Protective effects of tacrolimus on podocytes in early diabetic nephropathy in rats

TAO PENG¹, XIANGDI CHANG², JIE WANG³, JUNHUI ZHEN⁴, XIANGDONG YANG¹ and ZHAO HU¹

¹Department of Nephrology, Shandong University Qilu Hospital, Jinan, Shandong 250012; ²Department of Nephrology, Affiliated Hospital of Binzhou Medical School, Yantai, Shandong 264003; ³Department of Nephrology, Liaocheng People's Hospital, Liaocheng, Shandong 252000; ⁴Department of Pathology, Shandong University Qilu Hospital, Jinan, Shandong 250012, P.R. China

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Abstract. The aim of the present study was to investigate the protective effect of tacrolimus on early podocyte damage in rats with diabetic nephropathy (DN). A total of 38 normal male Sprague-Dawley rats were randomly divided into four groups: Normal control group (group N; n=8), DN group (n=10), tacrolimus (FK506) treatment group (group F; n=10), and benazepril (Lotensin) treatment group (group L; n=10). The rats in groups DN, F and L were administered with streptozotocin (STZ; 60 mg/kg) by intraperitoneal injection to establish the diabetic rat model. After 4 weeks, the diabetic rat model was established, and rats in the different groups were administered intragastically with the respective drugs. Blood glucose (BS), body weight (BW) and 24-h urine protein were detected every 4 weeks, serum creatinine (SCr), blood urea nitrogen (BUN) and kidney weight/body weight (KW/BW) were measured at the end of the 8 weeks of drug treatment. Renal pathological changes were observed under a light microscope and electron microscope. Expression of nephrin, which is a podocyte-specific marker, was detected using western blot analysis. The results showed that the levels of SCr, BUN, KW/BW and 24-h urine protein in groups D, F and L were significantly higher, compared with those in group N (P<0.05). No significant differences were found between groups F and L for the above indicators, with the exception of BS. However, all indices were significantly lower, compared with those in group DN (P<0.05). The results of the western blot analysis showed that the expression of nephrin decreased by 60.1% in group DN, compared with group N, and significant recovery in the expression of nephrin was observed in groups F and L (P<0.05). Tacrolimus reduced urinary protein and slowed the progression of DN, partially by recovering the protein expression of nephrin in the renal tissue of diabetic rats, and maintaining the integrity of the structure and function of podocytes.

Introduction

Diabetes mellitus (DM) has been identified as a major contributor in several important diseases, including the non-communicable diseases of cardiovascular disease and renal disease (1). Diabetic nephropathy (DN) is one of the most common microvascular complications of DM, and the prevalence of DN is increasing rapidly with the diabetes epidemic, due to the increase in obesity and the aging population (2). As an important cause of CKD, the proportion of DM in all causes of CKD has continued to increase.

DN is defined as albuminuria (albumin excretion rate >300 mg/24 h) and reduced renal function in a patient with known diabetes, in the absence of urinary tract infection or other renal disease (4). DN is a progressive, proteinuric renal disorder in patients with DM. It is a common cause of CKD worldwide, particularly in developed countries. DM is the underlying cause of micro- and macrovascular disorders, including DN, retinopathy, coronary artery disease and peripheral vascular disease (4). DN is a progressive disease in patients, which is functionally characterized by differing degrees of albuminuria and CKD. The early clinical manifestations of DN include high glomerular filtration rate and...
increased urinary albumin excretion (microalbumin) (5). Microalbuminuria is the earliest and most commonly used clinical index of DN, and is independently associated with cardiovascular risk in diabetic patients. Microalbuminuria is always followed by the appearance of clinical proteinuria (macroproteinuria), and renal injury subsequently becomes visible (6-8).

Proteinuria is an independent risk factor for the progression of several diseases, including DN, and structural and functional alterations of podocytes are an important pathological basis for proteinuria and glomerular sclerosis (9). Studies have suggested that an important mechanism in DN proteinuria may be that certain protein molecules in multiple podocyte slit diaphragms, including nephrin and podocin, are abnormally expressed and distributed (10,11). It has been reported that tacrolimus, a type of immunosuppressant, can mitigate urinary protein excretion in DN rats (12,13); however, few reports have discussed the association between the action of decreased proteinuria and protection of podocytes. The present study was designed to investigate the effect of tacrolimus, and examine whether it can regulate the expression of nephrin, reduce proteinuria and slow the progression of diabetes.

Materials and methods

Animals. A total of 38 healthy adult male Sprague-Dawley (SD) rats (clean grade; 190-210 g; Beijing Vital River Company, Beijing, China) were bred in the Animal Laboratory of Shandong University (Jinan, China). The room was maintained at a temperature of 20±1°C, relative humidity of 50-60% and 12-h light/dark cycle. All animal experimental procedures were performed in accordance with the Guidelines for Animal Experiments of Qilu Hospital of Shandong University (Shandong, China) and were approved by the Institutional Ethics Committee for the Laboratory Animal Care of Qilu Hospital, Shandong University.

Drugs and reagents. The following were used in the present study: Tacrolimus (FK506; batch no. J20090142; Astellas Pharma China, Ltd., Beijing, China), benazepril (Lotensin; batch no. H20030514; Beijing Novartis, Beijing, China), streptozotocin (STZ; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany), citrate buffer (0.1 mol/l; pH 4.2), rabbit anti-rat nephrin polyclonal antibody (Abcam, Cambridge, UK), horseradish peroxidase-labeled goat anti-rabbit IgG (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China).

Model establishment and treatment groups. A total of 30 male SD rats, which were randomly selected from the 38 male rats, were administered with STZ 60 mg/kg by intraperitoneal injection following fasting for 12 h with access to water. The remaining eight rats were included as the normal control group (group N; n=8). After 72 h, the whole blood glucose was measured using blood glucose meters from the rat tail vein randomly. When glucose was >16.7 mmol/l and lasted 3 days, the diabetic rat model was considered successfully established and remained as a diabetic rat model for at least 4 weeks. In week 5, when the 24-h urinary protein was >30 mg, establishment of the standard DN rat model was considered successful (14).

When the DN model was successfully established, the rats were randomly divided into a DN group (n=10), tacrolimus (FK506) treatment group (group F; n=10) and benazepril (Lotensin) treatment group (group L; n=10). The rats in group F were administered with tacrolimus (1 mg/kg daily) by gavage. At the same time, the rats in group L were administered with Lotensin (10 mg/kg daily) by gavage. The rats in the normal control group (group N; n=8) and group DN were administered with the same quantity of vehicle. All rats were fed a standard diet with free access to drinking water throughout the experiment, and without insulin or other antihyperglycemic drugs.

Specimen collection. All rats were sacrificed under anesthetic following 8 weeks of drug intervention. The urine of the rats was collected over the 24 h prior to sacrifice using metabolic cages, centrifuged at 8,056.8 x g, 4°C for 5 min and stored at -80°C in aliquots prior to protein measurement. Blood was collected from the inferior vena cava of the anesthetized rats by intraperitoneal injection of 10% chloral hydrate (4 ml/kg) to measure the blood sugar (BS), serum creatinine (SCr) and blood urea nitrogen (BUN). Pre-cooling saline (4°C) was used to rinse the kidneys through the duct of the right common carotid artery until the kidneys became pale in color. The left kidney was then removed, weighed and placed on ice to retain the renal cortex kidney tissue in 1 mm² blocks, which were fixed using 2.5% glutaraldehyde for electron microscopy. Kidney tissues in 8 mm³ blocks were fixed using 10% paraformaldehyde, embedded in paraffin and cut into slices of 4-µm thickness for HE, PAS and Masson staining. The remaining kidney tissues were cut into small sections, placed in liquid nitrogen and then transferred to -70°C prior to western blot analysis.

BS was measured from the tail vein using a glucose meter (clofibrate; Abbott Laboratories, Abbott Park, IL, USA) at the end of weeks 4, 8 and 12 of the experimental period. The 24 h-urinary protein was measured, and SCr and BUN were analyzed using an automatic biochemical analyzer (Olympus, Tokyo, Japan).

Light microscopy. Left kidney tissues were fixed in 10% paraformaldehyde, embedded in paraffin and then sliced into sections for HE, PAS and Masson staining. A light microscope was used (magnification, x400) to perform the pathological analysis. In addition, six similar-sized kidney pellets from each specimen were measured for their glomerular area (GA) and glomerular extracellular matrix (ECM) area, from which the ratio of ECM/GA was calculated using Image-Pro Plus 5.1 image analysis software (Media Cybernetics, Inc., Rockville, MD, USA).

Transmission electron microscopy. The left kidney cortex was cut into 1 mm³ sections and fixed in 2.5% glutaraldehyde to observe alterations in the ultrastructure of podocytes under electron microscopy. The average width of the foot processes, foot process fusion rate and the average thickness of the glomerular basement membrane (GBM) were calculated using Image-Pro Plus 5.1 image analysis software following electron microscopy. The width of the foot processes was determined as the average value of the distances between the projections on either side of the membrane of podocytes (15). The foot process...
fusion rate was calculated as follows: The total length of basement membrane was measured and termed X, and the length of the total foot processes fusion on the basement membrane was measured and termed Y. Finally, Y/X was calculated as the foot process fusion rate (16). The average thickness of the GBM was calculated using the following method. The basement membrane was divided into a number of points every 1 cm and the basement membrane thickness was measured at each point, with the sum of the basement membrane thickness of each point termed X and the number of points termed Y. Finally X/Y was calculated as the average thickness of the GBM (17).

**Western blot analysis.** The kidney tissues (100 mg) were placed into radioimmunoprecipitation assay lysis buffer, to which 1 ml phenylmethylsulfonyl fluoride was added. The mixture was then homogenized and centrifuged for 5 min at 4°C and 8,056.8 x g with a centrifugal radius of 5 cm, and the supernatant was used to measure protein concentration following the Bradford method. A 50 mg sample of supernatant was boiled for 5 min and was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% separation gel, 6% stacking gel), followed by electrical transfer onto a polyvinylidene fluoride membrane. The membrane was blocked for 1.5 h in 5% skim milk TBST solution at room temperature, following which the membranes were washed three times in TBST (5 min each). Subsequently, the membranes were incubated with rabbit-anti-mouse nephrin polyclonal antibody (1:400) at 4°C in a shaker overnight. The following day, the membrane was washed in TBST for 10 min five times, and was incubated with HRP-labeled secondary antibody (1:40,000) for 1.5 h at room temperature. Following washing of the membrane using TBST for 10 min five times, Millipore brightening agent (EMD Millipore, Billerica MA, USA) was added in a dark room, followed by X-ray film exposure for 1-5 min, development and fixing. The fixed hybridization signals were scanned using an optical density scanning image analysis system. The housekeeping gene β-actin was used as a protein loading control, which was compared with the other groups to obtain the relative quantity, and the average was calculated to analyze the results.

**Statistical analysis.** Values are presented as the mean ± standard deviation. Statistical analysis was performed using GraphPad Prism software (version 4.0; GraphPad Software, Inc., San Diego, CA, USA). One-way analysis of variance was performed where appropriate. *Post-hoc* Bonferroni pairwise comparisons were used to assess significant differences between two groups. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**General condition of the animals.** The daily eating and drinking habits, mental status and activities of the rats in group N were normal. However, polyuria, polydipsia and increased food intake were observed in the rats of groups DN, F and L from 4 weeks post-STZ injection. At week 4, the above symptoms became evident, and the symptoms were more marked with extended duration in groups DN, F and L. At week 12, the rats in the three groups exhibited darkened hair color, listlessness and reduced activity, and seven rats succumbed to mortality during the experiment, including three rats in group DN, two in group F and two in group L.

**Measured indicators.** There was a significant increase in BS and decrease in BW in the rats in groups DN, F and L, compared with group N (P<0.05) at the end of weeks 4, 8 and 12. However, no significant differences in BS or BW were found between groups F and L and group DN at the end of weeks 4, 8 and 12. The 24 h-urinary protein was significantly increased in the rats in groups DN, F and L 4 weeks following DN model establishment, and there were significant differences between groups DN, F and L and group N (P<0.05). With increased duration, the 24 h-proteinuria increased more markedly. Significant decreases in proteinuria were found in groups F and L, compared with that than in group DN at the same time point (P<0.05; Table I).

There were marked increases in Scr, BUN and KW/BW in groups DN, F and L, compared with group N at the end of week 12 (P<0.05); however, Scr, BUN and KW/BW in groups F and L were significantly decreased, compared with those in group DN (P<0.05; Table II).

**Pathological changes.** There were no changes in the glomerular, tubular or renal interstitium on evaluation of HE, PAS and Masson staining under light microscopy. However, compared with group N, the kidney pathology in the rats of group DN showed significantly increased glomerular volume, expanded glomerular capillary loop, proliferative mesangial cells, increased mesangial matrix, thickened basement membrane, tubular cell hypertrophy, vacuolar degeneration, tubular luminal narrowing and increased interstitial inflammatory cells on evaluation of HE, PAS and Masson staining under light microscopy.

In groups F and L, there were marginal pathological changes of mesangial cell proliferation, increased mesangial matrix, thickened GBM and interstitial inflammatory cell infiltration, compared with group DN (Figs. 1-3). There were significant increases in ECM area, GA and their ratios in groups DN, F and L, compared with group N (P<0.05). These indices were significantly decreased in groups F and L, compared with group DN (P<0.05; Table III).

**Ultrastructural changes.** In the rats in group N, the podocyte structures were complete and neatly arranged in foot processes, and the basement membrane was uniform without widening when visualized under an electron microscope. However, foot processes exhibited derangement, widening and fusion or had disappeared, and the basement membrane exhibited a degree of diffuse thickening in group DN. The above changes were significantly reduced in groups F and L, compared with group N (Table IV and Fig. 4).

**Protein expression of nephrin.** The results of the western blot analysis, determined by measuring the optical density of the tissue sections, showed that the expression of nephrin decreased by 60.1% in group DN, compared with group N (P<0.05) and significantly increased by 49.2 and 46.9% in groups L and F, compared with group DN (P<0.05). No significant differences were found between groups L and F (Fig. 5).
DN is one of the common and serious chronic complications of DM, and the early clinical manifestations of DN are microalbuninuria followed by the appearance of clinical proteinuria. Proteinuria is the primary clinical manifestations of DN and is also an independent risk factor for kidney damage. Therefore, it is particularly important to actively investigate proteinuria and is also an independent risk factor for kidney damage. Proteinuria is the primary clinical manifestations of DN.

Table I. Comparison of BS, BW and 24 h proteinuria.

<table>
<thead>
<tr>
<th>Group</th>
<th>Week</th>
<th>BS (c/mmoll⁻¹)</th>
<th>BW (m/g)</th>
<th>Proteinuria/ 24 h (mg)</th>
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</thead>
<tbody>
<tr>
<td>N</td>
<td>0</td>
<td>-</td>
<td>259.67±12.58</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>7.50±0.36</td>
<td>273.33±44.19</td>
<td>11.68±3.28</td>
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<td></td>
<td>8</td>
<td>7.40±0.26</td>
<td>264.33±27.97</td>
<td>12.12±2.61</td>
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<td>12</td>
<td>7.27±0.76</td>
<td>237.33±57.07</td>
<td>14.26±3.41</td>
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<tr>
<td>DN</td>
<td>0</td>
<td>-</td>
<td>257.00±20.66</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>22.13±5.10</td>
<td>196.67±17.47</td>
<td>32.74±5.36</td>
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<td>21.60±5.40</td>
<td>176.67±18.50</td>
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<td></td>
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<td>19.17±2.94</td>
<td>163.67±11.93</td>
<td>123.16±7.96</td>
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<tr>
<td>L</td>
<td>0</td>
<td>-</td>
<td>251.33±17.16</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>25.67±3.70</td>
<td>185.00±23.58</td>
<td>33.57±2.38</td>
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</tr>
<tr>
<td></td>
<td>12</td>
<td>25.53±1.97</td>
<td>148.00±10.39</td>
<td>96.47±6.87</td>
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<tr>
<td>F</td>
<td>0</td>
<td>-</td>
<td>252.00±21.21</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>26.72±2.15</td>
<td>188.50±17.35</td>
<td>31.59±6.43</td>
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<tr>
<td></td>
<td>8</td>
<td>23.62±3.45</td>
<td>187.00±13.64</td>
<td>43.82±0.95</td>
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<tr>
<td></td>
<td>12</td>
<td>20.82±1.97</td>
<td>161.25±25.46</td>
<td>94.39±8.04</td>
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</table>

Data are presented as the mean ± standard deviation. *P<0.05, vs. group N; **P<0.05, vs. group DN. N, normal control; DN, diabetic nephropathy; L, benazepril (Lotensin); F, tacrolimus (FK506); BS, blood sugar; BW, body weight.

Table II. Comparison of Scr, BUN and KW/BW.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Scr (c/µmol·l⁻¹)</th>
<th>BUN (c/µmol·l⁻¹)</th>
<th>KW/BW (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>8</td>
<td>37.85±5.36</td>
<td>5.89±1.83</td>
<td>4.22±0.41</td>
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<tr>
<td>DN</td>
<td>7</td>
<td>99.19±4.25</td>
<td>20.11±3.15</td>
<td>7.47±0.07</td>
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<tr>
<td>L</td>
<td>8</td>
<td>75.54±6.13</td>
<td>11.88±3.26</td>
<td>6.36±0.10</td>
</tr>
<tr>
<td>F</td>
<td>8</td>
<td>77.07±3.62</td>
<td>13.07±2.15</td>
<td>6.28±0.13</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± standard deviation. *P<0.05, vs. group N; **P<0.05, vs. group DN. N, normal control; DN, diabetic nephropathy; L, benazepril (Lotensin); F, tacrolimus (FK506); Scr, serum creatinine; BUN, blood urea nitrogen; KW/BW, kidney weight/body weight.

Discussion

DN is one of the common and serious chronic complications of DM, and the early clinical manifestations of DN are microalbuninuria followed by the appearance of clinical proteinuria. Proteinuria is the primary clinical manifestations of DN and is also an independent risk factor for kidney damage. Therefore, it is particularly important to actively investigate the mechanism of diabetic proteinuria. In previous years, studies investigating the mechanism of proteinuria provided encouraging results, and it was confirmed that podocyte lesions are important in the development and progression of proteinuria (18). Lin et al (19) reported that two key factors of proteinuria progression were a reduced number of podocytes and decreased expression of nephrin in podocytes. In 1999, Tryggvason (20) found that nephrin molecules are expressed by kidney podocytes, are positioned in the slit diaphragm of the membrane, belong to the immunoglobulin superfamily transmembrane protein and are involved in cell signal transduction, maintaining the normal morphology and function of podocytes. Abnormalities in the gene and/or the protein expression of nephrin are important in the pathogenesis of proteinuria. Previous reports have shown that abnormal distribution and expression of nephrin is involved in the pathogenesis of proteinuria in several glomerular diseases, and a reduction in the protein expression of nephrin may lead to loss of slit diaphragm holes in the membrane structure, proteinuria and CKD (20,21).

Several studies have confirmed that nephrin is significantly reduced in patients with DN, which is actively involved in DN. Langham et al (22) observed that, in 14 diabetic patients with proteinuria, the mRNA expression of nephrin was decreased by 62%, compared with control group, and increased linearly with proteinuria. Toyoda et al (23) confirmed that the mRNA expression of nephrin is decreased in human DN, which causes structural and functional damage to the podocyte slit diaphragm and can exacerbate with the deterioration of kidney disease. Koop et al (21) found that the expression of nephrin and podocin protein were significantly decreased in podocytes of patients with acquired renal disease, including proteinuria and CKD (20,21).
The results of the present study showed that the expression of nephrin were significantly decreased in the rats of group DN, compared with those of group N, and 24 h-urinary protein excretion was significantly higher in group DN, compared with group N (P<0.05). These results were consistent with the above findings.
Tacrolimus is a novel type of immunosuppressant, isolated in 1984 from a novel soil microbial medium (24). Tacrolimus is a potent immunosuppressant, and several animal experiments and clinical applications have demonstrated that it has a similar immunosuppressive effect to cyclosporin A (Cys A), with more potent immunosuppressive effects, compared with CysA (10-100-fold higher) and fewer side effects (25). Previous studies have found that tacrolimus not only forms a basis for immunosuppression to prevent kidney transplant rejection, but also for the treatment of chronic allograft nephropathy and lupus nephritis, which can significantly delay glomerulosclerosis and tubulointerstitial damage (26,27). Studies have shown that tacrolimus inhibits transforming growth factor-β-induced kidney tubular epithelial cell transdifferentiation, which may be an important mechanism of tacrolimus in treating organ rejection post-transplantation, and which also provides a theoretical basis for the prevention and treatment of renal fibrosis (28).

According to previous reports, immunosuppressants can reduce renal pathological damage and urinary albumin excretion rates, and the mechanism may be associated with the inhibition of renal macrophage infiltration (29). Gooch et al (30) found that Cys A, a type of calcineurin (CaN) inhibitor, can significantly inhibit the protein expression and activity of CaN, and reduce renal hypertrophy and ECM accumulation in the diabetic kidney. It has been shown that, in its application as a CaN inhibitor, tacrolimus also inhibits early diabetic renal hypertrophy and increases urinary albumin excretion, consistent with the results of Cys A (31). However, few studies have reported on whether there is an association between the protective effect of tacrolimus in renal tissues of a diabetic model and podocytes.

It has been confirmed that angiotensin converting enzyme inhibitor (ACEI) drugs can lower blood pressure and reduce significant proteinuria, protecting the kidneys and delaying the progression of kidney disease (32). Clinically, benazepril, a type of ACEI, is important in ameliorating diabetic kidney damage (33). In the present study, benazepril treatment was used as a positive control, and the results showed that tacrolimus and benazepril significantly reduced the levels of urinary protein, Scr and BUN in groups F and L, compared with those in group DN (P<0.05). The pathological changes observed under light microscopy were also significantly reduced in groups F and L, with no significant difference found between the therapeutic effects of the two drugs. The above results showed that the protective effect of tacrolimus on diabetic kidney tissue injury was similar to that of benazepril.

In the present study, the results of the electron microscopy and western blot analysis showed that the foot processes of podocytes manifested derangement, widening, fusion and even disappearance, and GBM showed diffuse thickening in DN. However, in groups F and L, the above-mentioned pathological alterations were significantly alleviated (P<0.05). The expression levels of nephrin in the kidneys of DN rats were significantly lower, compared with those in group N, however, the expression of nephrin in groups F and L were increased, compared with those in group DN (P<0.05). The results of the present study indicated that tacrolimus improved renal podocyte ultrastructure in diabetic rats, reduced DN renal damage and delayed the progression of kidney disease by upregulating the expression of nephrin.

The results of the present study indicated that tacrolimus may have partly regulated the expression of nephrin, and had a protective effect on kidney injury in DN. This may provide a novel theoretical basis for the treatment of DN with tacrolimus. Tacrolimus has renal toxicity and islet cell toxicity properties, which can lead to elevated BS. In the present study, no increase in BS was observed following the application of tacrolimus, and this was associated with the relatively short duration of experimentation and low drug cinentration. Although the nephrotoxicity of tacrolimus is lower than that of CysA, caution is required in using tacrolimus to treat non-renal transplant patients with DN, and the nephrotoxicity of tacrolimus on diabetic renal requires further investigation.

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References


