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Original Article

Effect of EPO gene modified human amniotic mesenchymal stem cell transplantation on renal function of rats with acute kidney injury

Qi Liu, Yi-Fan Zhang, Qiong Ye

Institute of Nephrology, Zhejiang Province Wenzhou Central Hospital, Wenzhou 325000, Zhejiang, China

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Abstract: To investigate the effect of EPO gene modified human amniotic mesenchymal stem cell transplantation on renal function and renal cell apoptosis in ischemic-reperfusion-induced acute kidney injury (AKI) rats. Human amniotic mesenchymal stem cells were cultured in vitro; retroviral virus PLXSN was used as the vector to mediate erythropoietin (EPO) gene transfection; it was divided into three groups: control group, negative transfection group, EPO transfection group. EPO protein in human amniotic mesenchymal stem cells was detected by Western blot after transfection; 68 SD rats were selected (male: female 1:1), weighing 220-260 g. Non-invasive artery clamp was used to clamp bilateral renal pedicle for 40 min; ischemia-reperfusion induced AKI model was established (after modeling there were a total of eight demyelination and death). The rats were divided into EPO-AM-MSCs (EPO-amniotic membrane-mesenchymal stem cells) group, AM-MSCs group and the control group, 20/group. On day 3, 28 after the transplantation left kidney specimens of 5 rats in three groups were stained by HE staining to observe renal tissue damage; TUNEL assay was used to detect renal cell apoptosis; on day 28 after transplantation fluorescence microscope was used to observe PKH-26 labeled AM-MSCs survival and distribution. In 1, 3, 14, 28 days after modeling serum creatinine (SCr) and blood urea nitrogen (BUN) were detected. At 3, 28 days after transplantation, HE staining examination showed that in EPO-AM-MSCs group tubular damage scores were 2.1 ± 0.12, 1.2 ± 0.10; in AM-MSCs group, tubular damage scores were 3.1 ± 0.12, 2.1 ± 0.11; in the control group tubular damage scores were 3.9 ± 0.16, 2.8 ± 0.13; the differences between two groups were statistically significant ($P < 0.05$). At 28 days after transplantation, PKH-26 positive cells: EPO-AM-MSCs group > AM-MSCs transplantation group > the control group; The difference between the groups was statistically significant ($P < 0.05$). At 3, 28 days after transplantation, TUNEL-positive apoptotic cells: the control group > AM-MSCs group > EPO-AM-MSCs group; The difference was statistically significant ($P < 0.05$) between groups. At 3, 14, 28 days after transplantation, serum levels of creatinine and blood urea nitrogen: EPO-AM-MSCs group < AM-MSCs group < control group ($P < 0.05$). EPO gene-modified human amniotic mesenchymal stem cell transplantation had a significant repair effect on rat acute kidney injury.

Keywords: EPO, human amniotic mesenchymal stem cells, transplantation, acute kidney injury, renal function, apoptosis

Introduction

Acute kidney injury (AKI) is a more serious and widespread kidney disease, seriously threatening human health, with high morbidity and mortality. Currently the main treatment in clinical is hemodialysis and kidney transplantation. But the cost of medical care of hemodialysis is high, and it only can partially replace kidney function; kidney transplantation is also facing a shortage of organs [1-4]. Therefore, to explore more effective treatment to prevent disease progression of renal injury is an important issue nowadays for kidney disease studies [5, 6]. In recent years, stem cell transplantation has been widely used in the treatment and research of kidney disease; more and more evidence confirms the efficacy of stem cells in acute kidney injury; stem cell transplantation in theory and technology has made great progress, and shows a broad clinical application prospect [7, 8]. But there are few studies on stem cell for the treatment of renal injury; if there is a clear therapeutic effect is uncertain. The stem cells can differentiate to endothelial cells and kidney cells, which may be able to solve the problems

of organ damage arising from the source [9, 10]. Therefore, we established rat acute kidney injury model and used EPO gene modified human amniotic mesenchymal stem cell transplantation to study the repair effect of stem cell on acute kidney injury.

Materials and methods

Design: a randomized controlled animal study

Time and place: Experiments are completed between February 2013 and 2014 October in Tianjin Medical University General Hospital animal laboratory.

Experimental animals and major reagents, instruments

Human amniotic membrane was provided by Obstetrics and Gynecology department, Tianjin Medical University General Hospital, peeled from fresh placenta in full-term caesarean. Maternal prenatal hepatitis B virus, hepatitis C virus, syphilis and human immunodeficiency virus were negative. Before the experiment maternal were informed consent, and signed informed consent. 70 SD rats, male and female 1:1, weighting 180-220 g, were purchased from the Animal Laboratory (animal quality certification number: SCXK20090028) in Chinese Academy of Medical Sciences; animal disposal methods were in line with animal ethic requirements. DMEM/F12 medium, 5% fetal bovine serum (FBS) (Gibco BRL company, USA); PKH-26, glutamine (Sigma); 25% trypsin (GibcoBRL Co.); (Sigma, USA); Trizol, Lipofectamine TM 2000 (Invitrogen Corporation): Western blot chemiluminescence reagent (Santa cruz Corporation); rabbit anti-human EPO antibodies, HRP goat anti-rabbit IgG, PVDF membrane (Pierce Corporation); 5 μL micro-syringe (Hamilton, United States); The main experimental apparatus included a carbon dioxide incubator (UK RS Biotech Inc.); Clean Benches (Suzhou Aetna air Technology Corporation) and fluorescence inverted microscope (Nikon Corporation of Japan).

Preparation and labeling of human amniotic charge mesenchymal stem cells

Placenta acquisition had get informed consent; in accordance with Ref. [11]. Human amniotic membrane was separated and obtained. Amniotic membrane was repeatedly washed with D-Hank's fluid to remove residual blood and mucus; amniotic membrane was cut into pieces, digested with 0.25% trypsin at 37°C under 200 r/min for 10 min to remove human amniotic epithelial cells; the digestion was discarded; after precipitation fresh digestion was added; experiment was repeated twice. Samples were digested with the mixture of 0.01% of collagenase and DNA enzyme at 37°C under 200 r/min for 1 h. When tissue was digested into fluff, the digestion was filtered by a stainless steel mesh, and the cell suspension was collected; cells were re-suspended in 10% FBS-DMEM medium, counted and placed in 25 cm^2 flasks, incubated in a 37°C, 5% CO_2 humidified incubator; After 40 h the medium was replaced and the non-adherent cells were discarded; when adherent cells covered 90% pf the bottom, cells were digested with 0.25% trypsin, and centrifuged to obtain Human amniotic membrane mesenchymal cells. Cell morphology was observed under an inverted microscope. Draw PKH-26 5 μL to 1.5 ml of EP tube; add 1ml complete medium, pipette uniform to prepare PKH-26 labeled solution. 90% confluent adherent cells were washed with PBS for 3 times, and incubated with 40 μL/cm^2 labeled solution in a 37°C, 5% CO_2, humidified incubator for 20 min; then labeled solution was discarded and 37°C complete medium 5 ml was added; 10 min later, complete medium was discarded and cells were washed twice with fresh medium. After 24 h, PKH-26 labeled effects and morphological changes were observed under an inverted fluorescence microscope. Five horizons were randomly selected; PKH-26 positive cells and the total cells were counted and labeled rate was calculated. Labeled rate = the total number of positive cells within the visual field/total number of cells within the visual field × 100%. Before transplantation cells were washed with PBS for three times; the final concentration was 1×10^{10}/L.

PcDNA3-EPO recombinant plasmid transfection and testing

PcDNA3-EPO recombinant plasmid stably transfected AM-MSCs: AM-MSCs of P3 generation were transfected with EPO gene by liposome-mediated transfection method, according to Lipofectamin CM 2000 reagent instructions. At 24 h after transfection, cells were seeded into 6-well plates (1:10) and incubated

with fresh complete medium. On the next day medium was changed; cells were cultured with culture medium containing 400 mg/L G418 for 6 d, and then cultured with the medium containing 200 mg/L G418; 2 weeks later, positive clone formed; individual clones were selected for expanding culture and subculture. Expression of EPO in EPO-AM-MSCswas detected by Western blot test: Well-grew AM-MSCs (1×10^7) which were stably transfected with pcDNA3-EPO were mixed with RIPA buffer (containing 10 g/L PMSF) and centrifuged at 4°C after ice bath; the supernatant was collected. 100 g/L SDS-polyacrylamide gel electrophoresis was performed after protein concentration was determined. After electrophoresis, proteins were transferred to PVDF membranes, closed with blocking solution, incubated with polyclonal rabbit anti-human EPO antibody (1:200); after washing the membrane, proteins were incubated with goat anti-rabbit HRP IgG (1:5000); after washing the membrane again, Western blot chemiluminescence reagent was added; 1 min later in a dark room, chemiluminescent white developing was performed with x-ray film; after washing an image analyzer was used for scanning.

Construction of ischemia-reperfusion-induced AKI model and cell transplantation

According to Reference [12], 70 SD rats were selected, male and female 1:1, weighing 250~300 g (after modeling totally 10 suffered to demyelination and death); the remaining 60 rats were randomly divided into three groups, 20/group. After intraperitoneal injection of 200 g/L urethane by 1.2 g/kg for anesthesia, abdominal incision was performed to expose kidney, with non-invasive artery clamp to clamp bilateral renal pedicle; 40 min later vessel clamp was released; after reperfusion cell transplantation was conducted. EPO-AM-MSCs group: 2×10^6 EPO-AM-MSCs were re-suspended in 100 ul of PBS; AM-MSCs group: 2×10^6 AM-MSCs were re-suspended in 100 ul of PBS; PBS group: with 100 ul of PBS, transplantations were performed respectively from upper, middle and lower renal parenchyma of the left kidney [13].

HE staining and histological observation and scoring

On day 3 and 28 after transplantation, left kidney specimens were drawn from 5 rats in each group for HE staining. Ischemia-reperfusion-induced AKI mainly performed as renal tubular damage, including tubular necrosis, brush border disappearance, tubular extension and tube formation. 0-normal kidney, 1-small injury (range < 5%), 2-moderate damage (range 5%~25%), 3-moderate-severe injury (range 25%~75%), 4-severe injury (> 75%) [14].

Immunofluorescence to observe PKH-26 positive cells

On day 28 after transplantation, tissue sections were observed by fluorescence microscopy. 10 fields were randomly selected for each slice in high magnification (× 200); PKH-26 positive cells in each horizon were calculated; the mean was taken as the number of PKH-26 positive cells in each group. Design, enforcement, and evaluation: experiment was designed by the first author; the intervention was conducted by the first author; assessment was performed by the second author. After formal training, blinded assessment was conducted.

TUNEL apoptosis and renal function tests

On day 3, 28 after transplantation, 5 rats were randomly selected in three groups; thoracotomy, left ventricular aortic cannulation and 4% paraformaldehyde perfusion were performed after anesthesia. The left kidney specimens were collected and paraffin slices were prepared; TUNEL assay was performed to count cells according to the German Roche kit operations. After hydration, 37°C proteinase K digestion for 10 min, and marking fluid labeling at 37°C, biotinylated digoxin reaction was performed for 30 min before SABC, DAB coloring. Films were mounted; around the damaged area five high power fields were randomly selected and cells with the nucleus containing brown particles were counted at 1, 3, 14, 28 days after the transplantation; 5 rats were selected in three groups, and eyelid venous blood was drawn to detect serum creatinine (SCr) and blood urea nitrogen (BUN).

Statistical analysis

Data were processed with SPSS17.0 software package. Measurement data were expressed as mean ± standard deviation ($\bar{x} \pm s$); Continuous variables between two groups were compared using t test; data among groups were compared using ANOVA and q test

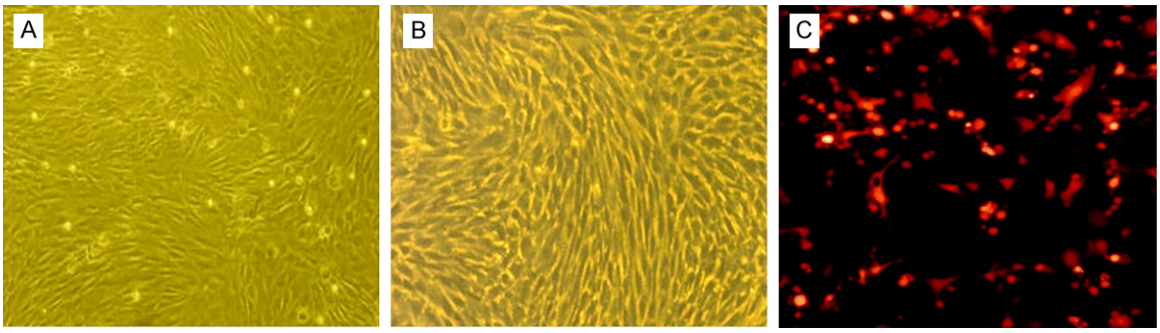

Figure 1. Morphology of AM-MSCs under microscope. A. Initially extracted AM-MSCs (inverted microscope, × 40), fusiform; B. AM-MSCs of the second generation (inverted microscope, × 100); C. At 24 h, PKH-26 labeled AM-MSCs (fluorescence microscope: × 100).

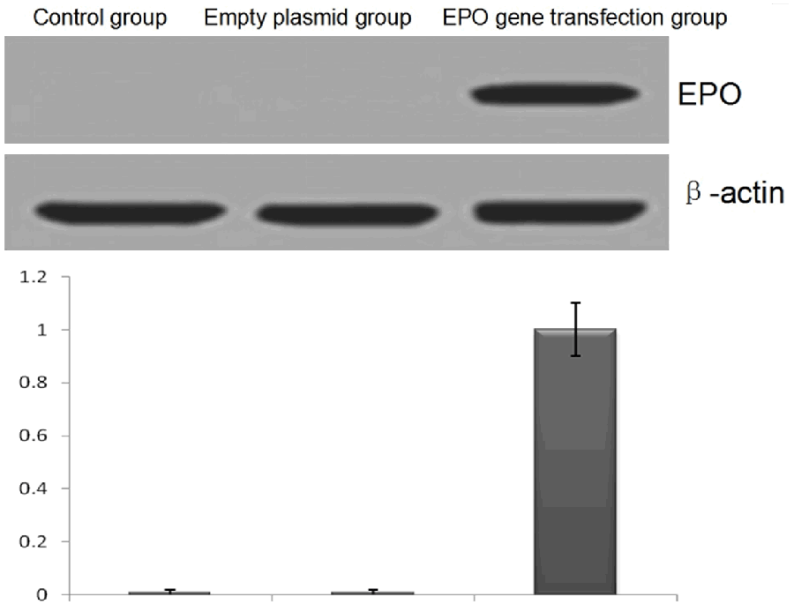

Figure 2. EPO protein expression in each group after 48 h.

(Newman-kueuls method); $P < 0.05$ indicated that there were significant differences.

Results

Morphology of AM-MSCs under microscope

At first, AM-MSCs showed adherent growth, morphological diversity, fusiform, polygons, stars, spindle or polygon (**Figure 1A**). After passaging to the second generation, AM-MSCs adherent speed increased; in eight hours cells were almost completely adherent; cells were uniform fibroblast-like cells, mostly showing single-radiation shape, long spindle or rotating nest-like growth, as shown in **Figure 1B**. Uniformity of AM-MSCs was good; purity was over 98%. At 24 h, PKH-26 labeled AM-MSCs showed red fluorescence under a fluorescence microscope; FCM detection showed that cell labeling rate was 100%, as shown in **Figure 1C**.

EPO protein expression in each group

At 48 h after pcDNA3-EPO recombinant plasmid was stably transfected with erythropoietin (EPO) gene, in EPO gene transfection group EPO protein expression was detected in AM-MSCs; in the control group and empty vector group EPO protein was not expressed; It indicated that the EPO gene was stably integrated into the EPO transfected AM-MSCs, and the protein can be stably expressed, as shown in **Figure 2**.

HE staining and histological score

On day 3 and 28 after transplantation, renal tissue injury was shown in **Figure 3**. On day 3 and 28 after transplantation, in EPO-AM-MSCs group tubular damage scores were 2.1 ± 0.12, 1.2 ± 0.10; in AM-MSCs group scores were 3.1 ± 0.12, 2.1 ± 0.11; in the control group scores were 3.9 ± 0.16, 2.8 ± 0.13; between two groups differences were statistically significant ($P < 0.05$). Ischemia-reperfusion-induced acute kidney injury mainly was tubular necrosis, tubu-

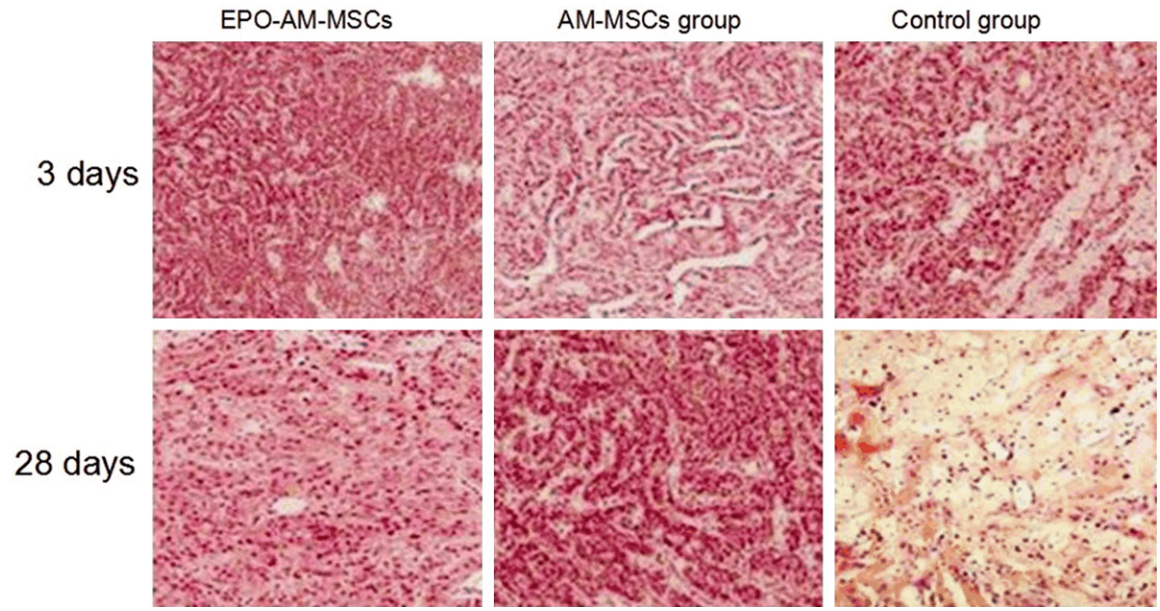

Figure 3. Ischemia-reperfusion-induced acute kidney injury was mainly as tubular necrosis, tubular formation, brush border disappearance and tubular expansion. Among EPO-AM-MSCs group, AM-MSCs group and control group there were statistically significant differences in renal tubular damage score on day 3, 28 after transplantation ($P < 0.05$).

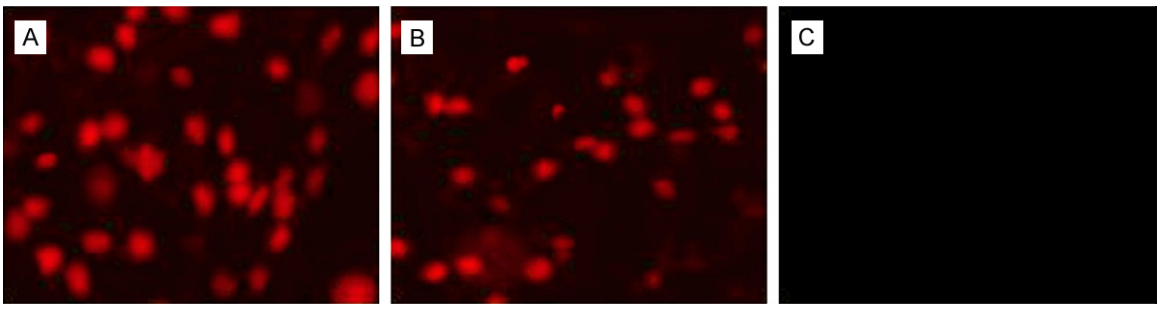

Figure 4. A. At 4 weeks after injury, in EPO-AM-MSCs group PKH-26 positive cells were the most (× 200); B. In AM-MSCs group, at four weeks after injury PKH-26 positive cells were visible (× 200); at 4 weeks after the injury in the control group no PKH-26 positive cells were observed (× 200); C. On day 28 after transplantation in renal tissue damage zone PKH-26-positive cells were observed (immunofluorescence, × 200).

lar formation, brush border disappearance and tubular expansion.

Comparison of PKH-26 positive cells

On day 28 after transplantation, PKH-26 positive cells in EPO-AM-MSCs group were the most (25.3 ± 4.42), followed by AM-MSCs transplantation group (16.74 ± 3.45); in the control group, no PKH-26 positive cells were observed (00.0 ± 0.00); the difference between the groups were statistically significant ($P < 0.05$). The results showed that on day 28 after cell transplantation, viable EPO gene-modified AM-MSCs in renal tissue damage zone cells were more than those in AM-MSCs cell transplantation, shown in **Figure 4**.

TUNEL to detect cell apoptosis

On day 3 after transplantation, the number of TUNEL positive apoptotic cells in EPO-AM-MSCs group was (23.65 ± 3.67), shown in **Figure 5A**; in AM-MSCs group, the number of TUNEL-positive apoptotic cells was (33.65 ± 4.32), shown in **Figure 5B**; in control group, the number of TUNEL positive apoptotic cells was (43.65 ± 5.67), shown in **Figure 5C**. On day 28 after transplantation, In EPO-AM-MSCs group, the number of TUNEL-positive apoptotic cells

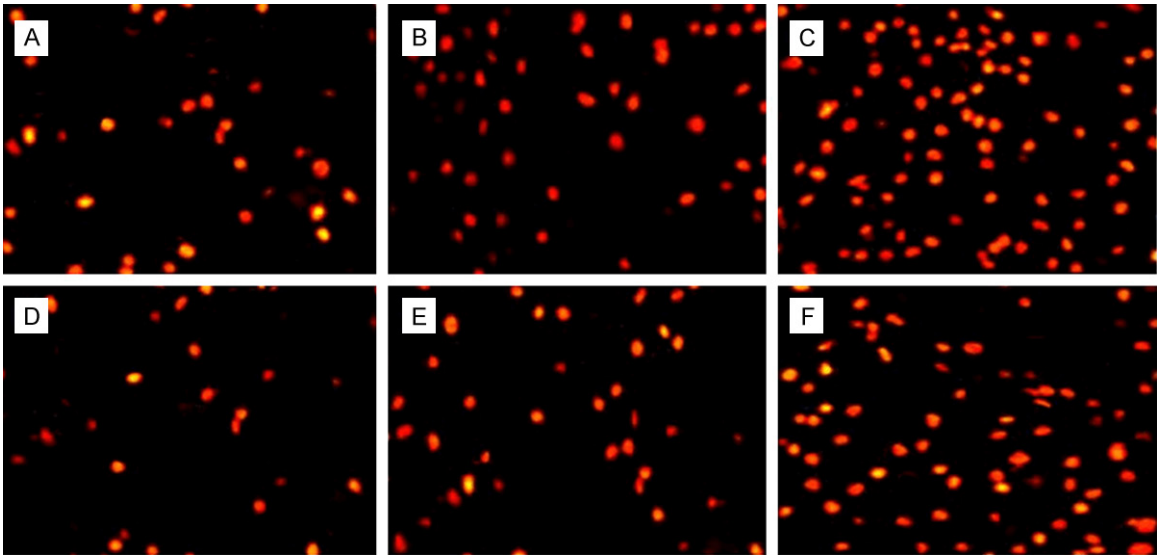

Figure 5. On d3 and d28 after transplantation, TUNEL-positive apoptotic cells were the most in the control group, followed by AM-MSCs group; apoptotic cells were the fewest in EPO-AM-MSCs group (× 200).

Table 1. Comparison of the three groups in renal function (± s, n=5)

Groups	SCr (umol/L)				BUN (mmol/L)			
	1 d	3 d	14 d	28 d	1 d	3 d	14 d	28 d
Control group	69.8 ± 4.9	55.3 ± 2.0	38.9 ± 3.6	34.7 ± 3.1	25.4 ± 2.2	21.8 ± 1.5	16.1 ± 2.2	13.2 ± 1.1
AM-MSCs	42.3 ± 3.2*	33.4 ± 2.7*	25.2 ± 1.9*	16.8 ± 1.5*	19.7 ± 1.8*	17.6 ± 1.3*	14.7 ± 1.6	11.3 ± 1.2
EPO-AM-MSCs	35.8 ± 2.7*	26.2 ± 3.2*	15.9 ± 2.6*	10.1 ± 2.0*	16.4 ± 1.7*	14.3 ± 1.1*	11.2 ± 1.2*	8.3 ± 1.0

*Compared to control group, P < 0.05.

was (13.42 ± 1.34), shown in **Figure 5D**; in AM-MSCs group, the number of TUNEL-positive apoptotic cells was (22.89 ± 2.75), shown in **Figure 5E**; in control group, the number of TUNEL positive apoptotic cells was (36.74 ± 4.85), shown in **Figure 5F**; at the two time points, among the three groups there were statistically significant differences (P < 0.05).

Kidney function tests

At 1, 3, 14, 28 days after cell transplantation, SCr in EPO-AM-MSCs group was (35.8 ± 2.7), (26.2 ± 3.2), (15.9 ± 2.6), (10.1 ± 2.0) umol/L; SCr in AM-MSCs group was (42.3 ± 3.2), (33.4 ± 2.7), (25.2 ± 1.9), (16.8 ± 1.5) umol/L, respectively; SCr in the control group was (69.8 ± 4.9), (55.3 ± 2.0), (38.9 ± 3.6), (34.7 ± 3.1) umol/L; BUN in EPO-AM-MSCs group was respectively (16.4 ± 1.7), (14.3 ± 1.1), (11.2 ± 1.2), (8.3 ± 1.0) mmol/L; BUN in AM-MSCs group was (19.7 ± 1.8), (17.6 ± 1.3), (14.7 ± 1.6), (11.3 ± 1.2) mmol/L, respectively; BUN in the control group was (25.4 ± 2.2), (21.8 ± 1.5), (16.1 ± 2.2), (13.2 ± 1.1) mmol/L, shown in **Table 1**; the differences were statistically significant (P < 0.05).

Discussion

Acute kidney injury is the kidney damage caused by renal structural or functional changes; it is a relatively common clinical syndrome, with high incidence and mortality [15-17]. Acute kidney injury can be induced by various reasons. The current treatment of kidney, such as dialysis and transplant, is facing various difficulties [18, 19]. In recent years, advances in stem cell technology enable human amniotic mesenchymal stem cells to effectively treat acute kidney injury [20, 21].

A number of studies indicate that stem cell transplantation is an effective treatment method in the repair of acute kidney injury. Now the more promising stem cells applied in renal replacement therapy mainly include umbilical cord mesenchymal stem cells, pluripotent stem cells, embryonic stem cells, human amniotic mesenchymal stem cells [13, 15]. Among them,

the human amniotic mesenchymal stem cells are a class of stem cells with wide range of sources; they are easy to store and collect, with the advantages of multi-differentiation, avoiding the ethical controversy and the ability to self-renewal. With the progress of stem cell research, human amniotic mesenchymal stem cell transplantation for acute kidney injury diseases have become one of the hotspots of medical research [16, 17]. A number of studies have shown that human amniotic mesenchymal stem cells may secret cytokines and trophic factors by paracrine or autocrine manner to make some damaged cells repair themselves; they also can fuse to the lesion tissue, replace or supply damage cells to restore damaged cells. Zhou et al. [13] found that in two weeks after the stem cell treatment, renal pathological changes were significantly reduced in mice. Immunohistochemistry prompted that TGF-β, FN and VEGF were downregulated, and deposition of complement C3 in renal tissue was reduced. And a number of studies on different animal models of progressive renal injury showed that mesenchymal stem cells had beneficial effects on renal tissue damage [15-17].

Recent studies have found that erythropoietin not only is a hematopoietic cytokine, but also has important protective effects on ischemia-reperfusion injury in a variety of tissues. The protection mechanism of Erythropoietin for acute kidney injury is complex, including anti-apoptotic, anti-oxidative, anti-inflammatory response, and promoting the regeneration of renal tubular cells; it is also involved in the interaction of a variety of cytokines, such as heme oxygenase-1, vascular endothelial growth factor, and heat shock proteins. Erythropoietin receptors are mainly expressed in the renal tubule and medullary collecting duct epithelial cells, mesangial cells; after binding to the erythropoietin, they play a protective role in ischemia-reperfusion injury; especially large dose of erythropoietin treatment before ischemia can significantly reduce kidney damage; meanwhile studies have shown that erythropoietin is related with up-regulation of stem cell factor and c-Kit [22-24].

In this study, the therapeutic effect of EPO gene-modified human amniotic mesenchymal stem cell transplantation on ischemia-reperfusion-induced acute kidney injury (AKI) was observed. The results showed that after EPO vector transfection, TUNEL assay showed that the average of apoptotic EPO gene-modified human amniotic mesenchymal stem cells decreased and average of cell proliferation increased; RT-PCR and Western blot found that after EPO transfection, in cord mesenchymal stem cells EPO gene protein and mRNA expression levels were significantly enhanced. On d3 after transplantation, HE staining examination showed that tubular damage score in EPO-AM-MSCs group was lower than that in AM-MSCs group and control group, and the difference was statistically significant ($P < 0.05$); meanwhile at 3, 14, 28 days after transplantation, levels of serum creatinine and blood urea nitrogen in EPO-AM-MSCs group were significantly lower than those in the AM-MSCs group and control group. In short, the umbilical cord mesenchymal stem cell transplantation can promote repair of acute kidney injury, mainly through promoting proliferation, anti-apoptosis and anti-inflammatory mechanism. Therefore, we believe that umbilical cord stem cell transplantation may offer a new approach for the treatment of acute kidney injury.

Disclosure of conflict of interest

None.

Address correspondence to: Yi-Fan Zhang, Institute of Nephrology, Zhejiang Province Wenzhou Central Hospital, Wenzhou 325000, Zhejiang, China. Tel: +86-0577-88070000; Fax: +86-0577-88070114; E-mail: zhangyfan_a@163.com

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