

Protection of mesenchymal stem cells on acute kidney injury

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Abstract. The aim of the present study was to determine the protection of allogeneic bone marrow mesenchymal stem cells (BMSCs) on an ischaemia/reperfusion (I/R)-induced acute kidney injury (AKI) rat model and to investigate the underlying mechanisms. The BMSCs were isolated and cultured from adult Sprague-Dawley (SD) rats and the I/R-induced AKI model was established by bilateral clamping of renal pedicles for 60 min. Following successfully establishing the AKI model, 1×10^6 BMSCs were administered by intrarenal injection. All animals were randomly divided into four groups (n=10 in each): 1 (sham control), 2 (I/R), 3 (I/R + culture medium) and 4 (I/R + BMSCs). Serum levels of creatinine (Cr) and blood urea nitrogen (BUN) were measured in all four groups at 24 and 72 h. Three days post-surgery, the level of inflammatory factors, including interleukin-6 (IL-6), tumour necrosis factor- α (TNF- α) and vascular endothelial growth factor (VEGF) in the kidney was analysed by quantitative polymerase chain reaction. Three days following surgery, mRNA expression levels of IL-6 and TNF- α were significantly lower, however, the expression level of VEGF was significantly higher in group 4 compared with groups 2 and 3 ($P < 0.05$). By contrast, the immunofluorescence results showed that the injected BMSCs differentiated into vascular endothelial cells. In conclusion, the present study identified that intrarenal administration of BMSCs improved I/R-induced AKI through the anti-inflammatory effect and a paracrine mechanism and therefore, may be hypothesised for the use in clinical trials.

Introduction

Acute kidney injury (AKI), also termed acute renal failure (ARF) (1), is mainly caused by toxic and ischemic injury (2). The common characteristics of AKI are functional and structural changes in the endothelium and proximal tubule cells, followed by regeneration of damaged renal tubular epithelial cells and rapidly progressive loss of kidney function (3-5). In

recent years, renal ischemic disease research has progressed, however, there remains a requirement for an effective therapy.

As previously reported, the kidney undergoes a regenerative response and novel cells replace the damaged cells (6). Previous experimental data shows that mesenchymal stem cell treatment in improving ischemia-related organ failure is safe and effective (7). The therapeutic potential of stem cells, including bone marrow-derived mesenchymal stem cells, hematopoietic stem/progenitor cells and adipose-derived mesenchymal stem cells, have been extensively investigated in experimental animals and patients with kidney disease. A number of experimental studies (8-11) have shown that stem cells protect glomerular and tubular structures, promote tubular epithelium regeneration, increase paracrine and systemic secretory functions and enhance peritubular capillary regeneration. However, the underlying mechanisms in the improvement of renal function remains unclear. Notably, a number of studies have demonstrated that the contribution of extrarenal cells to the regenerative renal response is minimal to none (6-12).

In the present study, the AKI rat model was established by bilateral clamping of renal pedicles for 60 min, as described previously, with some modifications (13). The allogeneic-bone marrow mesenchymal stem cells (BMSCs) were implanted by intrarenal injection into the ischaemia/reperfusion (I/R)-induced AKI rat model. The purpose of this study was to investigate the protective role of BMSCs on I/R-induced AKI and to discuss the potential underlying mechanism.

Materials and methods

Animals. Male Sprague-Dawley (SD) rats weighing between 200 and 300 g were purchased from the Animal Experimental Center of the Second Hospital of Shandong University, (Shandong, China). All animals received human care in compliance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institute of Health (NIH Publication no. 85-23, revised 1996). The study was approved by the Ethics Committee of the Shandong University, Jinan, China.

Isolation, culture, identification and labelling of BMSCs. The cells were isolated from the bone of adult SD rats using the methods described previously (14,15). Briefly, cells were isolated by flushing the cavity of femurs and tibias and transferred to a tissue culture dish 90 mm in diameter. To separate BMSCs and other cells, the Ficol1 (1.077) density gradient

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centrifugation method was used with lymphocyte separation medium (Boshide, Wuhan, China) and a low-speed tabletop centrifuge (Thermo Electron, Waltham, MA, USA). The white coat, composed of mononuclear cells from the upper layer and interface, was carefully collected and washed three times. The isolated bone marrow cells were cultured and expanded in low glucose culture containing Dulbecco's Modified Eagle's medium with Ham's nutrient mixture F-12, supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 g/ml streptomycin. BMSCs were primarily cultured for 7-10 days and then subcultured for 3-5 days. Cells at the third culture passage were transplanted. All culture experiments in this study were maintained at 37°C and in 5% humidified CO₂ and the cultures were replenished with fresh medium every 3 days. The cells were suspended with trypsin and 5x10⁵ cells were washed in PBS. Following centrifugation, the single-cell suspensions were stained with mouse anti-human monoclonal antibodies against CD34, CD44, CD45 and CD90 (Abcam, Cambridge, UK) for 30 min at 4°C on ice. The second polyclonal antibody [fluorescein isothiocyanate (FITC)-conjugated anti-mouse secondary antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA)] was added and incubated at 4°C for an additional 30 min in a dark room. An isotype-matched irrelevant antibody of the same species was used as a negative control. Following washing, cells were resuspended in phosphate-buffered saline (PBS) for fluorescence-activated cell sorting (FACS) analysis.

Prior to implantation, the cells were labelled with the cross-linkable membrane dye CM-DiI (2 µg/ml, Invitrogen Life Technologies, Carlsbad, CA, USA) according to the previously described manufacturer's instructions (16). Following labelling, 1x10⁶ cells were resuspended in 100 µl culture medium and maintained on ice prior to transplantation, the labelling efficiency reached >85%.

Rat AKI model and cell transplantation. The I/R-induced AKI rat model was established by bilateral clamping of renal pedicles for 60 min as previously described (17). Adult male SD rats were anaesthetised with Phenobarbital (50 mg/kg), following separation of the renal capsule and exposure of left kidney under sterile conditions, the bilateral renal pedicle was occluded with a nontraumatic microaneurysm clamp (DW Medical Equipment Company, Beijing, China) for 60 min and then the clamps were removed. The kidney reperfusion was performed for 6 h prior to the sacrificing of the animals by injection of excess pentobarbital sodium. Following the successful establishment of the AKI model, ~1x10⁶ BMSCs were administered by intra-renal injection. The animals (n=40) were randomly assigned to four groups (n=10 in each): 1 (control), 2 (I/R), 3 (I/R + culture medium only) and 4 (I/R + BMSCs).

Determination of renal function and histopathology. Prior to sacrifice, blood was collected from animals to determine serum levels of creatinine (Cr) and blood urea nitrogen (BUN) in all groups pre-I/R injury (IRI), and 24 and 72 h post-I/R injury, respectively, according to the manufacturer's instructions. The rats were euthanised and the kidney sections were fixed with 10% formaldehyde prior to embedding in paraffin.

Serial sections (5 µm) of the embedded tissue were stained with hematoxylin and eosin (H&E) and periodic acid-schiff stain (PAS, Baso Biotechnology, Shenzhen, China). The average of the 10 high-power fields (hpf) was randomly selected and the number of the positive cells in 100 cells in each field was counted at high magnification (x200). The percentages of 50 sections were counted by two examiners blinded to treatment assignments. The stained sections were captured as digital images using Nikon imaging software (Nikon, Tokyo, Japan).

Quantitative polymerase chain reaction analysis (qPCR). qPCR analysis was performed to detect the relative expression levels of interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α) and vascular endothelial growth factor (VEGF). Total RNA was extracted from kidney tissue homogenates with TRIzol Reagent (Invitrogen Life Technologies). The RNA sample was dissolved in RNase-free water and quantified spectrophotometrically. Primers were designed using the Primer Express software package (Applied Biosystems, Foster City, CA, USA) for the expression experiments in kidney tissue homogenates. Data were analysed with the ABI Prism 7900 sequence detection system software (Applied Biosystems) and Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control for input RNA.

Identification of the injected cells. Three days following cell transplantation, the rats were euthanised and the renal parenchyma was inflated with optimal cutting temperature compound (Sigma, St. Louis, MO, USA) and rapidly frozen in liquid nitrogen and stored at -80°C. Sections were cut into 5-µm slices and fixed in acetone for 10 min at -20°C. Survival of the transplanted BMSCs was demonstrated by observing the presence of CM-DiI-positive labelled cells by fluorescent microscopy. Immunohistochemical staining with antibodies against rabbit anti-human von Willebrand factor (vWF; 1:100) and VEGF (1:200), according to the manufacturer's instructions. FITC-conjugated antiserum (1:200; Abcam) was used as a secondary antibody. Following washing with PBS solution, the sections were observed and images were captured by fluorescent microscopy.

Statistical analysis. All data are presented as the mean ± standard error of the mean. Statistical analysis was performed by using the SPSS version 13.0 statistical software (SPSS Inc., Chicago, IL, USA). Student's t-test was used to compare the data between two groups and one-way analysis of variance was used to compare more than two groups, followed by the Scheffé's multiple-comparison test. P<0.05 was considered to indicate a statistically significant difference.

Results

Characterization and FACS of cultured BMSCs. The cells were monomorphic with a typical spindle-shaped appearance and attached to the tissue culture dishes (Fig. 1A). Following subculturing, the cells adhered tightly to the culture dish and proliferated rapidly in the culture medium (Fig. 1B). In the present study, FACS showed that the cultured cells were positive for CD90 and CD44 and negative for CD45 and CD34.

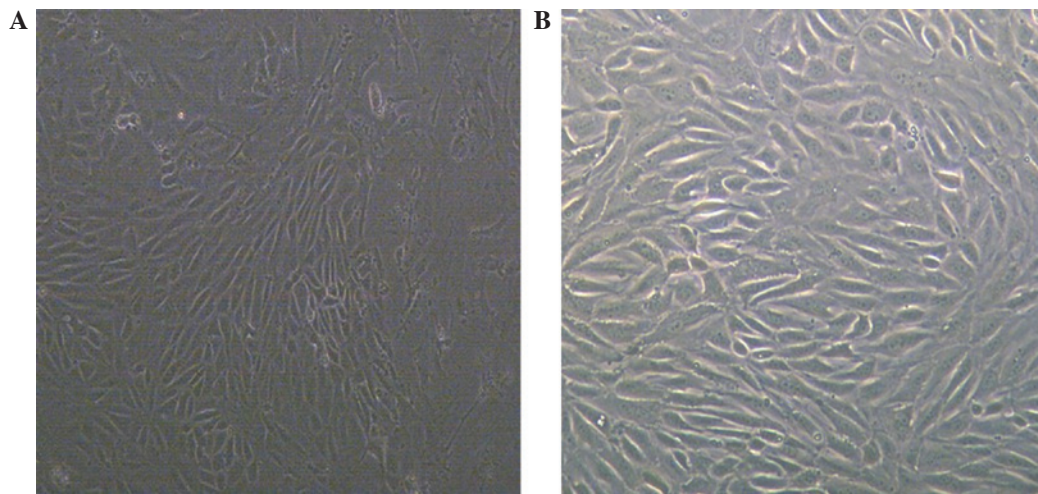


Figure 1. Cultured BMSCs. (A) Four days in primary culture. (B) Three days in subculture, the majority of cells were spindle-like (magnification, x100). BMSCs, bone marrow mesenchymal stem cells.

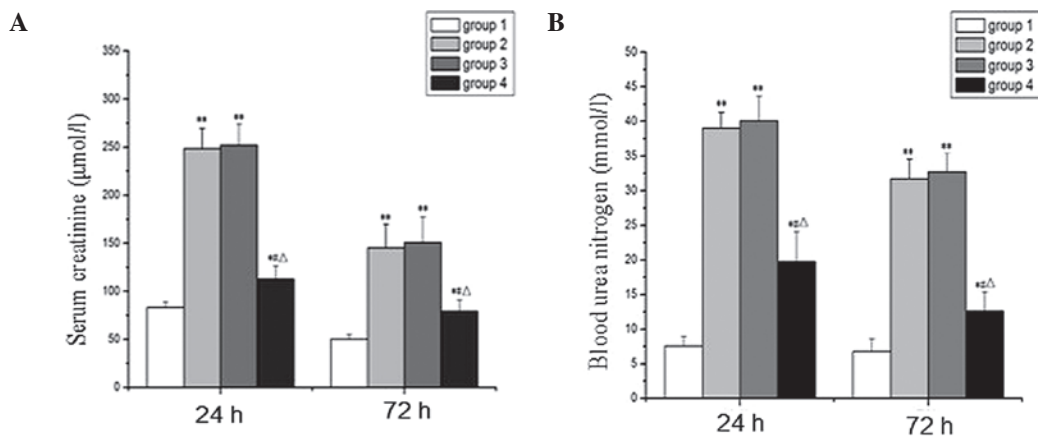


Figure 2. Expression levels of serum levels of Cr and BUN in all groups. The level of (A) Cr and (B) BUN was markedly higher in groups 2 and 3 compared with that in control group, but notably lower in group 4 at 24 and 72 h post-IRI. Group 1, control; group 2, I/R; group 3, I/R + culture medium; group 4, I/R + BMSCs. Data are presented as the mean \pm standard error (n=10). CR, creatinine; BUN, blood urea nitrogen; BMSCs, bone marrow mesenchymal stem cells; IRI, ischaemia/reperfusion injury. [#]P<0.05 and ^{**}P<0.01, vs. the control group, [#]P<0.05, vs. group 2, ^ΔP<0.05, vs. group 3.

Serum levels of Cr and BUN and histopathology analysis following IRI. The expression levels of serum Cr and BUN were determined at three time points, pre-IRI, and 24 and 72 h post-IRI. The levels of BUN and Cr were markedly higher in groups 2 and 3 compared with the control group (P<0.05), however, levels were notably lower in group 4 compared with groups 2 and 3 at 24 and 72 h post-IRI (P<0.05). The levels of BUN and Cr were not significantly different between groups 2 and 3 (P>0.05; Fig. 2A and B).

Microscopic analysis of PAS staining showed that kidney tissue exhibited severe pathological damage in groups 2 and 3, including tubular necrosis, loss of brush border, cast formation and tubular dilatation, compared with complete microstructure and normal morphology in the sham control group. Furthermore, this pathological damage of kidney was lower in group 4 compared with groups 2 and 3, three days following surgery. The H&E stain score was applied based on the aforementioned damage in 10 randomly selected, non-overlapping fields (7) (magnification, x200) and were as follows: 0 (none), 1, <10%; 2, 46-75%; 3, 48-76%; and 4 (36-50%). These results

also indicated that the AKI rat model was successful induced by I/R.

mRNA expression of inflammation factors. Three days after surgery, there was a significant increase of IL-6 and TNF- α and decreased VEGF mRNA expression in groups 2, 3 and 4 compared with the control. Notably, the mRNA levels of IL-6 and TNF- α were significantly lower and VEGF was significantly higher in group 4 compared with groups 2 and 3 (P<0.05; Fig. 3A and B). No significant differences were identified between groups 2 and 3 (P>0.05).

Identification of the transplanted BMSCs. Three days following cell transplantation, CM-DiI-labelled cells were observed at the transplanted area in group 4. Immunofluorescence demonstrated that in a number of regions the CM-DiI-positive BMSCs were observed to coincide with the green fluorescence spots of vascular endothelial cell specific markers vWF and VEGF, which suggested that BMSCs may have a potential angiogenesis effect (Fig. 4).

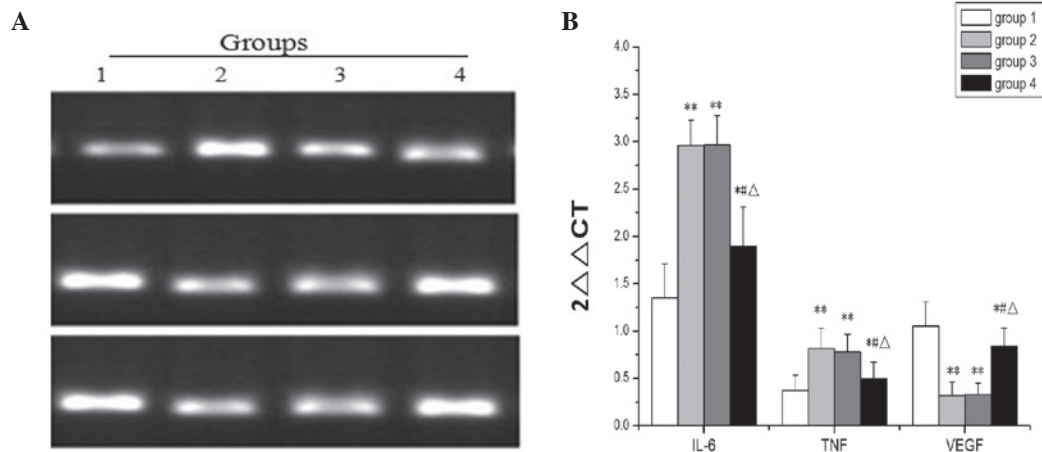


Figure 3. qPCR analysis. The mRNA expression of IL-6, TNF- α and VEGF 3 days following surgery. (A) There was significantly increased of mRNA expression of IL-6 and TNF- α in groups 2, 3 and 4 compared with the control group, but significantly lower in group 4 than that in groups 2 and 3; the level of VEGF in groups 2 and 3 compared with control group, but significantly higher in group 4 compared with groups 2 and 3. (B) Data are expressed as the mean of $2^{-\Delta\Delta CT} \pm$ standard error (n=10). Group 1, control; group 2, I/R; group 3, I/R + culture medium; group 4, I/R + BMSCs. qPCR, quantitative polymerase chain reaction; IL-6 interleukin-6; TNF- α , tumour necrosis factor- α ; VEGF, vascular endothelial growth factor; BMSCs, bone marrow mesenchymal stem cells; IRI, ischaemia/reperfusion injury. #P<0.05 and **P<0.01, vs. control group, #P<0.05, vs. group 2, ΔP<0.05, vs. group 3.

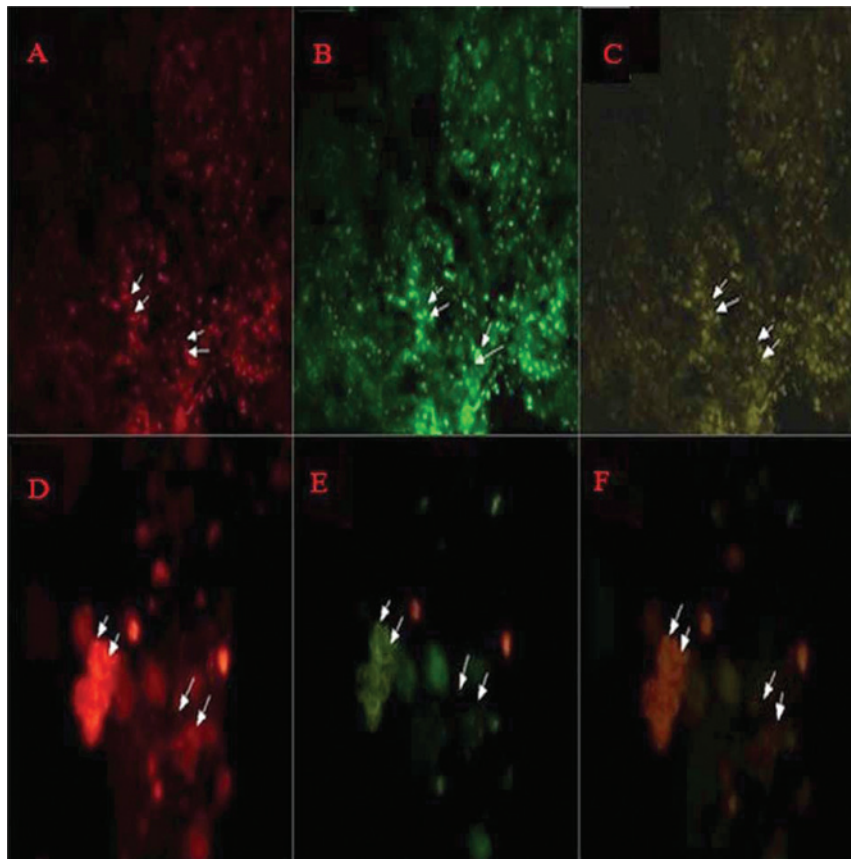


Figure 4. Identification of the transplanted BMSCs. (A and D) Three days following injection, transplanted cells survived in the injected area and the CM-DiI-labelled cells were identified by observing the distribution of red fluorescent-positive cells. (B and E) The sections stained with vWF and VEGF exhibited green fluorescence. (C and F, white arrow) The merged images of CM-DiI staining, and vWF and VEGF staining were yellow in color. The results suggest that injection of BMSCs resulted in differentiation into vascular endothelial cells *in vivo* (magnification, x400). BMSCs, bone marrow mesenchymal stem cells; VEGF, vascular endothelial growth factor; vWF, von Willebrand factor.

Discussion

The present study, to the best of our knowledge showed, for the first time, that intrarenal administration of allogeneic

BMSCs may improve kidney function during acute injury via an anti-inflammatory effect and a paracrine mechanism.

IRI is a major cause of AKI and acute renal failure (18-20) and is associated with prolonged hospitalization and high

mortality rates (21). Previous reports (7,22,23) have shown that there are functional and structural changes in I/R-induced AKI, including acute damage to the tubular-epithelium, loss of peri-tubular microvasculature, inflammation and leukocyte infiltration. Several studies have demonstrated that the inflammatory reaction is important in the pathophysiological changes resulting from ischemia (8,24,25). The primary mechanism is associated with endothelial cells, leukocytes and tubular cells produce inflammatory cytokines following ischemic injury. In previous years, a large number of studies regarding AKI have been performed; however, there remains a high mortality rate and therefore, it is important to perform further investigations. Novel research has shown that extra-renal stem cell administration is an effective treatment modality for AKI and IRI (9,11,26-28). The mechanism is highly complex and includes anti-oxidative activity, anti-inflammatory reactions, angiogenesis, stem cell homing and immuno-modulation effects (29).

A number of studies have indicated that the protective effect is attributed to the differentiation of stem cells for the replacement of damaged cells. Novel tubular cells are required to replace damaged cells, three possible sources of which are: i) Adjacent less-damaged tubular cells; ii) extra-renal cells, presumably of bone marrow origin, that home to the injured kidney or iii) resident renal stem cells (6). A recent study showed that only a small proportion of the tubular cells are derived from stem cells, suggesting that stem cells have a potential paracrine/endocrine effects (30). The underlying mechanisms of stem cells improving kidney function are complicated and remain unclear. Notably, a number of studies have shown that the contribution of extra-renal cells to the regenerative renal response is minimal to none (6,12). Therefore, allogeneic BMSCs were administered by intra-renal injection into the I/R induced AKI rat model and BMSCs were hypothesized to protect the kidney against inflammatory stimuli during acute renal injury.

CM-DiI-labelled allogeneic BMSCs (1×10^6) were injected intrarenally into the AKI injury rat model and the results showed that the expression levels of serum Cr and BUN were significantly decreased and the pathological damage to the kidney was significantly lower in the stem cell group compared with the AKI group. IL-6 and TNF- α are significant mediators in renal I/R injury (17). TNF- α was observed to reduce glomerular blood flow and the glomerular filtration rate by stimulating mesangial cells to produce a variety of vasoconstrictive mediators, including platelet activating factor, endothelin-1 and prostaglandins, and recruit neutrophils and monocytes to the kidney (31). The current study also demonstrates that the mRNA levels of IL-6 and TNF- α in I/R kidney were significantly lower three days following cell injection.

The paracrine/endocrine system is the main mechanism in stem cell therapy for ischemic organ dysfunction (32-34). A number of growth factors, including insulin-like growth factor-I, hepatocyte growth factor, angiopoietin-1, keratinocyte growth factor and VEGF exhibit a renal-protective function. Previous studies have shown that angiogenesis/vasculogenesis are key in the improvement of ischemic organ dysfunction following stem cell therapy and the efficacy of BMSCs was reduced significantly when VEGF was knocked down by small interfering RNA (siRNA). In the case of the present study, the mRNA level of VEGF was significantly higher in the stem cell

group compared with the AKI model rats. By contrast, immunofluorescence demonstrated that three days following cell transplantation, the CM-DiI-positive BMSCs were observed to coincide with the green fluorescence spots of vascular endothelial cell specific markers vWF, which suggested that BMSCs may have the potential to differentiate into endothelial cells *in vivo* and exhibit an angiogenesis effect. These results indicated that injected BMSCs by angiogenesis and vasculogenesis mechanisms may improve endothelial function.

In conclusion, BMSCs may protect the kidney against the I/R-induced AKI model via anti-inflammatory effects and a paracrine mechanism, these results provide sufficient evidence for potential use in clinical trials.

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