Original Research

Erythropoietin promotes the repair effect of acute kidney injury by bone-marrow mesenchymal stem cells transplantation

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Abstract

Bone-marrow mesenchymal stem cells (BMSCs) transplantation is effective for acute kidney injury (AKI) repair but with limited efficiency. In the present study, BMSCs marked by bromodeoxyuridine (BrdU) were transplanted to the AKI mouse model with erythropoietin (EPO) being subcutaneously injected. The blood urea nitrogen (BUN) and serum creatinine (Scr) levels, pathological changes, distribution of BMSCs, expressions of the cytokeratin 18 (CK18) and the stromal cell-derived factor 1 (SDF-1) in the nephridial tissues were measured. The directional migration of BMSCs to the AKI microenvironment *in vitro* was also tested. The results showed that BMSCs transplantation or EPO injection alone decreased the BUN and Scr levels and the acute tubular necrosis (ATN) scoring in varied degrees. The combination of these decreased the above indicators' levels significantly. $BrdU^+$ cells (BMSCs) were observed in the AKI nephridial tissues, and CK18 expressed in the cytoplasm of these cells. EPO injection increased the proportion of $BrdU^+$ cells with the enhanced expression of SDF-1 in the AKI nephridial tissues. EPO increased the migrating number of BMSCs to the AKI microenvironment *in vitro*, and additional anti-SDF-1 treatment with SDF-1 antibody neutralized this effect. Our results showed that EPO increased the number of the transplanted BMSCs in the injured nephridial tissues and enhanced the AKI repair effect of BMSCs transplantation. The enhanced kidney-homing efficiency for BMSCs mediated by the SDF-1/CXCR4 pathway is one of the possible mechanisms for EPO performance.

Keywords: Bone-marrow mesenchymal stem cells, acute kidney injury, erythropoietin, stromal derived factor-1, transplantation

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Introduction

Acute kidney injury (AKI) is a common clinical disease. At present, there is no specific therapeutic method to prevent the progress of AKI. Bone-marrow mesenchymal stem cells (BMSCs) have multi-directional differentiation potential and paracrine effect. Previous studies showed that BMSCs transplantation is a potential method for the treatment of AKI.[1–3] However, the efficiency could still be limited.[4,5] The limited kidney-directional homing ability for the transplanted BMSCs and the apoptosis of the homing BMSCs under the AKI microenvironment are the two main reasons.

Erythropoietin (EPO), a glycoprotein that can regulate erythroid cell growth and differentiation, has been suggested to be effective in treating AKI.[6–8] Our previous *in vitro* experiment[9] has found that EPO can inhibit the apoptosis of BMSCs cultured with the AKI kidney homogenate supernatant, which indicates that EPO can take positive effect on AKI repair by the BMSCs transplantation *in vivo*. In this study, AKI mouse models induced by ischemia/reperfusion (I/R) were constructed and the BMSCs transplantation was performed, in order to monitor the effect of exogenous EPO on AKI repair by the BMSCs transplantation. In addition, we also observed the effect of EPO on the kidney-directional homing of BMSCs in vitro as well as the changes by further neutralizing the stromal cell-derived factor 1 (SDF-1), for the purpose of investigating some other possible mechanism involved in the functioning of EPO.

Methods

Bromodeoxyuridine (BrdU) label of BMSCs

BMSCs, whose lines were purchased from Cyagen Biosciences (Guangzhou, China), were cultured in low-glucose DMEM (Invitrogen, NY) containing 10% foetal bovine serum (FBS) (GIBCO Inc., Montana) till 60% fusion. The culture medium was replaced with fresh DMEM containing 10 μmol/l BrdU (Sigma Inc, MO), and the cells were cultured for 72 h. The BrdU positive

($BrdU^+$) rate was determined by the immunohistochemistry (IHC) method. The $BrdU^+$ BMSCs (BrdU-BMSCs) were collected for use.

AKI mouse model preparation, experimental grouping and settlement

C57BL/6 mice used in the study were supplied by the Experimental Animal Center of the Second Military Medical University (Animal Production License No. SCXK (Shanghai) 2007-0003). One hundred C57BL/6 mice (20 ± 2 g) were fed for three days and divided into five groups randomly and evenly. Mice received anaesthesia through intraperitoneal injection of 2% nembutal sodium (Xi'an Wolsen Biological Technology Co., Ltd.) solution at a dose of 30 mg/kg. The treatments were as follows: (1) the control group: the kidneys were exposed for 30 min after anaesthesia, and 0.2 mL saline was injected into the tail vein; (2) the model group (AKI group): the AKI model was constructed by clamping bilateral renal pedicles for 30 min after anaesthesia and then closing the abdominal cavity, and 0.2 mL saline was injected into the tail vein; (3) the BMSCs transplantation group: after the AKI models were constructed, 0.2 mL PBS containing 2×10^6 BrdU-BMSCs was injected into the tail vein; (4) the EPO intervention group: after the AKI models were constructed, the mice received a subcutaneous injection of 3000 U/kg EPO for three days; (5) the EPO intervention plus BMSCs transplantation group (EPO-BMSCs group): after the AKI models were constructed, 0.2 mL PBS containing 2×10^6 BrdU-BMSCs was injected into the tail vein, and the mice also received a subcutaneous injection of 3000 U/kg EPO for three days. All mice were fed at favourable temperatures and humidity with unlimited supply of water and food. All procedures were performed in accordance with the principles of the Guidelines of Animal Experimentation at The Second Military Medical University. For each group, five mice per time were scarified at 1 d, 3 d, 7 d and 14 d after the model was constructed. Blood samples were collected from the eyes, and the serum was isolated and stocked at -20°C. After the blood vessel perfusion with 100 mL 0.01 mmol/l PBS via heart, the bilateral kidneys were separated and further perfused with PBS. Parts of the corticomedullary junction were frozen in liquid nitrogen to make frozen section for immunofluorescence detection. Other parts of the corticomedullary junction were fixed with 10% formalin for pathological and IHC analysis.

Serum indicator

Blood urea nitrogen (BUN) and Serum creatinine (Scr) concentrations were measured using the Beckman Automatic Biochemistry Analyzer (Beckman Coulter Inc.).

Pathological changes

The fixed samples were used to make paraffin sections. After HE staining, the degree of tubular necrosis was scored using the blinding method. Briefly, for each section, 15 view fields were chosen under the magnification of $200\times$ for scoring: 0 = normal, 1 = minor injury (injured tubular <5%), 2 = mild injury (injured tubular 5–25%), 3 = medium injury (injured tubular 25–75%), 4 = severe injury (injured tubular >75%). Semi-quantitative analysis was performed and the mean score value was used to indicate the degree of the acute tubular necrosis (ATN scoring).

BrdU expression in the nephridial tissues detected by IHC

The samples were fixed in 10% neutral formalin solution for 24 h and then embedded in paraffin. The sections were de-waxed with dimethylbenzene and dehydrated with graded ethanol. After endogenous peroxidase being blocked with 0.3% H_2O_2, the sections were placed in container filled with citrate buffer. After the antigen restored liquid being added, the container was heated using a microwave to expose the antigen (98°C for 3 min). The sections were incubated with BrdU monoclonal antibody (Sigma Inc., MO) at 4°C for 16 h. After washing with PBS, the sections were incubated with HRP labelled anti-mouse lgG (Santa Cruz Inc., CA) at 37°C for 1 h. Then the sections were incubated in DAB chromogenic substrate liquid for 15 min, and lining dyed with haematoxylin. After sealing with resin, the sections were stocked at room temperature for use. Fifteen non-overlapping view fields ($200\times$ magnification) were chosen for each section to determine the proportion of $BrdU^+$ cells in the epithelial tissue of the kidney tubules. The mean value of the proportion of $BrdU^+$ cells was used for statistical analysis.

Differentiation evaluation for the homing BMSCs

The frozen sections were fixed with acetone in the BMSCs transplantation group and the EPO-BMSCs group. After washing with PBS for 3 min and 0.3% Triton X-100 for 10 min, the sections were incubated with BrdU monoclonal antibody and rabbit anti-mouse cytokeratin 18 (CK18) monoclonal antibody (Santa Cruz Inc., CA) at 4°C overnight. After washing with PBS, Cy3 labeled rabbit anti-mouse IgG and FITC labeled goat anti-rabbit IgG (both from Jackson ImmunoResearch Labs Inc., West Grove, PA) were added and incubated for 2 h at room temperature. After PBS washing for three times and distilled water washing for 10 min, sections were mounted in the glycerol mounting medium. Observation was done for each section under the confocal laser scanning microscope.

SDF-1 level in the nephridial tissues of each group

The samples were treated with 1 mL detergent-containing lysis buffer and centrifuged at 20,000 rpm (4°C) for 10 min. The supernatant was taken for another 10 min centrifugation at 12,000 rpm (4°C). Protein concentration was measured. After SDS-PAGE electrophoresis, the protein was transferred to the NC membrane and blocked with TBST containing 1% BSA (10 mmol/l Tris-HCl, pH = 7.14, 150 mmol/l NaCl, 1% Tween-20) at room temperature for 4 h. The NC membrane was incubated with rabbit anti-mouse SDF-1 monoclonal antibody (Abcam Inc., Cambridge, UK) at 4°C overnight. Then HRP labeled goat anti-rabbit IgG (Santa Cruz Inc., CA) was added.

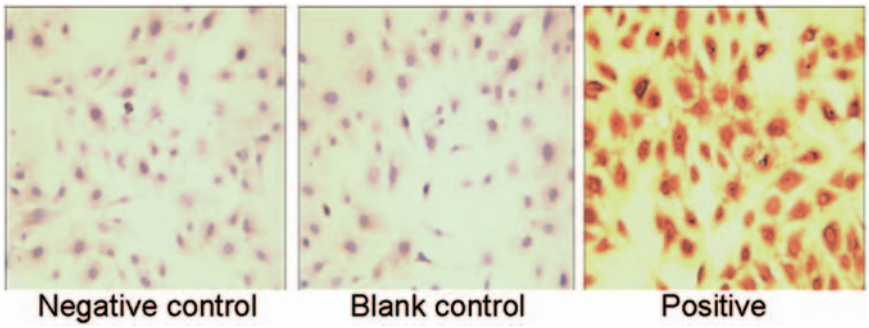

Figure 1 BrdU labelled BMSCs (Positive: BrdU was brown and surrounding the nucleus). (A color version of this figure is available in the online journal)

After incubation for another hour at room temperature, ECL was added to take effect for 5 min. Kodak *in vivo* imaging device was used for observation after exposure for 3 min and images results were automatically recorded by CCD camera. Gray value of the band was then measured. β-actin was used as the reference and the protein expression was the ratio of two gray values.

Directional migration of BMSCs *in vitro*

Preparation of the normal kidney homogenate supernatant and the AKI kidney homogenate supernatant. C57BL/6 mice and AKI mouse models were sacrificed separately. The corticomedullary junction of both kidneys were cut into pieces and made into 20 g/L homogenate with PBS. After centrifugation at 20,000 rpm (4°C) for 15 min, the supernatant was filtered with a 30-μm mesh-sized disposable sterile filter and stored at −20°C for use. Transwell chambers (Coring Incorporated, NY) were inserted into the six-well plates. BMSCs were cultured on the upper chamber with low-glucose DMEM containing 10% FBS, and the kidney homogenate supernatant was in the lower six-well plate. Four groups were included for the experiment: Group A (normal kidney homogenate chemotactic group), Group B (AKI kidney homogenate chemotactic group, for simulating AKI microenvironment *in vitro*), Group C (EPO group: 10 U/mL EPO was added to the AKI kidney homogenate supernatant in the six-well plates), and Group D (SDF-1 neutralization group: 5 μg/mL anti-mouse SDF-1 monoclonal antibody (R&D systems, Wiesbaden, Germany) and 10 U/mL EPO were both added to the AKI kidney homogenate supernatant). The vaccination density of BMSCs for each group is 1.8×10^5 per chamber, and the culturing time was 48 h. Upon the completion of culturing, the upper cells on the transwell micro-porous membrane were wiped out with a cotton bud. Staining was taken for BMSCs under the microporous membrane: After washing three times, the cells were stabilized with 4% paraformaldehyde for 20 min, and then 1% crystal violet was added for 30 min staining. PBS cleaning was finally carried out until the violet colour dimmed. Observation was taken for each membrane under microscope, counting the cell numbers in five non-overlapped visual fields with the mean value being obtained. Six duplicate wells were set for each group.

Statistical analysis

All measurement data were expressed as Mean ± Standard Deviation ($\bar{x} \pm s$). The significance test for multiple groups was performed using signal factor variance analysis. T-test was performed for comparison between two groups with SPSS16.0 statistical software.

Results

BrdU label of BMSCs

BMSCs were labelled using the BrdU label liquid. This method is not only simple and convenient, but also has a high positive label rate. The results of IHC indicated that BrdU was brown and distributed surrounding the nucleus of BMSCs (Figure 1). The positive label rate can be as high as 98.71 ± 0.32%.

The levels of BUN and Scr and the ATN socring

Compared to the control group, the BUN and Scr levels and the ATN scoring of the model group mice were significantly increased after the surgery, which reached the peak at 1 d after the models were constructed ($P < 0.01$) followed by a declining trend (Figure 2).

After BMSCs transplantation, the above indicators of the AKI mice were decreased at varied degrees. Compared to the model group, the AKI mice showed the greatest difference at 3 d and 7 d after the transplantation ($P < 0.05$). However, at a given time point, the BUN and Scr levels still showed remarkable increase compared to the control group ($P < 0.05$ or $P < 0.01$). With the prolonged transplantation time, the declining trend of these indicators was gradually weakened (Figure 2).

The EPO intervention also slightly decreased the BUN and Scr levels and the ATN scoring in the AKI mice but at an insignificant level (Figure 2).

If the BMSCs transplantation was combined with the EPO intervention, the BUN and Scr levels and the ATN scoring were sharply decreased ($P < 0.05$ or $P < 0.01$). Compared to the BMSCs transplantation group and the EPO intervention group, the EPO-BMSCs group showed obviously declined BUN, Scr and ATN scoring at 7 d and 14 d after the models were constructed ($P < 0.05$ or $P < 0.01$). With the transplantation time being prolonged, the declining trend was not weakened, and the BUN and Scr levels of

the mice were even near to those of the control group at 14 d (Figure 2).

Pathological changes in the renal tubules

In the model group mice, the HE staining revealed that the renal tubular epithelial cells (RTECs) in the S3 segment of the proximal tubule and the medullary thick ascending limb (mTAL) showed vacuolar degeneration and obvious brush border shedding, and the tubular basement membrane became partially naked with shed RTECs in the chamber (Figure 3(a,b)). These changes were the greatest at 1 d after the models were constructed. With the prolonged modelling time, the model group mice showed a

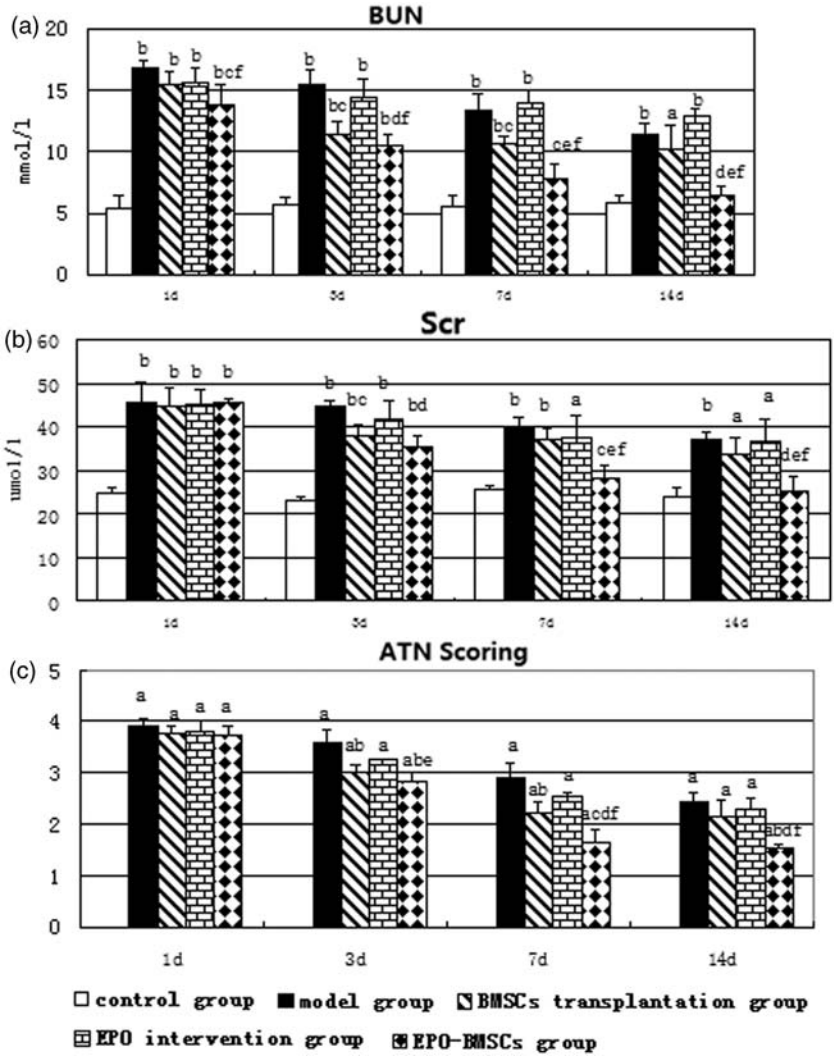

Figure 2 BUN and Scr levels and the ATN scoring in each group. vs. the control group : $^{a}P < 0.05$, $^{b}P < 0.01$; vs. the model group: $^{c}P < 0.05$, $^{d}P < 0.01$; vs. the BMSCs transplantation group: $^{e}P < 0.05$; vs. the EPO intervention group: $^{f}P < 0.05$

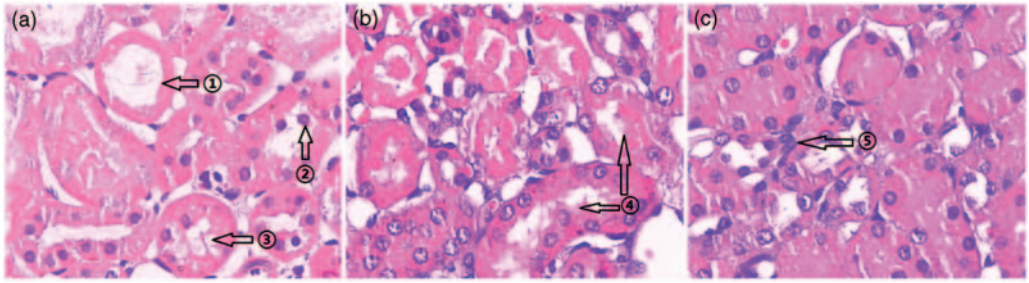

Figure 3 Pathological changes (x400). ①: naked basement membrane, ②: shed RTEC, ③: vacuolar degeneration, ④: brush border shedding, ⑤: regenerated RTEC with darker nucleus. (A color version of this figure is available in the online journal)

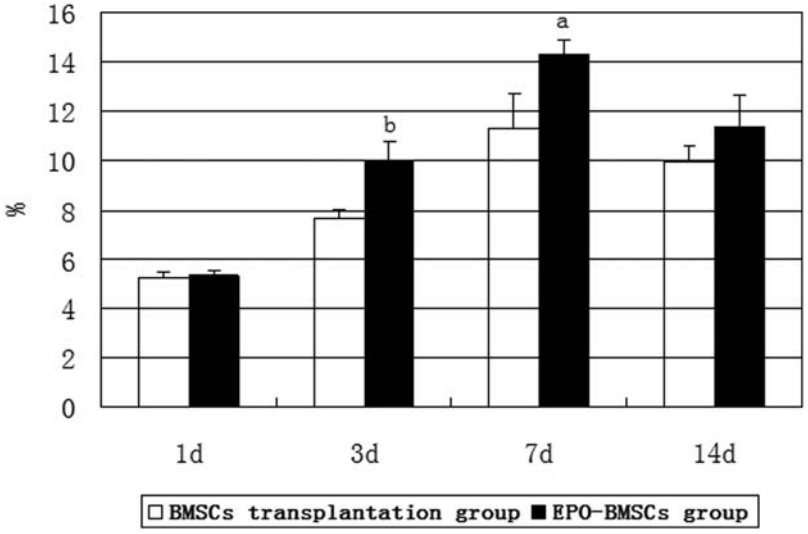

Figure 4 The proportion of $BrdU^+$ cell in the two transplantation groups vs. the BMSCs transplantation group: $^aP < 0.05$, $^bP < 0.01$

certain level of self-repair of the tubule injuries. In the BMSCs transplantation group and the EPO intervention group, the regenerated RTECs with darker nucleus were observed (Figure 3c). In the EPO-BMSCs group, the repair of injured tubules at a given time point was obviously faster compared to the BMSCs transplantation group and the EPO intervention group.

BMSCs distribution in the nephridial tissue

The microscopic results showed that the cells with brown nucleus were $BrdU^+$ ones. IHC indicated that for the BMSCs transplantation group, some cells in the epithelial tissue of the renal tubules showed brown nucleus, and the proportion of the cells with brown nucleus reached the maximum at 7 d after the models were constructed. The combination of BMSCs transplantation and EPO intervention increased the ratio of $BrdU^+$ cells in the epithelial tissue of renal tubules, which was the most obvious at 3 d and 7 d after the models were constructed ($P < 0.05$ or $P < 0.01$) (Figures 4 and 5).

CK18 expression of the transplanted BMSCs in the nephridial tissues

Based on the results of BMSCs distribution in the nephridial tissues detected by IHC, we selected the 3 d and 7 d after the models were constructed as the observation time point. Confocal laser scanning microscope was used for dual-fluorescence observation. Red fluorescence represented BrdU positive, i.e. nucleus of BMSCs. Green fluorescence represented CK18 positive. At 3 d after the BMSCs transplantation, the occurrence of dual fluorescence was detected in some cells of the epithelial tissue: the nucleus showed red fluorescence, and at the same time green fluorescence was also observed in the cytoplasm. The proportion of such cells increased at 7 d. The combination of BMSCs transplantation and EPO injection further increased the ratio of BrdU and CK18 double-positive cells in the epithelial tissue of renal tubules compared to the BMSCs transplantation group (Figure 6).

SDF-1 expression in the nephridial tissue

Western blot indicated that the SDF-1 level in the nephridial tissue was obviously increased after the models were constructed ($P < 0.01$) and reached the peak at 7 d (Figure 7). At 7 d, compared to the model group, the BMSCs transplantation group did not show significant difference in the expression of SDF-1 in the nephridial tissue. However, exogenous EPO increased the expression of SDF-1. Both the EPO intervention group and the EPO-BMSCs group showed a similar increase in the expression of SDF-1 ($P < 0.01$, compared to the control group) (Figure 8).

Directional migration of BMSCs *in vitro*

The number of migrating BMSCs for Groups A–D was (10.27 ± 1.88), (19.16 ± 2.54), (32.55 ± 5.21), (8.69 ± 1.45), respectively, under the microscopic field. Obviously, AKI kidney homogenate had more chemotactic effect on the directional migration of BMSCs compared to the normal kidney homogenate, and EPO intervention increased this chemotactic effect significantly. After SDF-1 was neutralized, this migration ability of BMSCs was blocked and the number of migrating BMSCs (Group D) decreased significantly, which was even lower than that of Group A but without significance (Figure 9).

Discussion

Stem cell transplantation is a new emerging cell biological engineering technology in the AKI treatment. BMSCs have been widely reported to be used as an ideal 'seed cell' for transplantation. The effect, however, is limited,[4,5] which in turn impedes its clinical application. The limited kidney-directional homing ability for the transplanted BMSCs and the negative effect of the AKI microenvironment on

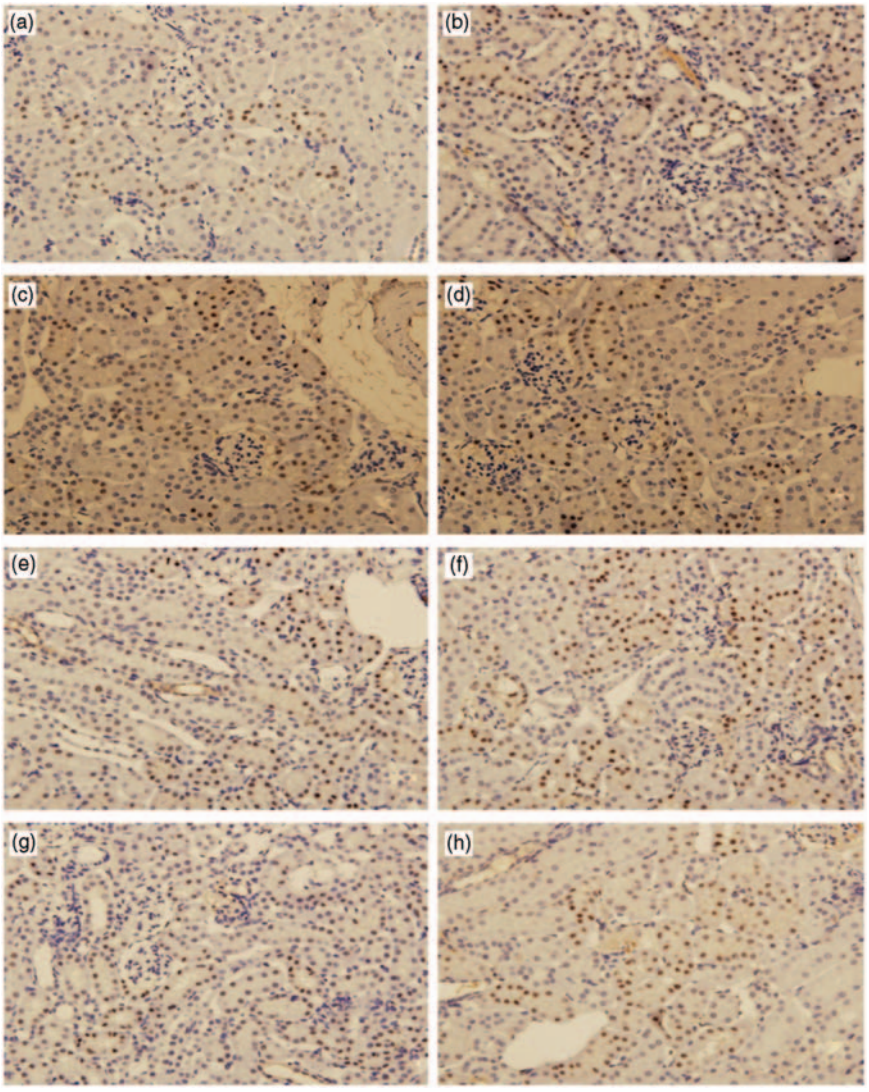

Figure 5 $BrdU^+$ cells in the two transplantation groups. IHC (×200). Brown nucleus indicates $BrdU^+$ cells. A: BMSCs transplantation group-1 d, B: EPO-BMSCs group-1 d, C: BMSCs transplantation group-3 d, D: EPO-BMSCs group-3 d, E: BMSCs transplantation group-7 d, F: EPO-BMSCs group-7 d, G: BMSCs transplantation group-14 d, H: EPO-BMSCs group-14 d. (A color version of this figure is available in the online journal)

the survival of homing BMSCs have been regarded as the two most concerned reasons.

At present, it is believed that EPO is not only the regulatory factor of erythrocyte production, but also can play the regulating role in multiple non-hematopoietic cells and tissues. Recently, our *in vitro* experiment[9] has proved that BMSCs express EPO receptor (EPOR), and the activation of EPOR by EPO could inhibit the apoptosis of cultured BMSCs and promote their proliferation under the simulative AKI microenvironment. Based on this phenomenon, the number of BMSCs in the injured kidney might be increased by EPO intervention when the limited number of BMSCs is transplanted for AKI repair. This is well in consistent with our experimental results. The proportion of $BrdU^+$ cells in the epithelial tissue of renal tubules was higher in the EPO-BMSCs group mice compared to that in the BMSCs transplantation group, and a significant difference was observed at specific time points. Observation with the confocal laser scanning microscope showed that the $BrdU^+$ cells (of which the nucleus showed red fluorescence) also expressed CK18 (with green fluorescence in the cytoplasm), which was the specific marker of RTECs.[10] This result indicated that the homing BMSCs incorporated into the epithelial tissue and differentiated into the functional RTECs. Interestingly, at 14 d after surgery, the proportion of $BrdU^+$ cells decreased in the EPO-BMSCs group. The reason could be that the self-repair of RTECs induced by the paracrine effect of the homing cells was enhanced with the prolonged transplantation time, resulting in a reduction of the proportion of $BrdU^+$ cells.

Therefore, the efficiency of kidney repair by the BMSCs transplantation via differentiation[11] and paracrine[12] could be promoted by EPO intervention. Here we tested the serum indicators and the pathological changes. BMSCs transplantation decreased the BUN and Scr levels and the ATN scoring. With the transplantation time being prolonged, the declining trend was gradually weakened. EPO intervention alone only slightly decreased the BUN and Scr levels and the ATN scoring in the AKI mice at an insignificant level, which was inconsistent with reports of various

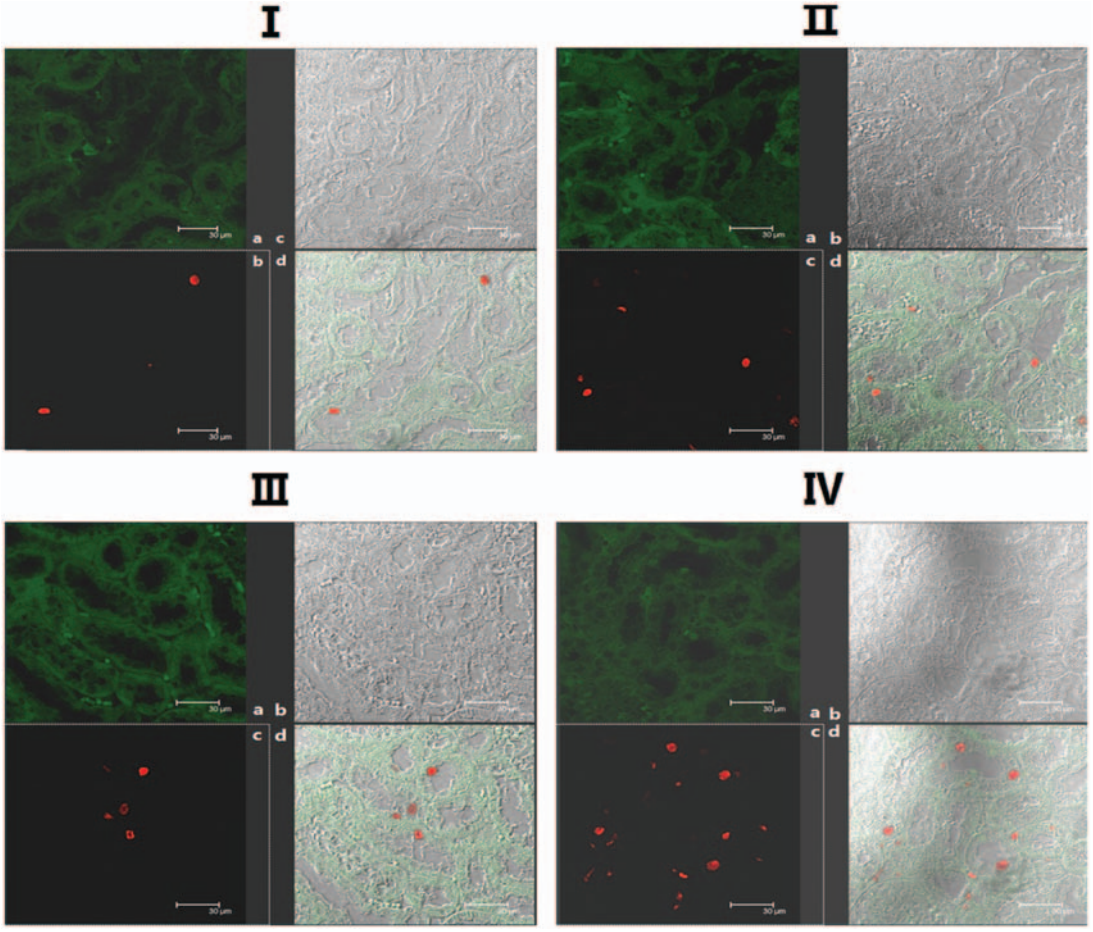

Figure 6 Dual-fluorescence observation in the two transplantation groups. Confocal laser scanning microscope was used (×400). I:BMSCs transplantation group-3 d,II:BMSCs transplantation group-7 d, III:EPO-BMSCs group-3 d, IV:EPO-BMSCs group-7 d. a: green fluorescence which represents CK18 diffuses in the cytoplasm. b: transmission image. c: red fluorescence which represents nucleus of $BrdU^{+}$ BMSCs. d: the superposition image: the nucleus of some cells shows red fluorescence and their cytoplasm shows green fluorescence, which means that these cells are BMSCs and have been differentiated into RTECs. (A color version of this figure is available in the online journal)

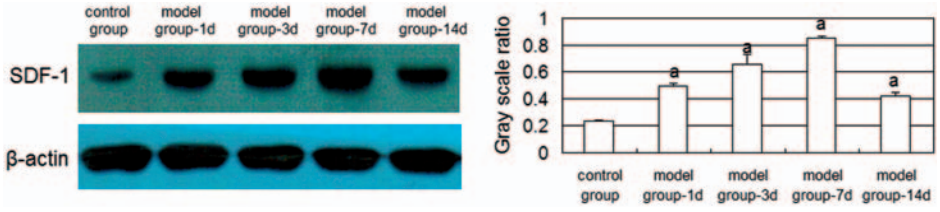

Figure 7 SDF-1 expression in the nephridial tissue of mice in the control group and model group at different time points vs. the control group: $^{a}P < 0.01$. (A color version of this figure is available in the online journal)

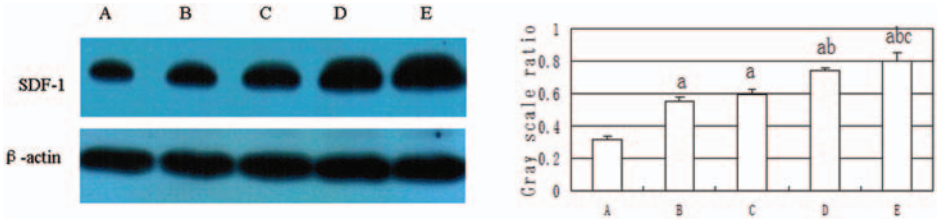

Figure 8 SDF-1 expression in the nephridial tissue of mice in all groups at 7 d after the model was constructed. A: control group-7 d, B: model group-7 d, C: BMSCs transplantation group-7 d, D: EPO intervention group-7 d, E: EPO-BMSCs transplantation group-7 d vs. the control group: $^{a}P < 0.01$; vs. the model group: $^{b}P < 0.01$; vs. the BMSCs transplantation group: $^{c}P < 0.01$. (A color version of this figure is available in the online journal)

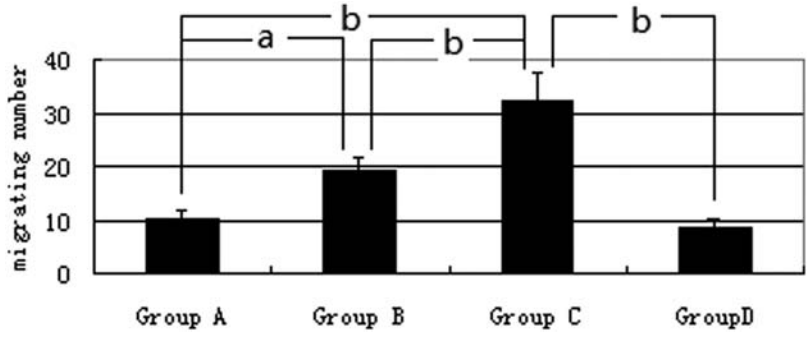

Figure 9 The number of migrating BMSCs *in vitro*. a: $P < 0.05$, b: $P < 0.01$

AKI models.[6,13] For this reason, we assumed that (i) EPO was used by subcutaneous injection in our experiment, and the absorption rate should be lower than that of direct intravenous injection used in those models; (ii) the dose used in our work was comparatively low. However, for the EPO-BMSCs group the BUN and Scr levels and the ATN scoring were all significantly lower than those in the sole BMSCs transplantation group and the EPO intervention group, and the kidney repair effect was not a simple summation of BMSCs transplantation and EPO intervention.

Preliminarily, it can be inferred that the increased number of $BrdU^+$ cells in the EPO-BMSCs group was directly due to EPO inhibiting the BMSCs apoptosis. However, the evident increase of SDF-1 expression was also observed in the AKI nephridial tissues. Similar results were reported in the acute myocardial infarction via interaction with EPOR.[14–16] SDF-1 expresses in many organs[17] and the ischemia microenvironment at the local injury will up-regulate its expression.[18–20] BMSCs can express CXCR4,[21] which is the receptor of SDF-1. SDF-1/CXCR4 pathway plays an important role in the BMSCs directional migration and homing.[22] Combining with the abovementioned fact that SDF-1 expression was increased, we consequently deduced that EPO might also increase the kidney-directional homing ability of the transplanted BMSCs and play a role in the increase of the proportion of $BrdU^+$ cells in the epithelial tissue of renal tubules. Therefore, we implemented *in vitro* experiment by using I/R kidney homogenate to simulate AKI microenvironment. We found that EPO intervention increased the number of migrating BMSCs to the AKI microenvironment, and additional anti-SDF-1 treatment neutralized this effect, suggesting that the effect induced by EPO was related to the increased SDF-1 and the SDF-1/CXCR4 pathway. These findings demonstrate that the enhanced kidney-directional homing ability of BMSCs induced by EPO also contributes to the increase of the number of BMSCs in the AKI nephridial tissues.

In conclusion, the combined EPO injection increased the repair effect when BMSCs transplantation was introduced for the AKI repair. Besides the inhibition of BMSCs apoptosis, the enhanced kidney-directional homing ability of BMSCs mediated by SDF-1/CXCR4 pathway also contributed to the effect. As a result, a preliminary conclusion can be reached that the combined therapy of BMSCs transplantation with EPO injection provides a novel effective treatment approach for the AKI repair.

Author contributions: NL designed the study, analysed the data and composed the article together with JT; NL, GH, JC, JH performed the study.

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