Protective effects and mechanism of quercetin on renal injury of diabetic nephropathy rats

Junsheng Wang¹²*, Jie Li³, Liexiang Zhang⁴⁵

1. Department of Nephrology, Suqian Hospital Affiliated to Xuzhou Medical University, Suqian 223800, Jiangsu Province, China
2. Department of Nephrology, Suqian People’s Hospital, Nanjing Drum Tower Hospital Group, Suqian 223800, Jiangsu Province, China
3. Department of Cardiothoracic surgery, Nanjing Drum Tower Hospital Clinical College of Nanjing Medical University, Nanjing210008, Jingsu, China
4. Department of Neurosurgery, Suqian Hospital Affiliated to Xuzhou Medical University, Suqian 223800, Jiangsu Province, China
5. Department of Nephrology, Suqian People’s Hospital, Nanjing Drum Tower Hospital Group, Suqian 223800, Jiangsu Province, China

*: Corresponding author

E-mail: liangweibang0925@126.com

Abstract: To evaluate the protective effects of quercetin on renal injury of diabetic nephropathy (DN) rats based on renal function parameters and histopathological examinations (HE staining), and explore its possible mechanism by establishing DN rat models induced by high-sugar high-fat diet combined with streptozotocin (STZ). The results showed that compared with the model group, the quercetin low and high dose groups (20, 80 mg/kg/d) could significantly improve serum creatinine, blood urea nitrogen, urine protein and other abnormal renal function parameters. HE staining result showed thickening of glomerular basement membrane, proliferation of mesangial cells and damages of podocyte structure in major rats of model group. However, the intervention of quercetin could effectively protect the glomerular injury. To explore its possible mechanism, the expressions of TGF-β1, fibronectin (FN) and collagen IV in renal tissues of rats in each group were detected by Western blot and
immunohistochemical assay, and the phosphorylation levels of downstream effect factors (Smad 2/3, p38MARK) of TGF-β1 were detected. The results showed that quercetin could effectively antagonize the activity of TGF-β1, lower the expressions of fibronectin (FN) and collagen IV extracellular matrix (ECM), and resist against the thickening of glomerular basement membrane. More importantly, its protective effect on renal injury in DN rats may be associated with interfering the conduction of Smad, MARK pathways and resisting against the TGF-β1-induced ECM accumulation.

**Key words:** Quercetin; Diabetic nephropathy (DN); Extracellular matrix (ECM); TGF-β1
Introduction

Diabetic nephropathy (DN) is glomerular sclerosis caused by microvascular lesions of diabetes mellitus. Glomerulosclerosis is caused by mesangial cell proliferation, mesangial extracellular matrix (ECM), and thickening of the glomerular basement membrane (1). The progressive accumulation of the extracellular matrix (ECM) of the renal mesangial cells is one of the rational characteristics of DN disease. At present, the pathogenesis of DN is well studied. It is known that its development involves multiple pathways, such as polyol pathway, protein kinase C pathway, non-enzymatic glycation end products (AGEs) pathway, and hexokinase pathway (2).

Activation of signaling pathways interacts with cytokines and inflammatory substances that eventually result in kidney damage. For example, AGEs and RAGE receptor binding had effects to activate inflammation and oxidative stress by promoting PKC pathway, meanwhile, NADH/NAD+ over-expression also activated PKC pathway, further TGF-beta 1 expression up-regulation induced by ECM in fibronectin and collagen IV protein accumulation leads to increased ECM and glomerular basement membrane thickening (3-4). The cytokine network DN complex, as the most important cytokines, transforming growth factor (TGF-beta 1) research has been paid attention to, their participation in the proliferation of glomerular hypertrophy, extracellular matrix metabolism, apoptosis, cell, and receptor binding activates the Smad pathway (including Smad2, Smad3, MARK) path very closely with the relationship of renal injury (7, 8).

Quercetin is a natural flavonoid, its chemical name is 3, 3', 4', 5', 7 - five hydroxyflavone, in the form of glycosides in plants are widely distributed, has anti-cancer, anti-inflammatory, antioxidant, anti-platelet aggregation and scavenging free radicals and other biological activity (9-12). However, it is not clear that the effects and mechanism of quercetin to improve DN in vivo study. In our present study, we investigate the mechanism of quercetin in improving diabetic nephropathy and provide a theoretical basis for the prevention and treatment of diabetic nephropathy.
Materials and methods

Materials

36 SPF level SD rats, body weight was 180~220g, 8 week age, were purchased from Nanjing Medicinal University. The quercetin was purchased from Sangon Biotech. Streptozotocin (STZ) was purchased from Sigma (USA); Blood glucose meter (Roche, Germany); primary antibody (Abcam, USA); Second anti-body (Abcam, USA). Aminoguanidine (Sigma, USA). Blood urea nitrogen reagent kit (Roche, USA).

Methods

Animal models were established and grouped into drugs

In this experiment, 36 rats were fed with free intake of water. The indoor illumination of animals was 12 h diurnal cycle and maintained at 22~25 C at room temperature. After 1 weeks of adaptation, The 36 rats were fed by high fat and high glucose diet. After 4 weeks, STZ solution prepared with sodium citrate buffer solution (0.1 mol/L, pH4.4) as 35 mg/kg, The STZ solution were injected into rat abdominal cavity. After modeling, 3d, 7d, 10 d, fasting, drinking water for 12 h, blood from the orbit of the dead rats were collected, and 24 h urine was collected. The fasting blood glucose, serum creatinine, urinary protein and body weight were measured, and the model rats were successfully determined. Collecting the successfully established rats which blood glucose ≥16.7mmol/L, 24h urinary protein mass fraction ≥20mg. The whole rats were successfully. The model successes 36 rats were divided into 4 groups according to the randomization method: Model group (treated with normal saline), Low group (treated with 20 mg/kg/d quercetin), High group (treated with 80mg/kg/d quercetin) and Positive Drug group (aminoguanidine which treated diabetes in clinical) (treated with 0.1g/kg/d AG). There were 9 rats in every group. The intragastric administration was continued for 8 weeks.

Sample collecting

During the experiment, the color, body weight and blood sugar of the rats in each group were observed. At the end of the 14 week, rats in each group fasted and
could not help water 12 h, and 24 h urine was collected in the metabolic cage. 10% chloral hydrate was injected intraperitoneally, abdominal aorta was taken 5 mL blood, centrifuging for 10 min by 3000 r/min, and blood serum was isolated. Fasting glucose, serum creatinine, urinary protein and blood urea nitrogen were measured strictly according to the operating instructions of the kit. Aseptic removal of kidney, renal tissue fixed record quality; 1/2 left kidney were stored in 4% Formaldehyde Solution, HE staining and immunohistochemistry; Taking a fixed 1 mm³ left renal tissue in 2.5% glutaraldehyde solution, change the transmission electron microscope was used to observe morphology; right kidney tissue is placed in the refrigerator freezer to save -80°C for Western blot assay.

Renal histopathological examination

Renal tissue in 4% formalin fixed for 24 h, embedded in paraffin, 4 m sections, 65 C baking 1 h, using xylene dewaxing, xylene I washed 20 min (xylene heated to 65 degrees, and then water bath), xylene II washing 15 min (room temperature); Then by using gradient water, ethanol xylene washing for 2 min, followed by 100%, 95%, 90%, 80%, 70%, 2 min 60% ethanol washing water; slice after soaking for 15 min in hematoxylin stain, RO/UP staining, water washed 1 min; 10 s 1% hydrochloric acid ethanol differentiation, alkaline water the solution (5% ammonia solution) back to blue 10 s; then washed with tap water for 10 min, followed by 60%, 70%, 80%, 90% ethanol dehydration, full hydration after slicing directly into the staining solution, 1 min staining cytoplasm; by gradient ethanol 95%, 100% I, II 100% ethanol xylene, xylene I, II xylene dehydration; finally with neutral balata, observed under the microscope. 

Immunohistochemistry

The kidneys of rats were removed and fixed with 10% formaldehyde for 24 h. The kidney tissue was dehydrated with gradient ethanol. After 3 h, the cells were embedded and sectioned continuously and cut into small pieces with a thickness of 5 m. The slices were heated and washed with 10 mmol L⁻¹ sodium citrate buffer, then treated with 4% hydrogen peroxide for 10 min. EDTA after boiling, add tissue sheet,
jet time 2 min, and cool. Adding anti TGF- beta 1 (1: 200), FN (1: 200), Col IV (1: 200), 37℃ water bath, 40 min. After the reaction, anti dumping a, washed with PBS, then adding two biotin labeled antibody, and incubated at room temperature for 20 min, PBS cleaning, color at room temperature dropping DAB, color effect under the microscope to brown with distilled water to suspend the color, hematoxylin staining, dehydration, two a transparent resin sheet, benzene, and finally by microscope observation results.

**Western blotting (WB) assay**

Kidney tissues were collected by cryogenic grinding ice PBS after rinsing, adding RIPA cell lysate, samples of 30 min, centrifugal for 3min at 4℃ as 15000r/min, collected by measuring protein concentration. The protein was denatured at 100 DEG C, and the same amount of total protein was used for protein electrophoresis of SDS-PAGE. The protein was transferred from SDS-PAGE gel to PVDF membrane, and 5% skimmed milk powder closed 1 h, Adding TGF-β1(1:400), FN(1:400), Col IV(1:400), Smad2/3(1:400), p38MARK (1:400) anti-body, cultured at 4 ℃ overnight, The next day a recovery anti membrane washing 30 min in TBST solution, after joining with 1: 5000 diluted two resistant, incubated at room temperature for 30 min, TBST was removed after washing the membrane, PVDF membrane, DAB was placed in color, color liquid, gel imaging system in fixing. GAPDH protein was used as a reference.

**Statistical processing and analysis**

Data of this study are presented as the mean ± standard deviation (SD) values. Statistical analyses between the two groups were evaluated using one way ANOVA with SPSS 19.0 (SPSS Inc., Chicago,USA). $P < 0.05$ was considered statistical significant.

**Results**

**Quercetin has effects to biochemical indexes of DN rats**

Renal function parameters are used to measure changes in renal function, and
the main test items include biochemical indicators in urine and blood (e.g. urea nitrogen, serum creatinine, urinary protein). After high and low doses of quercetin, the contents of blood glucose, serum creatinine, blood urea nitrogen and 24 urine protein in rats were significantly decreased (P<0.05, respectively), Abnormal changes in renal function parameters were improved to some extent. The data were shown in Figure 1.

Figure 1. Effect of quercetin on weight, FBG, renal function parameter in DN rats

*: P<0.05, compared with Model group

**: P<0.05, compared with Low group

Pathological observation of kidney in rats

At the end of the 14 week, the rats in the model group and the administration group were subjected to HE staining to observe the pathological changes of the kidney in DN rats. The results were shown in Figure 2. Renal tubular epithelial cells of rats in the model group fall off with tissue edema, focal inflammatory cell infiltration, hyperplasia of mesangial cells, most of glomerular basement membrane thickening, glomerular cysts showed less renal lesions in rats of model group were successfully established. After the treatment of quercetin, the renal lesions were improved obviously, and the high and low dose groups had protective effects on renal
injury in different degrees, which was similar to that of the positive drug aminoguanidine.

Figure 2. Effect of quercetin in renal tissue of DN rats (×200)

TGF-β1, FN and Col IV proteins expressions by IHC and WB assays in kidney tissues

In this study, we used immunohistochemistry and Western blot to evaluate the effect of quercetin on the expression of FN, TGF-β1 and Col-IV proteins in the kidney of DN rats. The results were shown that FN, TGF-β1 and Col-IV proteins expressions of quercetin treated groups were significantly increased compared with those of Model group (P<0.05,respectively). The data was shown in Figure 3- Figure 6.
Figure 3. Effect of quercetin on TGF-β1 expression level in DN rats by IHC

*: $P < 0.05$, compared with Model group

**: $P < 0.05$, compared with Low group

Figure 4. Effect of quercetin on FN expression level in DN rats by IHC

*: $P < 0.05$, compared with Model group
**P** < 0.05, compared with Low group

Figure 5. Effect of quercetin on Col-IV expression level in DN rats by IHC

*: P < 0.05, compared with Model group

**: P** < 0.05, compared with Low group

Figure 6. Effect of quercetin on TGF-β1, FN, Col-IV expression level in DN rats by WB assay
Quercetin has effects to regulate Smad2/3 and p38MARK in kidney tissues of DN rats

Compared with Model group, The p-Smad2/3 and p-p38MARK proteins expressions of quercetin treated groups were significantly suppressed in the kidney tissues (P<0.05, respectively). The relative data were shown in Figure 7.

Discussion

ECM is the major component of normal glomerular basement membrane and extra mesangial matrix. Under pathological conditions, the synthesis and secretion of ECM increase greatly, while the metabolism degrades slowly, which leads to the accumulation of ECM in glomeruli and eventually leads to glomerulosclerosis.
main components of ECM are collagen, elastin, proteoglycan, and adhesion glycoprotein (13). Fibronectin (FN) is an important component of ECM and plays an important role in mediating mesangial cell proliferation and deposition of other ECM components (14). In addition, collagen IV is another major component of ECM. Studies have shown that collagen content in the matrix is above 50% (15), is the scaffold that forms the basement membrane network of the glomerulus. Once the synthesis and secretion of type IV collagen are abnormal, it leads to abnormal accumulation in the glomerulus, resulting in a thickening of the basement membrane as the main feature. Especially in the early stage of diabetic nephropathy, the proliferation of collagen, the rate of conversion is accelerating, and binding with laminin to form a complete basement membrane (22, 23).

TGF-β1 is the cytokine that is most closely related to glomerular fibrosis, which can directly lead to the accumulation of extracellular matrix. Its mechanism of action is also very clear, including the promotion of mesangial cell synthesis of large amounts of ECM; Promote the secretion of plasminogen activator inhibitor and metalloproteinase, tissue inhibitor and other matrix degrading enzyme inhibitors, and reduce the degradation of ECM (18). Studies have found that in human and animal kidney lesions, TGF- beta 1 is abundantly expressed, and promotes the synthesis of fibronectin (FN) and IV collagen, thereby increasing the aggregation of ECM and leading to thickening of the glomerular basement membrane (19). TGF-β1 can act as a stimulator or an effector molecule, and closely participate in the development of diabetic nephropathy. The study found that the first TGF- beta 1 receptor binding, formation of ligand receptor binding, and promote Smad2 and Smad3 phosphorylation, phosphorylation of Smad2, Smad3, Smad4 form a heterotrimer, send a signal to the nucleus to regulate the expression of target genes (19). Report showed that MARK pathway activation and phosphorylation of Smad2 are interrelated, such as the expression of Smad2 is disturbed, will affect the MARK signal transduction pathway and the accumulation of ECM, suggesting the important role of the interaction of the MARK-Smad pathway in matrix synthesis in sediments (20).
Quercetin is a natural flavonoid found in many plants, flowers, leaves and fruits, with antioxidant, anti-inflammatory, anti-adhesion, anti-thrombotic, antiviral, anti-tumor, reducing blood fat, lowering blood pressure and immune regulation (21-23). In this study, high glucose and high fat diet combined with streptozotocin (STZ) induced diabetic nephropathy as a model for evaluation of renal function parameters of quercetin protective effect on renal injury of DN rats. The results showed that quercetin could significantly improve serum creatinine, blood urea nitrogen, urinary protein and renal function parameters. In addition, the glomerular basement membrane of rats in the model group the most thickening, destruction of podocyte structure and obvious proliferation of mesangial cells by HE staining, quercetin intervention can effectively protect the natural cell structure and function of glomerular injury. In order to further study the mechanism of the components of renal injury against DN rats, the experiment of TGF-β1 and fibronectin (FN) and determination of collagen albumen, and continue to study the TGF-β1 downstream effectors of Smad2/3, the phosphorylation level of p38MARK. It was found that the quercetin could effectively antagonize the activity of TGF-β1, and down regulate the expression of fibronectin (FN) and collagen-IV in the ECM induced by it, thus inhibiting the thickening of the glomerular basement membrane. The mechanism may be through inhibition of Smad and MARK pathways against TGF-β1 induced ECM synthesis, and Mudanpi glycoside / phenol component regulates Smad2/3 consistent effect of the phosphorylation level of p38MARK. Smad and MARK to verify the existence of side channel interaction.

In summary, quercetin has a protective effect on renal injury in DN rats, the mechanism for the inhibition of TGF-β1 Smad2/3 induced the activation of p38MARK pathway, and provide experimental basis for clinical prevention and treatment of diabetic nephropathy of quercetin.

References


