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Effects of autologous bone marrow-derived stem cell mobilization on acute tubular necrosis and cell apoptosis in rats

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Abstract. The aim of this study was to investigate the effects of stem cell factor (SCF) and granulocyte colony-stimulating factor (G-CSF) on bone marrow-derived stem cell (BMSC) mobilization in rat models of renal ischemia/reperfusion (I/R) injury. In addition, the effects of SCF and G-CSF on cellular apoptosis were explored in order to determine the protective mechanism of the two factors against renal I/R injury. A unilateral renal I/R injury model was established for the model and treatment groups. The treatment and treatment control groups were subcutaneously injected with SCF (200 μg/kg/day) and G-CSF (50 μg/kg/day) 24 h after the establishment of the model for five consecutive days. The total number of leukocytes in the peripheral blood and the cellular percentages of cluster of differentiation (CD)34$^+$, renal CD34$^+$ and apoptotic cells were detected. The total number of leukocytes in the peripheral blood and the percentages of CD34$^+$ cells in the treatment and treatment control groups reached maximum levels on the fifth postoperative day and were significantly higher than those in the normal control and model groups. The number of renal CD34$^+$ cells in the treatment group was significantly increased compared with that in the treatment control and model groups. The apoptotic indices (AIs) of the model and treatment groups were higher than those of the normal control and treatment control groups. The AI of the model group was significantly higher than that of the treatment group. In conclusion, the combined application of SCF and G-CSF can mobilize sufficient numbers of BMSCs and cause cellular 'homing' to the injured site, thus inhibiting apoptosis and promoting the repair of renal tubular injury.

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Introduction

Acute tubular necrosis (ATN) is the main cause of acute kidney injury (AKI), and the incidence of AKI in hospitalized patients has previously been reported as 2.41% (1). As such, the prevention and treatment of ATN is a major concern in the medical field. Blood purification therapy is the only remedial treatment for ATN once it occurs. This therapy replaces the work of kidneys to maintain a stable environment for the body until the renal tubular epithelial cells are able to repair themselves (1-3). When the renal tube is damage beyond complete repair, chronic renal failure may occur; patients with renal failure require a lifelong dependence on blood purification therapy or kidney transplantation. The prognosis of renal failure is not highly optimistic, and no drug or method to promote the repair of necrotic renal tubules or cure tubular necrosis has yet been determined.

Recent studies have shown that bone marrow-derived stem cell (BMSC)-mobilizing agents, such as granulocyte colony-stimulating factor (G-CSF) and stem cell factor (SCF), can mobilize autologous BMSCs to treat a variety of diseases, including kidney damage, myocardial necrosis, nerve and liver damage, and hematological malignancies (4-8). The flexibility of BMSCs presents a potential route for the treatment of renal failure.

Under normal circumstances, few BMSCs are known to enter the peripheral blood; therefore, the effect of these cells on the repair of renal tubular necrosis is limited. G-CSF and SCF can drive more BMSCs into the peripheral blood, thereby meeting the requirements for the treatment of renal failure (9). In the present study, a combinatorial treatment of G-CSF and SCF was administered, and the effects of the treatment on BMSC mobilization and cell apoptosis were observed in an ATN rat model. The aim of the study was to explore the roles of SCF and G-CSF in the promotion of ATN repair.

Materials and methods

Reagents. Recombinant human G-CSF injection (product name, Ji Sai Xin; batch no. 200804Y21) was purchased from NCPC GeneTech Biotechnology Development Co., Ltd. (Shijiazhuang, China), and SCF (batch no. 20080405) was obtained from Chengdu Diao Jiuhong Pharmaceutical Factory (Chengdu, China). Apoptosis kits (cat. no. 11684817910) were

purchased from Roche (Indianapolis, IN, USA), and the rabbit anti-rat cluster of differentiation (CD)34 polyclonal antibody (cat. no. bs-0754R) and ready-to-use Streptavidin-Peroxidase immunohistochemistry kit (cat. no. SP-0023) were obtained from Beijing Bioss Biosynthesis Biotechnology Co., Ltd. (Beijing, China).

Establishment of animal models and grouping. The present study was approved by the ethics committee of The First Affiliated Hospital of Xinxiang Medical University (Weihui, China). All animal experiments conformed to the Ethical Issues in Animal Experimentation guidelines (2009) (10). A total of 128 healthy male Sprague Dawley rats, weighing 250-280 g and aged 8-10 weeks, were provided by the Experimental Animal Center of Zhengzhou University (Zhengzhou, China). The rats were separately housed and fed *ad libitum*. Urine screening of the rats was completed, and rats that obtained negative results during the screening were randomly divided into four groups: Normal control, model, treatment and treatment control. Each group comprised 32 rats. The model and treatment groups were used to establish the unilateral renal ischemia/reperfusion (I/R) injury model according to the method described by Supavekin *et al* (11). The treatment group was subcutaneously injected with SCF (200 μg/kg, once/day) and G-CSF (50 μg/kg, once/day) for five consecutive days 24 h after modeling. Normal rats in the treatment control group were injected with SCF and G-CSF at the same time-points and with the same doses. The SCF and G-SCF doses injected into the rats were based on those described in the literature (12). Since BMSC mobilization causes a peak in the peripheral $CD34^+$ cell population 3-5 days after treatment (13), the fifth day post-intervention day was selected as the starting time-point of detection.

Specimen collection. Eight rats were randomly selected from each group to complete specimen collection on the fifth, 10th, 17th and 24th days post-treatment, respectively. Chloral hydrate solution (10%) at a dose of 3.5 ml/kg was intraperitoneally injected into the rats as anesthesia, and venous blood samples (2 ml) were obtained and preserved in EDTA-coated Eppendorf tubes. The left kidney of the rats was removed, washed with saline and then cut into pieces measuring 1.0x1.0x0.2 cm; the resultant tissues were fixed in 10% neutral formalin buffer.

Extraction of peripheral blood $CD34^+$ cells. Ficoll-Hypaque density gradient centrifugation was performed to isolate the mononuclear cells from the peripheral blood (14). Firstly, peripheral blood extraction of $CD34^+$ cells was completed by fold-diluting ~2 ml venous blood with Hank's solution (Sigma-Aldrich, St. Louis, MO, USA), prior to mixing. Ficoll-Hypaque lymphocyte separation medium (MP Biomedicals, Santa Ana, CA, USA) was then added to the blood via the tube wall, and the blood was centrifuged at 700 x g for 20 min. Mononuclear cells located in the tunica albuginea were subsequently pipetted from the blood using a capillary tube and placed in another sterile tube. The blood was washed with 2 ml Hank's solution and then centrifuged twice at 400 x g for 10 min. The supernatant was discarded. Subsequent to discarding the supernatant, RPMI-1640 containing 10% fetal calf serum (HyClone Laboratories, Inc., Logan, UT, USA) was added to the blood to resuspend the cells. A drop of the cell suspension and a drop of 0.2% trypan blue dye were mixed. The total cell numbers were then counted within four large squares, and the concentration of mononuclear cells was calculated using the following formula: Concentration of mononuclear cells (cell number/ml cell suspension) = total cell numbers in the four large squares/4 x 104 x 2 (dilution factor).

Flow cytometry. Fluorescein isothiocyanate-$CD34^+$ monoclonal antibody (~100 μl; R&D Systems, Inc., Minneapolis, MN, USA) was added and mixed for monoclonal antibody labeling in the dark at room temperature for 25 min. The antibody was then centrifuged twice in l ml phosphate-buffered saline (PBS) at 350 x g for 5 min. Following centrifugation, ~0.5 ml l% paraformaldehyde was used to resuspend the cells. Flow cytometry was used to detect the fluorescence of the cells. Exactly 105 cells were counted from each tube to detect the $CD34^+$ cells and the percentage was analyzed. The negative control group was treated in the same manner during this experiment.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method. Paraffin sections (4-μm) were conventionally dewaxed using H_2O_2-CH_3OH solution (0.3%) for 5 min at room temperature. PBS (0.01 mol/l) was then used to wash the cells, and proteinase K (20 mg/l; Sigma-Aldrich) was used to treat and incubate the cells at 37˚C for 30 min. The cells were subsequently washed with 0.01 mol/l PBS. Exactly 50 μl TUNEL reaction mixture (cat. no. 11684817910; Roche) was added to the cells, prior to incubation for 1 h at 37˚C. The cells were then washed once more with 0.01 mol/l PBS. A total of ~50 μl converter-POD solution (Roche) was added to the cells, and incubation was performed at 37˚C for 1 h. Following incubation, the cells were further washed with 0.01 mol/l PBS. 3,3'-Diaminobenzidine staining, hematoxylin re-staining, ethanol-hydrochloride differentiation, gradient ethanol dehydration, xylene hyalinization, neutral gum closure and microscopic observation were subsequently performed.

Calculation of the apoptotic index (AI). The numbers of positive cells in 10 consecutive high-power fields were observed under an ordinary optical microscope (magnification, x200). The cells were then counted to calculate the percentage of apoptotic cells among the total tubular epithelial cells, i.e. the AI (%) of the renal tubular epithelial cells.

Immunohistochemistry. The streptomycin avidin-biotin-peroxidase complex method (cat. no. SP-0023; Beijing Bioss Biosynthesis Biotechnology Co., Ltd.) was used for the immunohistochemical staining. The paraffin sections (4-μm) were conventionally dewaxed and hydrated, prior to undergoing microwave antigen retrieval with citrate buffer. The sections were incubated at 98˚C for 5 min and then at 20˚C for a further 5 min, followed by closure with 5% bovine serum albumin. Rabbit anti-rat $CD34^+$ polyclonal antibody (dilution, 1:200; cat. no. bs-0754R; Beijing Bioss Biosynthesis Biotechnology Co., Ltd.) was used as the primary antibody for overnight incubation at 4˚C. Subsequent to washing with PBS, goat anti-rabbit

Table I. Number of white blood cells in the peripheral blood (x10^9/l) and the ratio of $CD34^+$ cells among the mononuclear cells (%).

Time (days)	Index	N	Group			
			Normal control	Model	Treatment	Treatment control
5	Leukocyte	8	5.410±0.325	5.641±1.921	18.388±3.224[a]	13.340±3.309[a]
	$CD34^+$ cells	8	4.865±4.066	6.579±4.103	11.848±3.021[a]	9.585±1.054[a]
10	Leukocyte	8	5.400±1.032	5.828±2.493	9.738±4.136[a]	8.970±0.467
	$CD34^+$ cells	8	4.705±2.496	5.949±3.441	10.760±4.806[a]	8.235±1.435
17	Leukocyte	8	5.475±0.587	6.115±2.109	7.494±2.680	6.430±1.032
	$CD34^+$ cells	8	4.505±2.157	5.578±3.495	5.789±1.589	4.980±4.186
24	Leukocyte	8	5.495±0.219	5.896±1.821	6.573±2.354	5.410±0.438
	$CD34^+$ cells	8	4.225±0.247	4.013±1.413	4.300±1.779	4.775±2.991

Results are presented as the mean ± standard deviation. Compared with the control and model groups, [a]P<0.05. CD, cluster of differentiation.

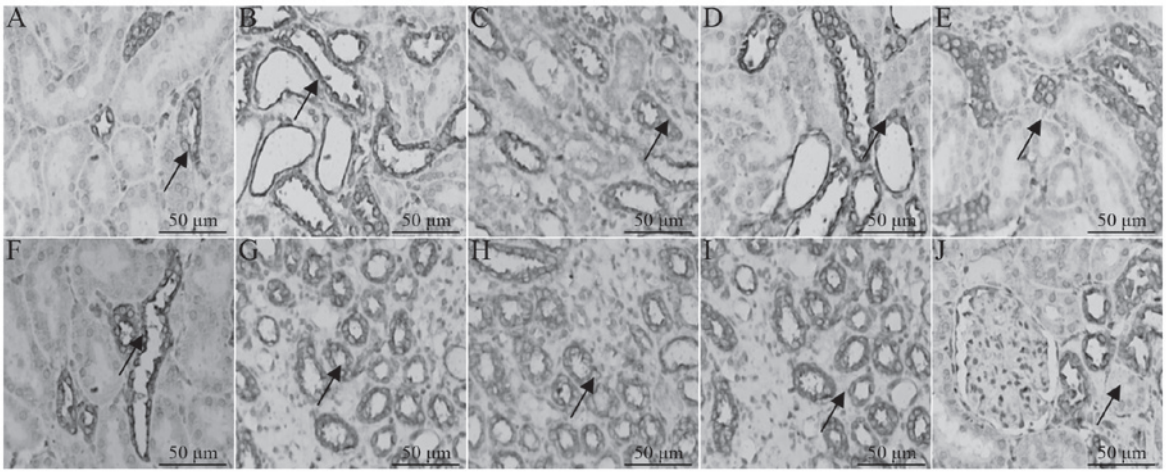

Figure 1. Number of cluster of differentiation 34^+ cells in four groups of rat renal tissues (arrows indicate immunohistochemical staining; magnification, x200). (A-C) The (A) normal control, (B) treatment control and (C) model groups at day 5; (D-F) the model group at (D) day 10, (E) day 17 and (F) day 24; (G-J) the treatment group at (G) day 5, (H) day 10, (I) day 17 and (J) day 24.

immunoglobulin G (dilution, 1:500; Zhongshan Goldenbridge Biotechnology Co., Ltd., Beijing, China) was added as a second antibody and incubation continued at 37˚C for 30 min. The sections were stained subsequent to washing with PBS. PBS was used instead of the primary antibody in the control group.

Evaluation of immunohistochemical results. Brown tissues or areas with brown granular deposition were observed as positively stained areas under a light microscope; 10 non-overlapping view fields of each slice were randomly selected at x200 magnification. An IDA-2000 computer image automatic analyzer (Olympus Corporation, Tokyo, Japan) was used to analyze the positive immunohistochemical signals in the selected fields of view.

Statistical analysis. All data are expressed as the mean ± standard deviation. The Student's t-test was used for intergroup comparisons, while Spearman's correlation was used to analyze and process inter-index associations. SPSS software (version 13.0; SPSS, Inc., Chicago, IL, USA) was used for the statistical analysis, and P<0.05 was considered to indicate a statistically significant difference.

Results

Changes in total peripheral blood leukocyte numbers and percentages of $CD34^+$ cells. The total peripheral blood leukocyte numbers and percentages of $CD34^+$ cells in the treatment and treatment control groups peaked on the fifth postoperative day compared with the normal control and model groups. The difference in these indices among the groups was statistically significant (P<0.05). The total peripheral blood leukocyte numbers and percentages of $CD34^+$ cells gradually decreased on the 10th postoperative day, although significant differences remained between the treatment and model groups (P<0.05). On the 17th and 24th postoperative days, the differences among the experimental groups showed no statistical significance (P>0.05). Comparisons between the model and normal control groups revealed no statistically significant differences at all the time-points studied (Table I).

Table II. Comparison of the apoptotic indices (%) of renal tissues from four groups of rats.

Time (days)	N	Group			
		Normal control	Model	Treatment	Treatment control
5	8	0.440±0.113	35.608±1.080[a]	30.611±0.781[a,b]	0.465±0.078
10	8	0.485±0.007	30.778±0.718[a,c]	20.876±1.207[a,b,c]	0.475±0.007
17	8	0.495±0.050	25.568±1.207[a,c]	5.536±0.698[a,b,c]	0.440±0.085
24	8	0.490±0.028	8.493±1.044[a,c]	3.869±0.359[a,b,c]	0.475±0.021

Results are presented as the mean ± standard deviation. Compared with the normal control group at the same time-point, [a]P<0.05; compared with the model group at the same time-point, [b]P<0.05; compared with the previous time-point of the same group, [c]P<0.05.

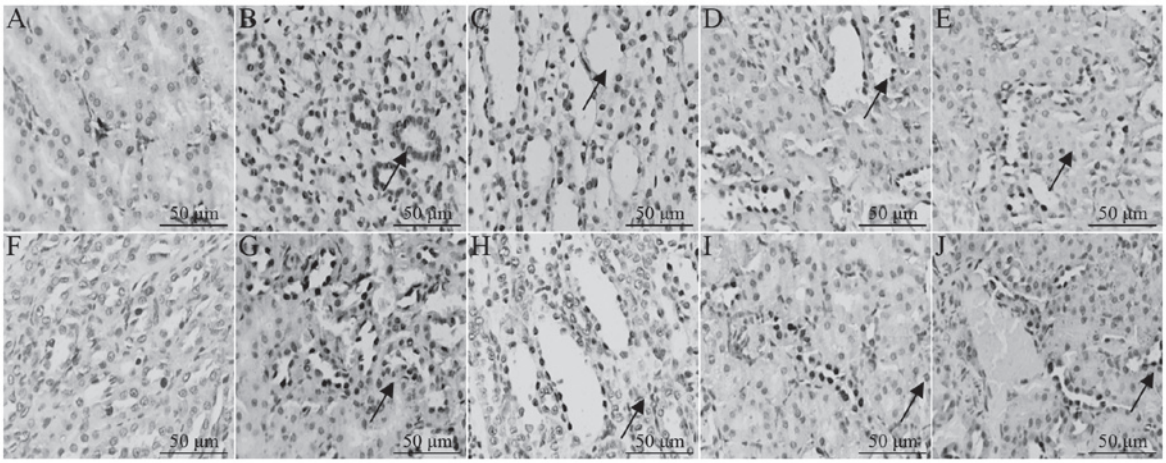

Figure 2. Number of apoptotic cells in four groups of rat renal tissues (arrows indicate terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining; magnification, x200). (A-C) The (A) normal control, (B) treatment control and (C) model groups at day 5; (D-F) the model group at (D) day 10, (E) day 17 and (F) day 24; (G-J) the treatment group at (G) day 5, (H) day 10, (I) day 17 and (J) day 24.

Expression of CD34⁺ cells in renal tissue. The number of CD34⁺ cells in the renal tissue of the model and treatment groups was significantly increased on the fifth, 10th and 17th postoperative days compared with that in the normal and treatment control groups (P<0.05). The increase in the number of cells in the treatment group was more significant than that in the model group (P<0.05) at the same time-points. The CD34⁺ cells in the renal tissue of the treatment group reverted to their usual state on the 24th postoperative day, while the number of cells remained high in the model group. At this time-point, the differences among the groups were not statistically significant (Fig. 1).

Comparison of apoptosis and the AI in renal cells. The AIs of the model and treatment groups were higher than those of the normal control and treatment control groups (P<0.05). The AI of the model group was significantly higher than that of the treatment group (P<0.05). The level of apoptosis in the renal tubular epithelial cells decreased over time (Table II and Fig. 2).

Discussion

A common cause of ATN is renal I/R injury, which is associated with high rates of morbidity and mortality (1,15). In recent years, significant clinical and laboratory studies have been performed to determine methods to treat ATN (1,12,16,17). In particular, the development of regenerative medicine has provided new avenues for the treatment of I/R injury.

BMSCs are multipotent stem cells. At least three types of BMSCs are known to exist in the bone marrow: Hematopoietic stem cells, endothelial progenitor cells (EPCs) and mesenchymal stem cells (18). The majority of studies have considered CD34⁺ cells as BMSCs; therefore, CD34⁺ cells have been treated as the source cell marker of the biological activities and clinical applications of BMSC mobilization (12,19,20). The differentiation of BMSCs exhibits a certain plasticity (21,22); BMSCs can be horizontally differentiated into other types of tissue cells, including renal tubular epithelial cells. Such differentiation provides a new means of treating ATN.

The number of BMSCs in bone marrow is meagre under normal circumstances, with BMSCs accounting for only 0.01% of the cells in the tissue. This number is even lower and may be virtually undetectable in the peripheral blood (23). Several factors can promote significant increases in the number of BMSCs in peripheral blood via a process known as mobilization. Kale *et al* (9) demonstrated that renal ischemic injury can significantly increase the number of BMSCs in the circulatory system. Besides endogenous ischemia and injury, which mobilize EPCs, other BMSC mobilizing agents include G-CSF, granulocyte macrophage colony-stimulating factor

(GM-CSF), SCF, vascular endothelial growth factor, angiopoietin-1, stromal cell-derived factor-1 and statins (24,25). Zhang *et al* (26) demonstrated that the combination of G-CSF and SCF, which are normally found in advanced progenitor cells and early hematopoietic stem and primitive hematopoietic progenitor cells, respectively, may encourage BMSCs to enter the peripheral blood. The mobilizing effect of G-CSF and SCF is more significant than that induced by each factor alone. SCF is an acid glycoprotein generated by stromal cells inside the bone marrow microenvironment (27). SCF is important in the survival, proliferation, differentiation and adhesion of hematopoietic stem cells, EPCs and the bone marrow microenvironment (28,29). SCF can combine various hematopoietic growth factors to promote and generate EPCs and increase the number of developing colonies. G-CSF is a growth factor that acts on advanced progenitor cells and promotes the resting stem cells into the cell cycle (25). G-CSF also induces BMSCs to leave the bone marrow and enter the blood circulation by reducing the number of cell surface adhesion molecules (30). The synergistic effects of G-CSF and SCF depend on phosphatidylinositol 3-kinase and mitogen-activated protein kinase (31,32). Signal transduction and transcription activator 3 could be the key factor in the downstream signal complement pathway of the SCF and G-CSF receptors (33,34).

In the present study, the total numbers of leukocytes and percentages of $CD34^+$ cells among the mononuclear cells in the peripheral blood were compared among four groups of rats at different time-points. Findings revealed that the total numbers of leukocytes and percentages of $CD34^+$ cells in the treatment and treatment control groups peaked on the fifth postoperative day. Furthermore, the number of $CD34^+$ cells in the peripheral blood positively correlated with the changes in the numbers of leukocytes; therefore, the combined application of G-CSF and SCF has significant mobilization effects on BMSCs, and these mobilization effects can be indirectly predicted by the number of leukocytes in the peripheral blood. Experimental results also showed that the number of $CD34^+$ cells in the peripheral blood exhibited a slight increase following simple renal I/R injury, which indicates that the self-mobilization of BMSCs during ischemia is a self-repair reaction of the body in response to ischemic injury; however, this self-repairing function is very weak.

Mobilized BMSCs must directly migrate to the ischemic area to completely repair the damage. Chemotactic factors of BMSC surface receptors, as well as several cell adhesion molecules, can precisely control the 'homing' of the BMSCs (35). Leukocyte chemokines and adhesion molecules can also precisely control the chemotactic movement of inflammatory cells when inflammation occurs (36-38). Inflammation occurs in the ischemic area subsequent to renal I/R. The conditions that are considered important for the homing of BMSCs include the release of various inflammatory mediators, e.g. interferon-γ, interleukin (IL)-2, IL-10, GM-CSF and tumor necrosis factor-α (39); invasion of mononuclear granulocytes; activation of mast cells; degradation of extracellular matrix proteins; and expression of adhesion molecules in endothelial cells and renal tubular epithelial cells. Matrix metalloproteinase-2 and slit homolog 3 protein have recently been identified as promoters of the release and homing of BMSCs from the bone marrow niche (40,41).

The present study showed that the homing of BMSCs is directed towards injured tubules and that this homing is positively correlated with the number of $CD34^+$ cells in the peripheral blood. Immunohistochemical results revealed that there were increased numbers of $CD34^+$ cells in the model and treatment groups compared with the normal and treatment control groups. The increase in $CD34^+$ cells in the treatment group, however, was notably more significant than that in the model group. The number of $CD34^+$ cells in the normal and treatment control groups did not increase, which indicates that G-CSF and SCF promote increases in BMSCs in the peripheral blood. Renal tissue injury appears to be an important factor influencing the homing of BMSCs.

In a number of studies it has been suggested that BMSCs can be trans-differentiated into renal tubular epithelial cells under certain conditions, thereby contributing to the repair of renal tubular injury (9,42-44). Li *et al* (45) demonstrated that BMSCs could be trans-differentiated into renal tubular epithelial cells under various physiological and pathological conditions and that trans-differentiation rates were associated with the extent of damage in the renal tube. Despite this, the mechanism through which BMSCs are differentiated into renal tubular epithelial cells to promote the rehabilitation of renal tubular injury remains unknown. The mechanism presented in the study by Li *et al* (45) was unclear; however, it is believed that the microenvironment is the deciding factor underlying directional differentiation following the direct homing of BMSCs towards the damaged renal tube. Numerous active cellular factors, e.g. epidermal growth factor, hepatocyte growth factor, insulin-like growth factor-I, bone morphogenetic protein and erythropoietin, are important in the regeneration process of renal tubes (46-48).

The present study used the TUNEL method to detect apoptotic cells in rat renal tissues. The findings revealed that the numbers of apoptotic cells in the model and treatment groups were significantly higher at different time-points than those in the normal control and treatment control groups. The findings also showed that the number of apoptotic cells in the treatment group was significantly lower than that in the model group, which indicates that the experimental intervention could inhibit apoptosis, reduce renal I/R injury and promote the restoration of renal tubes.

In conclusion, the present study has demonstrated that the combined application of G-CSF and SCF to rat models of I/R injury can mobilize the release of BMSCs into the peripheral blood and directly allow cellular homing towards the injured renal tubes for restoration. Further studies are required to investigate the mechanism underlying the mobilization and homing of BMSCs, as well as the mechanisms involved in the BMSC-mediated renal tube repair.

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