Rehmannia glutinosa ameliorates the progressive renal failure induced by 5/6 nephrectomy

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A B S T R A C T
Aim of the study: Rehmannia glutinosa, the steamed root of the Scrophulariaceae family, has been widely used in Asian countries for the treatment of renal diseases. In this study, we evaluated the renoprotective effect of aqueous extract of Rehmannia glutinosa in progressive renal failure.

Materials and methods: The effects of Rehmannia glutinosa on renal function, 24-h proteinuria, and the expression of angiotensin II, angiotensin II type 1 (AT1) receptor, TGF-β1, and type IV collagen in renal cortex were analyzed in progressive renal failure rats induced by 5/6 nephrectomy.

Results: Rehmannia glutinosa reduced the serum creatinine level, 24-h urinary protein excretion, and glomerulosclerosis, and it also inhibited the expression of angiotensin II, AT1 receptor, TGF-β1 and type IV collagen in the renal cortex.

Conclusions: These results suggest that the renoprotective effect of Rehmannia glutinosa might be mediated by suppressing the expression of angiotensin II and AT1 receptor and by regulating TGF-β1 and type IV collagen expression.

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1. Introduction

The progressive deterioration of kidneys by primary disease triggers a chain of events that result in glomerular and tubulointerstitial inflammation, progressive losses of the remaining nephrons, glomerulosclerosis and tubulointerstitial fibrosis, and development of chronic renal failure (Fogo, 1995, 2001). The central process in this phenomenon is the progressive accumulation of extracellular matrix (ECM) in the glomeruli and in the tubulointerstitial space. Accumulation of ECM occurs as a result of increased synthesis, decreased degradation, or some combination of these processes (Rodríguez-Peña et al., 2001). Angiotensin II and transforming growth factor-β1 (TGF-β1) seem to be involved in the accumulation of ECM proteins, thereby contributing to the progression of tissue inflammation, glomerulosclerosis or tubulointerstitial fibrosis (Border and Noble, 1994; Matsusaka et al., 1996). It has been shown that enhanced ECM synthesis due to angiotensin II is mediated by the generation of active TGF-β1, indicating that angiotensin II induces conversion of the latent form of TGF-β1 to the active form (Kagami et al., 1994). In addition, TGF-β1 contributes to renal fibrosis, which is characterized by the loss of renal tubules and interstitial capillaries, as well as the accumulation of ECM proteins, such as collagen type IV, fibronectin, and laminin (Eddy, 2000).

Rehmannia glutinosa (Gaertn.) Ligosch is a member of the Scrophulariaceae family whose roots have been widely used in Asian countries for the treatment of renal diseases, inflammatory-related diseases, or diabetes mellitus (Zhang et al., 2008). There have been many reports on the pharmacological efficacy of Rehmannia glutinosa with respect to its diuretic activity (Zhang et al., 2008), neuroprotective effects (Li et al., 2006), anti-allergic reactions (Kim et al., 1998), anti-inflammatory effect (Kim et al., 1999), hypoglycemic effect (Zhang et al., 2004), and activation of bone metabolism (Oh et al., 2003). The main constituents of Rehmannia glutinosa are sitosterol and mannotol; other constituents include a small amount of strigomerat, campes-terol, catapol, rehmannin, 2-formyl-5-hydroxymethylfurane, and rehmaglutin D (Kang et al., 2005). Recently, it was reported that Rehmannia glutinosa ameliorates renal dysfunction in rats with ischemia/reperfusion-induced acute renal failure (Kang et al., 2005). However, the renal protective effects of Rehmannia glutinosa have never been assessed in an animal model of progressive renal failure.

In this study, we evaluated the influence of an aqueous extract of Rehmannia glutinosa on the renal function, proteinuria, and the expression of angiotensin II, angiotensin II type 1 (AT1) receptor, TGF-β1, and type IV collagen in the remnant kidney of rats with progressive renal failure induced by 5/6 nephrectomy.
2. Materials and methods

2.1. Experimental design and animals

Adult male Sprague Dawley rats, obtained from a local breeding colony, with initial weights of 250–300 g were used in this study. Rats were housed under normal conditions with a 12-h light/dark cycle at 23 ± 1 °C with 40% humidity and 12 air exchanges per hour, and received standard rat powdered diet and water. Under anesthesia with pentobarbital sodium (50 mg/kg i.p.), a 5/6 nephrectomy (NTX) was performed on each rat by removing the whole right kidney and upper and lower poles of the left kidney, thus ensuring the removal of at least two-thirds of the left kidney. Sham-operated rats underwent anesthesia, ventral laparotomy, and manipulation of the renal pedicles, without any removal of renal mass. After recovering from anesthesia, the animals were returned to their original cages. The NTX rats were then divided into two groups as follows: the vehicle-treated control group (N = 7) orally received normal saline once per a day with normal rat chow powder for 8 weeks. The Rehmannia-treated group (N = 7) received 500 mg/kg aqueous extract power of Rehmannia glutinosa orally administered over the same period. Animals were monitored and sacrificed at 8 weeks after NTX. Kidneys were harvested for analysis of morphologic and molecular parameters.

2.2. Preparation of Rehmannia glutinosa aqueous extracts

Rehmannia glutinosa (Gaertn.) Ligosch was obtained from the Department of Pharmaceutical Preparation of Korean Medicine, Korean Medical Hospital, Kyung Hee University. The quality was tested according to the Korea Food & Drug Administration’s and our hospital’s standards. A voucher specimen (Code number HSHA/2007) was deposited at our department. The air-dried and crushed materials were added to distilled water, and extraction was performed by heating for 4 h at 100 °C. Then, the extract was concentrated with a rotary evaporator (Model NE-1, EYELA Co., Japan) and dried with a freeze dryer (Model FD-1, EYELA Co., Japan). The collection rate of the final aqueous extracts was 7.5%.

2.3. Measurements of serum and urine parameters

Twenty-four-hour urinary samples were collected with the aid of metabolic cages. The animals were placed in individual metabolic cages for 24 h to collect urine samples after receiving access only to tap water on the day before the experimental rats were killed. Total urinary volume and urinary protein were measured by conventional methods, and the urinary protein excretion was calculated. Blood was collected from the heart using ethylenediaminetetraacetic acid (EDTA) as an anticoagulant, and the plasma was stored at −20 °C. Serum creatinine, blood urea nitrogen (BUN), albumin, total-cholesterol, LDL-cholesterol, and triglyceride were measured using a commercial automatic analyzer.

2.4. Histology

The kidneys were immersion-fixed in 10% buffered formalin and embedded in paraffin for a light microscopic study. Two sections of 4-μm thickness (at an interval of 100 μm) per animal were stained with periodic acid-Schiff (PAS) reagent. The sections were photographed under an Olympus photomicroscope (Olympus BX-50, Olympus Optical, Tokyo, Japan). Mesangial matrix expansion was determined as PAS-positive materials presented in the mesangial region excluding cellular elements. The percentage of PAS-positive area in each glomerulus was analyzed using computer-assisted image analysis software (MetaVue, Molecular Devices, USA). Ten glomeruli, randomly selected in the two slides from each rat, were evaluated by two investigators without knowledge of the origin of the slides.

2.5. Immunohistochemistry

Sections 4-μm thick were placed onto slides, deparaffinized, and rehydrated. The following primary antibodies were used: polyclonal rabbit anti-rat TGF-β1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-type IV collagen (SouthernBiotech), and polyclonal rabbit anti-angiotensin II antibodies (Bachem, Peninsula). Sections were subjected to microwave irradiation in citrate buffer to enhance antigen retrieval and were incubated with the primary antibodies overnight at 4 °C in a humidified chamber. After rinsing, the slides were incubated in biotinylated antimouse/rabbit/goat IgG (H + L), made in horse (Vector Laboratories, Inc.). Tissue sections were consecutively stained with avidin–biotin horseradish peroxidase complex (Vectastain ABC ELITE kit; Vector Laboratories, Burlingame, CA) for 15 min before a substrate solution of 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma Chemical, St. Louis, MO) was added. Sections were then counterstained in hematoxylin and photographed under a photomicroscope. Semi-quantitative analysis of the percentage of TGF-β1, type IV collagen, and angiotensin II-positive staining area in the glomerulus was performed using computer-assisted image analysis software. For each kidney, more than 30 glomerular profiles, cut in equatorial section planes and successively appearing in the visual field of the microscope, were examined.

2.6. Evaluation of plasma TGF-β1 and angiotensin II concentrations

To perform the measurements of TGF-β1 and angiotensin II, rats from the sham-operated, vehicle-treated control and Rehmannia-treated groups, after being followed for 8 weeks, were killed by decapitation, and trunk blood was collected in chilled tubes containing either EDTA or a cocktail of enzyme inhibitors. The amounts of TGF-β1 and angiotensin II were determined by enzyme-linked immunosorbent assay (ELISA) using the TGF-β1 Emax ImmunoAssay System (Promega) and angiotensin II EIA Kit (Bachem, Peninsula) according to the manufacturers’ directions.

2.7. Semiquantitative reverse transcription polymerase chain reaction (RT-PCR) of TGF-β1, type IV collagen and AT1 receptor mRNA in the renal cortex

To evaluate the expression level of TGF-β1, type IV collagen and AT1 receptor mRNA, we performed a semiquantitative RT-PCR. The reaction mixture containing 1 μg RNA, PCR buffer, 5 mM MgCl2, 1 mM dNTP, 20 U of RNasin, 2.5 μM of Oligo (dT) and 100 U of Moloney murine leukemia virus reverse transcriptase was incubated at 42 °C for 50 min, then heated at 70 °C for 15 min. All experimental samples were reverse transcribed in the same set of experiments, and the efficiency of the reaction was controlled by β-actin amplification. PCR was carried out in a gradient PCR device (Eppendorf, Hamburg, Germany). Each sample mixture contained PCR buffer, 2.5 mM dNTP, 2 U Taq polymerase, and 5 pM of each primer: TGF-β1, 5′-CCCGCATCCAGAAGGCCTC-3′ and 5′-GCGG- GACTGGCCAGGCTTCTAG-3′; Collagen IVα1, 5′-TCCGGGAGATTTGTTTT-3′ and 5′-CACCTTGGACTGCGG-3′; AT1, 5′-CCGAAAAA-CAGTCCCCC-3′ and 5′-TACATTTCGGTGGATGACAG-3′. The PCR consisted of 35 cycles at 94 °C (for TGF-β1, Collagen IVα1 and AT1) and 30 cycles (for β-actin) at 55 °C. The reaction products were subjected to densitometry after electrophoresis on 2% agarose gel and staining with ethidium bromide.
Table 1  
General characteristics and the effects of *Rehmannia glutinosa* in 5/6 nephrectomized rats.

<table>
<thead>
<tr>
<th></th>
<th>Sham op. (n = 7)</th>
<th>NTX (n = 7)</th>
<th><em>Rehmannia</em>-NTX (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>461.66 ± 25.17</td>
<td>392.5 ± 41.46**</td>
<td>386.66 ± 23.86**</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>19.57 ± 2.04</td>
<td>53.53 ± 10.57**</td>
<td>41.33 ± 16.35**</td>
</tr>
<tr>
<td>Cr (mg/dl)</td>
<td>0.54 ± 0.03</td>
<td>1.35 ± 0.11***</td>
<td>1.00 ± 0.32**</td>
</tr>
<tr>
<td>Albumin</td>
<td>2.29 ± 0.19</td>
<td>1.97 ± 0.13</td>
<td>2.17 ± 0.11**</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>21.32 ± 3.63</td>
<td>21.32 ± 3.63</td>
<td>21.32 ± 3.63</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>67.50 ± 31.48</td>
<td>73.50 ± 39.03**</td>
<td>73.50 ± 39.03**</td>
</tr>
<tr>
<td>Urinary protein excretion (mg/d)</td>
<td>5.03 ± 2.28</td>
<td>147.80 ± 50.33**</td>
<td>147.80 ± 50.33**</td>
</tr>
<tr>
<td>Mesangial matrix areas of glomerulus (%)</td>
<td>8.13 ± 3.35</td>
<td>21.32 ± 4.90**</td>
<td>21.32 ± 4.90**</td>
</tr>
</tbody>
</table>

Results are presented as the mean ± S.D. Sham op., sham-operated rats; NTX, 5/6 nephrectomy plus vehicle-treated rats; *Rehmannia*-NTX, 5/6 nephrectomy plus *Rehmannia glutinosa*-treated rats. BUN, blood urea nitrogen; Cr, creatinine.

* * P < 0.05 compared with sham-operated rats.
** P < 0.01 compared with sham-operated rats.
*** P < 0.001 compared with sham-operated rats.
* P < 0.05 compared with vehicle-treated rats.
** P < 0.01 compared with vehicle-treated rats.
*** P < 0.001 compared with vehicle-treated control rats.

2.8. Statistical analysis

Statistical comparisons were performed using one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test. All data are presented as means ± standard deviations (S.D.). All P-values are two-tailed, and a P-value less than 0.05 was considered significant.

3. Results

3.1. Effects of *Rehmannia glutinosa* on renal function and biochemical parameters

The serum urea nitrogen (BUN), creatinine, total cholesterol, LDL-cholesterol, triglyceride level, and urinary albumin excretion were found to be significantly increased and albumin levels were decreased at 8 weeks after 5/6 nephrectomy compared with those of the sham-operated rats. *Rehmannia* treatment significantly inhibited the increase of serum creatinine level (P < 0.01), total cholesterol (P < 0.05), LDL-cholesterol (P < 0.01), and triglyceride (P < 0.05), and decreased the urinary protein excretion compared with the vehicle-treated control group (P < 0.01). Also, the albumin level was increased by *Rehmannia* treatment (P < 0.05). However, there was no significant decline in the BUN level, although the *Rehmannia* treatment group showed the effects of a decline in BUN. Although the mean body weight of the *Rehmannia*-treated group was slightly lower than that of the vehicle-treated control group at 8 weeks after 5/6 nephrectomy, the difference was not statistically significant (Table 1).

3.2. Histological changes

Fig. 1 shows light micrographs representing typical glomerular lesions from the kidneys of sham-operated rats, vehicle-treated control rats, and *Rehmannia*-treated rats. In sham-operated rats, the glomeruli and tubule were normal or almost normal. In the vehicle-treated control rats, severe matrix expansion was evident in the glomeruli, the number of glomeruli with sclerotic lesions was markedly increased, and interstitial fibrosis and mononuclear cell infiltration were also observed. Treatment with *Rehmannia* inhibited the progression of glomerulosclerosis and tended to prevent the appearance of interstitial fibrosis and mononuclear cell infiltration compared with the vehicle-treated control rats.

The mesangial matrix areas of the glomeruli in the vehicle-treated control rats were significantly increased compared with those of the sham-operated rats. Also, *Rehmannia* treatment significantly inhibited the increase compared with the vehicle-treated control rats (8.13 ± 3.35%; 21.32 ± 4.90% and 10.18 ± 3.15% for sham-operated, vehicle-treated, and *Rehmannia*-treated groups, respectively; P < 0.001)(Table 1 and Fig. 1).

Immunoreactive TGF-β1 was expressed in the glomeruli of the vehicle-treated control rats, and the area of expression was comparable to the location of the sclerotic lesions. In the semiquantitative analysis of the glomerular TGF-β1 expression, the percentage of glomeruli showing TGF-β1 expression in the vehicle-treated control rats increased. However, *Rehmannia* treatment did not show significant preventive effects (3.76 ± 1.93%, 18.24 ± 4.90% and 15.24 ± 5.96% for sham-operated, vehicle-treated, and *Rehmannia*-treated groups, respectively) (Fig. 1).

Type IV collagen-positive areas were observed throughout the nephron, particularly within the proximal tubules. In the semiquantitative analysis of the glomerular type IV collagen expression, *Rehmannia* treatment significantly inhibited the expression of type IV collagen in the glomeruli (2.93 ± 2.63%, 19.49 ± 4.41% and 14.22 ± 0.98% for sham-operated, vehicle-treated and *Rehmannia*-treated groups, respectively; P < 0.001)(Fig. 1).

Angiotensin II-positive cells in the sham-operated rats were detected almost exclusively in the glomerular arterioles and less consistently in interstitial or glomerular cells. However, in the vehicle-treated control rats, angiotensin II expression was detected in the afferent arterioles and in association with inflamed areas in the renal cortical interstitium, and this expression paralleled the severity of the renal damage. In the semiquantitative analysis of the interstitial angiotensin II expression, *Rehmannia* treatment significantly inhibited the expression of angiotensin II in the interstitium (0.23 ± 0.03 mm², 1.42 ± 0.91 mm² and 1.17 ± 0.94 mm² for sham-operated, vehicle-treated and *Rehmannia*-treated groups, respectively; P < 0.05)(Fig. 1).

3.3. TGF-β1 and angiotensin II concentrations in plasma

The plasma TGF-β1 and angiotensin II concentrations were 74.48 ± 16.81 pg/ml and 1.23 ± 0.15 ng/ml in the sham-operated rats, and the plasma levels of TGF-β1 and angiotensin II in the *Rehmannia*-treated rats (TGF-β1: 112.44 ± 48.72 pg/ml; angiotensin II: 2.97 ± 0.82 ng/ml) did not differ significantly from those of the vehicle-treated rats (TGF-β1: 111.33 ± 71.41 pg/ml; angiotensin II: 1.14 ± 0.27 ng/ml).

3.4. TGF-β1, type IV collagen and AT1 receptor mRNA expression in renal cortex

When the mRNA level in the vehicle-treated rats was adjusted to be 1, the TGF-β1 and type IV collagen mRNA levels were significantly inhibited by *Rehmannia* treatment (TGF-β1: 0.69 ± 0.01; type
IV collagen: 0.75 ± 0.48). The expression of AT1 receptor mRNA level was also reduced by Rehmannia treatment to 0.67 ± 0.18 (Fig. 2).

4. Discussion

In this study, we found that Rehmannia glutinosa inhibited the expression of angiotensin II and AT1 receptor, TGF-β1, and type IV collagen in the remnant kidney of progressive renal failure induced by 5/6 nephrectomy, consequently reducing urinary protein excretion and glomerulosclerosis and slowing the progression of renal function deterioration.

A steamed root of Rehmannia glutinosa Libosch (Scrophulariaceae), called ‘Sukjihwang’ in Korea, ‘Jukujio’ in Japan, and ‘Shudihuang’ in China, has been widely used in the East Orient for the treatment of renal diseases (Zhang et al., 2008). There have been reports on the renal protective efficacy of Rehmannia glutinosa. Rehmannia reduced the excretion of protein, improved glomeruli epithelial foot process fusion, and ameliorated kidney function in a nephropathy model induced by puromycin aminonucleoside (Zhang, 1999). Rehmanniae radix ameliorated the pathological condition of diabetic nephropathy in rats subjected to subtotal nephrectomy plus intraperitoneal administration of streptozotocin (Yokozawa et al., 2004). Recently, it was reported that Rehmannia glutinosa ameliorates renal dysfunction and leads to the up-regulation of protein levels of aquaporin 2 (AQP 2), Na, and KATPase and down-regulation of heme oxygenase-1 (HO-1) in rats with ischemia/reperfusion-induced acute renal failure (Kang et al., 2005). However, there has been no study including angiotensin II, TGF-β1 and type IV collagen, which play a central role in the progressive deterioration of the kidney.
The development of glomerulosclerosis to chronic renal failure is based on progressive changes of renal corpuscles and nephrons. The five-sixths nephrectomy or remnant kidney model in rats is a very useful and widely studied model for analyzing differences in the progression of chronic renal insufficiency. Regardless of the initial cause, the progression of chronic renal failure is characterized by the loss of renal cells and their replacement by ECM (Fogo, 1999, 2001; Rodríguez-Peña et al., 2001). Angiotensin II is involved in the accumulation of ECM proteins, thereby contributing to the progression of tissue inflammation, glomerulosclerosis, or tubulointerstitial fibrosis (Matsusaka et al., 1996). Many intervention strategies have been explored to inhibit angiotensin II, and therefore slow down or even reverse the progression of glomerulosclerosis (Brewster and Perazella, 2004). Angiotensin-converting enzyme (ACE) inhibitors and angiotensin II receptor type I antagonists (AT1RA) effectively reduce or even reverse the development of sclerotic lesions in animal models of progressive glomerular sclerosis (Brewster and Perazella, 2004). In this study, Rehmannia glutinosa showed an inhibitory effect on the expression of angiotensin II and AT1 receptor, which may support the traditional use of Rehmannia glutinosa as a treatment for progressive renal disease that could prevent the glomerulosclerosis as well as ACE inhibitors and AT1RA.

TGF-β1 exerts multifunctional regulation of cell proliferation and differentiation and acts as a key fibrogenic cytokine in many tissues by enhancing ECM synthesis (Massagué, 1990; Border and Noble, 1994). In addition, TGF-β contributes to the accumulation of ECM proteins, such as collagen types I, II, IV, and VII, fibronectin, and laminin by stimulating their synthesis (Ignotz and Massagué, 1986; Kim et al., 1998). In a rat model of experimental glomerulonephritis, TGF-β1 mRNA expression and protein production in the glomeruli are increased in parallel to increased mesangial matrix accumulation (Okuda et al., 1990); the increased matrix components can be prevented by injecting rats with TGF-β1 neutralizing antibodies (Border et al., 1990). In this study, Rehmannia glutinosa decreased TGF-β1 mRNA expression and type IV collagen accumulation, which induced the reduction of mesangial matrix areas and prevented the progression of glomerulosclerosis.

Based on these results, we assume that the renoprotective effect of Rehmannia glutinosa on progressive renal failure induced by 5/6 nephrectomy might be mediated by the suppression of angiotensin II and AT1 receptor expressions and by the regulation of TGF-β1 and type IV collagen expression.

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