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Original Article

Improved effect of adenovirus-mediated adiponectin on renal function of diabetic nephropathy rats

Yu Chen[1], Kun Men[2], Jian-Chao Guo[1], Zhen-Qian Fan[1]

Departments of [1]Endocrinology, [2]Laboratory, The Second Hospital of Tianjin Medical University, Tianjin, China

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Abstract: Objective: To investigate the improved effect of the recombinant adenovirus with local transfection of adiponectin gene in vivo on the renal function of diabetic nephropathy in rats. Method: Among 75 Wistar rats, 15 rats without treatment were randomly selected as the normal control group. The model of diabetic nephropathy rats was established by intraperitoneal injection of STZ and feeding with a high fat diet to the remaining 60 rats. And they were randomly and equally divided into the diabetic nephropathy group (saline), the recombinant adenovirus with local transfection of adiponectin gene group, and the empty virus vector group. After 6 weeks intervention, the expression levels of adiponectin in renal tissues of each group were detected by using RT-PCR and Western blot. The levels of blood glucose, serum insulin, SCr, BUN and 24 h urine protein were analyzed, and renal pathologic changes and renal ultra-structure changes were observed under the light microscope and electron microscope respectively. What's more, apoptosis of kidney cells was detected by TUNEL method, and body weight and kidney weight of rats were measured as well in the experiment. Result: The expression of adiponectin mRNA and adiponectin protein was higher in recombinant adenovirus with local transfection of adiponectin gene group than that in the normal control group, the diabetic nephropathy group and the empty virus vector group ($P<0.05$). Compared with the diabetic nephropathy group and the empty virus vector group, the levels of blood glucose, SCr, BUN and 24 h urine protein in recombinant adenovirus with local transfection of adiponectin gene group were decreased significantly, whereas the level of serum insulin was increased greatly ($P<0.05$). The level of blood glucose, SCr, BUN and 24 h urine protein in the normal control group was the lowest and the level of serum insulin was the highest ($P<0.01$). Rat kidney weight/body weight: the normal control group was the lowest ($P<0.05$), and the recombinant adenovirus with local transfection of adiponectin gene group was lower than the diabetic nephropathy group and the empty virus vector group ($P<0.05$). The TUNEL positive apoptosis rates of the diabetic nephropathy group and the empty virus vector group were the highest, followed by the recombinant adenovirus with local transfection of adiponectin gene group, and the apoptosis rate of the normal control group was the lowest ($P<0.05$). Compared with the diabetic nephropathy group and the empty virus vector group, pathologic changes and ultra structure changes of the kidney in recombinant adenovirus with local transfection of adiponectin gene group were reduced considerably. Conclusion: The recombinant adenovirus with local transfection of adiponectin gene can not only improve kidney pathomorphology and ultra structure of diabetic nephropathy rats, but also significantly decrease the level of blood glucose, SCr, BUN and 24 h urine protein. Meanwhile, it can help increase the level of serum insulin, and reduce cell apoptosis, which can improve renal dysfunction to a certain degree.

Keywords: Adenovirus, adiponectin, nephropathy, rat, diabetes, renal function

Introduction

Diabetic nephropathy (DN) is caused by type 2 diabetes and its pathogenesis and mechanism of diabetic nephropathy are complicated. And one of the most common DN complications is the systemic microvascular complication [1, 2]. Recently in China the number of patients with DR has shown an obvious upward trend [3], and DN complications have a serious impact on patients. Therefore, DN along with its complications has arisen more and more attention worldwide. At the early stage of DN, the glomerular filtration rate and levels of urine protein are increased, leading to the renal insufficiency [4]. The renal function of DN patients with such kidney damage fails much faster than those without such damage [5]. What's more, this disease is the main cause of late stage renal failure. Renal function cannot be improved through either clinic intervention or strict glycemic control [6].

Table 1. Primer design and length of the target gene

Target gene	Primer Sequence	Fragment length
Adiponectin	Upstream: 5'-TCA TTA TGA CGG CAG CAC-3'	162 bp
	Downstream: 5'-CCA GAT GGA GGA GCA CAG-3'	
β-actin	Upstream: 5'-CAA TTC CAT CAT GAA GTG TGA C-3'	184 bp
	Downstream: 5'-CCA CAC AGA GTA CTT GCG CTC-3'	

In recent years, there have been some reports studying on the correlation between disease progress and lipid metabolism disorder. Moreover, for DN patients, adiponectin is one of the influential factors of renal function [7, 8]. Adiponectin is a special protein secreted from adipocytes, whose molecular weight is 30 kDa [9, 10]. The previous studies provide a promising prospect for our study in renal function improvement, however there is a research gap about the influence of adiponectin on renal function improvement. This study aims to discuss the improved effect of adenovirus-mediated adiponectin on the renal function of diabetic nephropathy, by comparing rats' renal function among the diabetic nephropathy group, the empty virus vector group and the recombinant adenovirus with local transfection of adiponectin gene group through experiment.

Materials and methods

Design

Randomized controlled animal experiment study. Time and setting: The experiment was carried out at the Institute of Endocrinology, Tianjin Medical University between Feb. 2014 and Oct. 2015.

Experimental animals, main reagents and equipments

75 Wistar Rats with half males and half females, which weighted 190~230 g, were purchased from the animal laboratory of Chinese Academy of Medical Sciences (Animal Quality Certification No.: SCXK (Jin) 20090028). All the disposals in this study were in accordance with the guideline of animal ethics. Trizol, LipofectamineTM2000 (Invitrogen Company); Western blotchemiluminescence reagents (Santa Cruz Company); Rabbit anti-mice anti-Adiponectin, HRP-IgG and PVDF (Pierce Company); 5 μL microsyringe (Hamilton, USA); main equipments include CO_2 incubator (RS Biotech, UK); clean benches (Suzhou Antai Airtech Co., Ltd), Fluorescent Inverted microscope (Nikon Company, Japan) and Transmission Electron Microscope (Hitachi Limited, H600).

Experimental methods

Animal model construction and grouping: After adaptive breeding in the Institute of Endocrinology, Tianjin Medical University for 1 week, among 75 Wistar rats, 15 rats without treatment were randomly selected as the normal control group. The model of diabetic nephropathy rats was established by intraperitoneally injecting STZ (once per week, for 3 weeks) and feeding with a high fat diet for the remaining 60 rats. 72 hours after modeling, the blood glucose level was measured from the caudal vein and a level of 16.7 mmol/L indicated DM successful establishment. The mALB and UCr were analyzed after 1 week. mALB/UCr in the model group was significantly higher than that in the normal control group, indicating the DN model was successfully constructed. Subsequently, the model group was randomly and equally divided into the diabetic nephropathy group (150 μL saline was injected into the left kidney), the recombinant adenovirus with local transfection of adiponectin gene group (150 μL recombinant adenovirus vector with local transfection of adiponectin gene was injected into the left kidney), and the empty virus vector group (150 μL recombinant adenovirus vector with local transfection of adiponectin gene was injected into the left kidney).

Sq RT-PCR

Total RNA was extracted from renal tissues, and reversely transcribed into cDNA. The accuracy of the primer sequence was checked on Genebank database. Target genes were amplified by RT-PCR using the cDNA as the template. After analyzing the accuracy of the band positions of the oligonucleotide fragment and RNA in electrophoretic separation, the ratio of the target gene and the absorbance gray value were detected by using the gel imaging analysis system. The ratio reflected the relative expression level of the target gene, and the design and length of the target gene primer are shown in **Table 1**.

Detecting the expression of adiponectin protein by using western blot

PCR residuum and total protein were collected by centrifugation. Electrophoresis and transmembrane were performed on vertical electrophoresis apparatus from BioRad company. Hybridization was conducted based on the instruction of Western Blot KitBCIP/NBT System from KPL Company. The PVDF, with 5% volume fraction skim milk powder, was cultured in the shaking bed for 1 h. After 1 hour, skim milk powder was discarded, and 1:800 diluted rabbit anti-mouse anti-adiponectin antibodies were added into PVDF (diluted by skim milk powder). After 2 hours in the 37°C shaking bed, the primary antibodies were discarded and TBST was added, followed by shaking the bed for 10 min, three times. Then 1:200 diluted goat anti-rabbit IgG-HKP secondary antibodies were added (secondary antibodies were diluted by skim milk powder). After 1 hour in a 37°C shaking bed, the secondary antibodies were discarded and the TBST was added, followed by shaking the bed for 10 min, three times.

Chemiluminescence, chemical development, chemical fixing and gel image analysis: after scanning all the films, the absorbance value of target strips (adiponectin/β-actin) was analyzed by using gel analysis software Bandoscan 5.0.

The level of blood plasma, serum insulin, SCr, BUN and 24 h urine protein

6 weeks after transplantation therapy, rats' blood was taken from orbital and blood glucose was analyzed on blood glucose test strips. At the 8th week, blood plasma was collected by abdominal aortic method after rats were etherized. Based on the instruction of the ELISA kit, serum insulin in partial plasma of 5 groups' rats was analyzed. The serum and sediment of the rest plasma were separated in room temperature by centrifugation (5000 r/min, 3 min), and they were kept at -80°C for future usage. And SCr and BUN were measured by creatinine menstruation reagent kit (CRE) and BUN menstruation reagent kit (OPA). The creatinine menstruation reagent kit (CRE) included R1 0.2 mol/L sodium hydroxide and 25 mmol/L picric acid. The BUN menstruation reagent kit (OPA) included: R1 HADH and sodium azide; R2 α-oxpentanedioic acid, GLDH, UA and sodium azide. Coagulation indices were detected by an automatic biochemical analyzer. 8 weeks after transplantation therapy, 24 h urine of rats in all groups was collected and mixed. 5 ml urine was centrifuged (5000 r/min, 3 min) and the supernatant was separated. 24 h urine protein was analyzed in an automatic biochemical analyzer.

Rats' body weight and kidney weight measurement

6 weeks after intervention, the body weight of rats in each group (the normal control group, the diabetic nephropathy group, the empty virus vector group and the recombinant adenovirus with local transfection of adiponectin gene group) was measured 3 times. Then, the left kidney of all rats in above 4 groups was removed after anesthetized. The kidney was weighted in the Petri dish in order to calculate kidney weight/body weight.

Pathologic observation of rats' kidney

Routine pathological sections were fixed in 10% buffered neutral formalin, and stained by hematoxylin and eosin. The renal morphology was checked through light microscopy. 1 mm *1 mm *1 mm cortex from the right kidney upper pole was obtained and washed by PBS. Then the cortex was fixed in 2.5% glutaraldehyde and 1% osmic acid, which was infiltrated and imbedded by Epon812 epoxy resin. The prepared tissue was sliced at LKBI ultramicrotome, and stained by 3% uranyl acetate and lead citrate. Then the renal pathologic changes were observed under a Hitachi H600 transmission electron microscope.

Apoptosis and renal function assessed by the TUNEL method

The TUNEL method was applied in this experiment according to the instruction of Germany Roche Company. The paraffin sections of left kidney sample were digested by proteinase K for 10 minutes and marked by index liquid in 37°C. After 30-minute biotinylated dioxin reaction, SABC and DAB were added into paraffin sections, and afterwards the paraffin sections were sealed. In order to calculate apoptosis rates, 5 distinct high power fields were randomly chosen from damaged areas, and the number of cells with granular brown substance in the nuclei was counted in a MIAS-2000 high-resolution color graphic analysis system.

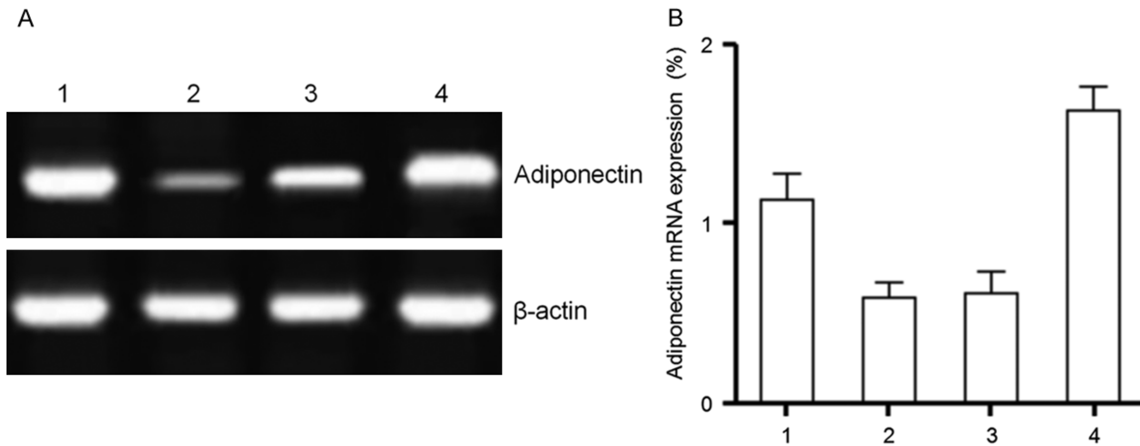

Figure 1. A: Adiponectin mRNA expression electrophoretogram of each group; B: mRNA expression of each group; Note: 1: Normal control group; 2: Diabetic nephropathy group; 3: Empty virus vector group; 4: Recombinant adenovirus with local transfection of adiponectin gene group.

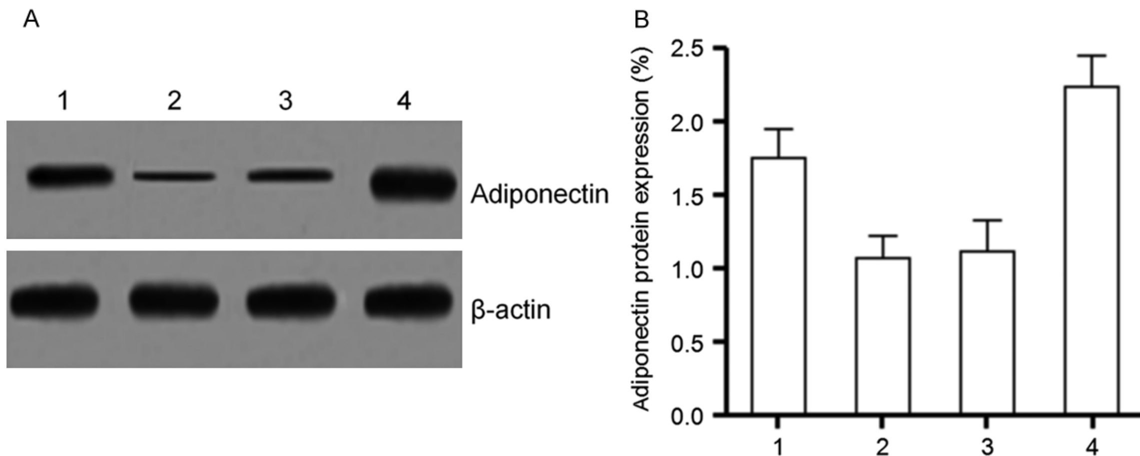

Figure 2. A: Adiponectin protein expression electrophoretogram of each group; B: Adiponectin protein expression of each group; Note: 1: Normal control group; 2: Diabetic nephropathy group; 3: Empty virus vector group; 4: Recombinant adenovirus with local transfection of adiponectin gene group.

Statistical analysis

All the metering data were analyzed by mean ± standard deviation, and the measurement data were compared with single factor analysis of variance. $P<0.05$ was considered as statistically significant. Statistical analysis was performed by using SPSS13.0 software.

Results

The expression of adiponectin mRNA

The adiponectin mRNA expression levels of the normal control group, the diabetic nephropathy group, the empty virus vector group and the recombinant adenovirus with local transfection of adiponectin gene group were detected by using RT-PCR. The result showed that, the adiponectin mRNA expression in the recombinant adenovirus with local transfection of adiponectin gene group was significantly higher than that in the normal control group, the diabetic nephropathy group and the empty virus vector group, with significant difference ($P<0.05$), as shown in **Figure 1**.

The expression of adiponectin protein

The adiponectin protein expression levels of the normal control group, the diabetic nephropathy group, the empty virus vector group and

Table 2. Comparison of blood glucose levels, serum insulin levels, SCr levels, BUN and 24 h urine protein levels ($\overline{x}\pm s$)

	Blood glucose	Serum insulin	SCr	BUN	24 h urine protein
Normal control group	3.48±1.01	22.25±1.08	28.25±1.21	10.16±1.27	24.67±2.13
Diabetic nephropathy group	25.15±2.68	13.35±0.67	42.86±2.16	17.35±2.58	32.52±2.06
Empty virus vector group	25.27±2.74	13.46±1.12	43.01±2.76	17.63±2.69	32.69±2.20
Recombinant adenovirus with local transfection of adiponectin gene group	6.24±0.98	21.02±2.91	33.64±3.52	9.20±2.23	26.08±2.02

Note: P<0.05.

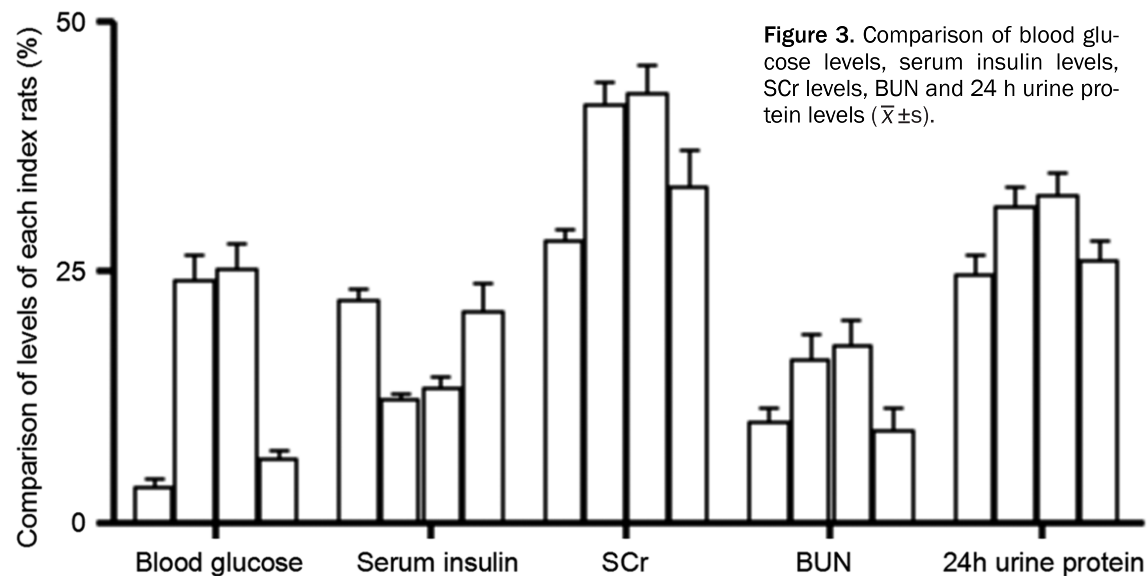

Figure 3. Comparison of blood glucose levels, serum insulin levels, SCr levels, BUN and 24 h urine protein levels ($\overline{x}\pm s$).

the recombinant adenovirus with local transfection of adiponectin gene group were detected by using Western blot. The result showed that the adiponectin protein expression in the recombinant adenovirus with local transfection of adiponectin gene group was significantly higher than that in the normal control group, the diabetic nephropathy group and the empty virus vector group, with significant difference (P<0.05), as shown in **Figure 2**.

The changes of blood glucose levels, serum insulin levels, SCr levels, BUN and 24 h urine protein levels

Compared with the diabetic nephropathy group and empty virus vector group in the recombinant adenovirus with local transfection of adiponectin gene group, the levels of blood glucose, SCr, BUN and 24 h urine protein were decreased significantly, while serum insulin levels were increased obviously (P<0.05). In the normal control group, the levels of blood glucose, SCr, BUN and 24 h urine protein were the lowest while the serum insulin level was the highest (P<0.01), as shown in **Table 2** and **Figure 3**.

Rat body weight and kidney weight of each group

By measuring and tracking changes of rats' body weight and kidney weight, body weight and kidney weight in recombinant adenovirus with local transfection of adiponectin gene group were nearest to those in the normal control group. Meanwhile, compared with the diabetic nephropathy group and the empty virus vector group, the body weight in recombinant adenovirus with local transfection of adiponectin gene group was increased obviously. Rat kidney weight/body weight, also called kidney index, was the lowest in normal control group, and it was lower in recombinant adenovirus with local transfection of adiponectin gene group compared to the diabetic nephropathy

Table 3. Body weight, kidney weight and kidney weight index ($\overline{x}\pm s$)

	Body weight/g	Kidney weight/g	Kidney weight index
Normal control group	217.36±5.38	1.27±0.24	0.56±0.26
Diabetic nephropathy group	169.24±4.17	1.53±0.33	0.97±0.48
Empty virus vector group	164.13±3.95	1.50±0.36	0.90±0.42
Recombinant adenovirus with local transfection of adiponectin gene group	185.24±8.15	1.41±0.68	0.78±0.35

Note: P<0.05.

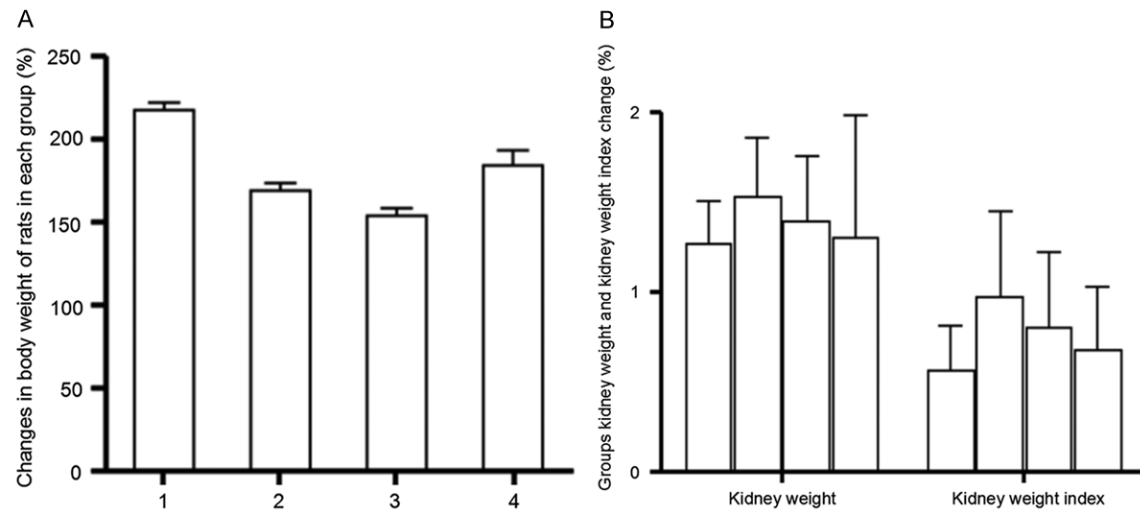

Figure 4. A: Body weight changes of each group ($\overline{x}\pm s$); B: Kidney weight and kidney weight index changes of each group ($\overline{x}\pm s$).

group and the empty virus vector group. There was significant difference among groups ($P<0.05$), as shown in **Table 3**; **Figure 4A**, **4B**.

Kidney pathologic changes under the light microscope

Kidney pathologic changes of each group were observed and compared under the light microscope. In the normal control group, the barrier of glomerular filtration was clear, thickness was uniformed, glomerular podocyte neatly attached to basement membrane and the fenestrated capillary endothelium was clear. The glomerular volume in the diabetic nephropathy group and the empty virus vector group was enlarged, the glomerular capillary loop was opened incompletely, glomerular mesangial cells were proliferative, the glomerular mesangial matrix was increased and the mesangial areas were widened. Compared with the diabetic nephropathy group and the empty virus vector group, kidney pathologic changes in recombinant adenovirus with local transfection of adiponectin gene group were reduced considerably, as shown in **Figure 5**.

Kidney ultra-structure

In the normal control group, the glomerular ultra-structure was normal, the basement membrane was intact, the glomerular podocyte was clear, and the structure of both the proximal convoluted tubule and the distal convoluted renal tubule was normal. The cell junction was clear, the cell villi were present and the organelles were complete. In the diabetic nephropathy group and the empty virus vector group, the glomerular ultra-structure was lack of clarity, the basement membrane was thickened equally, glomerular podocytes were fused, glomerular mesangial cells were proliferative and the glomerular mesangial matrix was increased. Compared with the diabetic nephropathy group and the empty virus vector group, the glomerular ultra-structure changes in re-

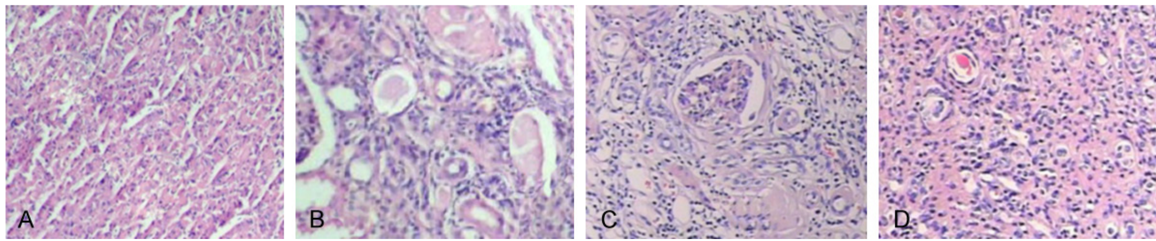

Figure 5. Kidney pathologic changes under light microscope. Note: A: Normal control group (HE×200); B: Diabetic nephropathy group (HE×200); C: Empty virus vector group (HE×200); D: Recombinant adenovirus with local transfection of adiponectin gene group (HE×200).

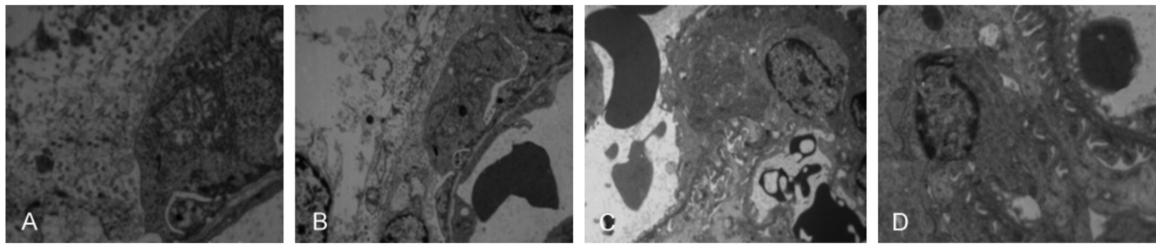

Figure 6. Kidney ultra-structure changes of each group; Note: A: Normal control group (×8000); B: Diabetic nephropathy group (×40); C: Empty virus vector group (×8000); D: Recombinant adenovirus with local transfection of adiponectin gene group (×8000).

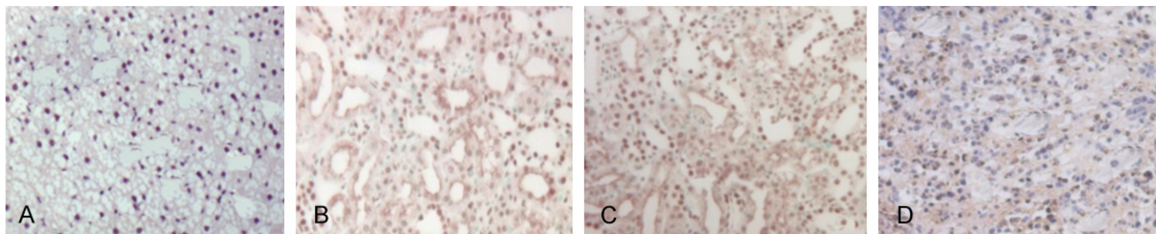

Figure 7. Kidney cell apoptosis of each group ($\overline{x}\pm s$). Note: A: Normal control group (TUNEL×200); B: Diabetic nephropathy group (×200); C: Empty virus vector group (TUNEL×200); D: Recombinant adenovirus with local transfection of adiponectin gene group (TUNEL×200).

combinant adenovirus with local transfection of adiponectin gene group were reduced remarkably, the glomerular basement membrane was thickened segmentally, glomerular podocytes were partially fused and glomerular mesangial matrix was increased slightly, as shown in **Figure 6**.

Detection of apoptosis by the TUNEL method

Specificity brownish yellow granules could be found in the nuclei of apoptotic renal cells. In the normal control group, there were only a small number of brownish yellow granules stained renal cells under the light microscope and the apoptosis rate was (0.27±0.09). The most apoptotic renal cells were founded in the diabetic nephropathy group and the empty virus vector group, and the apoptosis rates were (17.82±3.24) and (18.75±3.32), respectively. Compared with the diabetic nephropathy group and the empty virus vector group, the apoptosis rate in the recombinant adenovirus with local transfection of adiponectin gene group was relatively low (7.14±1.13) ($P<0.05$), as shown in **Figure 7**.

Discussion

Nowadays in China, one of the most important causes of late stage renal failure is diabetic nephropathy [11, 12], and in USA, based on literature statistics investigation, around 42% of diabetic patients develop into renal failure [13].

The primary pathological mechanism of renal dysfunction is the blood perfusion and the amount of filtered protein in kidney increase. Then, the glomerulus extracellular matrix is accumulated in the glomerular mesangial area and the basement membrane is thickened. Eventually glomerulosclerosis and renal tubulointerstitial fibrosis will lead to renal function damage and renal failure [14].

Adiponectin, as an adipocytokine with extensive biological effects, has already became a research hotspot in recent years. And it is regulatory when the body is in a high-fat and a high-glucose condition [15]. Many articles point out [16-18] that adiponectin can be used for predicting vascular endothelial dysfunction at the early stage and late stage of diabetes with heavy proteinuria. Some studies show that, the increase of the adiponectin level can protect tubular injury, and it plays an important role in anti-inflammation and anti-atherosclerosis [19], which is also considered as a physiological reaction in DN prevention. There are some previous studies about the correlation between adiponectin and diabetes, while it is still a noteworthy topic currently. Moreover, there are studies confirming that the lack of an adiponectin gene can accelerate the development of rats' diabetic nephropathy [20]. However the effect of adiponectin on renal function remains to be further studied.

Adenovirus-mediated transfection technology was used in this experiment. And the expression levels of adiponectin in each group were analyzed by RT-PCR and Western blot, as well as blood glucose, SCr, BUN and 24 h urine protein. SCr can be used for judging renal damage degree, and blood glucose, BUN and 24 h urine protein can also help comprehensive analysis of renal damage. The result showed that these 4 indices of the rats with local transfection of adiponectin were decreased significantly; indicating the renal function had been improved. Meanwhile the level of serum insulin in recombinant adenovirus with local transfection of adiponectin gene group was increased remarkably, which reduced the damage of high blood glucose to kidney. Through the light microscope and electron microscope observation, the kidney pathologic morphology and ultra structural changes had been reduced considerably. The renal cell apoptosis and kidney weight/body weight measured by TUNEL were relatively low in transfection of adiponectin gene group, which proved the renal function improvement. The above experimental results proved that adenovirus-mediated adiponectin can effectively increase its expression in kidney cells and improve renal function, which provides a new direction in future diabetic nephropathy treatment.

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Disclosure of conflict of interest

None.

Address correspondence to: Yu Chen, Department of Endocrinology, The Second Hospital of Tianjin Medical University, Tianjin 300211, China. Tel: +86-15222053066; Fax: +86-15222053066; E-mail: yuchencyu@163.com

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