

Rhein lysinate protects renal function in diabetic nephropathy of KK/HIJ mice

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Abstract. The purpose of the present study was to assess the protective effects of rhein lysinate (RHL) in a KK/HIJ mouse model of diabetic nephropathy (DN) and to explore its mechanism of action. A total of 4 groups were established: C57BL/J control, the KK/HIJ model and 25 and 50 mg/kg/day RHL-treated KK/HIJ groups. The KK/HIJ mouse model of DN was established by streptozotocin injection, followed by maintenance on a specific diet. The albumin-to-creatinine ratio (ACR) was determined at 5 weeks and at 16 weeks, the kidneys were harvested, and morphological examination and immunohistochemical analysis were performed. The levels of malondialdehyde (MDA), as well as superoxide dismutase (SOD) and glutathione peroxidase (GSH-px) activities in the kidneys were measured using appropriate assay kits. The expression of inflammatory factors and associated proteins was analyzed using western blot analysis. At 5 weeks, the levels of ACR in KK/HIJ mice were increased, which was inhibited by treatment with RHL. Treatment with RHL (50 mg/kg/day) decreased the body weight of KK/HIJ mice. Compared with the C57BL/J control, the KK/HIJ model mice had a significantly lower activity of SOD and GSH-px in the kidneys, but had significantly higher levels of MDA. Treatment of KK/HIJ mice with RHL significantly increased the activities SOD and GSH-px, and reduced the MAD level in the kidneys. Renal tubular epithelial cell edema was observed in KK/HIJ mice but not in C57BL/J mice. RHL decreased the incidence of renal tubular epithelial cell

edema and significantly decreased the expression of TNF- α and IL-6 as well as the expression and phosphorylation of NF- κ B in the kidneys. Therefore, DN is associated with the expression of inflammatory factors, renal tubular epithelial cell edema and renal dysfunction in KK/HIJ mice. RHL improves renal function by decreasing kidney inflammation.

Introduction

Diabetes mellitus is an increasing global health problem. Estimates for 2013 by the International Diabetes Federation (IDF) indicate that a total of 382 million have diabetes in 2013, and the number is expected to rise to 592 million by 2035 (1-3). Diabetic nephropathy (DN) is one of the most common complications of diabetes. An estimate of 30-50% of patients with diabetes develop renal manifestations (4-6). DN often leads to chronic kidney disease (CKD). Nearly 44% of end-stage renal disease (ESRD) patients that require hemodialysis are diabetic nephropathy patients (7). Inflammation and oxidative stress are associated with the pathogenesis of DN (8). Transcription factors such as nuclear factor κ of activated B cells (NF- κ B) (9), pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α) and interleukin 1 (IL-1) (10) are associated with inflammatory pathways in DN. Thus, it is important to treat DN using anti-inflammatory drugs.

Research using experimental models of type 2 diabetes with nephropathy may provide an enhanced understanding of this complication in this multifactorial disease (11). Qi *et al* (12) reported that KK/HIJ mice are more prone to DN, whereas the most widely used C57BL/6J mice are relatively resistant to developing DN. Rhein lysinate (RHL) is the lysine salt of rhein, which is one of the active components of rhubarb root (*Rheum palmatum* Linn or *Rheum tanguticum* Maxim) (13). A previous study by our group found that RHL reduced the levels of TNF- α , IL-6 and NF- κ B, decreased the incidence of glomerulonephritis and prolonged the median survival time of senescence-prone inbred strain 10 mice (14). However, to the best of our knowledge, the effect of RHL on DN has not been previously reported. The present study investigated the effect of RHL on DN in KK/HIJ mice.

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Materials and methods

Chemicals and reagents. Rhein lysinate (RHL) was synthesized at the Oncology Department of the Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College (Beijing, China; patent no. 2008100890258). The structural formula of this compound is presented in our previous study (13). Streptozotocin (STZ) was obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GSH-px) kits were obtained from Nanjing Jiancheng Co. (Nanjing, China). Antibodies targeting TNF- α (3707s), IL-6 (12912s), NF- κ B (8242s) and phosphorylated (p)-NF- κ B (3033s) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibody targeting β -actin (sc-8432) was provided by Santa Cruz Biotechnology (Dallas, TX, USA). The appropriate anti-mouse (7076s) and anti-rabbit (7074s) horseradish peroxidase-conjugated secondary antibodies were obtained from Cell Signaling Technology, Inc. Prestained protein marker p7708V was provided by New England Biolabs, Ltd. (Ipswich, MA, USA). Immobilon™ western kit and polyvinylidene difluoride (PVDF) membranes were obtained from Millipore (Billerica, MA, USA).

Animals and induction of DN in inbred mice. Induction of DN was performed according to a previous protocol (12). The inbred male C57BL/J mice (n=12) and KK/HIJ mice (n=36) (weight, 18–24 g; age, 8 weeks) and mouse food used in the present study were purchased from Beijing HFK Bioscience Co. (Beijing, China). The diet contained water ($\leq 8\%$), crude protein ($\geq 18\%$), crude fat ($\geq 6\%$), crude fiber ($\geq 5\%$), crude ash ($\leq 7\%$), as well as minerals and trace elements. All protocols were approved by the institutional animal care and use committee of Beijing Hospital (Beijing, China). Mice were housed in an environmentally-controlled facility maintained on an automatic 12-h light/dark cycle. Food and water were provided *ad libitum* throughout the study. The study used the following 4 groups: C57BL/J control mice (n=12), the KK/HIJ model mice (n=12), the 25 mg/kg/day RHL-treated KK/HIJ mice (n=12) and the 50 mg/kg/day RHL-treated KK/HIJ mice (n=12). At 10 weeks of age, STZ was administered to KK/HIJ mice by intraperitoneal injection (50 mg/kg/day, made freshly in 0.1 mol/l citrate buffer, pH 4.5) for 5 consecutive days with normal diet and mice received the diabetic diet for the remaining treatment time. After all of STZ injections, 25 or 50 mg/kg/day RHL was respectively administered to the animals by gavage in the 25 or 50 mg/kg/day RHL-treated KK/HIJ group for 15 weeks.

Analysis of urinary albumin. Urinary albumin was assessed by determining the albumin-creatinine ratio (ACR) in morning urine. Urine was collected monthly by a home-made mouse urine collection device which used a 96-well plate as the floor. Mice were able to move freely on this 96-well plate until they naturally urinated. Urine was collected by a pipette without contamination by feces. Urinary albumin and creatinine concentrations were determined using a microalbumin/creatinine reagent kit (SIEM-6011A) supplied by Siemens medical solutions diagnostics (Tarrytown, NY, USA).

Measurement of laboratory parameters in serum. Serum was collected at the end of the experiment. Serum creatinine, urea nitrogen and blood glucose were measured in the clinical laboratory using standard protocols.

Analysis of antioxidant activity. At the end of the experiment, mice were anesthetized by intraperitoneal injection of 10% chloral hydrate and sacrificed; the kidneys were quickly removed and placed in cold PBS. Renal tissue (50 mg) was dissected and homogenized in a glass Teflon homogenizer containing 450 ml precooled PBS and the homogenate was then centrifuged at 3,000 x g for 15 min at 4°C. The activities of SOD and GSH-px and the MDA content in the obtained supernatant were measured using test kits, referring to the supplier's manual.

Histological and immunohistochemical analysis. For histological examination, kidney tissues fixed with 4% buffered paraformaldehyde were dehydrated in ethanol and embedded in paraffin wax. The embedded kidney tissues were serially sectioned into 3- μ m slices and stained with hematoxylin (staining 5 min)-eosin (staining 2 min) (Beijing Solarbio Science & Technology Co., Ltd., Beijing China) at room temperature. To better characterize the inflammation of kidney tissues, immunohistochemistry detection of TNF- α and IL-6 was performed. After dewaxing with xylene, samples were incubated with 3% H₂O₂ to block endogenous peroxidase. All slices were incubated with 5% bovine serum albumin (Sigma Aldrich; Meck KGaA, Darmstadt, Germany) at 37°C for 1 h to block non-specific binding. Subsequently, the slices were incubated with anti-TNF- α (1:1,000) or anti-IL-6 (1:1,000) antibodies at 4°C overnight according to the manufacturer's protocol. As a negative control, the primary antibody was replaced with 5% bovine serum albumin at 4°C overnight. Positive staining was identified by visual observation of a yellow/brown pigmentation under the light microscope. Images were captured with an Olympus IX81 inverted microscope (Olympus, Tokyo, Japan).

Western blot analysis. The expression of TNF- α , IL-6 and NF- κ B, as well as the phosphorylation of NF- κ B in the kidneys were determined by western blot analysis using a procedure identical to that used in a previous study by our group (14).

Statistical analysis. Values are expressed as the mean \pm standard deviation. Statistical analysis was performed using SPSS 11.0 for Windows (SPSS, Inc., Chicago, IL, USA). Differences between groups of values were compared using a one-way analysis of variance and the Student-Newman-Keuls test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

RHL improves the kidney function of mice with DN. The ACR in mice in each group was determined at 5 weeks. Compared with that in the C57BL/6J control group, the ACR was significantly increased in the KK/HIJ model group ($P < 0.05$; Table I). Compared with those in the KK/HIJ model group, RHL (25 and 50 mg/kg/day) significantly decreased the ACR ($P < 0.05$; Table I). The ACR was 40.8 ± 8.6 , 486.5 ± 82.9 , 424.7 ± 78.6 ,

Table I. Effect of RHL on ACR, blood glucose, creatinine and urea.

Groups	C57BL/6J control	KK/HIJ		
		Model	RHL 25 mg/kg/day	RHL 50 mg/kg/day
ACR ($\mu\text{g}/\text{mg}$) 5 weeks	40.8 \pm 8.6	486.5 \pm 82.9 ^a	424.7 \pm 78.6 ^{a,b}	385.1 \pm 52.4 ^{a,b}
ACR ($\mu\text{g}/\text{mg}$) 15 weeks	45.12 \pm 10.5	553.6 \pm 82.1 ^a	442.3 \pm 61.5 ^{a,b}	310.5 \pm 49.7 ^{a,b}
Blood glucose (mmol/l)	6.53 \pm 0.51	12.36 \pm 3.0 ^a	9.53 \pm 2.81 ^{a,b}	8.50 \pm 1.90 ^{a,b}
Creatinine (mmol/l)	65.5 \pm 24.8	130.0 \pm 28.8 ^a	92.5 \pm 26.7 ^{a,b}	70.0 \pm 25.1 ^b
Urea (mmol/l)	7.0 \pm 2.1	12.5 \pm 3.2 ^a	11.7 \pm 1.8 ^a	9.6 \pm 2.2 ^{a,b}

^aP<0.05, compared with C57BL/J control group. ^bP<0.05, compared with KK/HIJ control group. Values are expressed as the mean \pm standard error (n=12). RHL, rhein lysinate; ACR, albumin-to-creatinine ratio.

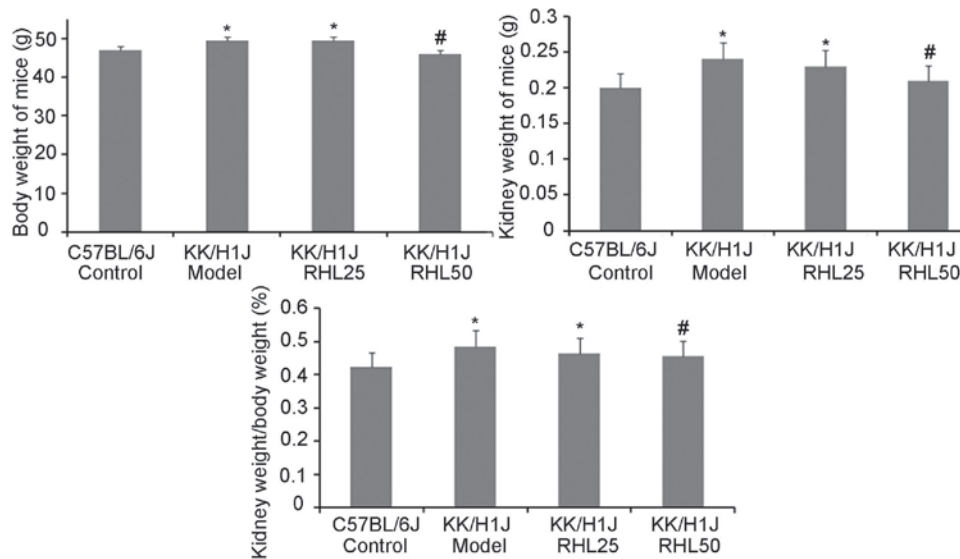


Figure 1. Kidney and body weights of the mice and the kidney weight to body weight ratio. The diabetic nephropathy model group mice were intraperitoneally injected with streptozotocin for 5 consecutive days and received a specific diet for 16 weeks. Values are expressed as the mean \pm standard deviation. *P<0.05 vs. C57BL/J control group; #P<0.05 vs. KK/HIJ model group.

385.1 \pm 52.4 $\mu\text{g}/\text{mg}$ in the C57BL/6J control group, KK/HIJ model group, 25 and 50 mg/kg/day RHL treatment groups, respectively at 5 weeks (Table I).

RHL protects kidney damage in diabetic KK/HIJ mice.

To assess the effects of RHL on DN in KK/HIJ mice, the effect of RHL on body and kidney weight was investigated (Fig. 1). Compared with those in the C57BL/6J control group, the body and kidney weights in the KK/HIJ model group and the 25 mg/kg/day RHL-treated group were increased. Compared with those in the KK/HIJ model group, the body and kidney weights in the 50 mg/kg/day RHL-treated group were decreased. The kidney weight-to-body weight ratio in KK/HIJ and C57BL/6J mice was also determined. As presented in Fig. 1, a significant increase in the kidney weight-to-body weight ratio was observed in all KK/HIJ mice, compared with that in C57BL/6J mice. The kidney weight-to-body weight ratio in the RHL 50 mg/kg/day group was decreased compared with that in the KK/HIJ model group. In addition, renal hypertrophy and hydronephrosis were observed in the KK/HIJ groups, including the RHL treatment groups.

RHL decreases the ACR, as well as blood glucose, creatinine and urea in mice with DN. To assess the effect of RHL on the kidney function of KK/HIJ mice with DN, the ACR, as well as blood glucose, creatinine and urea were detected at 15 weeks (Table I). Compared with those in the C57BL/6J control group, the ACR, as well as blood glucose, creatinine and urea were increased in the KK/HIJ model group, and certain parameters were also increased in the 25 and 50 mg/kg/day RHL-treated groups. Compared with those in the KK/HIJ model group, RHL (25 and 50 mg/kg/day) decreased the ACR, as well as blood glucose and creatinine, and RHL at 50 mg/kg/day also decreased the levels of urea (Table I).

RHL improves kidney function in mice with DN. In the present study, it was observed that the size and weight (0.05 g) of one of the kidneys from one mouse was decreased in the KK/HIJ model group. However, this was not observed in the other groups. Hematoxylin-eosin staining revealed that the characteristic changes of the kidney in the KK/HIJ mouse models of DN were renal tubular epithelial cell edema (Fig. 2), which demonstrated that renal tubular epithelial cell edema was shallow. No

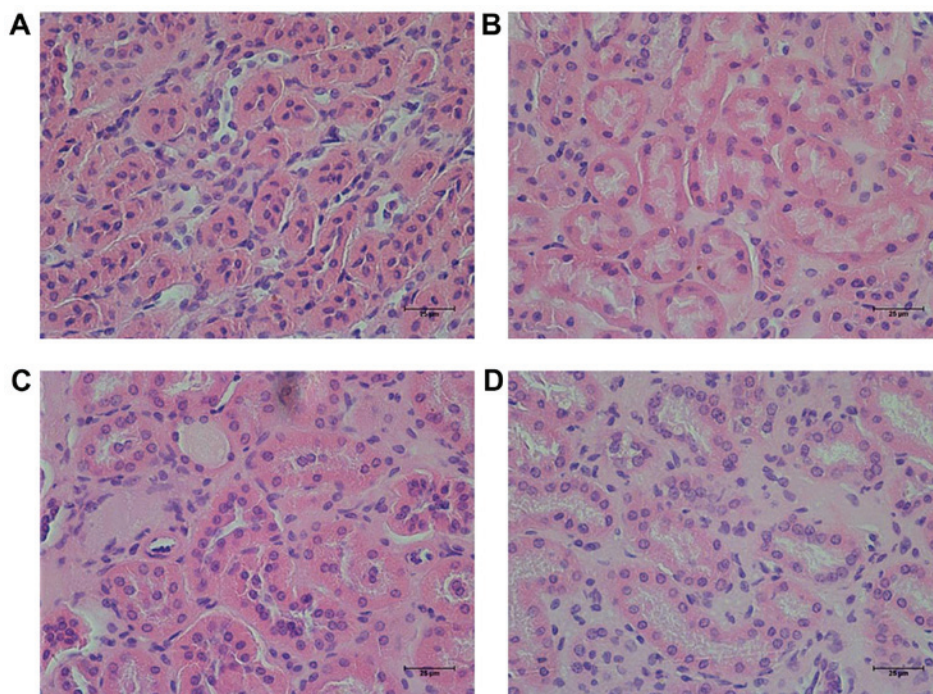


Figure 2. Effect of RHL on changed of the histopathological profile of the kidneys of mice, as determined by hematoxylin and eosin staining (magnification, x200). Renal sections from (A) the C57BL/6J control group, (B) the KK/H1J model group, (C) the 25 mg/kg/day RHL-treated KK/H1J group and (D) the 50 mg/kg/day RHL-treated KK/H1J group. Renal tubular epithelial cell edema was present in the KK/H1J control mice. RHL, rhein lysinate.

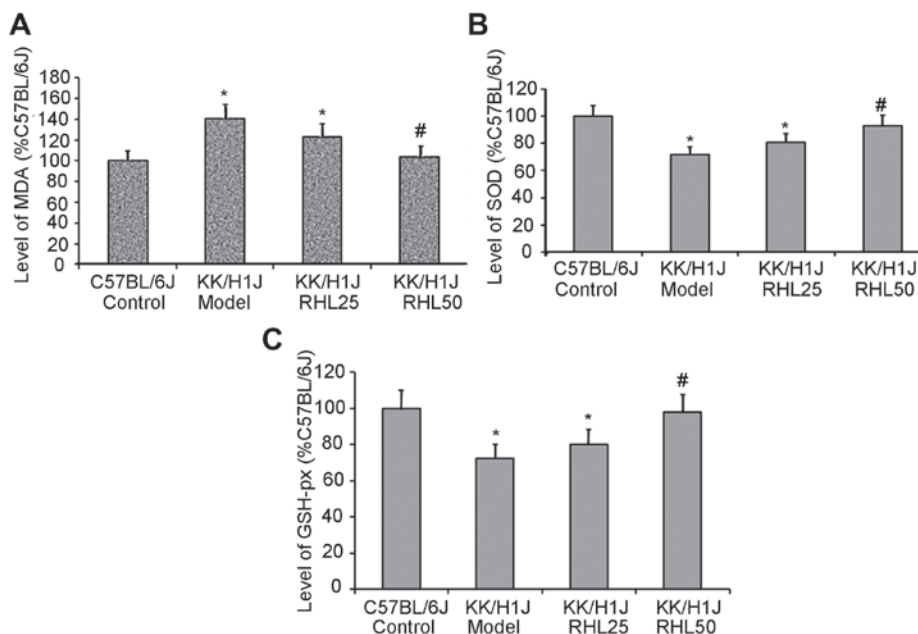


Figure 3. Effects of RHL on the MDA content and on the activity of SOD and GSH-Px in the kidney tissues of mice in each group. (A) MDA content and the activities of (B) SOD and (C) GSH-Px were determined using test kits. * $P < 0.05$ vs. C57BL/6J control group; # $P < 0.05$ vs. KK/H1J model group. RHL, rhein lysinate; SOD, superoxide dismutase; MDA, malondialdehyde; GSH-Px, glutathione peroxidase.

other structural changes were observed in the DN model group. In comparison, administration of RHL (25 and 50 mg/kg/day) improved renal tubular epithelial cell edema (Fig. 2).

RHL increases the activity of SOD and GSH-Px and decreases the levels of MDA in kidney tissue. In the KK/H1J model group, the activities of SOD and GSH-Px were 28 and 27% lower, respectively, than those in the C57BL/6J control group;

however, the MDA levels were 40% higher. In the KK/H1J group treated with 25 mg/kg/day RHL, the activities of SOD and GSH-Px were 12 and 10% higher, respectively, than those in the KK/H1J model group; however, the MDA levels were 13% lower. In the KK/H1J group treated with 50 mg/kg/day RHL, the activities of SOD and GSH-Px were 30 and 35% higher, respectively, than those in the KK/H1J model group; however, the MDA levels were 26% lower (Fig. 3).

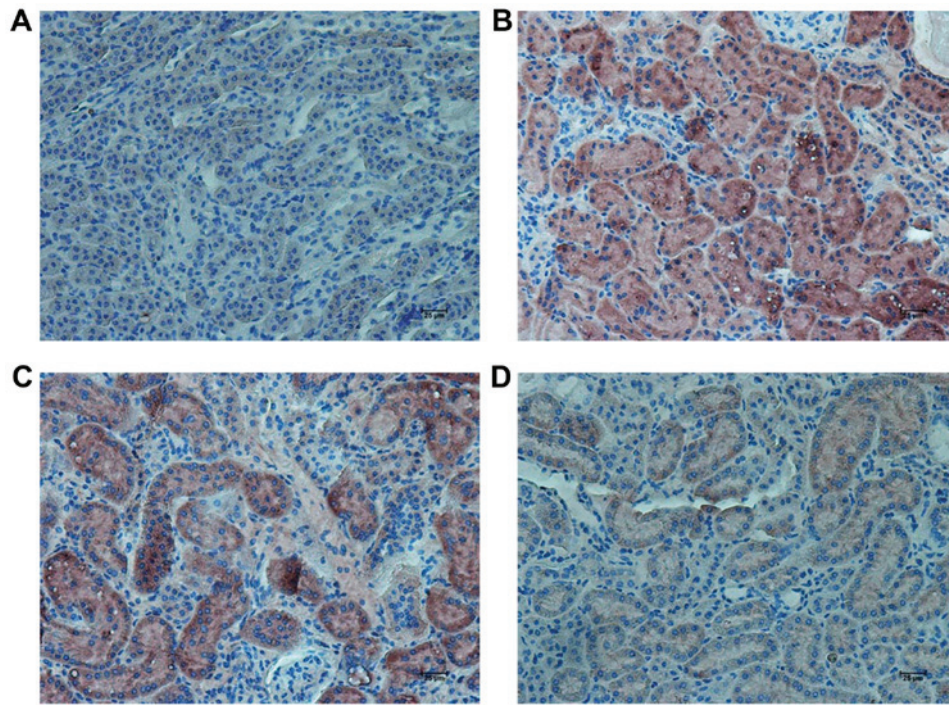


Figure 4. Effects of RHL on the expression of TNF- α in mouse kidney tissue. Representative immunohistochemically stained renal tissue sections from (A) the C57BL/6J control group, (B) the KK/HIJ model group, (C) the 25 mg/kg/day RHL-treated KK/HIJ group and (D) the 50 mg/kg/day RHL-treated KK/HIJ group (magnification, x200). TNF- α expression was indicated by brown staining. TNF, tumor necrosis factor; RHL, rhein lysinate.

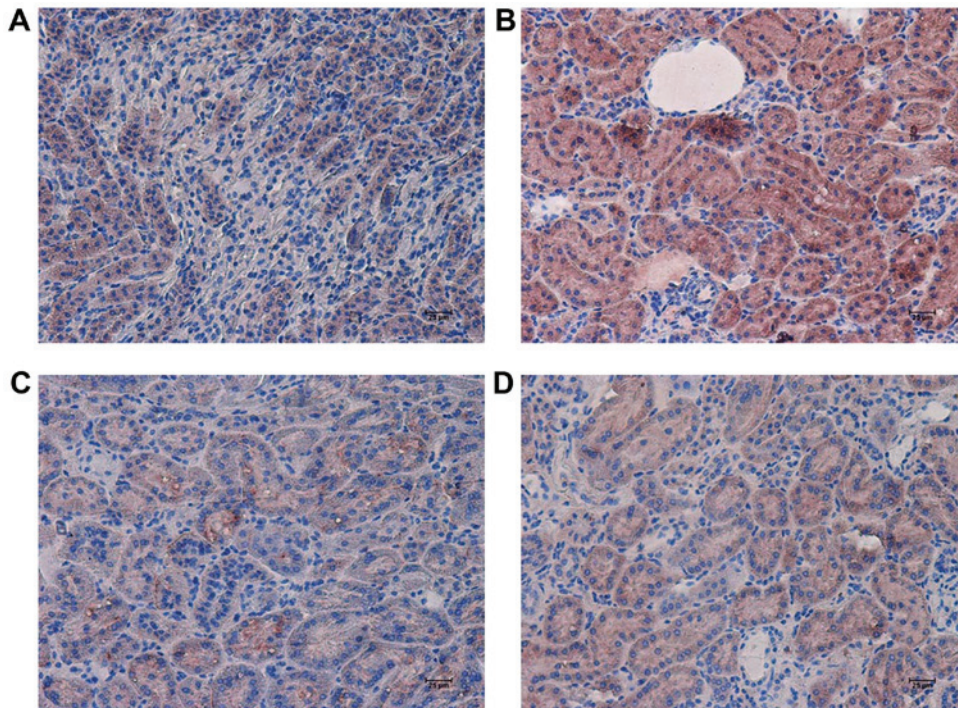


Figure 5. Effects of RHL on the expression of IL-6 in the mouse kidney tissue. Representative immunohistochemically stained renal tissue sections from (A) the C57BL/6J control group, (B) the KK/HIJ model group, (C) the 25 mg/kg/day RHL-treated KK/HIJ group and (D) the 50 mg/kg/day RHL-treated KK/HIJ group (magnification, x200). IL-6 expression was indicated by brown staining. IL, interleukin; RHL, rhein lysinate.

RHL suppresses the expression of inflammatory factors and associated proteins in the kidney of mice with DN. In the KK/HIJ model group, the TNF- α expression levels were higher than those in the C57BL/6J control group. RHL treatment (25 and 50 mg/kg/day) decreased the expression of TNF- α and

IL-6, as indicated by immunohistochemical and western blot analysis (Figs. 4-6). In addition, compared with the C57BL/6J control group, the KK/HIJ model group exhibited increased expression and phosphorylation of NF- κ B. Compared with the KK/HIJ model group, the RHL groups (25 and 50 mg/kg/day)

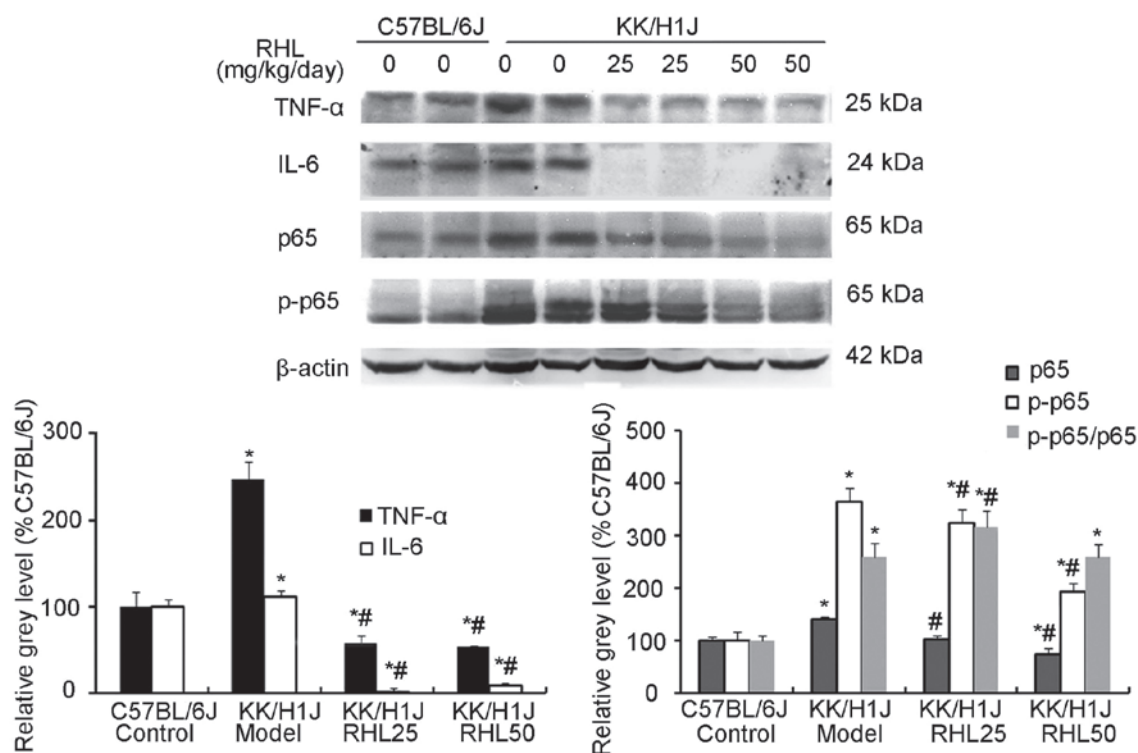


Figure 6. Effects of RHL on the expression of proteins associated with inflammation-associated genes in the kidney were assessed by western blot analysis. β -actin was used as the internal control. Values are expressed as the mean \pm standard deviation. * P <0.05 vs. C57BL/6J control group; # P <0.05 vs. KK/H1J model group. RHL, rhein lysinate.

exhibited decreased phosphorylation and expression of NF- κ B. Compared with the C57BL/6J control group, the KK/H1J model and RHL groups (25 and 50 mg/kg/day) exhibited increased p-NF- κ B/NF- κ B ratios; however, compared with the KK/H1J model group, 25 mg/kg/day RHL treatment exhibited increased p-NF- κ B/NF- κ B ratios and 50 mg/kg/day RHL treatment exhibited no significant effect on the p-NF- κ B/NF- κ B ratios (Fig. 6).

Discussion

The role of rhein and its analogues in the management of chronic kidney disease (CKD) has been addressed in several previous studies. Rhein improved the symptoms of nephropathy through decreasing the production of proinflammatory cytokines, including IL-1 β , prostaglandin E2 and TNF- α and inhibiting the expression of transforming growth factor- β 1 (15). A previous study by our group also observed that RHL protects the kidney from impairment in a senescence-prone inbred strain 10 mice (14). However, the effect of RHL, the lysin salt of rhein, on DN has remained elusive. In the present study, a KK/H1J mouse model of DN was induced by STZ injection and a specific diet. The ACR was detected at 5 weeks; compared with that in the C57BL/6J control group, the ACR was increased in the KK/H1J model group and in the KK/H1J RHL treatment groups. It was demonstrated that the kidney function in the KK/H1J model group and the KK/H1J RHL treatment groups was impaired. The levels of ACR were also detected at 15 weeks; overall, it was demonstrated that RHL improved kidney function impairment of KK/H1J mice at 5 and 15 weeks. After 16 weeks of treatment, the mice were

sacrificed, and kidney weights and the kidney weight-to-body weight ratio in the KK/H1J model group were revealed to be higher than those in the C57BL/6J control group, and to be reduced by treatment with RHL at 50 mg/kg/day. A previous study by our group reported that RHL decreased the body weight and blood glucose in high-fat diet and STZ-induced diabetic mice (16). In the present study, it was also observed that RHL treatment at 25 and 50 mg/kg/day decreased blood glucose, and RHL at 50 mg/kg/day also decreased the body weight, compared with that in the KK/H1J model group. Blood biochemistry analysis indicated that creatinine and urea, the biomarkers of kidney function, were increased in the KK/H1J model group, compared with those in the C57BL/6J control group, which was decreased by RHL treatment.

The pathophysiological characteristics of DN include renal hypertrophy, decrease of renal function, glomerular and tubular basement membranes thickening, mesangial matrix expansion, ultimately causing glomerulosclerosis and interstitial fibrosis (17-19). In the present study, a renal function decrease, renal hypertrophy and renal tubular edema were observed, while glomerulosclerosis and interstitial fibrosis were not seen. It may be speculated that DN in KK/H1J mice induced in the present study was at its early stage. Thus, early DN may be cured by RHL; however, if glomerulosclerosis and interstitial fibrosis had been present, DN would have hardly been cured. Oxidative stress has been reported to be responsible for the development of peripheral diabetic neuropathy (20,21). MDA, SOD and GSH-px are indicators of the oxidative stress status (22,23). In the present study, the activities of SOD and GSH-px in the KK/H1J model group were lower than those in the C57BL/6J control group; however, the levels of MDA in the KK/H1J model group were

higher than those in the C57BL/6J control group. These results indicated that RHL increased the levels of SOD and GSH-px and decreased the levels of MDA in kidney tissues of KK/HIJ mice. Thus, it may be deduced that RHL protects the kidney by reducing the levels of reactive oxygen species. These results were similar to those of a previous study by our group (14).

Inflammatory factors reportedly have a role in diabetes and in the progression of DN (24-26). Furthermore, diabetes is associated with increased levels of inflammatory biomarkers, including IL-6 and TNF- α (27,28). The TNF- α -308G/A polymorphism was reported to be associated with the expression levels of TNF- α and may be as a genetic susceptibility factor for DN (29). However, the effect of RHL on inflammatory factors associated with DN has remained elusive. The present results demonstrated that the levels of TNF- α and IL-6 in the KK/HIJ model group were higher than those in the C57BL/6J control group, and the expression levels of TNF- α and IL-6 were markedly suppressed in RHL-treated KK/HIJ mice. Therefore, it was speculated that inflammatory factors (TNF- α and IL-6) take part in the progression of DN in KK/HIJ mice and that RHL treatment decreases the production of inflammatory factors to thereby protect renal function.

TNF- α is one of the key proinflammatory cytokines and is involved in a number of inflammatory processes. TNF- α activates the NF- κ B transcription factor. NF- κ B has two functions: First, in malignant cells, it promotes cell survival and proliferation. Furthermore, NF- κ B activates the immune response, particularly the production of proinflammatory cytokines (30), and inflammation mediated by NF- κ B has a critical role in the pathogenesis of DN (31). The present study revealed that TNF- α was involved in renal tubular edema via the TNF- α /NF- κ B biochemical pathway. RHL decreased the expression of TNF- α and NF- κ B, and inhibited the phosphorylation of NF- κ B downstream of TNF- α . Therefore, RHL had the ability to inhibit the immune response by directly or indirectly blocking the TNF- α /NF- κ B biochemical pathway.

In conclusion, oxygen free radicals and inflammatory factors took part in the progression of DN in KK/HIJ mice. RHL (25 and 50 mg/kg/day) significantly decreased kidney inflammation by reducing the levels of oxygen free radicals, blocking the TNF- α /NF- κ B biochemical pathway and reducing renal function impairment. This finding is expected to inspire future investigations on the efficacy of RHL in DN.

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