Bone mesenchymal stem cells pretreated with erythropoietin accelerate the repair of acute kidney injury

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song zhou
Zhijiang Hospital

Yu-ming Qiao
Zhijiang Hospital

Yong-guang Liu
Zhijiang Hospital

Ding Liu
Zhijiang Hospital

Jian-min Hu
Zhijiang Hospital

Jun Liao
Zhijiang Hospital

Min Li
Zhijiang Hospital

Ying Guo
Zhijiang Hospital

Li-pei Fan
Zhijiang Hospital

Liu-Yang Li
Zhijiang Hospital

Ming Zhao

Corresponding Author

zhaoming02@hotmail.com
Abstract

Background Mesenchymal stem cells (MSCs) represent a promising treatment option for acute kidney injury (AKI). The main drawbacks of MSC therapy including the lack of specific homing following systemic infusion and early death of the cells in the inflammatory microenvironment, directly affect the therapeutic efficacy of MSCs. Erythropoietin (EPO)-preconditioning promotes the therapeutic effect of the MSCs, although the underlying mechanism remains unknown. In this study, we sought to investigate the efficacy and mechanism of EPO on bone marrow mesenchymal stem cells (BMSCs) for the treatment of AKI.

Results We found that incubation of BMSCs with ischemia/reperfusion (I/R)-induced AKI kidney homogenate supernatant (KHS) caused apoptosis in the BMSCs, which was decreased following EPO pretreatment indicating that EPO protected the cells from apoptosis. Further, we found that EPO upregulated SIRT1 and Bcl-2 expression, and downregulated p53 expression. The EPO-mediated anti-apoptotic mechanism in pretreated BMSCs may be mediated though the SIRT1 pathway. In a rat AKI model, our data showed that 24 h following intravenous infusion, GFP-BMSCs were predominantly in the lungs. However, EPO pretreatment reduced the lung entrapment of BMSCs, and increased the distribution of the BMSCs to the target organs. AKI rats infused with EPO-BMSCs had significantly lower levels of serum IL-1β and TNF-a and significantly higher level of IL-10 compared to rats infused with BMSCs. The administration of EPO-BMSCs after reperfusion was more effective in reducing serum creatinine, blood urea nitrogen, and pathological scores in the I/R-AKI rats than BMSCs.

Conclusions Our data suggest that EPO pretreatment enhances the efficacy of BMSCs in improving renal function and pathological presentation in I/R-AKI rats.

Background

Acute kidney injury (AKI) is one of the most clinically impactful diseases with high morbidity and mortality [1, 2]. Multiple injuries such as those resulting from sepsis, ischemia/reperfusion (I/R), and drug administration may induce AKI. I/R injury is a major cause of human AKI, which is associated with tubular necrosis, cast formation, tubular dilation, loss of brush border, and inflammation [3]. AKI remains a worldwide public health concern due to the increased risk for subsequent development of
chronic kidney disease (CKD) [4]. The annual medical expenses associated with AKI treatment places a heavy burden on the public health care system, and yet AKI lacks an established treatment strategy. Therefore, there is an urgent need to find innovative and effective therapeutic strategies for AKI. The application of mesenchymal stem cells (MSCs) has been suggested as a potentially promising treatment strategy for AKI [5, 6].

In the recent years, MSCs have become an area of intense research in the field of stem cell therapy. MSCs are adherent, fibroblast-like cells, derived from different tissues and organs including bone marrow, umbilical cord blood, adipose tissue, and solid organs that have the potential for multidirectional differentiation and self-renewal. The tissue reparative, regenerative, and immunoregulatory properties of MSCs make them good candidates for cell therapy and tissue regeneration. A large number of basic science studies and clinical trials have demonstrated the safety, feasibility, and effectiveness of MSCs in the treatment of myocardial infarction, spinal cord injury, diabetes, and kidney disease [7–9]. MSCs have reno-protective effect in both acute and chronic kidney injury. This effect seems to be associated with immunomodulation, anti-apoptotic effects, and reduction of disease-related inflammation [10, 11].

The main drawbacks of MSCs therapy are early pulmonary entrapment and the lack of specific homing to target tissues following systemic infusion [12, 13]. Even when the MSCs reach the target organ, many of the cells undergo apoptosis mainly due to the inhospitable local microenvironment associated with conditions such as hypoxia, oxidative stress, and inflammation, which directly affect the therapeutic efficacy of the MSCs [14, 15]. Application of pretreated MSCs is a novel strategy to enhance the capacity of the MSCs to migrate and promote tissue repair in AKI [16, 17]. In our previous study, we demonstrated that pretreatment with 500 IU/ml erythropoietin (EPO) for 48 h prior to infusion markedly increased the homing and healing abilities of bone marrow-derived mesenchymal stem cells (BMSCs). These BMSCs significantly inhibited the apoptosis of HK2 cells from the toxicity of cyclosporin A (CsA). Moreover, the one-time infusion treatment with EPO-BMSCs significantly improved the renal function of CsA-induced chronic toxic renal injury, and promoted the repair of renal fibrosis in rats [18].
EPO has been reported to stimulate the silent information regulator 1 (SIRT1) pathway to promote mitochondrial function and protect against oxidative stress in white adipose tissue [19]. EPO has also been demonstrated to protect against the chemotherapy drug doxorubicin (DOX)-induced cardiotoxicity by activating SIRT1 and enhancing mitochondrial function [20]. SIRT1 is an nicotinamide adenine dinucleotide (NAD+)-dependent protein deacetylase that plays an important role in maintaining the self-renewal and differentiation of mouse embryonic stem cells (ESCs) and hematopoietic stem cells (HSCs). Studies on MSCs have also revealed that SIRT1 maintains the proliferation and self-renewal of stem cells, especially under stress conditions [21, 22]. SIRT1 has positive effects on senescence and apoptosis of MSCs [23, 24].

In this study, we sought to investigate the efficacy and mechanism of EPO pretreatment on BMSCs for the treatment of AKI. We demonstrate that EPO promotes the survival of transplanted BMSCs in an inflammatory microenvironment through a SIRT1-dependent pathway.

Results

Cell culture and characterization of BMSCs

BMSCs were transduced with a lentiviral vector expressing the green fluorescent protein (GFP) gene (GFP-BMSCs) and verified to retain stable GFP expression after proliferation. The passage 8 (P8) GFP-BMSCs were observed under a fluorescence microscope (Fig. 1A). Both GFP-BMSCs and EPO-pretreated GFP-BMSCs were capable of osteogenic and adipogenic differentiation when cultured in the appropriate inducing media. After three weeks of incubation, GFP-BMSCs and EPO-pretreated GFP-BMSCs differentiated into osteoblasts and adipocytes, respectively (Fig. 1B). Flow cytometric analysis confirmed that both groups of cells were CD45-negative and positive for the phenotypic markers CD90 and CD44 (Fig. 1C). Both groups of cells maintained their stem cell characteristics.

AKI-KHS induced apoptosis in BMSCs

To study the anti-apoptotic effect of EPO on BMSCs, BMSCs were pretreated with EPO. BMSCs or EPO-BMSCs were incubated with AKI-KHS (or N-KHS). After culture for 24 h, flow cytometric analysis was performed to assess apoptosis in each group (Fig. 2A). The apoptotic rates were significantly higher in both BMSCs/AKI-KHS and EPO-BMSCs/AKI-KHS groups when compared with the control group. AKI-KHS
resulted in apoptosis of the BMSCs, but EPO pretreatment protected the cells against this apoptotic effect. The apoptotic rate in EPO-BMSCs/AKI-KHS group was significantly lower compared to that in the BMSCs/AKI-KHS group (Fig. 2B).

**Western blot analysis**

Results of the flow cytometric analysis demonstrated that AKI-KHS treatment had an adverse effect on the survival of BMSCs while the EPO pretreatment improved BMSCs survival in the AKI microenvironment. To further investigate the anti-apoptotic mechanism of EPO pretreatment of the BMSCs, we analyzed the expression of the anti-apoptotic factor Bcl-2, apoptotic factor p53, and silent information regulator 1 (SIRT1). All cytokines were examined using western blot analyses (Fig. 2C). SIRT1 and Bcl-2 protein expression in both BMSCs/AKI-KHS and EPO-BMSCs/AKI-KHS groups was significantly increased compared to that in the control group (p < 0.05), especially in the EPO-BMSCs/AKI-KHS group (p < 0.05). The expression of p53 protein was significantly higher in the BMSCs/AKI-KHS group compared to the other two groups (p < 0.05, Fig. 2D).

**Treatment of AKI in rats**

**Blood biochemical indicators**

To compare the effect of the treatment with BMSCs and EPO-BMSCs in AKI rats, blood urea nitrogen (BUN) and serum creatinine (SCr) were measured on days 1 and 5 after treatment. There was a significant increase in BUN and SCr levels on days 1 and 5 after the induction of AKI. Treatment of AKI rats with EPO, BMSCs, and EPO-BMSCs showed varying therapeutic effects. All three treatments reduced the level of BUN and SCr on days 1 and 5 compared to the control group treated with the vehicle, however only BMSCs and EPO-BMSCs treatment showed significant effect (p < 0.05). Rats in the EPO-BMSCs group had significantly lower BUN and SCr levels compared to those in the BMSCs group (p < 0.0, Fig. 3A and B).

**Detection of cytokines**

Twenty-four hours after the treatment of the AKI rats with vehicle, EPO, BMSCs and EPO-BMSCs, the level of serum proinflammatory cytokines IL-1β and TNF-α, and that of the anti-inflammatory cytokine, IL-10, were tested using enzyme-linked immunosorbent assay (ELISA). There was a significant
increase in the level of IL-1β, TNF-α, and IL-10 on day 1 after the induction of AKI. The level of the cytokines in the EPO group was not significantly different compared to the model group. Infusion of BMSCs significantly reduced serum IL-1β (Fig. 3C), and TNF-α (Fig. 3D) level, while it increased serum IL-10 level (Fig. 3E). Furthermore, the AKI rats that underwent EPO-BMSCs infusion had significantly lower serum IL-1β and TNF-α levels, and significantly higher IL-10 levels compared to the rats that received BMSCs (Fig. 3C-E).

**GFP fluorescence in frozen sections**

Our data showed that 24 h following the intravenous infusion, majority of the GFP-BMSCs were trapped inside the lungs, with minimal fluorescence detected in the spleen, liver, and kidneys in the BMSCs group. However, in the EPO-BMSCs group, the fluorescence intensity was significantly lower in the lungs, while the other organs, especially the kidneys showed increased number of fluorescent cells. In both the groups, GFP-BMSCs were found predominantly in the lungs, and EPO preconditioning reduced the lung entrapment of the BMSCs, and increased the distribution of the fluorescent BMSCs to the target organs (Fig. 4).

**Histological analysis**

Hematoxylin and eosin (HE) staining of kidney sections revealed tubular necrosis, cast formation, tubular dilation, and loss of brush border in the model group (Fig. 5A). These parameters were assessed using the pathological scores following treatment with EPO, BMSCs, and EPO-BMSCs on days 1 and 5. The pathological scores in the BMSCs and EPO-BMSCs treatment groups were significantly lower than that in the model group on days 1 and 5 (p < 0.05). Both BMSCs and EPO-BMSCs infusion reduced tubular injury. EPO-BMSCs administration led to significantly better improvement of the tubular injury on day 5 compared to infusion with BMSCs (Fig. 5B).

**Discussion**

In the present study, we reveal several novel findings regarding the efficacy and mechanism of EPO-BMSCs in I/R-AKI. Firstly, the I/R-AKI microenvironment clearly caused apoptosis in the BMSCs, but EPO pretreatment protected the cells against this apoptotic effect. We found that EPO upregulated SIRT1 and Bcl-2 expression, and downregulated p53 expression. The anti-apoptotic mechanism
mediated by EPO pretreatment of BMSCs may occur though the SIRT1 pathway. Secondly, EPO pretreatment reduced pulmonary entrapment, and increased the number of cells reaching the target organ. Furthermore, EPO pretreatment reduced the expression of disease-related inflammatory cytokines. The AKI rats that underwent EPO-BMSCs infusion had significant lower serum IL-1β and TNF-α levels, and significant higher IL-10 levels compared to the rats that received BMSCs. Our data suggest that EPO pretreatment enhanced the efficacy of BMSCs in the improving the renal function and pathological presentation of I/R-AKI rats.

The protective effect of MSCs in acute and chronic renal injury may be through a paracrine/autocrine mechanism, which is related to immune regulation, anti-apoptosis, and reduction of disease-related inflammation. Several studies have shown that the survival and retention of MSCs in target organs or tissues is closely related to the therapeutic effect mediated by MSCs. The main drawbacks of MSCs therapy are the early pulmonary entrapment and the lack of specific homing after systemic infusion. Most of the MSCs undergo cell death after transplantation, mainly due to local adverse microenvironment, and this directly affects the therapeutic efficacy of the MSCs [14, 15]. Increasing the dose of the MSCs infusion represents a viable option to achieve better therapeutic effect. However, this has some unfavorable side effects, such as microvascular embolization and potential risk of long-term tumor differentiation. For these reasons, current studies on MSCs-related therapies have focused on minimal infusion amounts to achieve the maximum therapeutic effect possible. Cell-free treatments including the microvesicles, exosomes, specific cytokines, miRNA, and the pretreatment of MSCs represent viable alternatives to address the issue of long-term negative effects [25–27]. Application of pretreated-MSCs is a novel strategy to enhance the capacity of MSCs to migrate and promote tissue repair in the treatment of kidney injury[28]. Pretreatment with cytokines such as transforming growth factor β1 (TGF-β1), interleukin-17A (IL-17A), and melatonin before infusion can increase the number of MSCs that home to the injured kidney, promote the recovery of renal function, and ameliorate impairments to the renal structure [3, 17, 29]. Similar result was obtained by pretreatment of MSCs with EPO.

EPO is a glycoprotein hormone that promotes the proliferation and differentiation of bone marrow
HSCs, and hematopoietic function of bone marrow through EPO receptor (EpoR). BMSCs and bone marrow HSCs are homologous and express the EpoR. Many other cell types, including neurons, endothelial cells, cardiomyocytes, and renal tubular cells also express the EpoR and respond to EPO treatment[30]. Studies have shown that EPO is suitable for the treatment of a variety of diseases, including cerebral ischemia, myocardial infarction, and chronic congestive heart failure, as well as renal injury [20, 31]. Moreover, few studies suggest that overexpression of EPO by gene-transfection in MSCs could further enhance the protective effect [32, 33]. Compared to other pretreatments or improvement strategies aimed at improving the therapeutic effect of MSCs, in vitro pretreatment with EPO has significant advantages in terms of clinical feasibility. Firstly, EPO is a commonly used therapeutic drug with few side effects and is widely used in the clinical treatment of anemia, especially in patients with CKD. Secondly, it has anti-oxidative and anti-inflammatory effects. Our data showed that 24 h following intravenous infusion, GFP-BMSCs were found predominantly in the lungs. EPO preconditioning reduced the lung entrapment of BMSCs, and increased the distribution of the fluorescent BMSCs to the target organs. In our previous study, we showed that pretreatment of BMSCs with an optimal concentration of EPO for an appropriate time induced a marked change in their proliferation rate and cytoskeletal rearrangement. After incubation with EPO, most of the cells exhibited parallel-oriented filaments organized along the axis of the cells. We observed that CXCR4 which was a pivotal mediator of migration and engraftment of MSCs was upregulated following EPO treatment. These changes enhanced the migration ability of BMSCs [18]. Homing of MSCs to the injured tissues is very critical in cell therapy. There are various methods of MSCs infusion, such as peripheral intravenous infusion, arterial infusion of target organs, and local injection. Local injection increase risks and side effects such as bleeding and tissue injury, while direct arterial administration can result in occlusion and embolization. Following these observations, MSCs are mostly administered through a standard intravenous route. Pulmonary cell entrapment is a major problem after intravenous infusion. Harting et. al. demonstrated that less than 4% of the infused cells were likely to traverse the pulmonary microvasculature and reach the arterial circulation, a phenomenon termed the “pulmonary first-pass effect”, which limits the efficacy of this therapeutic
approach [34]. Some studies showed that smaller microspheres (4-5 µm) can pass through the pulmonary system while the majority of the 20 µm microspheres and MSCs (15-19 µm) were trapped inside the lungs. Lung entrapment may be due to the small capillary size, large capillary network, and strong adhesion properties of the MSCs [35-37]. A variety of molecules may have a role in the lung entrapment of systemically infused cells and the composition of the cell surface molecules like α4, α5, and α6 integrins likely affect the migratory behavior of the therapeutic cells [13, 37]. We found that following intravenous infusion, most of the stem cells were trapped inside the lungs, but EPO pretreatment reduced pulmonary entrapment, and increased the number of cells reaching the target organ.

We also found that EPO upregulated SIRT1 and Bcl-2 expression, and downregulated p53 expression. SIRT1 is NAD+-dependent deacetylase belonging to the class III histone deacetylases, and is known as the longevity protein in mammals. SIRT1 regulates a variety of cellular signaling pathways by modifying the acetylation status of target proteins, including p53, members of the forkhead family of transcription factors (FOXO), nuclear factor NF-κB, among others. Following activation, it participates in various cellular processes such as cell senescence, apoptosis, DNA damage repair, cell cycle, antioxidative stress, energy metabolism regulation, tumor generation, and other physiological and pathological processes [38-40]. P53 plays a vital role in the apoptotic signaling pathways, including membrane apoptotic signaling and mitochondrial apoptotic pathways, and affects the transcription and expression of many apoptosis-related cytokines in the nucleus [41]. SIRT1 reduces the transcriptional activity of p53, and blocks p53-dependent cell apoptosis caused by DNA damage [42]. In tumor studies, SIRT1 inhibits the apoptosis of tumor cells by regulating p53 and Bcl-2 [43]. EPO has been demonstrated to protect against chemotherapy drug doxorubicin (DOX)-induced cardiotoxicity by activating SIRT1 to enhance mitochondrial function [20]. Hong et. al. revealed a novel mechanism of alleviation of hepatic steatosis by EPO by activation of autophagy through SIRT1-dependent deacetylation of LC3 in the treatment of hepatic steatosis [38]. The data from the present study demonstrated that the anti-apoptotic effect following EPO pretreatment of BMSCs may be mediated though the SIRT1 pathway.
The mechanism through which EPO-pretreated BMSCs accelerate the repair of AKI involves three facets. Firstly, after incubation with EPO, most of the BMSCs exhibit parallelly-oriented filaments organized along the axis of the cells, and showed increased CXCR4 expression. These changes reduce the lung entrapment of the BMSCs, and increase the homing of the BMSCs to the target organs. Secondly, EPO upregulated SIRT1 and Bcl-2 expression, and downregulated p53 expression in the BMSCs. SIRT1 inhibited the apoptosis of BMSCs cells by regulating p53 and Bcl-2. Thirdly, the direct anti-inflammatory effect of EPO-BMSCs is also likely to be involved in the process. However, there are some limitations in the present study. Our study did not elucidate the mechanism underlying the EPO-mediated activation of SIRT1 signaling in BMSCs, which needs future investigations.

Conclusion
In conclusion, the administration of EPO-BMSCs was more effective in the reduction of SCr, BUN, and pathological scores in I/R-AKI rats after reperfusion than untreated BMSCs. These results suggest that EPO pretreatment may be a potential novel alternative to untreated BMSCs for the management of AKI.

Materials And Methods

Cell culture
Sprague-Dawley (SD)-derived BMSCs (P5) (Cyagen Biosciences Inc., USA) that stably express GFP were propagated at 37°C under 5% CO₂ in Dulbecco’s Modified Eagle Medium (DMEM, Gibco, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS, Gibco, USA) and 1% antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin, Gibco, USA). The P8-GFP-BMSCs were used for in vivo and in vitro experiments.

Characterization of BMSCs incubated with EPO
GFP-BMSCs were trypsinized after incubation with or without EPO (500 IU/ml) for 48 h, and washed three times with phosphate buffered saline (PBS). Cell surface markers were examined by immunostaining with the following antibodies: phycoerythrin (PE) conjugated anti-CD45 (BD Biosciences, USA), fluorescein isothiocyanate (FITC) conjugated anti-CD44 (BD Biosciences, USA), and FITC conjugated anti-CD90 (BD Biosciences, USA) antibody. The labeled cells were analyzed using a
flow cytometer (BD LSRFortessa™). The osteogenic and adipogenic differentiation potential of the cells in the two groups was examined according to the manufacturer’s protocol (Cyagen, China).

**Animals**

Adult female Sprague–Dawley (SD) rats (200–250 g) purchased from the animal house of the Faculty of Medicine, Southern Medical University (Guangzhou, China) were used for the study. Animals were maintained under specific pathogen-free laboratory conditions with a 12 h dark–12 h light cycle and pelleted food and tap water were supplied *ad libitum*. Animals were left to acclimatize to the housing conditions for one week. All procedures were performed in strict accordance with the principles of the Guidelines for Animal Experimentation of Southern Medical University (Guangzhou, China).

**I/R-AKI kidney homogenate supernatant (KHS) preparation**

An AKI model was generated using the SD rats by clamping both renal pedicles for 45 min followed by clamp-release to allow reperfusion. Both the kidneys of the rats were obtained 60 min after the reperfusion, cut into small pieces and a 20 g/L homogenate was prepared in PBS using a glass homogenizer. The homogenate was then centrifuged at 20,000 rpm for 15 min at 4°C. The supernatant was filtered with a 30 μm mesh-sized disposable sterile filter to obtain AKI-KHS, which was then stored at −80°C until further use. Normal KHS (N-KHS) obtained from healthy SD rats was used as the control.

**Anti-apoptotic effect of BMSCs pretreated with EPO**

To study the anti-apoptotic effect of BMSCs pretreated with EPO, BMSCs or EPO-BMSCs were incubated with AKI-KHS (or N-KHS). Cells were seeded at 4 x 10⁵ cells/well in six-well plates in DMEM/F12 with 2% FBS. Transwell chambers with 0.4 μm pore size polycarbonate filter (Corning Incorporated, NY, USA) were introduced in the wells for the interventions. Three groups were set up: the control group (BMSCs + N-KHS group, BMSCs were plated in the 6-well plates and 1.5 ml N-KHS was added to the upper chamber); the BMSCs/AKI-KHS group (BMSCs were plated in the 6-well plates and 1.5 ml AKI-KHS was added to the upper chamber); and the EPO-BMSCs/AKI-KHS group (EPO-BMSCs were plated in the 6-well plates and 1.5 ml AKI-KHS was added to the upper chamber). All
groups were incubated at 37°C for 24 h in a humidified atmosphere with 5% CO₂. Then, apoptosis of the BMSCs and EPO-BMSCs was analyzed by flow cytometry, and cells were subsequently harvested for western blot analyses.

**Western blotting**

After the indicated treatments, the BMSCs and EPO-BMSCs were washed twice with PBS, harvested, and the proteins were extracted. The following antibodies were used to analyze the protein expression: anti-BCL-2 (1:1000, Santa Cruz), p53 (1:1000, Santa Cruz), SIRT1 (1:1000, Santa Cruz), and anti-β-actin (1:2000, Bioworld, Shanghai, China). Protein bands were developed and quantified by densitometry analysis using an Alpha Innotech imaging system. Protein levels were normalized to β-actin using Image J analysis software.

**Animal grouping and treatment**

AKI models were generated in the SD rats. The animals were anesthetized using intraperitoneal injection of 2% pentobarbital sodium (50 mg/kg), abdominal incisions were made, and the two renal pedicles were bluntly separated. A microvascular clamp was used to clamp both the renal pedicles for 45 min followed by clamp-release to allow reperfusion. Then the abdominal incision was closed. Intervention treatments were administered to the mice through tail vein injections after the reperfusion.

To determine the effect of treatment with BMSCs and EPO-BMSCs, the rats (n = 50) were randomly divided into the following five groups (n = 10 for each group): Sham group (kidneys of the SD rats were exposed for 45 min, 1 ml of low-glucose DMEM was injected); Model group (both renal pedicles were clamped for 45 min, 1 ml low-glucose DMEM was injected); EPO group (both renal pedicles were clamped for 45 min, 1 ml EPO (500 IU/ml) was injected); BMSCs group (both renal pedicles were clamped for 45 min, 1 x 10^6 BMSCs was injected); EPO-BMSCs group (both renal pedicles were clamped for 45 min, 1 x 10^6 EPO-BMSCs was injected). All rats were housed at favorable temperatures and humidity with an unlimited supply of water and food post-surgery. Then the rats were sacrificed 1 and 5 days after the treatment (5 rats for each time point), and blood samples and tissues from the
kidneys, lung, spleen, and liver were collected for the subsequent tests. Blood samples were collected through the inferior vena cava and the serum was separated and stored at −80°C until use. Both kidneys of each rat were immediately excised and cut into two coronal sections each. Two pieces of kidney were fixed in 4% paraformaldehyde at room temperature. In the BMSCs and EPO-BMSCs group, the lung, spleen, liver, and the remaining part of kidney were tested immediately.

**Renal functional analysis**

Renal functions were estimated using diagnostic kits for BUN and SCr. SCr was measured using a colorimetric microplate assay based on the Jaffe reaction (Quantichrom Creatinine Assay; BioAssay Systems). BUN was measured using a colorimetric assay kit according to the instruction protocol (Quantichrom Urea Assay; BioAssay Systems).

**ELISA**

The concentrations of inflammatory factors IL-1β and TNF-α, and that of the anti-inflammatory cytokine IL-10 in the serum were measured by ELISA (R&D Systems, Minneapolis, MN). The assay was performed according to the manufacturer’s instructions.

**GFP fluorescence in frozen tissue sections**

To verify the *in vivo* distribution of the BMSCs after intravenous infusion, fast frozen sections from kidney, lung, spleen, and liver were prepared 24 h after the BMSCs or EPO-BMSCs infusion and the sections were observed immediately under fluorescence microscope.

**Renal histological analysis**

To detect injury to the kidneys, the samples were fixed in 4% neutral-buffered paraformaldehyde for histological assessment, embedded in paraffin, and cut in 3-μm-thick slices. Then, the sections were stained with hematoxylin and eosin (HE, Servicebio, China). Histological examinations were performed in a blinded manner for acute tubular necrosis (ATN) scores regarding the grading of tubular necrosis, cast formation, tubular dilation, and loss of brush border. Ten non-overlapping fields (×200) were randomly selected and scored as follows: 0, no damage; 1, patchy isolated necrosis ≤ 10%; 2, tubular necrosis between 10% and 25%; 3, tubular necrosis between 25% and 50%; 4, tubular necrosis > 50% [3].
**Statistical analysis**

The results are expressed as mean ± standard deviation (SD). Student’s t test was performed to analyze the differences between two groups. Multiple-group comparison was performed using one-way analysis of variance (ANOVA) test. SPSS 19.0 statistical software was used for the analysis. P < 0.05 was considered statistically significant.

**Declarations**

**Authors contributions**

SZ designed the overall study, executed the experiments, analyzed data, and wrote the manuscript; YMQ executed the experiments, prepared the manuscript; YGL designed the overall study, supervised the project and edited the manuscript; DL, JMH, JL, ML, YG, LPF, LYL provided suggestions for the project and edited the manuscript. MZ: designed the overall study, analyzed data and final approval of manuscript. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

Not applicable.

**Availability of data and material**

Not applicable.

**Competing Interests**

The authors declare that they have no conflicts of interests.

**Consent for publication**

Not applicable.

**Funding**

This work was supported by the Initiative Research Projects of Southern Medical University in
Acknowledgments

Not applicable.

Author details

1Department of Organ Transplantation, Zhujiang Hospital, Southern Medical University, No.253, Industrial Avenue, Haizhu District, Guangzhou 510280, Guangdong Province, China;

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Figures
Cell culture and characterization of BMSCs. (A) The P8 GFP-BMSCs with stable fluorescence expression were observed under fluorescent microscope. (B) GFP-BMSCs and EPO-pretreated GFP-BMSCs differentiated into osteoblasts and adipocytes, respectively. (C) Flow cytometric analysis confirmed that both groups of cells were CD45-negative and positive for the phenotypic markers CD90 and CD44.
Figure 2

AKI-KHS induced apoptosis in BMSCs. (A) After culture for 24 h, flow cytometric analysis was performed to assess apoptosis in each group. (B) The apoptotic rates in each group; (C) Anti-apoptotic factor Bcl-2, apoptotic factor p53, and SIRT1 were examined using western blot analyses. *p < 0.05 versus BMSCs+N-KHS; #p < 0.05 versus BMSCs+AKI-KHS;
To compare the effect of the treatment with BMSCs and EPO-BMSCs in AKI rats, SCr(A) and BUN(B) were measured on days 1 and 5 after treatment. Twenty-four hours after the treatment of the AKI rats with vehicle, EPO, BMSCs and EPO-BMSCs, the level of serum proinflammatory cytokines IL-1β(C), and TNF-α(D), and that of the anti-inflammatory cytokine, IL-10(E), were tested using ELISA.*p < 0.05 versus Model group; #p < 0.05 versus BMSCs group;
GFP fluorescence observed in frozen sections. Intravenous infusion of BMSCs and EPO-BMSCs after 24h, Lung, liver, spleen, and kidney of rats frozen sections were observed immediately under fluorescence microscope.
Figure 5

Histological analysis. (A) In different groups, the kidney sections stained with hematoxylin and eosin (HE) on days 1 and 5. (B) After treatment with EPO, BMSCs, and EPO-BMSCs for 1 day and 5 days, pathological parameters were assessed. *p < 0.05 versus Model group; #p < 0.05 versus BMSCs group;