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Original Article

Protective effects of low-dose rapamycin combined with valsartan on podocytes of diabetic rats

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Abstract: The aim of this study was to study the impacts and the mechanisms of low-dose rapamycin combined with valsartan on the renal functions of diabetic nephropathy (DN) rats. 50 SD rats were randomly divided into the normal control group (group A, n=10) and the DN model group (n=40), the DN model group was intraperitoneally injected streptozocin (STZ) for the modeling, which were then equally divided into the DN group (group B), the rapamycin group (group C, orally administrated rapamycin 1 mg/kg/d), the valsartan group (group D, orally administrated valsartan 30 mg/kg/d) and the combined therapy group (group E, orally administrated rapamycin 1 mg/kg/d + valsartan 30 mg/kg/d). Group A and group B were orally administrated the same amount of 0.5% carboxymethylcellulose. After 8-week treatment, the rats of each group were killed for the renal functional and pathological detection, as well as the expression detection of nephrin and podocin of kidney tissues. Compared with group A, the renal functions of the DN model groups were all decreased, and the pathological changes were significant. Meanwhile, the expressions of nephrin/podocin were reduced ($P<0.05$); among which group B exhibited the most serious changes, while the situations of group E were improved after the combined treatment, the expressions of nephrin/podocin were increased. Low-dose rapamycin and valsartan could enhance the expressions of nephrin and podocin, reduce kidney damages, thus achieving the protective effects towards the kidneys, and the effects of the combined therapy were superior to those of monotherapy.

Keywords: Podocytes, rapamycin, valsartan, diabetic nephropathy

Introduction

Diabetic nephropathy (DN) was one major microvascular complication of diabetes [1], and one of the most common chronic complications of diabetes in China currently [2], with the gradual increasing incidence of diabetes, the incidence of DN was also increased, and it had become a major cause of mutilation and death of diabetes [3], and from the global perspective, DN was also one of the major diseases that would lead to the end-stage renal disease, as well as the leading cause of end-stage renal disease (ESRD) in Europe and other countries [4]. The pathogenesis of DN was complex, which used the sustained clinical proteinuria as the main indicator [5]. However, the nephrin-CD2AP-podocin complex was the key unit that could rivet the slit membrane onto the actin cytoskeleton of podocytes, and the necessary condition for maintaining the normal glomerular filtration functions [6], therefore, the expression and distribution abnormalities of its related proteins could lead to the imperfection of slit membrane of podocytes, which would ultimately lead to the proteinuria, especially the abnormal expressions of nephrin and podocin, podocyte slit membrane-related proteins, played an important role in the occurrence of proteinuria and development of diabetes [7], the promotive roles of downregulation and loss of nephrin protein in the DN process towards the proteinuria had been confirmed [8]. Clinically, besides controlling the primary diseases, namely the active antidiabetic treatment [9], upregulating the levels of nephrins and podocin was also used to reduce the damage extent of DN, as well as to reduce the excretion of proteinuria [10]. As an immunosuppressant, rapamycin was reported to be able to reduce the renal lesions of DN, and delay the progression of nephropathy [11]. In addition, because the high blood pressure

could contribute to the development of renal failure, the antihypertensive therapy could slow the reduction of glomerular filtration rate, and reduce the excretion of proteinuria [12]. So, this research studied the effects of low-dose rapamycin combined with valsartan in intervening the expressions of nephrin and podocin, detected the changes of renal pathological and related indicators in DN rats, aiming to explore the protective effects of low-dose rapamycin combined with valsartan towards the podocytes of diabetic rats.

Materials and methods

Grouping of animal models

Male SD rats, weighed 180~200 g body weight, were selected from the Henan Experimental Animal Center. After adaptive feeding for one week, the rats were randomly divided into the control group (group A, n=10) and the DN model group (n=40) by the random number table. The rats in the DN model group were fasted for 12 h, then intraperitoneally injected streptozotocin 60 mg/kg (Sigma, USA), after 48~72 h, the random blood glucose >16.7 mmol/L indicated that the diabetic model was successfully prepared. 4 weeks later, the rats with urinary protein >30 mg/24 h were seen as the successfully modeled DN rats. All the rats were successfully prepared the DN model, which were then randomly divided into the DN group (group B, n=10) and the rapamycin group (LC Labs, USA) (group C, n=10), the valsartan Group (Novartis, Switzerland) (group D, n=10) and the combined treatment group (group E, n=10). Group C was orally administrated rapamycin (1 mg/kg/d), group D was orally administrated valsartan (30 mg/kg/d), group E was orally administrated rapamycin (1 mg/kg/d) + valsartan (30 mg/kg/d), group A and group B were orally administrated the equal volume of 0.5% carboxymethyl cellulose, the body weights of rats were weekly weighed to adjust the dosage. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Zhengzhou University.

Sample collection

Urine specimens: After 8-week drug intervention, the 24 h urine of rats of each group were collected 2 days before the harvest, an performed the immunoturbidimetric assay to detect the urinary albumin after the centrifugation, and the urine creatinine was detected by the alkaline picric acid assay, the ratio of urinary albumin/creatinine was then calculated (ACR, μg/μmol).

Blood specimens: The body weights of rats were weighed before the harvest, the blood specimens were sampled from the heart, the serum BUN, Cr, glucose (Glu) were detected by the automatic biochemical detector.

Detection of renal pathology

After the renal tissues were weighed, the kidney weight index was calculated, KWI (mg/g)= kidney weight (mg)/body weight (g). 1/4 of renal tissues was fixed in 10% neutral formalin, the renal tissues were then performed the dehydration, hyalinization, wax dipping, embedding and slicing, then observed under the ordinary light microscope for the renal histological changes which were revealed by the HE and Masson staining.

RT-PCR

Total RNA was extracted from renal cortex, the concentration of total RNA was measured, and the cDNA was synthesized. The PCR test was performed to amplify the genes of nephrin and podocin, with β-actin as the internal reference. The amplification conditions were: pre-denaturation at 94°C for 3 min, denaturation at 94°C for 30 s, annealing at 57°C for 40 s, extension at 72°C for 45 s, with a total of 30 cycles, extension at 72°C for 5 min. The PCR products were performed the 1.5% agarose gel electrophoresis, then the gel image analysis software was used to detect the absorbance values of electrophoretic images, the optical density ratios of nephrin and podocin towards β-actin were set as the relative expression levels of the detection indicators and for the statistical process. The primers of nephrin, podocin and β-actin were synthesized by Nanjing jinsite Co, and the primers' sequences and product sizes were: β-actin primer sequences: Upstream: 5'-CTGAACCCTAAGGCCAACC-3'; Downstream: 5'-CTGAACCCTAAGGCCAACC-3', the amplified fragment length was 309 bp; nephrin primer sequences: Upstream: 5'-TACCACCAGCATTTC-CACG-3'; Downstream: 5'-GGGCTCGGCTGTAT-GTATT-3', the amplified fragment length was

Table 1. Impacts of RPM on general indexes of each group ($\bar{x} \pm s$)

Item	Group A (n=6)	Group B (n=6)	Group C (n=6)	Group D (n=6)	Group E (n=6)
Glu (mmol/L)	5.14 ± 1.08	27.69 ± 4.55[b]	26.03 ± 5.30[b]	26.61 ± 3.21[b]	26.39 ± 2.77[b]
BUN (mmol/L)	4.44 ± 0.36	10.92 ± 2.03[b]	9.21 ± 1.98[a]	9.71 ± 2.13[b]	7.17 ± 2.05[b]
Scr (umol/L)	40.56 ± 3.43	90.44 ± 10.31[a]	69.52 ± 8.12[a]	71.66 ± 5.03[a]	58.34 ± 5.12[a]
TC (mmol/L)	0.91 ± 0.25	2.49 ± 0.37[a]	2.31 ± 0.31[a]	2.41 ± 0.26[a]	2.56 ± 0.48[a]
TG (mmol/L)	0.68 ± 0.29	1.67 ± 0.31[a]	1.61 ± 0.33[a]	1.65 ± 0.27[a]	1.57 ± 0.43[a]
ACR (μg/μmol)	53.50 ± 7.88	178.27 ± 21.01[b]	100.3 ± 8.02[a,c]	118.2 ± 7.66[a,c]	70.8 ± 6.09[a,c,d]
KWI (mg/g)	5.04 ± 0.24	9.41 ± 0.83[b,c]	7.78 ± 1.07[b,c]	7.17 ± 1.16[a,c]	6.25 ± 1.03[a,c,d]

Note: Compared with group A, [a]$P<0.05$, [b]$P<0.01$; compared with group B, [c]$P<0.05$; compared with group C and D, [d]$P<0.05$.

299 bp; podocin primer sequences: Upstream: 5'-AGCGAATCAGCACCTACGGACTYC-3'; Downstream: 5'-GGTTCCACTCCACCAGCCTTCTTC-3'; the amplified fragment length was 200 bp.

Western blotting

50 μg protein of renal cortex was firstly isolated on the SDS-polyacrylamide gel, then transferred onto the nitrocellulose membrane, after closed with 5% skim milk for l h, the goat anti-rat nephrin antibody and goat anti-rat podocin antibody (1:50, diluted by TBS) (Santa Cruz, USA) were added, and incubated overnight at 4°C, followed by TBST-washing for three times; added the secondary antibody (1:100, diluted by TBS) (zymed, USA), TBST washed for three times; ECL luminescence, developed and fixed, after the films were scanned, the grayscale analysis was performed with Bandscan 5.0 software to analyze the relative contents of Nephrin and Podocin.

Statistical analysis

The SPSS13.0 software package was used for the statistical analysis. The measurement data were expressed as $\bar{x} \pm s$. the intergroup difference was compared using ANOVA, the paired comparison between two groups used the LSD test, with the test level a set as 0.05, and $P \leq 0.05$ was considered as the statistical significance.

Results

Blood and urinary biochemical indicators

Compared with group A, the serum BUN, Scr, Glu, total cholesterol (TC), triglyceride (TG), ACR and KWI of group B, C, D and E were all significantly increased ($P<0.05$ or $P<0.01$); compared with group B, ACR, KWI and Cr of group C, D and E were decreased ($P<0.05$ or $P<0.01$); compared with group C and D, ACR and KWI of group E were significantly decreased ($P<0.05$). The data are shown in **Table 1**.

Renal pathological changes

Compared with group A, the glomerular diameters of group B, C, D and E were increased, the mesangial matrix was increased, while the capillary lumens were narrowed, with focal invasion of lymphocytes and monocytes seen inside the renal interstitium. Compared with group B, C and D, the proliferation of mesangial matrix of group E was reduced, the capillary loops were opened well, the infiltration of inflammatory cells were reduced, and the glomerular diameter was narrowed (**Figure 1**).

RT-PCR

Compared with group A, the mRNA expressions of nephrin and podocin inside the renal cortex of group B, C, D and E were downregulated, while that of group E was higher than group B, C and D ($P<0.05$, **Figure 2**).

Western blot

The Western blot results showed that compared with group A, the protein expressions of nephrin and podocin inside the renal cortex of group B, C, D and E were downregulated, and the differences were statistically significant ($P<0.05$); and compared with group B, C and D, the protein expressions of nephrin and podocin inside the renal tissues of group E were increased, and the differences were statistically significant ($P<0.05$, **Figure 3**).

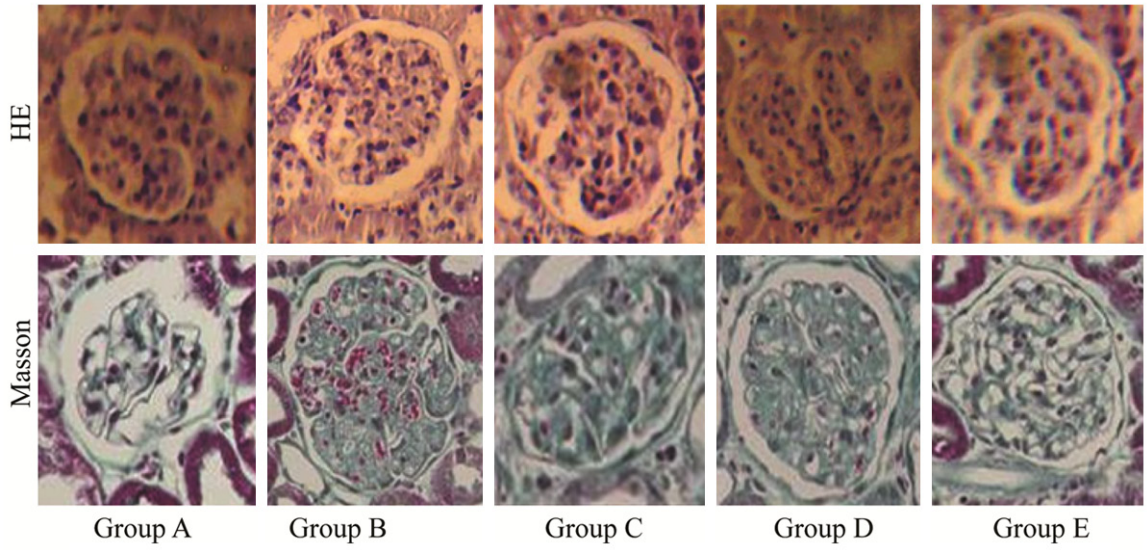

Figure 1. Renal pathological changes of each group. Group A, normal control group; Group B, DN group; Group C, rapamycin group; Group D, valsartan group; Group E, rapamycin + valsartan group.

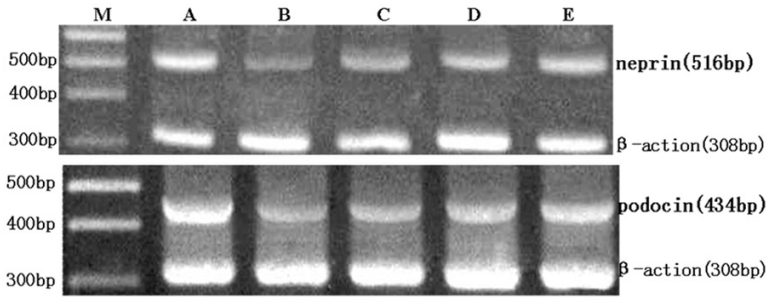

Figure 2. mRNA expressions of nephrin and podocin inside renal tissues of each group detected by RT-PCR. M, Marker; A. Normal control group; B. DN group; C. Rapamycin group; D. Valsartan group; E. Rapamycin + valsartan group.

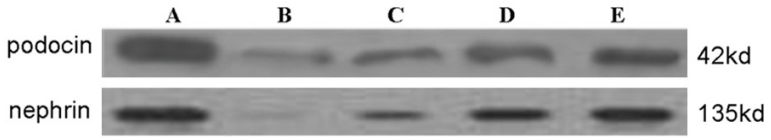

Figure 3. Protein expressions of nephrin and podocin inside renal tissues of each group detected by Western blot. A. Normal control group; B. DN group; C. Rapamycin group; D. Valsartan group; E. Rapamycin + valsartan group.

Discussion

The proteinuria was a common clinical manifestation of DN, and also the main factor that might contribute to the progress of diabetes [13-15]. The glomerular filtration membrane was composed of visceral epithelial cells, basement membrane and endothelial cells. The visceral epithelial cells were also known as the podocytes, which were the main constituents of glomerular filtration membrane and attached to the lateral side of basement membrane. The podocytes had the essential components for the synthesis of basement membrane, and participated the repairing of damaged basement membrane, they could form the autophagosomes, thus maintaining the stabilities of cellular functions through the autophage of damaged and aged organelles. Presently, many studies had shown that the damages and malfunctions of podocytes were closely related with the formation of proteinuria [16]. There existed the bridged linkage of slit membranes among the processes of podocytes, the slit membranes were composed of various protein molecules, and were the important barrier of glomerular filtration membrane. The slit membrane proteins, namely nephrin and podocin, participated the transduction of a variety of cellular sig-

nals, and exhibited the significant meaning in maintaining the normal glomerular filtration functions [17]. Certain study found that the podocyte injury appeared in the early stage of diabetes [18], the destruction of podocytes led to the damages of integrity of filtration barrier, which was an important reason for the occurrence ad development of kidney diseases [19, 20]. In DN, a variety of factors such as hyperglycemia, non-enzymatic protein glycosylation, angiotensin II (Ang II) and transforming growth factor β (TGF-β), etc., could cause the downregulation of nephrin, while the transportations of podocin and nephrin were closely related to the internal signaling transduction of podocytes [21, 22], the deficiencies of nephrin and podocin would cause the rearrangements of cytoskeleton and foot processes, thereby undermined the structural and functional integrities of glomerular filtration membrane, the gaps among the slit pores might be increased, as well as the protein might leak out, which were also the main reasons that constituted to such chronic renal proteinuria as DN [23-25]. The results of this study showed that compared with the normal control group, the blood glucose, ACR and Scr of the model group were increased, while the protein and mRNA expressions of nephrin and podocin of the DN model groups were reduced, the glomerular diameters were increased significantly, the mesangial matrix was increased, while the capillary lumens were narrowed, the renal interstitium exhibited the focal infiltration of lymphocytes and monocytes. The low-doses of rapamycin and valsartan could reduce ACR and Scr of corresponding treatment groups, upregulate the protein and mRNA expressions of nephrin and podocin within the renal cortex, reduce the proliferation of mesangial matrix, the capillary loops were thus opened well, the infiltration of inflammatory cells was then decreased, and the glomerular diameters were also decreased. Furthermore, the indicators of the combined therapy group were better than those of the monotherapy group. The most obvious pathological changes of DN were the thickened basement membrane, proliferated extracellular matrix and damaged small arteries, which would eventually exhibit the sphered hardening. Certain study had shown that the mTOR signaling pathway played an important role in the DN process [26]. mTOR was the target molecule of rapamycin, as well as an important signaling molecule, which could coordinate the synthesis of a variety of growth factor-induced proteins. mTOR existed inside the living body with two forms of complexes, i.e. mTORCl and mTORC2, and mTORCl was sensitive towards the inhibition of rapamycin. The hyperglycemia could activate the mTORC1 pathway, leading to such vascular complications as the damages of microvascular endothelial cells [27-29]. This study revealed that the protein and mRNA expressions of nephrin and podocin inside the renal tissues of DN rats were significantly lower than the normal rats, the glomerular functions and structures were all pathologically changed, while when the protein expressions of nephrin and podocin were upregulated, the renal glomerular functions and structures were improved, indicating that the abnormal expressions of nephrin and podocin were related with the damages of filtration barrier in DN. Rapamycin could inhibit the activities of mTORCl of podocytes, enhance the expressions of podocyte-specific protein (nephrin) and increase the autophagy, thus maintaining the homeostasis of podocytes, maintaining the integrities of glomerular filtration barrier and podocyte structures, playing the roles of reducing the proteinuria in various chronic kidney diseases, and slowing the progression of DN [30, 31]. The angiotensin II (Ang II) was the main active medium of renin-angiotensin-aldosterone system (RAAS), widely distributed in the kidney tissues, when DN occurred, accompanied by the abnormal activation of RAAS, the Ang II levels of local renal tissues might be increased [32-34]. Ang II could promote the cytoskeletal rearrangement of podocytes, which were shown as the fusion and disappearance of foot processes, damages of filtration barrier and macroalbuminuria [35], certain study showed that nephrin could inhibit the Ang II-induced apoptosis of mouse podocytes [36]. Valsartan could selectively competitively combine with Ang II receptor subtype 1 (AT1) and block the effects of Ang II, and there was evidence indicating that when Ang II within the ATl receptor antagonist-treated diabetic rats was downregulated, the nephrin mRNA expression level of podocytes could be upregualted to the normal level [37]. In this research, by upregulating the expressions of nephrin and podocin, rapamycin and valsartan could maintain the integrities of protein structures of podocyte slit membrane, reduce the glomerular injuries, and

these effects were independent from the regulation of blood sugar. Currently, there had been many studies about the ARB drugs in reducing the proteinuria leakage in DN, this study applied low-doses of rapamycin and valsartan, explored the combinative effects of immunosuppressive drugs combined with ARB drugs in interfering the functions of podocytes of DN rats, tried to minimize the side effects caused by the high-dose of single medication, and tried the multi-factorial intervention factors for the greater therapeutic effects, the results showed that the combination of these 2 drugs had much more prominent protective effects towards the glomerulus of diabetic rats, and could provide a reference for the further clinical applications.

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Disclosure of conflict of interest

None.

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