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Protective effects of rutin on kidney in type 1 diabetic mice

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Abstract: Diabetic nephropathy is one the most serious diabetic microangiopathies, which is the main cause of mortality in diabetic patients. Our research investigated the protective effects of rutin on kidney of the type 1 diabetes mice induced by streptozotocin (STZ). The levels of kidney weight index (KWI), postprandial plasma glucose (PPG), creatinine (Cre), blood urine nitrogen (BUN) , the activity of super oxide dismutase (SOD) , malondialdehyde (MDA) and glutathione per oxidase (GSH-Px) were all measured. The histological morphology of kidney tissues was observed by hematoxylin-eosin (HE) staining, masson staining and electron microscope. The collagen I (COL-I) and transforming growth factor-β_1 (TGF-β_1) levels were estimated by immunohistochemistry, western blot and Real-Time PCR respectively. The results revealed that the levels of SOD and GSH-Px all increased, while the levels of KWI, PPG, Cre, BUN and MDA all decreased in diabetic mice after the rutin treatment for eight weeks. Moreover, the histological morphology of kidney tissues was also improved. Furthermore, the expression of COL-I and TGF-β_1 in kidney tissues increased significantly in the diabetic mice, which were antagonized by the rutin treatment. Together, the result suggested that rutin can improve kidney injury of the type 1 diabetic mice.

Keywords: Diabetic nephropathy, rutin, collagen I, TGF-β_1.

INTRODUCTION

Recently, the incidence of type 1 diabetes has been increasing as a result of insufficient insulin secretion, which is often associated with multiple organs including heart, brain, nervous system and kidney. As one of the most common and severe micrangium complications of diabetes mellitus, diabetic nephropathy (DN) has been a critical reason that contributes to cripple and mortality (Li *et al*., 2010; Parving *et al*., 2015). Therefore, it is of great significance to delay the developing of DN when it is in the early stage through the treatment to inhibit the pathological changes of early DN patients and slow down the rate of progression to end stage kidney disease. The histological characteristics of DN are diffuse glomerular sclerosis, interstitial fibration and augmentation of glomerular extra cellular matrix (ECM) (Makino *et al*., 2006). The elevation of transforming growth factor-β1 (TGF-β_1) level is regarded as an important factor to cause renal disease (Tang *et al*., 2011). It was reported that the abnormal activation of TGF-β_1 signal transduction pathway was the main mechanism of kidney inflammation and fibrosis (Lan *et al*., 2011; Lan, 2011). The inhibition of the activation of TGF-β_1 was a key to prevent renal fibrosis (Oh *et al*., 2012; Shen *et al*., 2013). Moreover, the increase of TGF-β_1 expression could raise the expression of collagen I (COL- I), which could strengthen and support many tissues in the body.

There is a lack of effective way to treat DN and reverse established renal fibrosis nowadays, so it is critical to develop drugs to treat DN. An important approach to find new drugs for DN is to obtain the bioactive components from Chinese herbal medicines. Previous research reported that flavonoids could decrease blood lipid and blood glucose, resist oxidative stress and inflammatory response (Jubie *et al*., 2015; Kaskoniene *et al*., 2015; Hatamnia, 2015; Lan *et al*., 2011). Meanwhile, it could alleviate kidney injury and had a good effect in the treatment of diabetes and DN. Rutin is one of the flavonoids which exist in many traditional Chinese medicines and common plants, such as apples and black tea. It is considered as a promising traditional Chinese medicine to DN treatment. In this study, we established the model of DN in mice to explore the protective effects and mechanism of rutin on kidney in type 1 diabetic mice.

MATERIALS AND METHODS

Animals

Seventy healthy male mice (35±10g) were provided by academy of military medical science (license number 0000349). All animal procedures and experiments were conducted in accordance with the official recommendations of the chinese community guidelines.

Establishment of the type 1 diabetic mice model

After one week adaptive feeding in the animal experimental center, type 1 diabetic mice model were established by intraperitoneally injecting streptozotocin (STZ) (Sigma-Aldrich Co., St Louis, MO, USA) at a dose of 62.5mg/kg once a day for five days. The postprandial blood glucose levels of the blood samples from the tail vein were measured by blood glucose meter after 72 hours. The type 1 diabetic model was successfully established as the postprandial blood glucose level of mice exceeded 16.6mmol/L.

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Grouping and administration

Twelve mice selected from the total mice were set as the normal group. The other mice were administrated STZ (62.5 mg/ kg) by intraperitoneal injection once a day for five days to establish the diabetic mice model. The type 1 diabetic mice were randomly divided into the model group, the low-dose high- dose rutin groups, and the irbesartan group which were administrated with rutin 50 and 100mg/kg and irbesartan (45mg/kg). While the model group and the normal group received 0.1% Carboxyl Methyl Cellulose (CMC) sodium solution (Sinopharm Chemical Co., Ltd, Beijing, China) 10mg/kg per day, other groups were given rutin (Abcam Co., Ltd. Shanghai, China) by intragastric administration accordingly. All mice were administrated once a day for eight weeks.

Measurement of experimental index

The blood samples obtained from eyeball after the mice treated with rutin for eight weeks, then which were centrifuged at 3000rpm for 10 minutes at 4℃... The levels of creatinine (Cre), blood urine nitrogen (BUN) in serum was determined t by Rayto automatic biochemical analyzer (Chemray 240; Rayto Co., Shenzhen, China).Further, the activity of super oxide dismutase (SOD), malondialdehyde (MDA) and glutathione per oxidase (GSH-Px) in serum and kidney tissues were measured by test kit. All procedures were conducted in accordance with manufacture's instruction. The kidney weight index (KWI) was calculated as the following: KWI=kidney weight / body weight (mg/g). Finally, the kidney tissues were fixed with 4% paraformaldehyde and 2% glutaraldehyde, which were used for histological examination respectively.

Histological analysis

The kidney tissues fixed in 4% paraformaldehyde were dehydrated with ethanol of gradient concentration,were cleared in xylene and embedded in paraffin sections, at last stained by hematoxylin-eosin(HE) staining kit and Masson kit (Solarbio Co., Beijing, China). The kidney histology and fibrosis degree were observed by light microscope (BX-53, Sunny Hengping Co., Shanghai, China). The ultrastructure of kidney ultrathin frozen sections was observed by electron microscope (H-7650; Hitachi, Tokyo, Japan).

Immunohistochemistry assay

Western Blot assay

The samples were homogenized in RIPA (Radio-Immunoprecipitation Assay) lysis buffer containing protease and phosphatase inhibitors. Protein concentration was determined by a bicinchoninic acid (BCA) method (Beyotime Biotechnology Co., Shanghai, China). About 60 μg protein of each sample was separated on SDS-PAGE (Beyotime Biotechnology Co., Shanghai, China) gels under the electrophoresis and then transferred to PVDF membrane. Subsequently, the membranes were washed in TBST and blocked in 5% nonfat milk for 2 hours. Then the membranes were incubated at 4°C overnight, followed by an incubation with the anti-rabbit secondary antibody (1:4000 dilution, KPL Scaffold Inc.) for 1 hour at room temperature. The primary antibodies were as follows: COL-^{I} (1:300, Boster Biotechnology Co.), TGF-β_1 (1:3000, Abcam Co., Ltd) and GAPDH (1:5000, Bioworld Technology, Inc., St. Louis ,USA). After washed in TBST, the blots were visualized with ChemiDocXRS machine using enhanced chemiluminescence (ECL) (Solarbio Co.) method. The electrophoresis bands were converted into gray level by Image J software to display relative protein content. The expression of TGF-β_1 or COL-^{I} was expressed as ratio expression rate of TGF-β_1 / GAPDH or COL-^{I}/ GAPDH.

Real-time PCR assay

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, United States) according to the instructions. The extracted RNA was reversely transcribed into using cDNA SYBRE ScriptTM RT-PCR kit (Takara Biotechnology, Dalian, China). Add 2 μl of the cDNA to the primers and the SYBR pre-mixed solution. qPCR was carried out according to the manufacturer's procedures (Takara SYBR Premix Taq TM^{II}). The results were expressed as the OD ratio relative to GAPDH. The following primer pairs were used: GAPDH, forward -GGTGAAGGTCGGTGTGAACG-, reverse –CTCGCTC CTGGAAGATGGTG-; COL-I, forward –GCGAGTGCT GTGCTTTCTG- , reverse –CATAGGACATCTGGGAA GCAA-; TGF-β_1, forward-GTGGAAATCAACGGG ATCAG-, reverse -ACTTCCAACCCAGGTCCTTC-. The target gene expression was calculated by $2^{-\Delta\Delta Ct}$.

STATISTICAL ANALYSIS

All the data are expressed as means ± SD and analyzed with SPSS 17.0 software. Multiple were compared using one-way ANOVA. Differences were considered significantly if $P<0.05$.

RESULTS

Effects of rutin on metabolic and biochemical parameters in diabetic mice induced by STZ

As shown in table 1, compared with the nomal group, the levels of PPG, Cre, BUN in serum and KWI were significantly higher in the model group ($P<0.01$), which were all attenuated after treatment of low- and high-dose rutin for eight weeks ($P<0.05$, $P<0.01$). There was no significant difference among low-, high-dose rutin and irbesartan groups ($P>0.05$). These data indicated that rutin could decrease PPG and improve the renal function of diabetic mice effectively.

The level of oxidative parameters of various groups

Compared with the nomal group, SOD and GSH-Px levels all decreased and MDA level increased

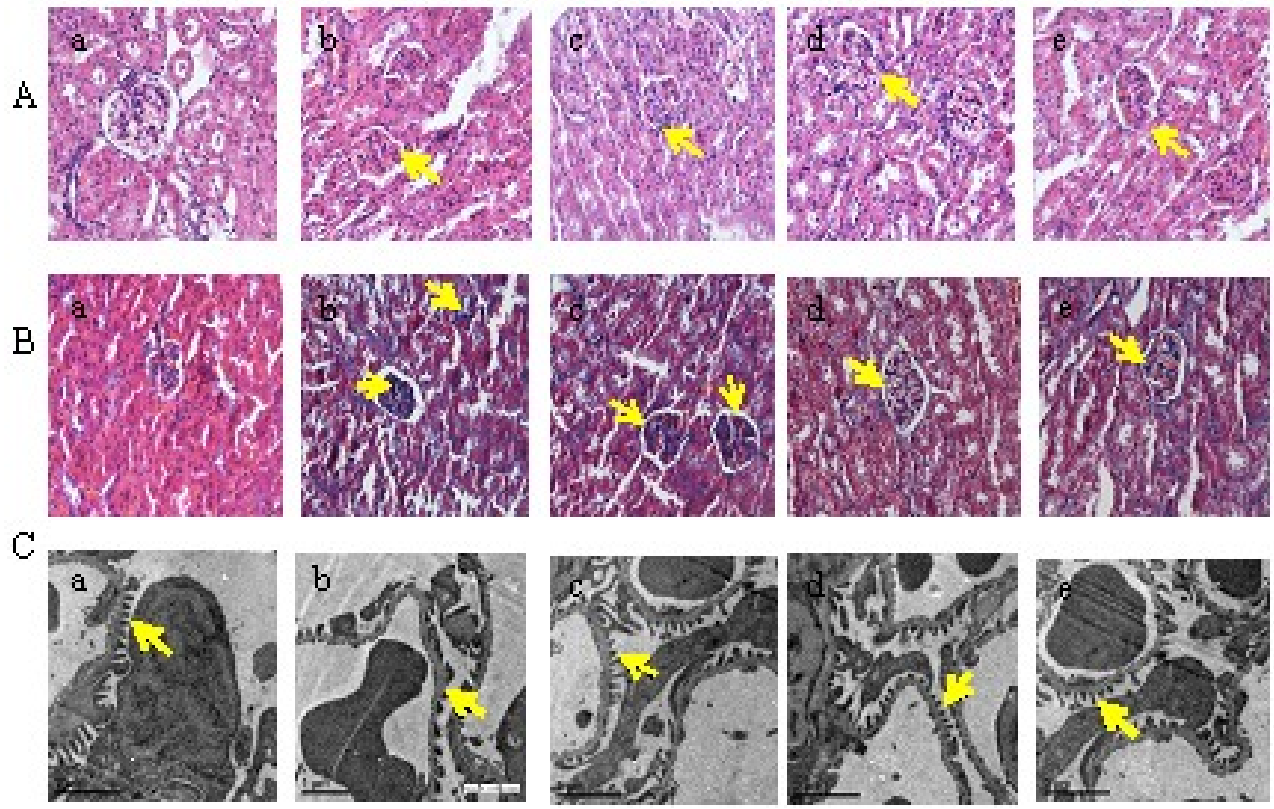

Fig. 1: (A) Results of HE staining of kidney histopathological in various groups (original magnification ×400, n=4), the glomerular condensed and the capsular space enlarged (yellow arrow); (B) Results of Masson of kidney histopathological in various groups (original magnification ×400, n=4) , the collagen fibers are dyed blue (yellow arrow) ; (C) Ultrastructure changes of the glomeruli observed by electron microscope in various groups (original magnification ×10000, n=4), the myofilaments are distorted, ruptured and partially dissolved, and the mitochondria are swollen and partially vacuolated (yellow arrow). (a: normal group; b: model group; c: low dose of rutin group; d: high dose of rutin group; e: Irbesartan group)

significantly in serum and kidney tissues in model dose group (P<0.05, P<0.01). All the indexes were improved significantly by rutin in dose-dependent manner (P<0.05, P<0.01), but irbesartan had no effect on them (P>0.05) (table 2).

Effect of rutin on histological structure of kidney

It was shown in fig. 1A, there was a severe histopathological changes like nucleus increased, glomerular mesangial area widened, the glomerular condensed and the capsular space enlarged in the model group, while which dramatically ameliorated in different rutin dose groups. Also, it was shown in fig. 1B, more collagen fiber was stained blue at the base of the glomerulus in the model group relative to different rutin dose groups with the high-dose group having the most obvious improvement. The results suggest that rutin could efficiently prevent kidney injury and kidney fibrosis in diabetic mice.

In normal mice, the thickness of glomerular basement membrane was uniform and smooth, the structure of podocytes was complete and the arrangement of the foot process was clear and orderly without fusion. As showed in fig. 1C, glomerular basement membrane diffusely thickened, the number of podocytes reduced and the foot process was irregular and broadened, or even disappeared in the model group. In the low-, high- dose rutin group, the above phenomena were improved to some extent. In high dose group, the improvement was most obvious.

The expression of COL-I and TGF-$\beta_1$observed by immunohistochemistry

There was a small amount of TGF-β_1 expression in the kidney of normal mice, the positive expression of which was stained brown. Compared with the model group, the expression of TGF-β_1 was significantly decreased in the low-, high- dose ruin group and irbesartan group (fig. 2A). There was a low level of COL-I in glomeruli, tubules and kidney interstitium in the normal group, but the expression of which was significantly increased in the model group. Compared with the model group, the expressions of COL-I in the low-, high- dose rutin group and irbesartan group were decreased to varying degrees. The effect of high-dose rutin was more obvious (fig. 2B).

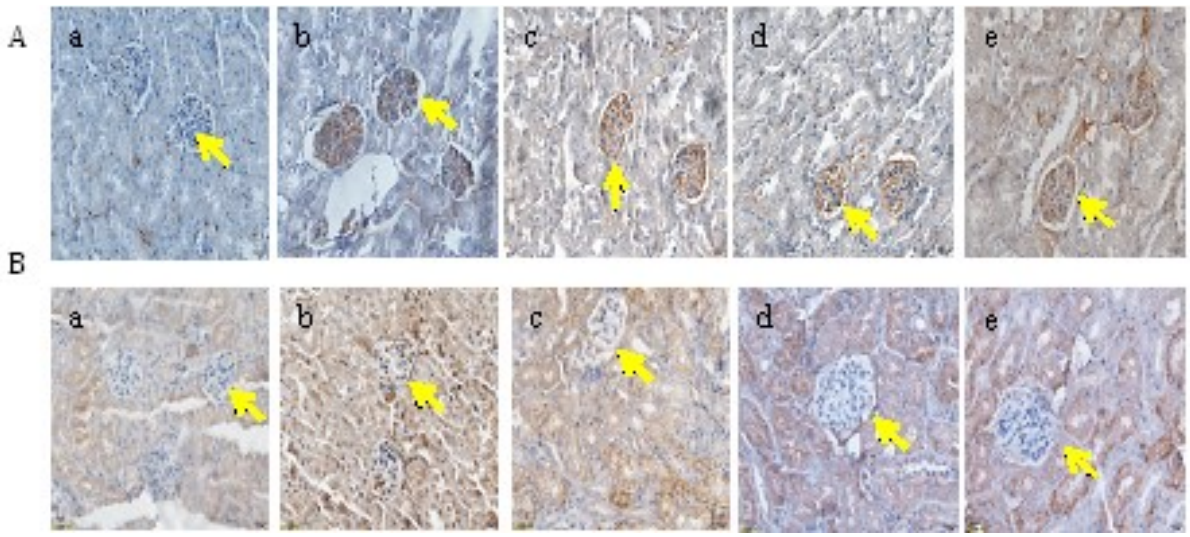

Fig. 2: The expression of TGF-β_1 (A) and COL- Ⅰ (B) determined by immunohistochemistry(original magnification ×400, n=4). The brown staining of the cytoplasm and the mesenchyme in kidney tissue represents positive immunostaining for TGF-β1 and Collagen I (yellow arrow). (a: normal group; b: model group; c: low dose of rutin group; d: high dose of rutin group; e: Irbesartan group)

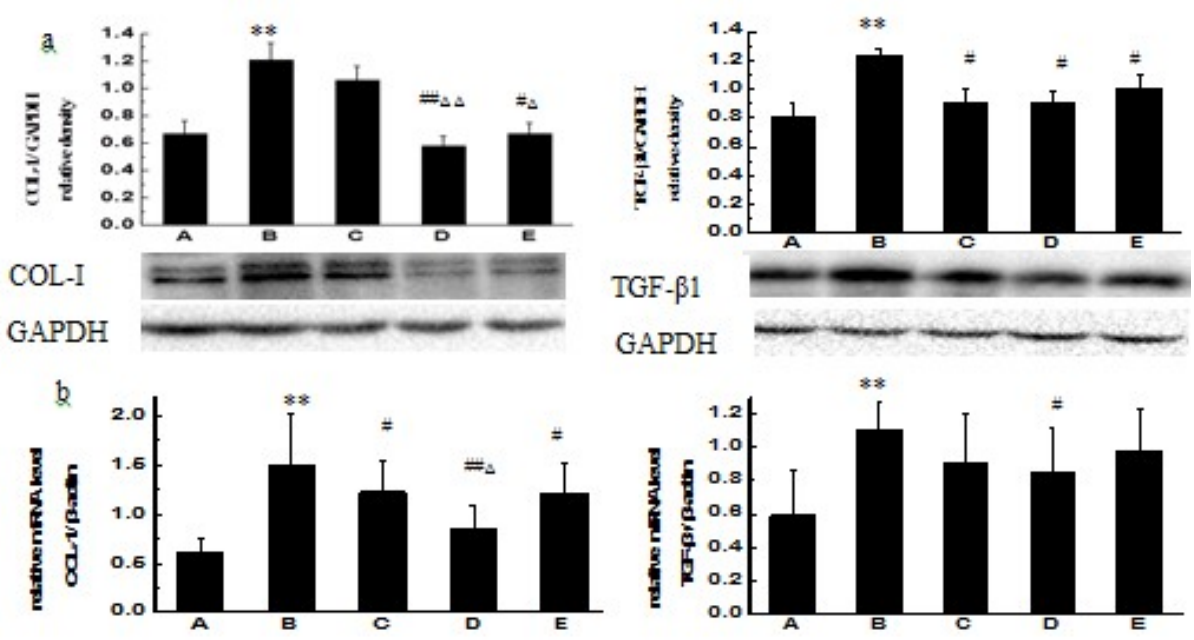

Fig. 3: (a) Effect of rutin on the protein expression of TGF-β1 and COL-I in kidney tissues (n=4); (b) Effect of rutin on the mRNA expression of COL-I and TGF-β_1 in kidney tissues (n=6). A: normal group; B: model group; C: low-dose rutin group; D: high-dose rutin group group; E: Irbesartan group. *P<0.05, **P<0.01 vs normal group; $^{\#}$P<0.05,$^{\#\#}$P<0.01 vs model group; $^{\Delta}$P<0.05， $^{\Delta\Delta}$P<0.01 vs low-dose rutin group

Table 1: Effects of rutin on metabolic and biochemical parameters in STZ-induced diabetic mice (n=12, means ± SD)

Group	PPG(mM)	Cre(μM)	BUN (mM)	KWI (%)
normal	6.88±1.50	25.2±3.4	8.67±1.60	6.22±0.63
model	24.4±2.63**	37.0±6.3**	23.93±7.16**	24.4±2.63**
low-dose rutin	21.64±1.43**$^{\#}$	29.83±5.67$^{\#}$	14.61±7.51$^{\#\#}$	7.26±0.73$^{\#\#}$
high-dose rutin	22.10±1.73**	29.20±4.60$^{\#}$	14.07±5.84$^{\#\#}$	6.98±0.69$^{\#\#}$
irbesartan	24.40±3.41**	27.00±4.00$^{\#}$	18.16±4.90*$^{\#}$	7.13±0.82$^{\#\#}$

*P<0.05, **P<0.01VS normal group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 VS model group

Table 2: The level of SOD in serum and kidney tissue in various groups (n=12, means ± SD)

Group	SOD (serum, U/ml)	SOD (tissue, U/ml)	MDA (serum, nmol/mL)	MDA (tissue, nmol/mL)	GSH-Px [serum, λB/ (U/ml)]	GSH-Px [tissue, λB/ (U/ml)]
normal	67.7±5.33	25.57±5.32	10.76±1.83	5.12±0.15	475.95±21.43	133.27±5.13
model	58.03±7.54*	16.05±1.90**	19.73±2.12**	10.45±1.62**	419.21±15.85**	109.72±7.16*
low-dose rutin	54.55±6.29*	20.87±3.46*	15.65±3.24*$^{\#}$	8.54±1.61*$^{\#}$	443.65±12.29*$^{\#}$	127.25±6.36*$^{\#}$
high-dose rutin	68.06±6.33$^{\#\Delta}$	23.75±4.07$^{\#}$	12.94±2.76$^{\#\Delta}$	7.03±0.89$^{\#\Delta}$	464.73±18.62$^{\#\#\Delta}$	128.53±8.34$^{\#}$
irbesartan	61.47±9.74*	18.89±1.94**	18.49±1.65**	10.73±1.50**	409.92±8.93**	111.39±9.78*

*P<0.05, **P<0.01VS normal group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 VS model group; $^{\Delta}$P<0.05 VS low-dose rutin group.

The expression of COL-I and TGF-β_1 was detected by western and PCR

The results showed that the expression of COL-I and TGF-β_1 in the model group increased significantly either in protein or mRNA expression level (P<0.01, fig. 3). After the rutin treatment, the expression of COL-I and TGF-β_1 decreased (P<0.01 and P<0.05) in the protein and mRNA levels, which were more obvious in the high-dose group.

DISCUSSION

Diabetes is a widespread chronic disease characterized by high plasma glucoses, which leads to various complications in many organs and tissues throughout the body. DN is a severe complication of diabetes and has become the most common reason to cause end-stage kidney disease all over the world. After rutin treatment for 8 weeks, the levels of PPG, Cre, BUN and MDA in mice were all attenuated, while the levels of SOD and GSH-Px increased compared with the model group. These experiment data shows that rutin can downgrade the plasma level and alleviate kidney function, indicating that rutin has an obviously positive effect on DN.

It was reported that hyperglycemia was a major cause of the increased proteins and lipids glycation, which in turn enhanced the generation of reactive oxygen species (ROS) (Lee *et al*., 2010; Sayed *et al*., 2010; Aljofan *et al*., 2012). Thus, diabetes is usually accompanied with an increased ROS production and impaired antioxidant defense mechanisms, which lead to increase the oxidative stress. Vinod *et al* reported that SOD could protect superoxide anions and convert them to H_2O_2. Further, it can reduce the harm of the oxygen free radicals to human and improve the imbalance of oxidation and antioxidant (Vinod *et al*., 2011). MDA is the metabolite of lipid peroxidation of oxygen free radical and unsaturated fatty acid, which has a significant effect on the free radical damage. What is more, GSH-Px also as an antioxidant factor can reduce the injury of oxygen free radical to the body (Chiang *et al*., 2006; Hou *et al*., 2010). Our results releaved that, the SOD and GSH-Px levels were all elevated in the type 1 diabetic mice, but the MDA level decreased, which may be the main cause leading to renal injury in the diabetic mice. After the rutin treatment, all the factors above attenuated in different degree, suggesting that rutin can protect the renal injury by anti-oxidation effect.

The author conducted histological examination, an intuitive and reliable method, to detect the kidney morphological changes and kidney damage. HE staining was used to observe the Histopathological changes of kidney tissue. Collagen fibers in kidney tissue could not be seen clearly by HE staining, which make it difficult to distinguish mild fibrosis. Hence, the author used Masson staining as an assistant method to determine the degree of fibrosis. As a traditional method of collagen fiber staining, Masson staining plays a significant role in pathological diagnosis, disease identification and research. The ultra structure of kidney tissue was observed by electron microscope. The histopathological observation indicated that kidney tissues damaged severely in the model group, while the degree of kidney tissues damage were improved in the low-, high- dose ruin group and irbesartan group, especially in high-dose rutin group.

Kidney fibrosis is a common symptom of DN, which is the main pathological feature from chronic diabetes to kidney failure (Kowalski *et al*., 2014). TGF-β_1 is often recognized as a key mediator in the initiation and progression of fibrosis (Lasky *et al*., 2000). It was reported that, in the diabetic animal models, interruption of TGF-β signal significantly decreased the expansion of glomerular mesangial matrix (Wang *et al*., 2007). Recent researches indicated that mesangial cell caveolae played a vital role in the fibronectin overproduction in the diabetic models, which may result in the pathogenesis of DN (Liu *et al*., 2014). Meanwhile, it has been demonstrated that TGF-β_1 can induce the autophagy of kidney tubular epithelial cells (podocyte) by themselves and promote the apoptosis of kidney tubular epithelial cells which further cause kidney tubular injury (Xu *et al*., 2012). Podocyte are highly differentiated and nonrenewable cells and podocyte injury is critical in diabetic nephropathy. The destruction of glomerular filtration membrane caused by the decrease of podocytes and kidney basement membrane thickening cannot be completely restored. Moreover, DN is associated with extracellular matrix (ECM). In diabetic nephropathy patients, glomerular basement membrane thickened and ECM protein

accumulated in the glomerular mesangial area, resulting in glomerular lumen stenosis and blockage, which can determine the rate of decline in renal function (Liu *et al*., 2010; Huang *et al*., 2012; Xie *et al*., 2013). TGF-β_1 is the main factor in promoting ECM accumulation. One way to promote ECM accumulation is increasing the number of components, such as COL-I. In summary, all these indicate that it is high glucose that initially induces the increased expression in TGF-β1, ultimately accounting for the accumulation of COL-I. In other words, the increased accumulation of ECM is caused by the activated TGF-β1 signaling pathway. TGF-β1/Smads/ECM is obviously a linked response. Therefore, it is of great significance to detect the expression of TGF-β1 and COL-I when study the impairment of kidney in diabetic mice after treating them with rutin. Consistent with this, the COL-I and TGF-β1 expression in both the protein and mRNA levels were all evaluated, which were all decreased after the rutin treatment, and the effect of the high-dose rutin group was more obvious,. Our results indicated that rutin could significantly improve the DN by decreasing the levels of COL-I and TGF-β1 in the diabetic mice.

Together, the results above suggested that rutin can protect kidney injury in type 1 diabetic mice, the mechanism of which may be that it can decrease blood glucose, resist oxidative stress and down regulate the TGF-β_1 and COL-I expression. Therefore, it is significant to study the therapeutic effect of rutin on DN mice.

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