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Intravenous administration of bone marrow mesenchymal stem cells alleviates renal failure in diabetic mice

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Abstract. – OBJECTIVE: To study the protective effect and mechanism of intravenously administered bone marrow mesenchymal stem cells (BMSCs) on renal failure in diabetic mice.

PATIENTS AND METHODS: BMSCs were obtained from the bone marrow of mice, identified by flow cytometry and tri-line differentiation test. Diabetic model (DM) mice were established using STZ and infused with mice BMSCs intravenously, followed by the analysis of fasting blood glucose and proteinuria levels, renal tissue damage by optical microscope and electron microscope, and PI3K/AKT signaling protein expression in kidney by Western blot and endocrine function by immunofluorescence staining.

RESULTS: DM mice exhibited gradually increased albumin excretion rate with time, and the MSC-treated diabetic mice presented significantly reduced proteinuria levels. HE staining indicated that MSC administration mitigated renal damage as proved by smaller tubular dilatation, reduced glomerulosclerosis and trace protein cylinders, and recovered kidney ultrastructure as shown by improved mesangial dilatation and podocyte loss. Further, BMSCs treatment activated PI3K/AKT signaling, which was downregulated in diabetic mice. However, MSC administration failed to improve pancreatic endocrine function in DM mice.

CONCLUSIONS: Intravenous administration of MSCs can effectively prevent renal failure in diabetic mice by activating PI3K/AKT signaling pathway.

Key Words:
MSCs, Intravenous administration, Diabetes, Renal failure.

Introduction

Diabetic nephropathy (DN) is one of the main chronic complications of diabetes and also the main cause of the end-stage renal disease (ESRD). Currently, China has 92 million diabetic patients and 148 million prediabetes patients. With the global increase in diabetes patients, the prevalence of DN also increases. DN is the leading cause of ESRD in the West (about 35%). DN in type 1 diabetes prevalence is about 33-40%, and prevalence is about 20-25% in type 2 diabetes[1-3]. Clinical and experimental research in recent years indicated that the disorder of glucose metabolism (formation of glycosylation end products, polyol pathway), increased protein kinase C activity and changes in renal hemodynamics as well as signaling pathways (mTOR, JAK/STAT, MCP-1, VEGF, endothelial nitric oxide synthase) plays an important role in DN pathogenesis[4-6]. Furthermore, abnormal level of a variety of cytokines, genetic susceptibility, oxidative stress and other factors are also involved in DN pathology. To explore the pathogenesis of DN in order to find effective prevention and treatment methods has always been a hot topic in the field of diabetes research.

Bone marrow mesenchymal stem cells (BMSCs) have self-renewal ability with the potential of multidirectional differentiation, whose differentiation direction breaks through the restriction of the germ layer and during the long-term culture process, the potential of multidirectional differentiation can always be maintained[7]. BMSCs have a wide range of sources, low immunogenic-

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ity, and is relatively easy to obtain. It is widely used in the treatment of diabetes complications, cirrhosis, bone and joint diseases, etc. MSC contributes to kidney regeneration in animal models of kidney injury. Transplantation of MSC into tubular epithelium can promote the recovery of necrotic segments in drug-induced acute renal failure. MSC can also be used in the treatment of severe renal diseases, including glomerular nephropathy and Alport syndrome[8-10]. Moreover, in type 2 DM models, administration of a large number of human MSC through the heart can lead to homing of donor cells to the kidney, which is beneficial to the recovery of renal function in immunodeficient non-obese mice[11-14]. MSC can upregulate IL-10 by JAK/Stat3 signaling to activate the signal transduction pathway, thus protecting the renal microenvironment and avoiding immune destruction of renal cells. However, the effect and mechanism of intravenous administration of mesenchymal stem cells on the prevention of renal failure in diabetic mice have not been reported. Therefore, our study intends to explore the preventive effect and mechanism of MSC on renal failure in diabetic mice.

Materials and Methods

Animals

C57BL/6J wild-type and C57BL/6-Tg1OS-B/J mice were purchased from Zunyi Medical University Animal Experimental Center (Zunyi, China), maintained in the appropriate amount of natural light, free feeding and drinking. Experiments were performed after adapting feeding for a week to the laboratory environment.

Diabetes Model Establishment

The model was induced by multiple low dose STZ (Sigma-Aldrich, St. Louis, MO, USA) administration. Routine adaptation feeding after 2 weeks, the model mice were injected intraperitoneally for 5 consecutive days with streptozotocin (STZ) solution (60 mg /kg), fasting 8 h, before injection. When injected, STZ was dissolved in 0.1 mol/L citric acid buffer (pH 4.5) and light-free ice after dissolution.

Isolation and Culture of BMSCs

BMSCs isolation: mice were treated with 75% ethanol immediately after cervical dislocation. The femur and tibia were separated by sterilized tweezers and scissors under sterile conditions, and the bone surface muscles were removed and washed twice with PBS solution. The femur and tibia were cut from the middle, the DMEM/F12 solution was extracted from the bone marrow cells with a 2 ml sterile syringe, then, blown with a needle, and the pipette was inhaled into the centrifuge tube followed by centrifugation at 1000 r/min and resuspending cells with PBS. Slowly add to a 15 mL centrifuge tube, which was pre-loaded with 5 mL of the lymphocyte isolate and keep the cell suspension in the upper layer of the lymphocyte isolate. The solution was divided into 4 layers after centrifugation at 2000 rpm for 15-25 min. The intermediate bone marrow stromal cell layer (white turbid) was absorbed and washed three times with PBS. The DMEM/F12 containing 15% fetal bovine serum and streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) were inoculated and cultured. After 24 hours, the culture solution was discarded (see cell adhesion) and washed with PBS 2-3 times to remove un-adherent cells, and the culture medium was added to continue the culture. After 0.25% trypsin digestion, 1:2 passage (inoculation density $1*10^5/cm^2$), well-grown third generation cells were used for follow-up experiments.

Identification of BMSCs: to identify the source of cultured cells, the surface antigen B220, CD4, CD8 (both from Nantong Yishi Biotechnology Co., Ltd., Nantong, China) was detected by flow cytometry. To identify the differentiation ability of cultured cells, the third generation of cultured cells was stained with alizarin red, Alizarin blue, and oil red O.

MSC Tail Vein Injection

After trypsin digestion, when the cells became round in inverted microscope, the medium containing bovine serum was added to stop digestion and the cell suspension was transferred in a centrifuge tube and centrifuged to discard supernatant, followed by suspending cells and adjusting MSC-GFP to 0.5×10^6 cell/0.2 ml to be used for cell transplantation. MSC cells were administrated 30 days and 51 days after diabetes, and the untreated DM mice were injected with 0.2 mL saline containing 5% mouse plasma as a control.

Detection of Biochemical Indicators

Rat tail vein blood was taken before and after MSCs transplantation to detect blood glucose and insulin levels by Olympus AU6000 automatic biochemical analyzer (Olympus, Shinjuku, Tokyo, Japan).

Determination of Urinary Albumin and Urine Creatinine

24 h of urine were collected and 24 h of urinary albumin were detected by Clinical Chemistry Kit (Cayman Chemical, Ann Arbor, MI, USA).

Histopathological Observation of Pancreas and Kidney

After injection, the spinal cord was severed, and the pancreas and kidney were isolated. Kidney tissue underwent routine dehydration and paraffin embedding. The steps are as follows: (1) Immerse fully fixed specimens in a gradient concentration of alcohol for series of dehydration. (2) Specimens were placed in xylene solution for transparent for 15 min. (3) Embedded tissue with paraffin. (4) Underwent continuous sections of paraffin-embedded tissue for 4 um thickness. Then, the sections were stained with H&E and analyzed under a microscope.

Analysis of Renal Tissue Electron Microscopy

Appropriate renal tissue was put in 2.5% glutaraldehyde at room temperature then replaced with PBS for glutaraldehyde. Then, electron microscopy was used for examination of the thickness of the glomerular basement membrane for the above tissue.

Double-label fluorescence assay of pancreatic tissue

Pancreatic sections were incubated with anti-human insulin and anti-pig glucagon antibody for 2 hours. After washing, they were incubated with Alexa 594 bound mice IgG or Alexa 488 labeled anti-rabbit (Thermo Fisher Scientific, Waltham, MA, USA) for 1 hour, the sections were examined under a laser scanning confocal microscope.

Western Blot

RIPA Lysis Buffer (Beyotime Biotechnology, Nantong, China) was added into Kidney tissue for 30 min at 4°C, then centrifuged at 12000 g, 4°C for 10 min to collect the supernatant followed by addition of 10 mg protein with loading buffer and being boiled for 10 min. Then, the protein was separated on SDS-PAGE for Western blot using primary antibodies against p-PI3K, p-AKT (Cell Signaling Technology, Danvers, MA, USA). The membrane was developed after adding the ECL mixture (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical Analysis

All data were expressed as mean ± SEM and assessed by an analysis of variance (ANOVA) or paired *t*-test. $p < 0.05$ suggests a statistical significance.

Results

Establishment of Diabetes Model

STZ injected DM mice showed a significant increase in blood glucose and urine protein level as well as a decrease in blood insulin content (Figure 1A-C), indicating damage to renal function (Figure 1D), which was also confirmed by histopathological changes with glomeruli and renal tubules (Figure 1E).

BMSCs Identification

Microscope observation indicated that BDMSCs grew adherent to the plastic and the cell body became basically prism with elevated protruding similar to that of fibroblasts. Besides, ADMSCs exhibited a negative expression of B220, CD4, and CD8, and positive expression of SCA1 and CD90. Oil red O staining (bright red indicated lipid droplets) and alizarin red staining (bright red for calcium nodules) assay certified the adipogenic differentiation and osteogenic differentiation capability, respectively (Figure 2).

MSC Administration Improved Renal Function and Structure of DM Mice

DM mice exhibited a gradually increased albumin excretion rate with time, and the MSC treated diabetic mice presented a significantly reduced proteinuria level (Figure 3). HE staining indicated that MSC administration mitigated renal damage as proved by smaller tubular dilatation, reduced glomerulosclerosis, and trace protein cylinders (Figure 4) (Table I). Further, the electron microscopy analysis showed that MSC administration recovered the kidney ultrastructure as certified by improved mesangial dilatation and podocyte loss (Figure 5) (Table I).

MSC Administration Significantly Activates PI3K/Akt Signaling in Rat Kidney Tissue

Western blot analysis showed that the pi3k/akt signal transduction pathway in diabetic mice was downregulated compared with that in nor-

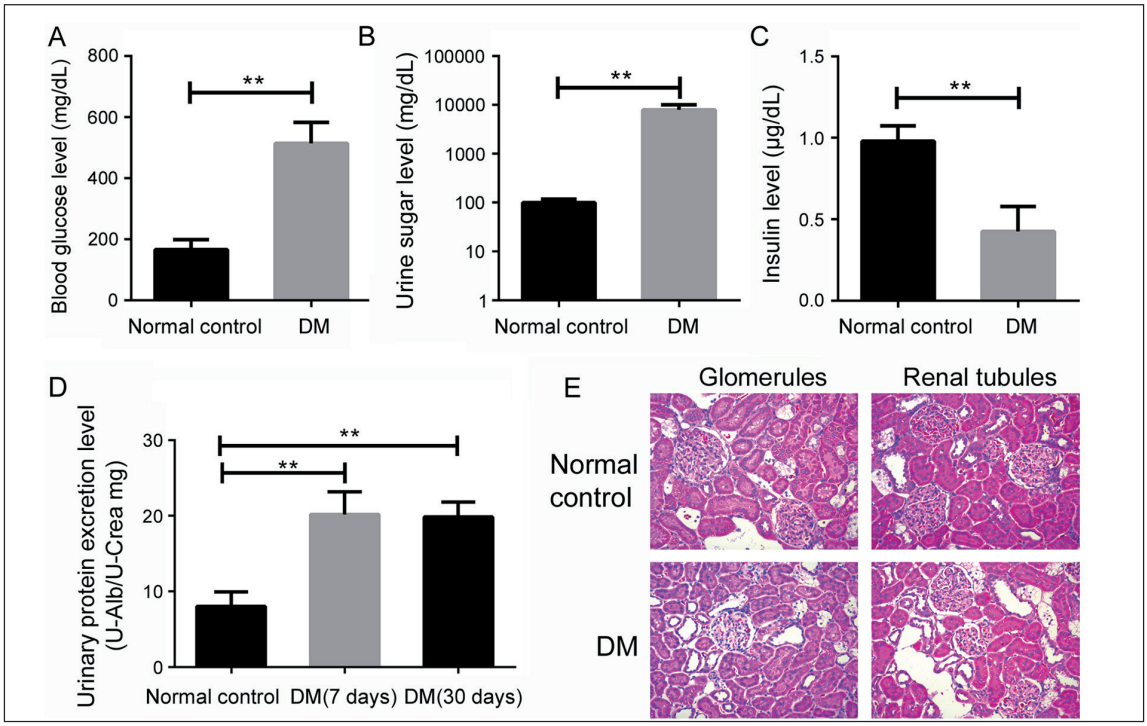

Figure 1. Serum biochemical indexes and renal pathological changes of diabetic mice. (**A**) Blood, (**B**) urine glucose levels, (**C**) blood insulin levels, and (**D**) urine albumin excretion levels in normal and diabetic mice. (**E**) Representative images of glomerular and tubular structure in kidney sections of mice (original picture ×60).

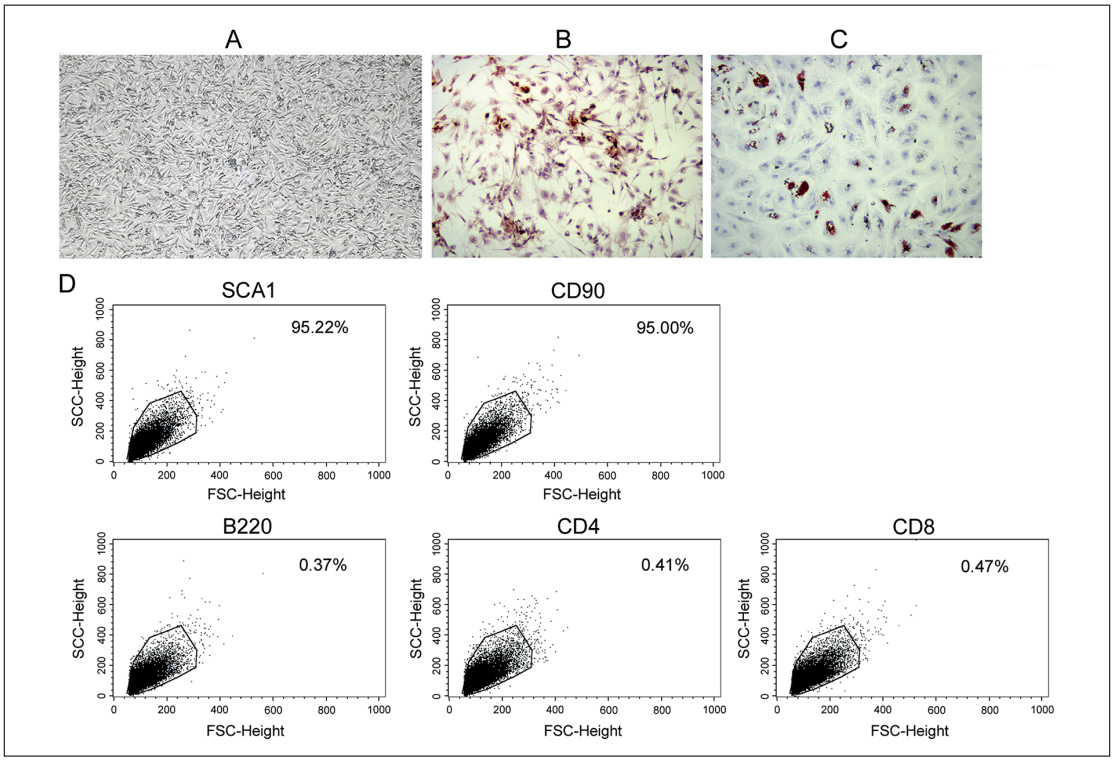

Figure 2. Characterization of mouse BMSCs. **A,** Representative images of MSC cell proliferation *in vitro* (original X40). Representative images of adipogenic (**B**) or osteogenic (**C**) differentiation of MSC cells (original ×40). **D,** Analysis of MSC cell surface CD90 and B220, CD4, and CD8 expression by flow cytometry.

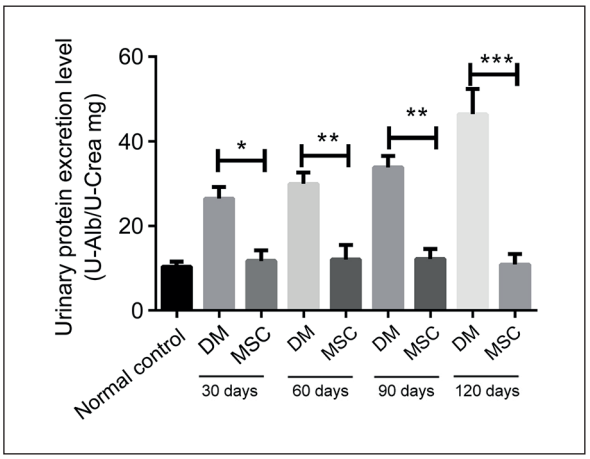

Figure 3. Levels of albumin in mouse urine.

mal control mice, but after MSC treatment, the PI3K/AKT signal transduction pathway in the kidney of DM mice was significantly activated (Figure 6).

MSC Administration Failed to Improve Pancreatic Endocrine Function in DM Mice

5 days after STZ injection, blood glucose levels in DM mice were increased and maintained at a high level, and the insulin level was downregulated, numbers of islet beta-cells were significantly reduced, the glucagon-producing cells were significantly increased while insulin-producing cells were decreased significantly. However, MSC administration failed to improve these above index-

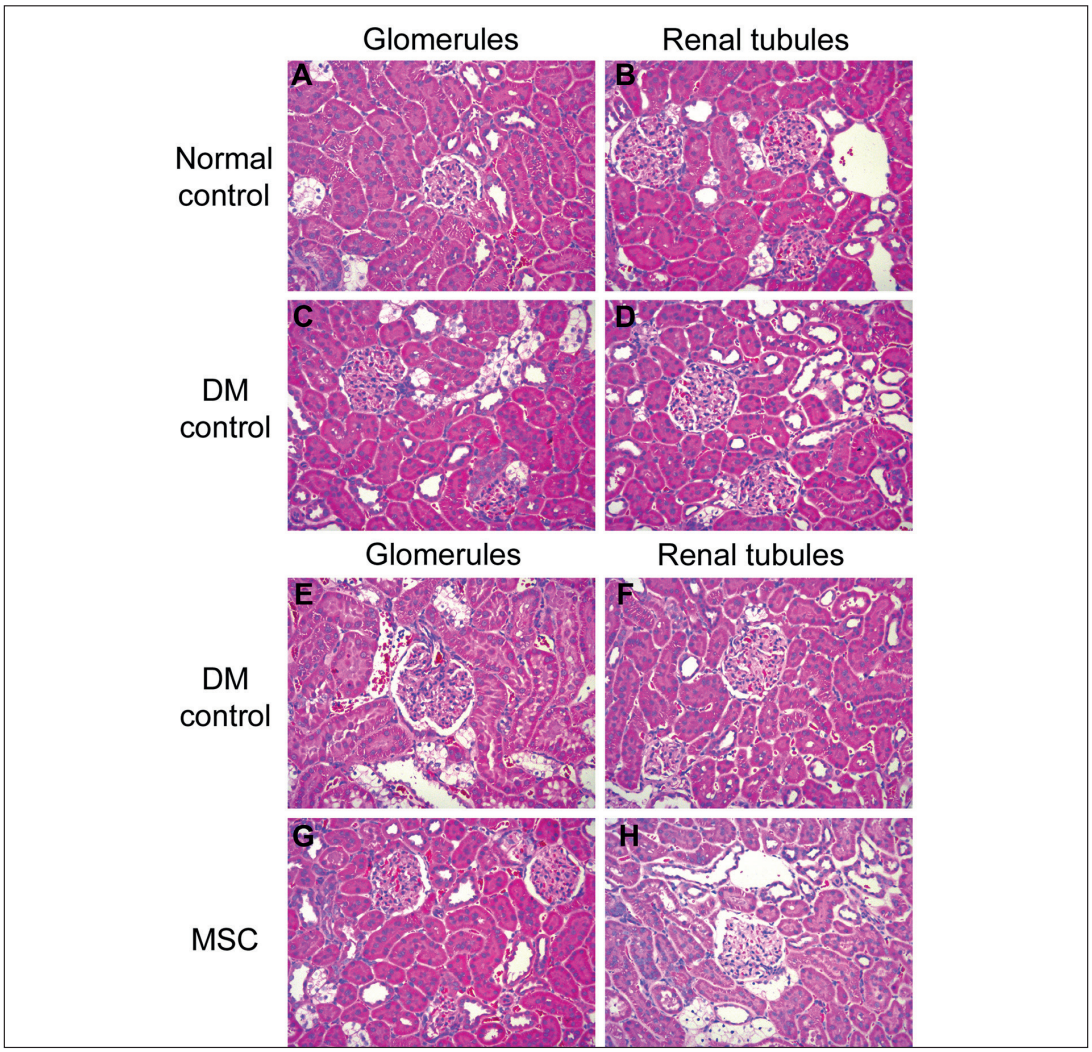

Figure 4. MSC treatment improved kidney injury in DM mice. Representative images of glomeruli (**A, C, E, G**) and renal tubules (**B, D, F, H**) in the kidney of normal control group, DM group, and MSC group, respectively (original ×100).

Table I. Quantification of glomerular and tubular injury in untreated and MSC treated DM mice.

Groups	Glomerular injury		Tubular injury
	Diabetes control group (%)	Mesangial dilatation (%)	Score
DM control group	23.6 ± 3.4	14.3 ± 3.5	2.1 ± 0.6
MSC+DM group	2.3 ± 1.1*	8.1 ± 2.6*	0.7 ± 0.1*

Compared with the diabetic control group, *$p < 0.05$.

es for pancreatic endocrine function in DM mice (Figure 7).

Discussion

At present, clinical treatment cannot effectively prevent the occurrence and development of diabetic nephropathy. Once pathological glomerular basement membrane thickening and mesangial proliferation or clinical proteinuria occur, diabetic nephropathy will slowly change toward renal failure. Studies[15,16] have shown that MSC can undergo chemotaxis and homing in acute kidney injury models to the damaged kidney and reduce the inflammatory response of the damaged kidney, promoting and improving the endogenous repair of the kidney. Our results showed that MSC administration failed to improve these above indexes for pancreatic endo-

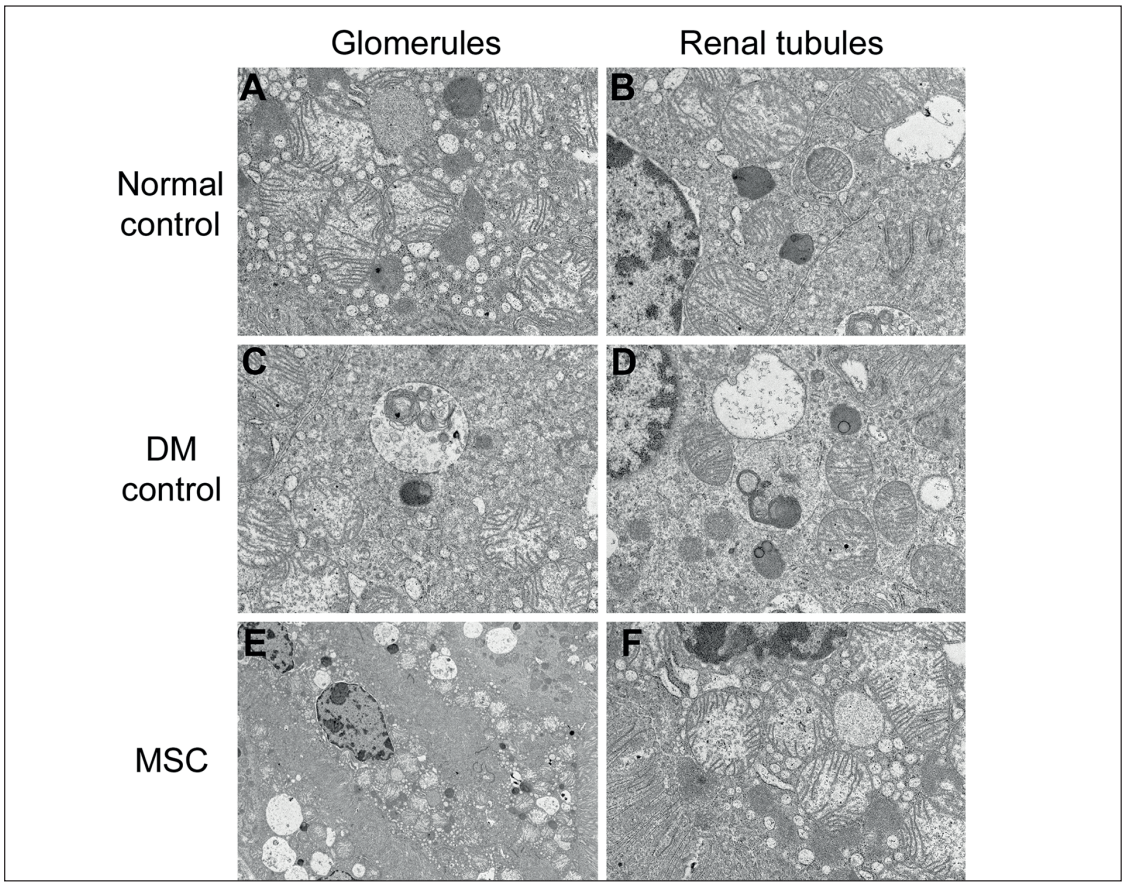

Figure 5. MSC treatment improved changes of renal structure in DM mice. Representative electron microscopy images of glomeruli (**A**) and renal tubules (**B**) in normal nondiabetic animals, representative electron microscopy images of renal tubular cells with (**C**) glomerular mesangial dilatation and podocyte loss and (D) cytoplasmic vacuoles in untreated DM mice, improved (**E**) glomeruli and (**F**) renal tubules in MSC group.

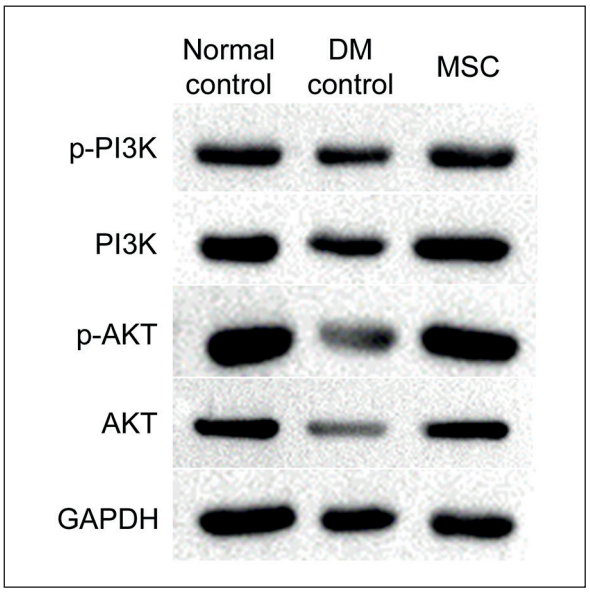

Figure 6. Western blot analysis of PI3K/AKT pathways in mouse kidney tissue.

crine function in DM mice. However, intravenous administration of MSCs can effectively prevent renal failure in diabetic mice by activating PI3K/AKT pathway.

Researcheas[17-19] shows that MSCs can be induced into islet cells; the PDX-1-positive MSCs differentiated cells were implanted into STZ diabetic nude mice under the renal capsule, which leads to blood sugar recovered to normal level within two weeks, and was maintained more than 42 d. On this basis, these differentiated and β cells have similar functions and can replace β cells. It has been reported that rats MSCs exposed to high glucose plus nicotinamide and other conditions can differentiate into typical islet-like cells, and their subcutaneous implantation into STZ diabetic Wistar rats can reduce blood sugar level. However, our results showed that MSC administration failed to improve these above indexes for pancreatic endocrine function; it may be due to different MSC treatment manners as in this experiment, MSC was intravenously administrated.

Previous literature[20,21] has shown that MSC cells retained in the kidneys of diabetic animals may have therapeutic effects because of their ability to produce anti-fibrotic or anti-inflammatory cytokines. MSCs can secrete a variety of cytokines, promote mitosis and inhibit apoptosis and abnormal morphological changes of renal cells induced by PI3K/Akt activation. On the other hand, MSCs may promote the remodeling

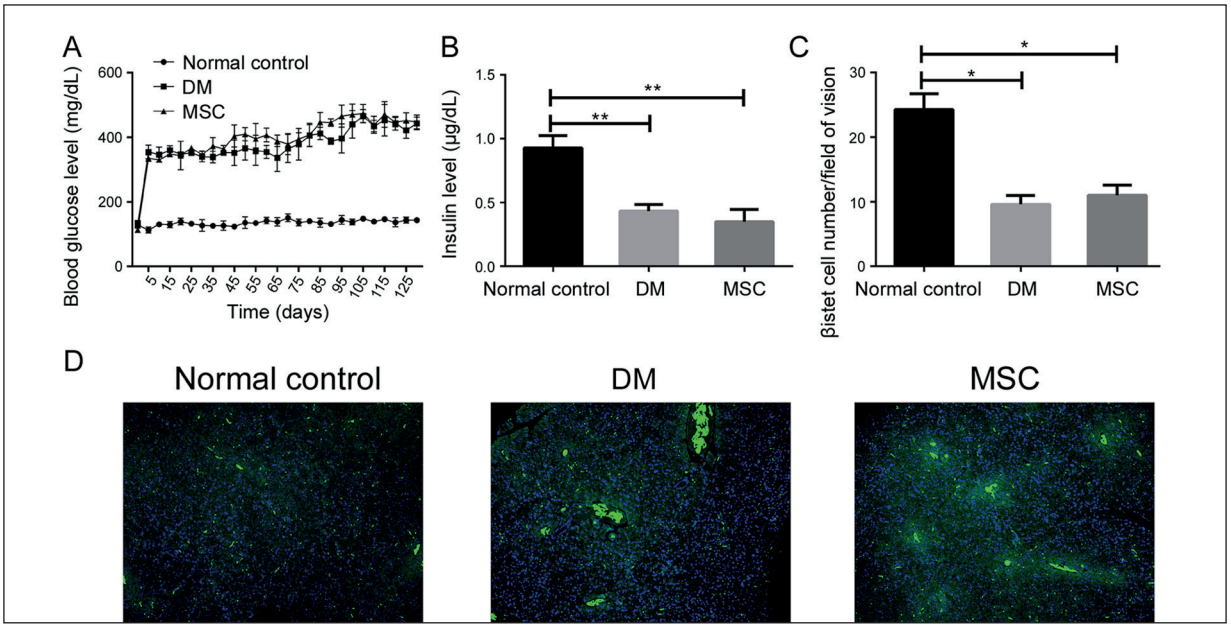

Figure 7. MSC administration failed to improve pancreatic endocrine function in DM mice. **A,** Blood glucose levels in mice, (**B**) blood insulin levels in fasting mice after 120 days of STZ injection, (**C**) the number of β islet cells in three groups of mice, and (**D**) representative images of insulin (red) and glucagon-producing cells (green) in three groups of mice (original picture ×100).

of the extracellular matrix through the synergistic action of many signal transduction pathways, thus eliminating renal structural damage[22-24]. The results of this study confirmed that the activation of PI3K/Akt signal pathway in the kidney was significantly inhibited when hyperglycemia was induced in mice, while it was significantly activated after MSC cell therapy. The histological examination further showed that after activation of PI3K/Akt pathway, glomerular (glomerulosclerosis, mesangial dilatation) and renal tubular injury, as well as proteinuria level, were significantly reduced, indicating that PI3K/Akt signal pathway was activated. Therefore, MSC cells can effectively protect the renal structure and function of diabetic mice, thus preventing the occurrence of diabetic nephropathy.

Conclusions

Our findings for the first time show that intravenous administration of MSC can prevent chronic kidney disease secondary to diabetes by activating PI3K/Akt signaling, which has not been reported and is the novelty of our study, indicating that MSC might be served as an effective cell therapy strategy. However, there are some limitations. For example, no gene knockout or PCR was carried out to detect the detailed action and transcriptional level of other related signaling pathways, and the distribution of MSC cells *in vivo* was not detected.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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