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Original Article

Protective effect of exosomes derived from bone marrow mesenchymal stem cells on rats with diabetic nephropathy and its possible mechanism

Shaobo Wang[1,2], Liwen Bao[2], Wenjin Fu[3], Li Deng[2], Jianmin Ran[1,4]

[1]Department of Endocrinology, The First Affiliated Hospital of Ji'nan University, Guangzhou, Guangdong Province, China; Departments of [2]Endocrinology, [3]Laboratory Medicine, Houjie Hospital, Dongguan, Guangdong Province, China; [4]Department of Endocrinology, Guangzhou Red Cross Hospital, Guangzhou, Guangdong Province, China

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Abstract: Objective: To investigate the effect of exosomes derived from bone marrow mesenchymal stem cells (BMMSC-Exos) on diabetic nephropathy (DN) rats and its possible mechanism. Methods: Thirty rats were divided into the following three groups of 10 rats each: the NC group (normal rats), the DN group (rats with DN), and the BM group (DN rats injected with BMMSC-Exo). Blood glucose level, renal function, blood lipid level, and plasma viscosity of the rats were detected. Renal tissue morphology was observed using hematoxylin-eosin staining. Expression levels of JAK2 and STAT3 in rats' kidneys were measured by RT-PCR and western blot. Results: The rats in the DN group had higher levels of blood glucose, blood lipids, and blood viscosity, worse renal function, and lower body weight than those in the NC group (all $P<0.05$). After treatment with BMMSC-Exos, rats in the BM group had markedly decreased levels of blood glucose, blood lipids, and blood viscosity, improved renal function, and higher body weight compared to those in the DN group (all $P<0.05$). The renal tissues in the NC group had intact structure, and no hyperplastic or hypertrophic cells were observed. In the DN group, the renal glomerulus and mesangial matrix were abnormal, and the capillary lumen and renal tubule lumen were depressed and blocked, accompanied by interstitial edema. Pathologic changes in the renal glomerulus and tubule in the BM group were less severe than those in the DN group. The DN rats had higher expression levels of JAK2 and STAT3 than normal rats, and the rats treated with BMMSC-Exos had lower levels of JAK2 and STAT3 compared to the DN rats (all $P<0.05$). Conclusion: BMMSC-Exo can achieve a good therapeutic effect in DN, which may be due to its ability to lower the blood glucose level, improve renal function, and inhibit JAK2/STAT3 expression.

Keywords: Diabetic nephropathy, exosomes derived from bone marrow mesenchymal stem cells, JAK2/STAT3, renal function

Introduction

Diabetic nephropathy (DN) is a major cause of end-stage renal disease and its incidence rate has been rising. The major clinical symptoms of DN include edema and an increase in urinary protease content, which can bring severe burdens to patients both physically and emotionally [1]. Statistics from the World Health Organization showed that by 2019, there were 114 million patients with diabetes and 493 million people with prediabetes in China; moreover, it was estimated that the number of patients with diabetes will rise to 130 million by 2025. DN is the major cause of end-stage renal disease and the main reason for dialysis treatment in western countries [2]. According to the data from the national dialysis registration quality control in China in 2018, 19.4% of new hemodialysis patients and 18.5% of the new peritoneal dialysis patients were patients with DN [3]. Based on incomplete statistics, one-third of the diabetic patients in China may develop DN each year, and the pathogenesis of DN may be related to heredity since most of the patients have a family history of DN [4]. Currently, patients with DN are mainly treated by medication, and the treatment mainly focuses on controlling the levels of blood glucose, blood pressure, and blood lipids,and suppress-

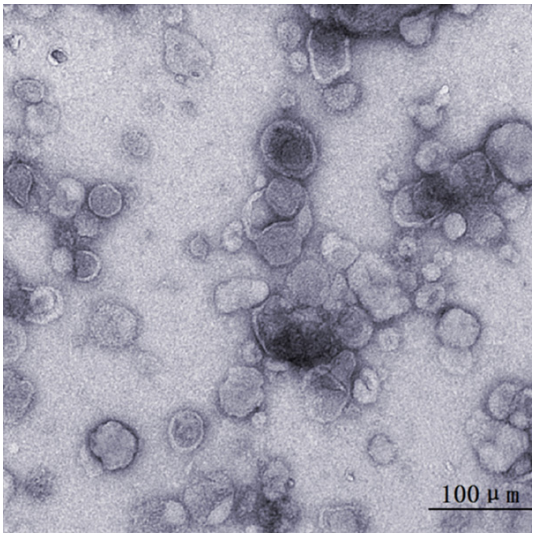

Figure 1. Morphology of BMMSC-Exos (100×). BMMSC-Exo: exosomes derived from bone marrow mesenchymal stem cells.

ing the renin-angiotensin system. However, this method cannot achieve excellent results and can cause many side effects. Therefore, it is essential to find a safer and effective method for treating DN.

The bone marrow mesenchymal stem cell (BMMSC) is a type of stem cell with differentiation ability. The cell is considered a desirable seed cell in tissue engineering due to its low immunogenicity, easy obtainability, and strong self-renewal ability. Meanwhile, it has been found that the exosomes secreted by the stem cells can regulate the function of tissue cells and effectively repair the damaged tissues including the kidney and spinal cord [5, 6]. Some studies have revealed that exosomes derived from BMMSCs (BMMSC-Exos) can achieve a good outcome in treating cartilage injury, brain injury, and renal fibrosis. The JAK/STAT pathway, which has been widely studied in recent years, is closely related to the occurrence and development of DN. STAT signaling pathway can be activated by a kinase reaction and phosphorylated. A phosphorylated STAT can cause the target genes to be transcribed, thereby affecting the proliferation, differentiation, and apoptosis of renal cells [7, 8]. Currently, the mechanism of BMMSC-Exos in DN remains unclear and there are few studies on the effects of BMMSC-Exos on the JAK/STAT pathway. Therefore, we aimed to investigate the effect of BMMSC-Exos on DN rats and its possible mechanism.

Materials and methods

Model creation and grouping

Thirty Sprague-Dawley male rats (Topgene Biotechnology, Hubei, China) were selected for the study and weighed. Of them, 10 were assigned to the NC group and were fed with a normal diet, whereas 20 were fed with a high-fat high-sugar diet for one month before receiving an intraperitoneal injection of streptozotocin (STZ, Xinyu Biotech, Shanghai, China) to construct the rat model of diabetes [9]. The STZ solution was prepared immediately before injection (solvent: trisodium citrate buffer, pH=4.5, 0.1 mol/L; injection dosage: 30 mg/kg). The DN model was considered to be established successfully if the urine volume of the rat more than doubled, and the blood glucose level was over 16.7 mmol/L. The creation of the disease model was successful in 18 rats and failed in one rat; another rat died during the modeling. The 18 model rats were then divided into a DN group (n=9, rats with DN) and a BM group (n=9, rats with DN treated with BMMSC-Exos). The rats in the BM group were injected with 5 mL of BMMSC-Exos ($4*10^6$ BMMSCs) through the tail vein once every 3 days, 6 times of injection in total). Meanwhile, the rats in the DN and NC groups were given the same dose of normal saline. The study was approved by the ethics committee of the hospital.

Preparation of BMMSC-Exos

BMMSCs were plated in cell culture flasks (Huzhen Biotechnology, Shanghai, China) containing 6 mL of complete medium at 37°C in 5% CO_2. The medium was changed every 3 days, and the non-adherent cells were removed. When the cell confluence reached 80%, the culture medium was replaced. The supernatant was collected after 48 h and 2 mL of exosome extraction reagent were added, followed by centrifugation at 4°C for 30 min. The supernatant was removed and 1 mL of precipitate was kept for centrifugation again for 5 min. The supernatant was discarded and the BMMSC-Exos were collected and preserved at -80°C. The morphology of exosomes was observed under a transmission electron microscope (**Figure 1**). Western blot was conducted to detect the surface markers of the exosomes. The exosomes (15 μL) were denatured at 95°C for 5 min. The BMMSC protein was used as a

Table 1. Primer sequences

Gene	Primer	Sequence
JAK2	Forward	5'-TTGAAGACCGGGATCCTACACA-3'
	Reverse	5'-AGGGTCATACCGGCACATCTC-3'
STAT3	Forward	5'-GCAGCTGACTACACTGGCAGAGA-3'
	Reverse	5'-ATTGTCCAGCCAGACCCAGAA-3'
β-actin	Forward	5'-GAGAGGGAAATCGTGCGTGAC-3'
	Reverse	5'-GACGTAGCACAGCTTCTCCTTAATG-3'

positive control, and the expression levels of CD63 and TSGI01 were detected by western blot to identify the surface markers of the exosomes. Zeta potential analysis and video counting were performed to measure the concentrations of BMMSC-Exos.

Hematoxylin-eosin (H&E) staining

The rats in the DN and NC groups were injected intraperitoneally with normal saline, and the rats in the BM group were injected intraperitoneally with the BMMSC-Exos. After six weeks of treatment, the rats were sacrificed and their kidneys were taken out. The renal tissues were fixed with formaldehyde (Guozheng Chemical, Shandong, China) and dehydrated with ethanol solution (Danhua Chemical, Guangdong, China). After paraffin embedding, the samples were sectioned at a thickness of 4 μm. The sections were sealed in goat serum (Yuanye Biotechnology, Shanghai, China) and stained with hematoxylin for 6-8 min (Macklin, Shanghai, China) and eosin for 10 s (Yuanye Biotechnology, Shanghai, China). The changes in the structure of the renal tissues were observed under a microscope (XSP-6c binocular biological microscope, Tianshi Scientific Instruments, Shanghai, China).

Detection of blood glucose level, body weight, renal function, blood lipid level, and blood viscosity

After model creation, the blood glucose level and body weight of the rats in each group were measured before injection of exosomes and 8 weeks after injection of the exosomes. Meanwhile, 10 mL of inferior vena cava blood was collected from each rat. A blood sample (5 mL) was centrifuged to separate the serum, and the values of blood urea nitrogen (BUN), serum creatinine (Scr), total cholesterol (TC), and triglyceride (TG) were measured with an automated biochemical analyzer. Meanwhile, plasma viscosity (PV) was detected in another 5 mL of the blood sample (blood rheometer: BV-100, TND, Beijing, China). A 24-hour urine sample was collected, followed by immediate centrifugation for 3 to 5 min (3,500 rpm). The supernatant was collected, and the value of kidney injury molecule-1 (KIM-1) was detected using ELISA (Boster, Hubei, China).

RT-PCR

RT-PCR was performed to detect the expression levels of JAK2 and STAT3 in the renal tissue. The renal tissues of the rats were digested by trypsin (Jinri, Shanghai, China), washed in PBS, and treated with 0.9% sodium chloride. The total RNA was extracted using Trizol (Invitrogen, USA) and was reversely transcribed into cDNA (Reverse transcription kit: Servicebio, Wuhan, China) according to the manufacturer's instructions. SYBR Green I was used for the detection of the expression levels of JAK2 and STAT3, and β-actin was used as an internal reference. The conditions of PCR (gradient PCR instrument: Takara Bio, Japan) were as follows: predenaturation at 60°C for 10 min, denaturation at 95°C for 30 s, annealing at 72°C for 30 s, and extension at 95°C. The cycle was repeated 40 times. Each sample was detected 3 times; the expression levels of JAK2 and STAT3 were calculated using the $2^{-\Delta\Delta Ct}$ method (**Table 1**).

Western blot

Western blot was performed to detect the protein expression levels of JAK2 and STAT3 in the renal tissues. The renal tissues of the rats were washed with PBS three times, cut into pieces, and treated with 1 mL of lysis buffer. The total protein was extracted, and the protein concentration was measured before conducting SDS-PAGE (Baiaolaibo, Beijing, China) at 100 V. Bradford method was applied for protein quantitation. After electrophoresis, the proteins were transferred to a polyvinylidene fluoride membrane, sealed with 10% goat serum for 0.5 h, and incubated with anti-JAK2 and anti-STAT3 antibodies (Cell Signaling Technology, USA) at 4°C overnight. Subsequently, the samples were incubated with secondary antibodies at room temperature for 1 h. The membrane was washed three times at room temperature and treated with chemiluminescence solution. The image was visualized and photographed. A

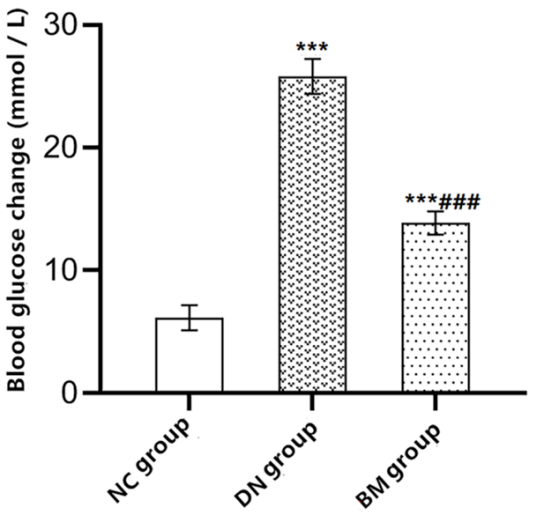

Figure 2. Blood glucose levels in each group after intervention. Compared with the NC group, ***P<0.001; compared to the DN group, ###P<0.001. NC group: normal rats; DN group: rats with diabetic nephropathy; BM group: DN rats injected with BMMSC-Exo.

grayscale image of the protein bands was analyzed with GAPDH as the internal reference.

Statistical analysis

SPSS 20.0 was applied for statistical analysis. The variables were presented as mean ± standard deviation ($\bar{x}$ ±sd). Comparison among the three groups was conducted by one-way analysis of variance and Bonferroni post hoc test. Comparison between pre- and post-treatment was conducted by paired samples t-test. P<0.05 indicated a significant difference.

Results

Blood glucose levels in each group

The diabetic rats had higher blood glucose levels than the normal rats. Compared with the DN group, the rats treated with BMMSC-Exos in the BM group had lower glucose levels (all P<0.001, **Figure 2**).

Weight changes in each group

Before the intervention, the rats in the NC group had higher body weights than those in the DN and BM groups (both P<0.05). After the intervention, the body weight was highest in the NC group and lowest in the DN group (all P<0.001, **Table 2**).

Renal function in each group

Compared with the NC group, the DN and BM groups had higher levels of Scr, BUN, and KIM-1 (all P<0.001). Compared with the DN group, the BM group had lower levels of these renal function markers (all P<0.001, **Table 3**).

Levels of blood lipids and blood viscosity in each group

Compared with the NC group, the levels of TC, TG, and PV in the DN and BM groups were higher (all P<0.05). Compared with the DN group, the levels of these markers in the BM group were lower (all P<0.05, **Table 4**).

H&E staining

The cells of the renal tissues in the NC group had intact structure, and no hyperplastic or hypertrophic cells were observed. In the DN group, the renal glomeruli and mesangial matrix were abnormal, and the capillary lumen and renal tubule lumens were depressed and blocked, accompanied by interstitial edema. The pathologic changes in the renal glomerulus and tubule in the BM group were less severe than those in the DN group (**Figure 3**).

JAK2 and STAT3 mRNA expression levels in the renal tissues

Compared with the NC group, the mRNA expression levels of JAK2 and STAT3 were higher in the DN and BM groups (all P<0.05). The BM group had lower expression levels of these mRNAs than the DN group (both P<0.05, **Figure 4**).

JAK2 and STAT3 protein expression levels in the renal tissues

Compared with the NC group, the protein expression levels of JAK2 and STAT3 were higher in the DN and BM groups (all P<0.05). Compared with the DN group, the BM group had lower expression levels of these proteins (both P<0.05, **Figure 5**).

Discussion

Diabetic nephropathy (DN) is a microvascular disease with a high prevalence among diabetic patients worldwide. In China, the incidence rate of DN has been increasing each year, and about

Table 2. Body weight in each group

Group (n=10)	Before intervention (g)	After intervention (g)	t	P
NC group	265.23±17.44	270.69±17.21	0.704	0.491
DN group	235.56±15.71*	213.26±14.22***	3.328	0.003
BM group	236.48±15.65*	252.36±16.78***,###	2.189	0.042
F	10.735	33.102		
P	<0.001	<0.001		

Note: Compared with the NC group, *P<0.05, ***P<0.001; compared with the DN group, ###P<0.001. NC group: normal rats; DN group: rats with diabetic nephropathy; BM group: DN rats injected with BMMSC-Exo.

Table 3. Renal function in each group

Group (n=10)	Scr (μmol/L)	BUN (mmol/L)	KIM-1 (μg/L)
NC group	42.74±5.02	7.05±2.21	1.19±0.12
DN group	112.02±12.31***	14.64±5.04***	3.78±0.35***
BM group	98.11±8.65***,###	8.97±3.75***,###	2.46±0.26***,###
F	160.211	10.542	243.457
P	<0.001	<0.001	<0.001

Note: Compared with the NC group, ***P<0.001; compared with the DN group, ###P<0.001. Scr: serum creatinine; BUN: blood urea nitrogen; KIM-1: kidney injury molecule-1; NC group: normal rats; DN group: rats with diabetic nephropathy; BM group: DN rats injected with BMMSC-Exo.

Table 4. Levels of blood lipids and blood viscosity in each group

Group (n=10)	TC (mmol/L)	TG (mmol/L)	PV/mPa.s
NC group	1.95±0.34	0.85±0.22	0.62±0.21
DN group	3.61±0.83*	1.57±0.37*	1.37±0.42*
BM group	2.47±0.67*,#	1.12±0.31*,#	1.00±0.35*,#
F	17.26	14.100	12.30
P	<0.001	<0.001	0.043

Note: Compared with the NC group, *P<0.05; compared with the DN group, #P<0.05. TC: total cholesterol; TG: triglycerides; PV: plasma viscosity; NC group: normal rats; DN group: rats with diabetic nephropathy; BM group: DN rats injected with BMMSC-Exo.

30% of diabetic patients can develop DN [6]. The clinical manifestations of DN include kidney hypertrophy, excessive bleeding in renal glomeruli, and thickening of the glomerular intima. When the blood glucose level increases in the human body, there can be a sugar metabolism disorder, which can promote polyols activation, activate the protease pathway, and increase the production of advanced glycosylation, thus promoting the progression of DN [10, 11]. STAT3 is in a state of continuous tyrosine oxidation in DN patients. The overactivation of STAT3 can lead to the disorder of the downstream target gene expression, which can increase the inflammatory cells and aggravate the disease [12]. BMMSCs are a type of stem cells with differentiation ability. It has been documented that the application of BMMSC-Exos may be a method for treating DN [13].

In this study, the DN rats had higher blood glucose levels, lower body weight, worse renal function, and higher blood lipid and blood viscosity levels than the normal rats. After treatment with BMMSC-Exos, the levels of these markers partially improved in the rats. Patients with DN can have an abnormal level of blood lipids and accelerated oxidation in the body. The free radicals formed by oxidation can accumulate to cause kidney damage. Since the kidney works as a central hub for lipid metabolism, abnormal metabolism of lipids is closely related to poor renal function, and the abnormal blood lipid metabolism can be reflected in the relevant blood lipid levels [14, 15]. Some studies have reported that an increase in blood viscosity can activate vascular endothelial tissue, increase the inflammatory factors to disuturb the balance in the body, and induce the occurrence of microvascular diseases, thus increasing the incidence of DN [16]. The renal tissue damage in DN patients involves cellular apoptosis and the inflammatory response. BMMSC-Exos can inhibit renal cellular apoptosis and are anti-inflammatory and anti-oxidant, thereby serving in a protective role in the kidney. These findings are consistent with our study results [17].

In the present study, the structure and morphology of cells in the renal tissues of normal rats were relatively intact, and no hyperplastic or hypertrophic cells were observed. In contrast, the growth of renal glomeruli and mesangial matrix was abnormal, and the capillary lumina and renal tubule lumina were depressed

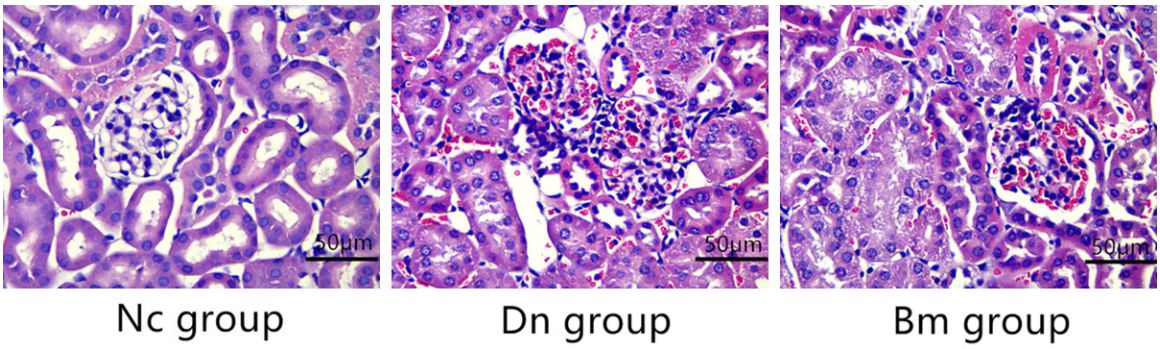

Figure 3. H&E staining (200×). In the NC group, the renal cells were intact without hyperplasia and hypertrophy. In the DN group, the renal cells were abnormal, and renal tubule lumina were depressed and accompanied by interstitial edema. In the BM group, the renal cells were moderately intact, and the capillary lumina were slightly depressed. H&E: hematoxylin-eosin staining. NC group: normal rats; DN group: rats with diabetic nephropathy; BM group: DN rats injected with BMMSC-Exo.

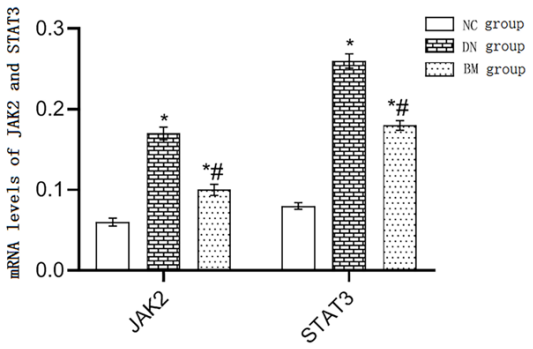

Figure 4. JAK2 and STAT3 mRNA levels in renal tissues. Compared with the NC group, *P<0.05; compared with the DN group, #P<0.05. TC: total cholesterol; TG: triglycerides; PV: plasma viscosity; NC group: normal rats; DN group: rats with diabetic nephropathy; BM group: DN rats injected with BMMSC-Exo.

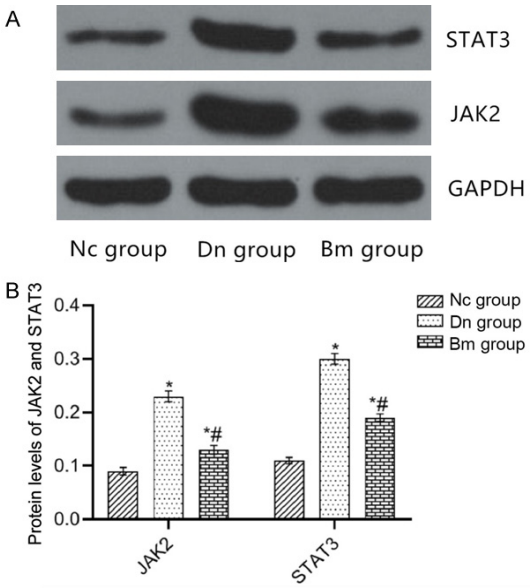

Figure 5. JAK2 and STAT3 protein expression levels in renal tissues. A: Protein bands of STAT3 and JAK2 in the renal tissues of rats in the three groups; B: Comparison of protein expression levels of JAK2 and STAT3 in the three groups. Compared with the NC group, *P<0.05; compared with the DN group, #P<0.05. TC: total cholesterol; TG: triglycerides; PV: plasma viscosity; NC group: normal rats; DN group: rat with diabetic nephropathy; BM group: DN rats injected with BMMSC-Exo.

and blocked, accompanied by interstitial edema in the DN rats. These pathologic changes of the glomerulus and renal tubules were alleviated markedly in the DN rats treated with BMMSC-Exos. Compared to the normal rats, the DN rats had higher mRNA and protein levels of JAK2 and STAT3. However, the levels of these markers were reduced partially in the DN rats treated with BMMSC-Exos. Some studies have confirmed that the JAK/STAT signaling pathway is involved in regulating the growth and development of various tissues, cells, and organs, and serves an essential role in the inflammatory response. JAK2/STAT3 can promote angiogenesis, vascular endothelial cell migration, and the formation of cellular microtubules [18]. During the progression of DN, the JAK/STAT signaling pathway can affect tissue and cell injury and the formation of the microvasculature. Inhibition of the JAK/STAT signaling pathway can attenuate renal tissue damage in DN patients [19]. It has been reported that BMMSC-Exo has an important role in the repair and regeneration of nerve tissue and can alleviate

renal damage in DN patients. Its mechanism may be related to the fact that exosomes can carry cellular contents and regulate the growth and development of renal tissue cells in a variety of ways [20]. Studies have confirmed that BMMSC-Exo has protective effects on myocardial injury, acute liver injury, acute kidney injury, and nerve injury; and the low immunogenicity makes the application of BMMSC-Exo safer compared to the direct application of BMMSC [21].

However, in the study had some limitations. The phosphorylation levels of the JAK2 and STAT3 pathways and the downstream molecules were not investigated thoroughly, and apoptosis in renal tubular epithelial cells and glomerular cells in the renal tissues was not detected. Therefore, more studies need to be carried out in the future to provide more of an experimental basis for the treatment of DN.

In conclusion, BMMSC-Exo can achieve marked therapeutic effects in treating DN, which may be related to its ability to lower blood glucose, improve renal function, and inhibit JAK2/STAT3 pathway activity.

Disclosure of conflict of interest

None.

Address correspondence to: Shaobo Wang and Jianmin Ran, Department of Endocrinology, The First Affiliated Hospital of Ji'nan University, No. 613 West Huangpu Avenue, Tianhe District, Guangzhou 510630, Guangdong Province, China. Tel: +86-0769-85080882; Fax: +86-0769-85080882; E-mail: wangshaobojd03@163.com (SBW); Tel: +86-13826267656; E-mail: ranjianmin6t3e@163.com (JMR)

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