment group.

The diabetic model was produced by intraperitoneal injection of STZ at week 10 (diluted to 1% in a 10 mmol/L citrate buffer, pH 4.5) at 25 mg/kg body weight after the rats were fasted for 12 h. Blood glucose was measured 72 h after STZ injection, and rats with blood glucose over 11.1 mmol/L were considered to be diabetic. All the blood glucose of the rats injecting with STZ reached the diagnostic criteria. Rats in the control group were injected with an equal volume of the citrate buffer. RH was administered by intragastric gavage, and rats not receiving RH were given normal saline through the same route.

Body weight, blood glucose, oral glucose tolerance test (OGTT), urinary protein and biochemical parameters

Body weight was measured once a month and blood glucose once a week throughout the experimental period. Blood samples were collected from the tail vein. The fasting blood glucose (FBG) was measured after fasting for 12 h, and postprandial blood glucose (PBG) was measured at 2 h after meal. The oral glucose tolerance test was measured on overnight-fasted rats and rats were given an oral glucose dose of 2 g/kg by gavage at the end of the study [13]. Blood glucose level was measured with One Touch Ultra test strips and blood glucose meter (Johnson & Johnson Medical Ltd., Shanghai, China).

Table 1. Primer sequences

RT-qPCR primers	
PTEN F	5'-AACCGATACTTCTCTCCAAAT-3'
PTEN R	5'-TTCATCAAAAGGTTCATTCTC-3'
FN F	5'-CCAGCTTTGGACACTCCCAT-3'
FN R	5'-ATTCAGGCCTGGCCAATCAA-3'
α-SMA F	5'-CATTGCTGACAGGATGCAGAA-3'
α-SMA R	5'-GAAGCATTTGCGGTGGACAA-3'
Collagen IV F	5'-GTTGGTCTACCGGGACTCAA-3'
Collagen IV R	5'-GTTGGTCTACCGGGACTCAA-3'

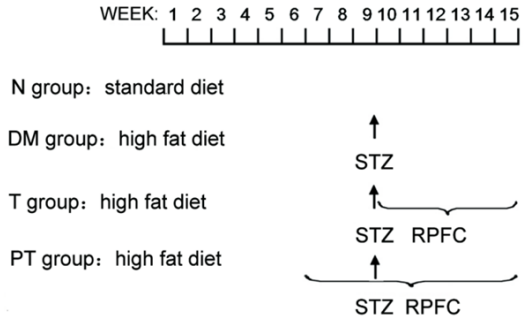

Figure 1. Schematic of experimental group design.

Clinically, microalbuminuria was defined as urine albumin at 30-300 mg/g creatinine [14]. Rats were housed individually in metabolic cages for 24 h to collect urinary samples. The urinary protein was determined by MODULAR P800 Automation Biochemist Analyzer (Roche, Basel, Switzerland).

In order to measure biochemical parameters, blood samples were collected from the femoral artery into EDTA-anticoagulant tubes after a 12 h fasting at the end of study, and then centrifuged to collect the serum for detection of serum insulin, total protein (TP), high-density lipoprotein cholesterol (HDL-C), low-density lipoproteins (LDL-C), total cholesterol (TC), triglycerides (TG), ureanitrogen (BUN), creatinine (CREA) and uric acid (UA) with a MODULAR P800 Automation Biochemist Analyzer (Roche, Basel, SWIT). At last, rats were sacrificed and kidney tissues were collected for histological staining and RNA and protein extraction.

Renal histological analysis

Kidney tissues were fixed for 48 h, dehydrated with graded ethanol, paraffin imbedded routinely, and cut into 3 μm sections. The sections were subjected to hematoxylin and eosin (H&E), periodic acid schiff (PAS), and Masson trichrome staining and observed under a microscope.

Real time reverse transcriptase polymerase chain reaction (RT-qPCR)

Total RNA was extracted from kidney tissues using TRIzol reagent (Invitrogen, US). One ug of total RNA was used for reverse transcription. Polymerase chain reactions were performed in a Lightcycler 480 II Authorized Thermal Cycler (Roche, Basel, SWIT) using the following protocol: denaturation at 95°C for 5 min, followed by 45 cycles of 95°C 10 s, 60°C 20 s, and 72°C 30 s. The primer sequences are detailed in **Table 1**. Relative mRNA levels were calculated with the 2-ΔΔCt method using the gene β-actin as the internal reference.

Western blot

Forty microgram renal tissues were lysed with radioimmunoprecipitation assay (RIPA) lysis buffer (Solarbio, Beijing, China) supplemented with 1 mM phenylmethane-sulfonyl fluoride for protein extraction. Samples (50 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membrane, blocked and then incubated with primary antibodies overnight. The primary antibodies are as follows: PTEN (Abcam, Cambridge, MA, 1:1000), HRP-labeled secondary antibodiesy (Beyotime, China, 1:1000). The immunoreactions were visualized with an enhanced chemiluminescence (ECL) reagent using a gel imaging and analysis system (Tanon, Shanghai, China). The density values of protein bands were quantified using the software Image J (NIH, Maryland, US).

Immunohistochemical staining

Kidney slides were used to observe α-SMA and collagen IV on renal tissue sections examined by immunohistochemical staining with the following primary antibodies: monoclonal mouse anti-rat α-SMA (Abcam, Cambridge, MA, 1:50) and polyclonal rabbit anti-rat collagen IV (Abcam, Cambridge, MA, 1:200). The sections were then incubated with HRP-labeled secondary antibodies for 30 min. Color reaction was developed with diaminobenzidine (DAB, Boster, China) and counterstained with hematoxylin. Quantitative analysis of the brown positive staining was performed using Image-Pro Plus 6.0 (Media Cybernetics, USA).

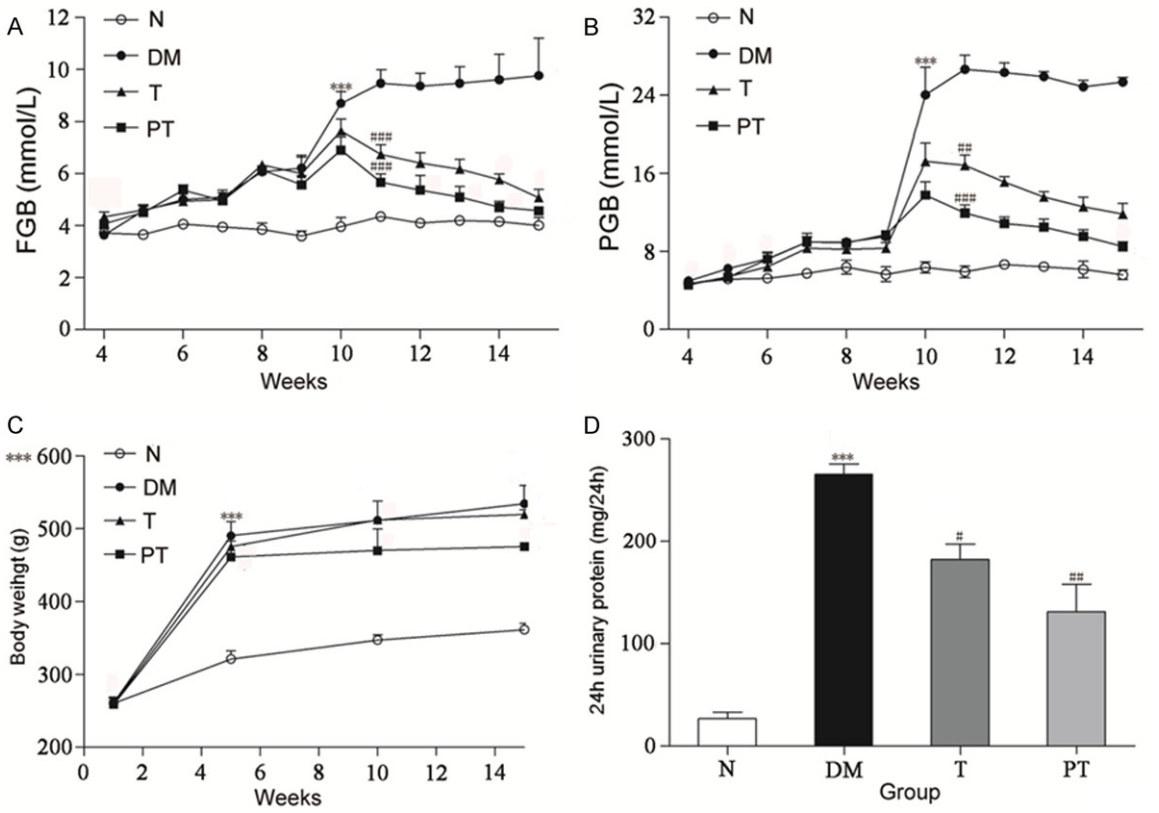

Figure 2. Both RH prevention and treatment decrease serum glucose and urinary protein effectively in diabetic rats. Fasting blood glucose (FBG) in (A) and postprandial blood glucose (PBG) in (B) were measured. Body weight (C) was measured every two weeks. 24 h urinary protein (D) was measured at the end of the study. Results are presented as mean ± SD; n=3. ***Indicates *P*<0.001 compared with normal group. #Indicates *P*<0.05 compared with DM group. ##Indicates *P*<0.01 compared with DM group. ###Indicates *P*<0.001 compared with DM group.

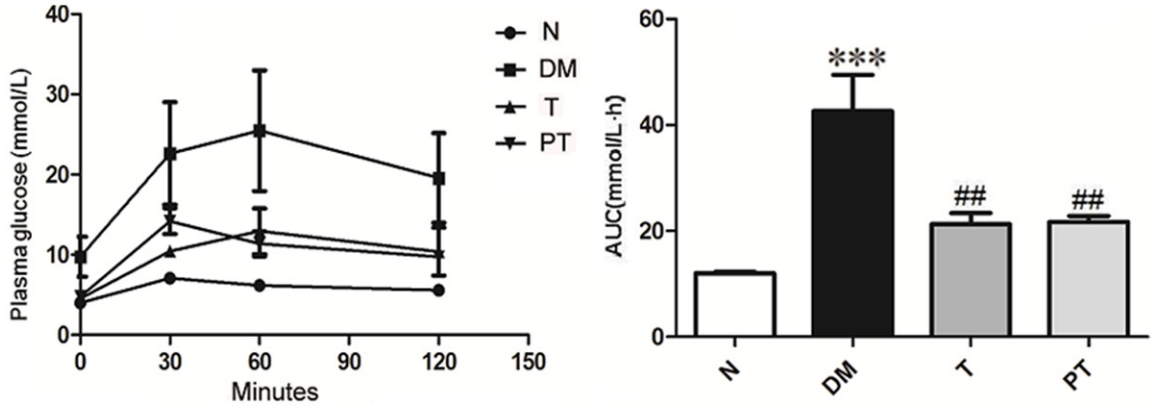

Figure 3. RH significantly improves glucose intolerance of diabetic rats. Plasma glucose during oral glucose tolerance test (OGTT) and its area under the curve (AUC).***Indicates P<0.001compared with normal group. ##Indicates *P*<0.01 compared with DM group.

Statistical analysis

All the data are presented as mean ± standard deviation of the mean (S.D). The differences among groups were analyzed by randomized block design analysis by one-way ANOVA using SPSS 13.0 software, followed by LSD (Least-Significant Difference) test, while for comparisons between two groups, student's t-test (normally distributed) or Mann-Whitney test

Table 2. Biochemical parameters for all rats at the end of the study

	N	DM	T	PT	*p* value	F
TP	71.00±10.47	69.73±4.95	79.25±6.29	74.80±3.96	0.127	2.856
HDL-C	0.86±0.20	1.13±0.11	1.00±0.32	1.15±0.15	0.531	2.815
LDL-C	0.55±0.02	0.58±0.04	0.46±0.21	0.55±0.08	0.677	0.533
TC	1.48±0.01	1.82±0.04	2.55±0.88	1.82±0.87	0.222	1.959
TG	2.44±1.73	1.98±0.86	3.20±1.28	2.23±0.54	0.710	0.481
BUN	5.40±0.86	4.83±0.91	5.57±0.45	5.30±0.14	0.477	0.967
CREA	23.00±4.24	23.00±2.00	18.33±0.58	21.00±1.41	0.628	0.619
UA	76.50±17.68	101.00±10.58	115.50±33.23	115.50±12.02	0.248	1.897

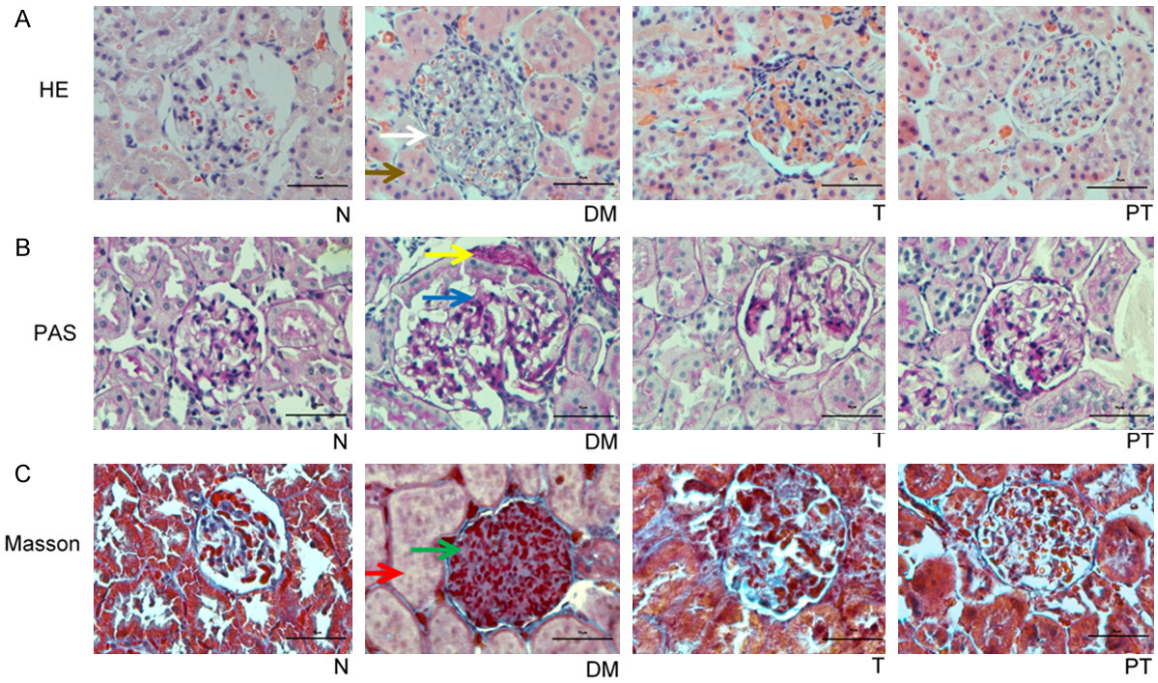

Figure 4. RH protects renal damage in diabetic rats (scale bar: 2.5 μm). Representative pictures of (A) H&E, (B) PAS, and (C) Masson trichrome staining are presented, respectively.

(non-normally distributed) was used. *P* value less than 0.05 was considered statistically significant.

Results

Effects of RH on blood glucose, body weight, and urinary protein and OGTT

Rats were randomly divided into four groups. All of the rats were fed on high fat diet and injected with STZ at week 10 except the rats in the normal controls. Rats in normal group (N) and diabetes mellitus (DM) group were gavaged with normal saline; DM plus RH treatment group (T) gavaged with RH at week 10-15; DM plus RH pretreatment and treatment group (PT), gavaged with RH at week 7-15 (**Figure 1**). FBG were measured from week 4 to 15 (**Figure 2A**). The rats in DM, T and PT groups showed higher FBG than the N group from week 5 to 15. FBG increased after injection of STZ at week 9. However, FBG in T and PT groups declined slowly after STZ injection, and showed significant statistical differences (p_T=0.0064, p_{PT}=0.0012) with DM group from 11 weeks to the end of the experiment. Moreover, FBG in T and PT groups were comparable to those in the normal group at the end of the study. PBG were also measured starting from week 4 (**Figure 2B**). The rats in DM, T and PT groups showed higher PBG than the normal group from week 7 to 15. PBG

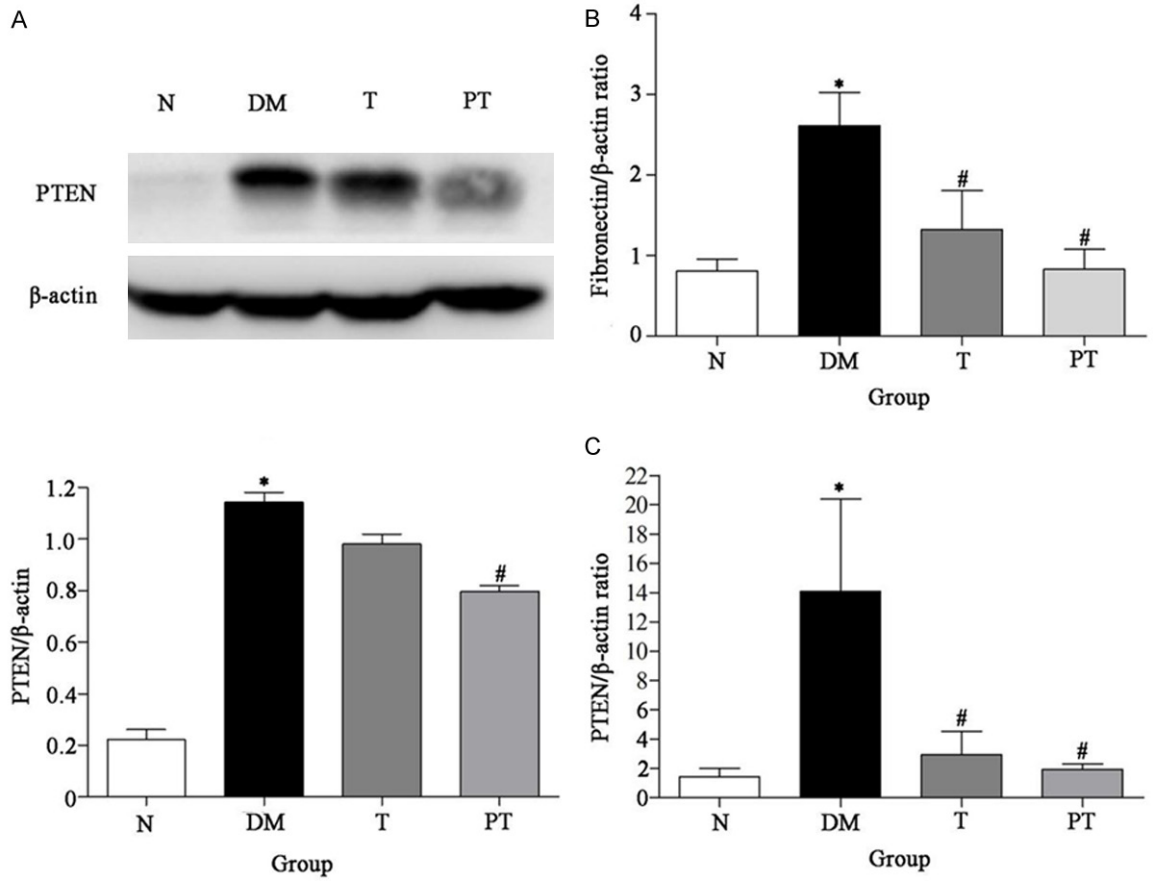

Figure 5. RH reduces the expression of PTEN and FN in the renal of diabetic rats. A. Protein expression of PTEN in renal was determined by Western blot. B and C. Renal mRNA expression of PTEN and FN were determined by real-time RT-PCR. *Indicates $p<0.05$ compared with normal group. #Indicates $P<0.05$ compared with DM group.

increased significantly ($P<0.001$) after injection of STZ in DM, P and PT groups. However, PBG in T and PT groups declined slowly from week 10, and were significantly lower ($p_T=0.0019$, $p_{PT}<0.001$) than DM group from 11 weeks to the end of the study. Based on the above results, we conclude that both RH prevention and treatment can decrease serum glucose effectively in the experimental diabetic rats.

As shown in **Figure 2C**, body weights increased significantly ($P<0.001$) in the DM, T and PT groups as compared with the normal group. However, the body weights of the three diabetic groups were not influenced by STZ, and RH did not change the body weight, either. Compared with the normal group, urinary protein was significantly higher in all other groups. Urinary protein in the T group and PT group were significantly decreased ($p_T=0.0144$, $p_{PT}=0.0024$) than in the DM group (**Figure 2D**). So, both prevention and treatment of RH decrease urinary protein effectively.

In the OGTT, blood glucose in the normal and PT groups reached peak levels at 30 min after the rats were given oral glucose. While in the other two groups, blood glucose reached peak levels at 60 min after rats were given oral glucose. Rats in the T and PT groups showed significantly statistical differences compared with rats in the DM group ($p_T=0.0054$, $p_{PT}=0.0037$), illustrate the RH has significant hypoglycemic effect (**Figure 3**). These results indicating that treatment, especially pretreatment of RH could improve glucose intolerance of diabetic rats.

Effects of RH on biochemical parameters

In order to observe the effects of RH on biochemical functions, we measured the biochemical parameters in all rats at the end of the study. All parameters selected showed no sig-

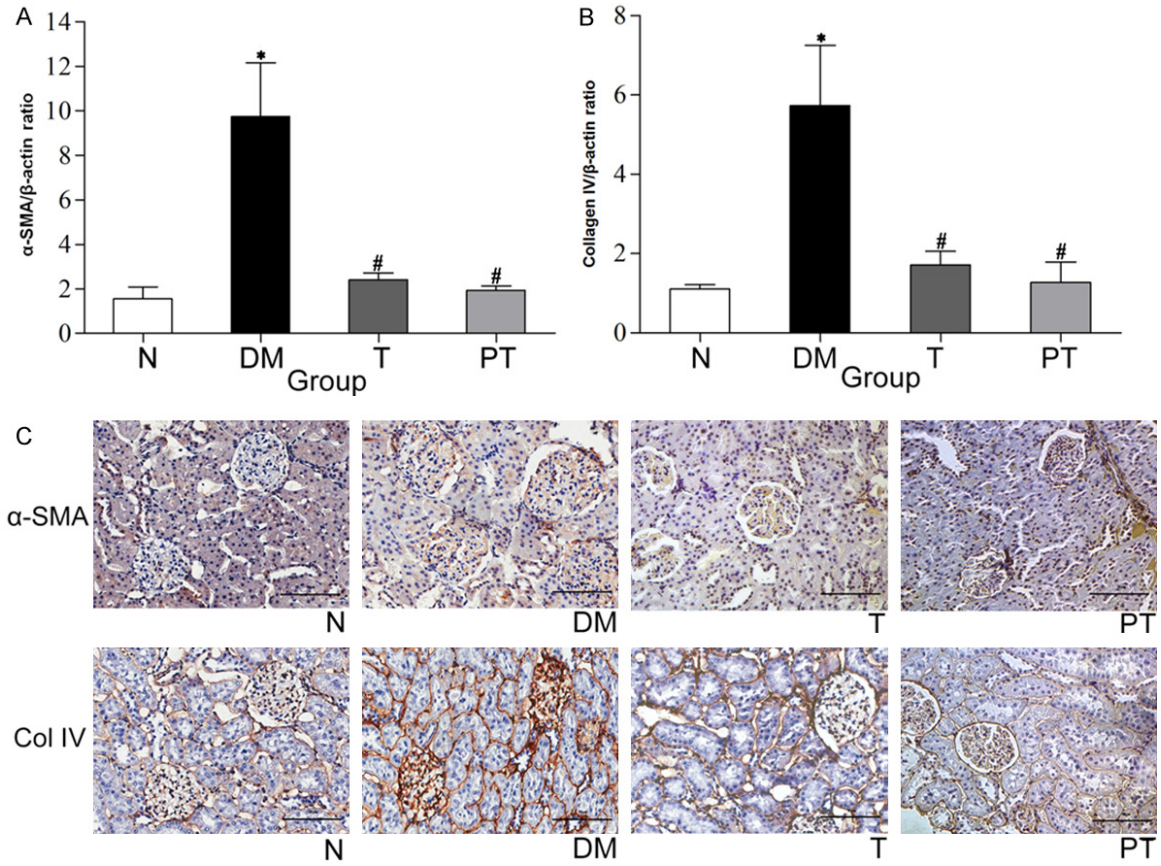

Figure 6. RH reduces the expression of α-SMA and collagen IV in the renal of diabetic rats (scale bar: 5 μm). The mRNA expression of (A) α-SMA and (B) collagen IV in renal were determined by real-time RT-PCR. Renal protein expression of α-SMA and collagen IV (C) were determined by immunohistochemistry. *Indicates $P<0.05$ compared with normal group. #Indicates $P<0.05$ compared with DM group.

nificant differences among the groups (**Table 2**). These data may indicate that RH compound did not affect the indexes of lipid level and kidney functions measured.

Protective effects of RH on renal damage induced by high-fat diet and low-dose STZ

Compared with the kidney morphology in the normal group, glomerular hypertrophy, glomerular extracellular matrix accumulation, and renal capsule constriction were observed in the DM group with H&E staining (**Figure 4A**) and observed under optical microscope. Although mesangial matrix expansion and renal capsule constriction were visible in both RH treated and pretreated (T and PT) groups, renal damage was much better than the corresponding RH untreated groups (**Figure 4**).

Sedimentary glycogen was observed with PAS staining while the collagen accumulation was observed with Masson trichrome staining on rat kidney sections. For the DM group, the mesangial cells with severe hyperplasia (**Figure 4B**, blue arrow) and tubule-interstitial expansion were observed, and collagen deposition (**Figure 4C**, green arrow) was particularly obvious. Fortunately, these performances of renal damage were reduced after RH pretreated and treated (**Figure 4A-C**). In addition, the DM group showed visible balloon adhesions (**Figure 4B**, yellow arrow) and collapsing of capillary loops. Moreover, the renal tubules appeared foamy (**Figure 4C**, red arrow) because of the accumulation of fats and glycogen, while RH treatment reduced its accumulation in the kidney compared to the DM group. Overall, RH pretreated and treated reduced renal pathologic damage.

RH alleviate renal fibrosis in diabetic rat

PTEN and FN play a significant role in renal fibrosis [15]. We quantified the expression levels of PTEN and FN by RT-PCR and Western blot analysis. The protein levels of PTEN were sig-

nificantly improved (P=0.019) in rats of the DM group than in normal group (**Figure 5A**). Notably, the mRNA expression levels of PTEN and FN were lower (PTEN*: p_T=0.1, p_{PT}=0.045;* FN*: p_T=0.027, p_{PT}=0.013*) in rats of the T and PT groups than in DM group (**Figure 5B**, **5C**). These results indicate that RH may alleviate renal fibrosis through decreasing the expression levels of PTEN and FN in diabetic rats.

α-SMA and collagen IV are the common molecular markers for fibrosis [16]. We therefore compared the expression of α-SMA and collagen IV in renal tissues of all groups. Treatment with RH in rats of the T and PT groups significantly reduced (α-SMA: p_T=0.026, p_{PT}=0.032; collagen IV: p_T=0.035, p_{PT}=0.022) the mRNA expression of α-SMA and collagen IV as compared with the DM group (**Figure 6A**, **6B**). The results of the immunohistochemical staining demonstrated that, in the normal group, α-SMA-positive staining was limited to the vascular walls, whereas significantly marked immune staining was observed in glomerular capillaries in the DM groups. In the T and PT groups, the expression levels of α-SMA were considerably decreased (**Figure 6C**). Overall, these data strongly suggest that RH alleviate renal fibrosis in diabetic rats.

Discussion

Regardless of the current treatment interventions of patients with DN using Angiotensin-Converting Enzyme Inhibitors (ACEIs) and Angiotensin Receptor Blocker (ARBs) [17], cases of diabetes are on the rise, and its renal complications are causing morbidity and mortality of more and more people [10]. So there is an urgent need to develop new therapies for DN. In recent years, more and more studies have shown that some herbs can treat chronic nephritis albuminuria, including astragalus, polygonum and ebony.

Pueraria is the root of Pueraria Leguminosae family of plants [8], and it has been reported to effectively reverse the fibrotic process [18]. Moreover, pueraria improves insulin resistance and lowers blood sugar and lipids levels. The possible mechanism of its effect was thought to be through the inhibition of the TGF-β1/Smad2 pathway [19]. Another potential anti-diabetic herb is hawthorn. To date, the research on hawthorn has focused on its role in reducing blood lipids and cholesterol levels, and lowering blood pressure, but no study on hawthorn and renal diseases has been conducted.

Previous studies suggested that deregulation of PTEN occurs in lung and skin fibrosis, diabetes and renal injury [20], but the potential role of PTEN and its associated mechanisms in the progression of renal fibrosis remain unknown. The PTEN expression in kidney tubule and interstitium was dramatically increased in STZ-mediated injury [20]. We hypothesized that RH may contribute to alleviate kidney injury in the diabetic rats through decreasing PTEN. FN is an extracellular matrix protein that plays an important role in cell adhesion, migration, tumor invasion and fibrosis [21]. Reduced expression of both PTEN and FN has been noted in diabetic rats pretreated and treated with RH (**Figure 6**).

It is generally known that α-SMA is involved in chronic kidney disease changes [22] and it expresses at low level in mesangial cells but not in podocytes normally. It becomes widely expressed when mesangial cells and podocytes are damaged and show phenotypic changes. Moreover, increased level of renal α-SMA in diabetic rats is associated with tubule-interstitial fibrosis in DN [23]. The presence of α-SMA will induce synthesis and secretion of ECM leading to renal fibrosis. Collagen IV is synthesized and secreted by a variety of cells in the kidney (glomerular mesangial cells, endothelial cells, epithelial cells, and tubular epithelial cells). The deposition of collagen IV might contribute to alterations in glomerulus and tubular function and play an important role in the development of tubule-interstitial fibrosis and glomerular sclerosis [24]. Many studies have confirmed that the processes of glomerulus hypertrophy, and glomerulosclerosis induced by long-term high glucose have a close relationship with accumulation of ECM proteins such as fibronectin, collagen, and laminin [25]. Our findings in the current study showed that the expression of α-SMA and collagen IV were significantly increased in diabetic rats, while renal α-SMA and collagen IV expression were decreased in rats with treatment of RH. We therefore speculate the therapeutic activity of RH in diabetic rats was partly mediated by inhibition of PTEN expression. Since PTEN is known to activate FN and α-SMA expression, our results are consistent with previous studies showing that the high glucose milieu can

increase secretion of FN and the synthesis of α-SMA [26]. Moreover, it was reported that rats with diabetes exhibited markedly increased renal Smad1 and collagen IV expression and ECM deposition [27]. Thus, our data suggest that RH played a role in inhibiting PTEN, which indirectly leads to decrease of α-SMA and collagen IV.

Here we proposed a potential mechanism to explain how herbal compound of pueraria and hawthorn alleviate diabetic renal injury in diabetic fatty rats. It would also be necessary to determine whether RH could help ameliorate renal injury in patients with advanced DN and other nephropathies in future studies. Based on the current study, RH is safe and causes no side-effect in rats. However, it has been indicated that pueraria is not suitable for people who have gastritis diseases, and if given intravenously could cause itching and nausea, while hawthorn is not recommend for anyone who has digestive diseases. RH is a traditional medicine which has been in use for a long time with promising prospect of treating diabetes, although careful clinical evaluations will be needed.

In summary, our data illustrate that herbal compound of pueraria and hawthorn could decrease blood glucose and urinary protein effectively, and reduce glycogen, collagen and fats deposition in the renal. Compared with untreated diabetic rats, pretreated and treated with RH alleviate glomerular hypertrophy, glomerular extracellular matrix accumulation, renal capsule constriction, as well as mesangial matrix expansion. Additionally, RH alleviated renal fibrosis obviously in diabetic rats, possibly by inhibiting PTEN and decreasing expressions of α-SMA and collagen IV. Our study demonstrated for the first time that RH was effective in alleviating renal injury in diabetic fatty rats, which provide support for the clinical applications of RH for treating DN.

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Disclosure of conflict of interest

None.

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