Effect of bone marrow stem cell mobilisation on the expression levels of cellular growth factors in a rat model of acute tubular necrosis

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Abstract. The aim of the present study was to observe the mobilisation effects of stem cell factor (SCF) and granulocyte colony-stimulating factor (G-CSF) on bone marrow stem cells (BMSCs) in rats with renal ischaemia-reperfusion injury. In addition, the effects of the BMSCs on the expression levels of hepatocyte growth factor (HGF) and epidermal growth factor (EGF) were investigated, with the aim to further the understanding of the protective mechanisms of SCF and G-CSF in renal ischaemia-reperfusion injury. The model and treatment groups were established using a model of unilateral renal ischaemia-reperfusion injury, in which the treatment group and the treatment control group were subcutaneously injected once a day with 200 μg/kg SCF and 50 μg/kg G-CSF, 24 h after the modelling, for five consecutive days. The $CD34^+$ cell count was measured in the peripheral blood using flow cytometry. The mRNA expression levels of HGF and EGF were determined using polymerase chain reaction analysis, while the protein expression levels of HGF and EGF were detected using immunohistochemistry. The $CD34^+$ cell count in the peripheral blood of the treatment and treatment control groups was significantly higher compared with that in the model group ($P<0.05$). However, CD34 expression levels in the cells from the renal tissues of the model and treatment groups were significantly higher compared with that of the control and treatment control groups ($P<0.05$), with the greatest increase observed in the treatment group. The mRNA and protein expression levels of HGF and EGF in the treatment group were significantly higher compared with the model group ($P<0.05$). Therefore, the results indicated that a combination of SCF and G-CSF can promote the repair of acute tubular necrosis. This combination, which can mobilise sufficient numbers of BMSCs to migrate back to the injured site, is a key factor in promoting the repair of renal tubular injury. Upregulation of HGF and EGF was also shown to promote the repair of renal tubular injury.

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Introduction

Acute renal failure (ARF) has a high incidence, and acute tubular necrosis (ATN) is the main pathological manifestation of this condition, which is also one of the leading causes of mortality in critically ill patients. Numerous studies have shown that bone marrow stem cells (BMSCs) possess the properties of migrating and homing towards the injured tissues, and these stem cells may be distinguished into renal intrinsic parenchymal cells, including mesangial cells, tubular epithelial cells and podocytes (1,2). A previous study applied BMSCs to the repair treatment of acute tubular damage (1); however, recent experiments investigating the promotive effects of BMSCs on kidney damage repair are primarily based on *in vitro* cellular transplantation, which has a long preparation period and multiple involvements (3,4). Therefore, these studies lack clinical feasibility in the treatment of ARF. A number of studies have found that BMSC mobilisation agents, such as granulocyte colony-stimulating factor (G-CSF) and stem cell factor (SCF), are able to mobilise autologous BMSCs to promote the healing of diseases, including kidney damage, myocardial necrosis, nerve damage, liver damage and haematological malignancies (3-7).

Numerous scholars have hypothesised that when ATN occurs, the renal tubular regeneration process is also involved in various endogenous regulatory factors (8-10). Tögel *et al* (11) hypothesised that the period in which BMSCs produce a renal protective effect may be achieved through a pathway similar to paracrine or autocrine secretion. For instance, when hepatocyte growth factor (HGF), epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1) are administered exogenously, the cell cycle is accelerated, thereby promoting cell proliferation and accelerating the recovery from ATN. In the current study, ATN was simulated experimentally in a laboratory, and SCF and G-CSF were subcutaneously injected

to mobilise the autologous BMSCs, with the aim to observe the distribution of BMSCs in the renal tissues, as well as the changes in HGF and EGF expression during the repair process of ATN tubular epithelial cells. Thus, the aim of the present study was to investigate the effects of mobilisation agents on the level of BMSCs, as well as the possible mechanisms underlying ATN repair.

Materials and methods

Establishment of models and grouping. A total of 128 healthy male Sprague-Dawley rats (age, 8-10 weeks; weight, 250-280 g) were provided by the Experimental Animal Centre of Zhengzhou University (Zhengzhou, China). The rats were housed separately and provided with food and water *ad libitum*. Following urine screening to identify non-healthy rats, the healthy rats were randomly divided into four groups, which included the control, model, treatment and treatment control groups. The study was conducted in strict accordance with the recommendations from the 'Guide for the Care and Use of Laboratory Animals' of the National Institutes of Health (Bethesda, MD, USA). The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Xinxiang Medical University (Xinxiang, China). The model and treatment groups were established using the unilateral renal ischaemia-reperfusion injury model, based on the method outlined by Supavekin *et al* (12). After 24 h of modelling, the treatment group was subcutaneously injected once a day with 200 μg/kg SCF (Chengdu Di'ao Jiuhong Pharmaceutical Co., Chengdu, China) and 50 μg/kg G-CSF (North China Pharmaceutical Jintan Biotechnology Co. Ltd, Shijiazhuang, China) for five consecutive days (13). In the treatment control group, the normal rats were injected with SCF and G-CSF at the same time points and with the same doses. As BMSC mobilising agents typically cause the number of peripheral stem cells to peak between days three and five following treatment (14), postoperative day five was selected as the starting time point for detection.

Specimen collection. Upon specimen collection on day 5, 10, 17 and 24, eight rats from each group were randomly selected for specimen reservation. For anaesthesia, 10% chloral hydrate solution (3.5 ml/kg) was intraperitoneally injected into the rats. Subsequently, 2-ml venous blood samples were collected and preserved in an EDTA-coated Eppendorf tube. The left kidney was removed, and after washing with saline, the kidney was cut into tissue sections measuring 1.0x1.0x0.2 cm. A number of pieces were quickly placed into liquid nitrogen, whereas the remaining sections 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 (15). Briefly, 2 ml venous blood was fold diluted with Hank's solution (Sigma-Aldrich, St Louis, MO, USA), then mixed and added to the Ficoll-Hypaque lymphocyte separation medium (MP Biomedicals, LLC, Santa Ana, CA, USA) along the tube wall. The mixture was centrifuged at 700 x g for 20 min. The mononuclear cells aggregated and formed a white film following centrifugation, which was pipetted with a capillary tube and moved into an additional sterile tube. The cells were washed with 2 ml Hank's solution, and centrifuged twice at 400 x g for 10 min, after which the supernatant was discarded. Subsequently, 10% foetal calf serum-containing RPMI 1640 (GE Healthcare Life Sciences, Logan, UT, USA) was added for the resuspension of the cells. A drop of cell suspension and a drop of 0.2% trypan blue dye (Sigma-Aldrich) were mixed, and the cell numbers were counted within the four squares. The concentration of the mononuclear cells was calculated as follows: (Cell number/1 ml cell suspension) = [Cell number (in the four squares)/4] x 10^4 x 2 (dilution factor).

Detection of peripheral blood CD34$^+$ cells. For CD34$^+$ cell detection, 100 μl fluorescein isothiocyanate (FITC)-CD34$^+$ monoclonal antibody (bs-0646R-FITC; Beijing Bioss Biotechnology Co., Ltd., Beijing, China) was added, and mixed at room temperature in the dark for 25 min. The primary antibodies were incubated with mononuclear cells extracted from peripheral blood. The mixture was centrifuged twice in 1 ml phosphate-buffered saline (PBS) at 200 x g for 5 min. Next, 0.5 ml paraformaldehyde (1%) was added to resuspend the cells, and a flow cytometric method (Epics-XL; Beckman Coulter, Inc., Brea, CA, USA) was used to detect the fluorescence value. From each tube, 10^5 cells were counted using flow cytometry to detect the number of CD34$^+$ cells and analyse the percentage in the peripheral blood. A negative control was also performed during the experiment, using 100 μl PBS instead of the primary antibody.

Polymerase chain reaction (PCR). Primers were designed according to the sequences in GenBank and were as follows: HGF upstream, 5'-CTTCTGCCGGTCCTGTTG-3' and downstream, 5'-CCACTTGACATACTATTG-3'; EGF upstream, 5'-AGACCAGGAACTGTCAG-3' and downstream, 5'-AACTCAGAAGAACACGG-3'; and β-actin upstream, 5'-CCTCGCCTTTGCCGATCC-3' and downstream, 5'-TGATGGAGTACTTCTAGG-3'. All the primers were synthesised by Shanghai Biological Engineering Co., Ltd. (Shanghai, China). TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA) was used to extract the total mRNA from the kidneys of the rats, and cDNA was synthesised using a reverse transcription kit (Takara Biotechnology Co., Ltd., Dalian, China). The thermocycling conditions for the PCR-amplified products were as follows. For HGF, initial denaturation was performed at 94˚C for 5 min, followed by 35 cycles of denaturation at 94˚C for 30 sec, annealing at 55˚C for 30 sec and elongation at 72˚C for 45 sec. A final elongation was conducted at 72˚C for 10 min. For EGF, the initial denaturation was performed at 94˚C for 5 min. Subsequently, 32 cycles of denaturation at 94˚C for 30 sec, annealing at 51˚C for 30 sec and elongation at 72˚C for 45 sec was performed, followed by a final elongation at 72˚C for 10 min. Finally, the β-actin reaction underwent initial denaturation at 94˚C for 5 min, and then 30 cycles of denaturation at 94˚C for 30 sec, annealing at 55˚C for 30 sec and elongation at 72˚C for 45, followed by a final elongation at 72˚C for 10 min. The final storage temperature was 8˚C. The reaction products were photographed under ultraviolet light following electrophoresis. The electrophoresis strips were analysed using image analysis software (Olympus Stream Version 1.9; Olympus Corporation, Tokyo, Japan) and the intensity of the strips was calculated by

Table I. Percentage of CD34+ mononuclear cells in the peripheral blood.

Time (days)	Cases (n)	Control group (%)	Model group (%)	Treatment group (%)	Treatment control group (%)
5	8	0.13±0.01	0.90±0.06[a,c]	2.07±0.08[a,b,c]	1.41±0.04[a]
10	8	0.13±0.02	0.56±0.44	1.61±0.07[a,b,c]	1.03±0.04[a]
17	8	0.13±0.02	0.42±0.49	0.88±0.05[a,b,c]	0.54±0.18
24	8	0.13±0.02	0.22±0.03	0.31±0.77	0.24±0.02

Results are expressed as the mean ± standard deviation. [a]P<0.05, vs. control group; [b]P<0.05, vs. model group; [c]P<0.05, vs. treatment control group.

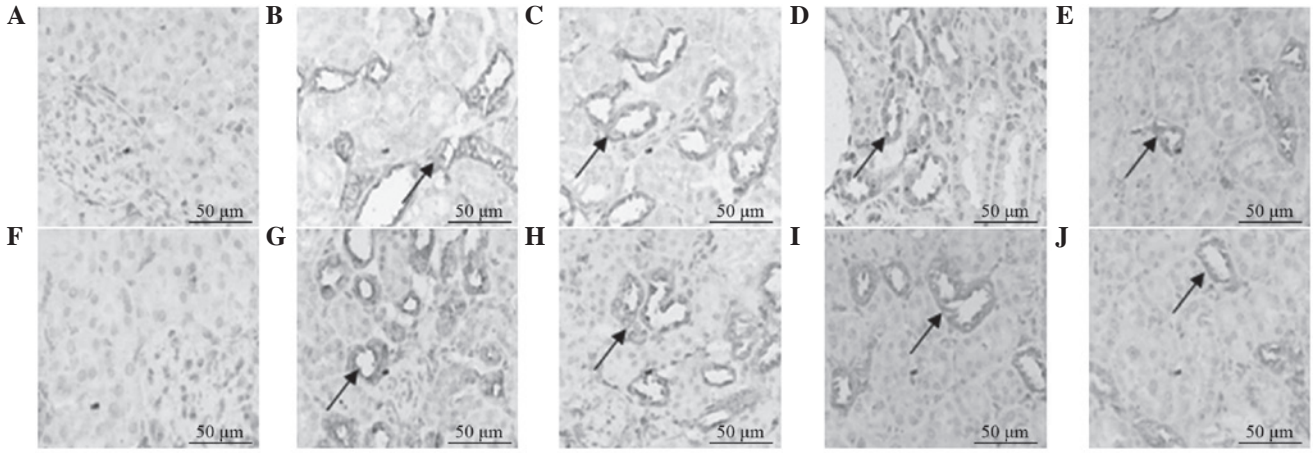

Figure 1. Expression of CD34 (arrows, immunohistochemistry staining; magnification, x200) in the renal tissue of the (A) control group; model group on days (B) 5, (C) 10, (D) 17 and (E) 24; (F) treatment control group; and treatment group on days (G) 5, (H) 10, (I) 17 and (J) 24. The percentages of CD34+ cells in the renal tissues of the model and treatment groups increased, and the increased degree in the treatment group was higher compared with the model group.

multiplying the area and grey scale. To determine the HGF and EGF gene expression levels, the ratio of the signal intensity of HGF and EGF was calculated against that of β-actin.

Immunohistochemistry. For immunohistochemistry, the streptomycin-avidin-biotin-peroxidase complex method (no. SP-0023; Beijing Bioss Biotechnology Co., Ltd.) was used. Paraffin sections of 4 μm were conventionally dewaxed and hydrated, and the citrate buffer was microwave repaired. The sections were placed under high fire for 5 min and low fire for 5 min, followed by closure with 5% bovine serum albumin (Sigma-Aldrich). Subsequently, rabbit anti-rat CD34+ (1:200; no. bs-0646R; Beijing Bioss Biotechnology Co., Ltd.), rabbit anti-rat HGF (1:200; no. bs-1025R; Beijing Bioss Biotechnology Co., Ltd.) and rabbit anti-rat EGF polyclonal antibodies (1:100; no. bs-2008R; Beijing Bioss Biotechnology Co., Ltd., China) were used as the primary antibodies for overnight incubation at 4˚C. The sections were washed with PBS, and a secondary horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:500; Zhongshan Goldenbridge Biotechnology Co, Ltd., Beijing, China) was added and incubated at 37˚C for 30 min. After washing with PBS, the sections were stained using diaminobenzidine. PBS was used instead of the primary antibody in the control group.

Evaluation of the immunohistochemical results. The staining results for CD34, HGF and EGF were evaluated from ten non-overlapped view fields of each slice that were randomly selected under x200 magnification (Bx51/Bx52; Olympus). An IDA-2000 computer image automatic analysis system (Olympus) was used to investigate the positive immunohistochemical signals in the aforementioned selected view fields. The percentages of cells positive for CD34, HGF or EGF relative to the total number of renal tubular cells in the selected fields were calculated, with the mean values shown as the immunohistochemical results.

Statistical analysis. SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis, and all the data are expressed as the mean ± standard deviation. Intergroup comparisons were performed using one-way analysis of variance, whereas pairwise intergroup comparisons were conducted using the least significant difference method. P<0.05 was considered to indicate a statistically significant difference.

Results

Percentage changes in the CD34+ cell count. On postoperative day five, the percentages of CD34+ mononuclear cells in the model, treatment and treatment control groups reached a peak. Compared with the control group, the differences were statistically significant (P<0.05), and the ratio for the treatment group was significantly higher compared with that for the model and

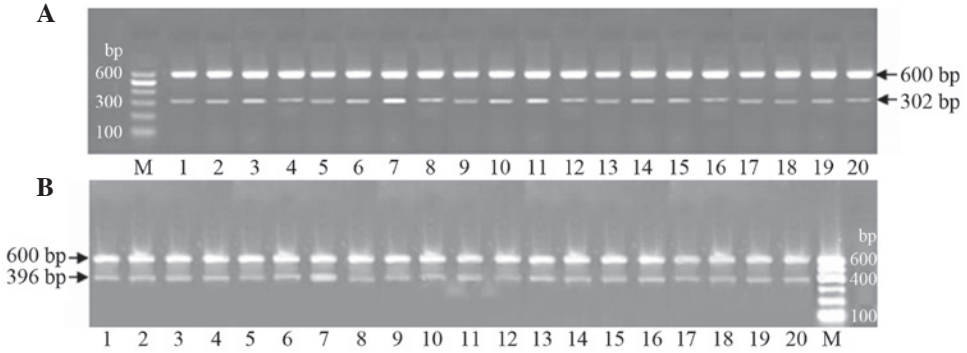

Figure 2. mRNA expression levels of (A) hepatocyte growth factor (HGF) and (B) epidermal growth factor (EGF) in the four experimental groups. HGF mRNA was found to be 302 bp in length, while EGF mRNA was shown to be 396 bp. β-actin was used as the internal reference (length, 600 bp). M, marker; 1-4, control, model, treatment and treatment control groups on day five, respectively; 5-8, control, model, treatment and treatment control groups on day 10, respectively; 9-12, control, model, treatment and treatment control groups on day 17, respectively; 13-16, control, model, treatment and treatment control groups on day 24, respectively. The mRNA expression levels of HGF and EGF in the model and treatment groups increased on postoperative day five, and peaked on postoperative day 10 and 17 in the treatment and model groups, respectively. The mRNA expression of HGF was significantly higher in the treatment group compared with the model group.

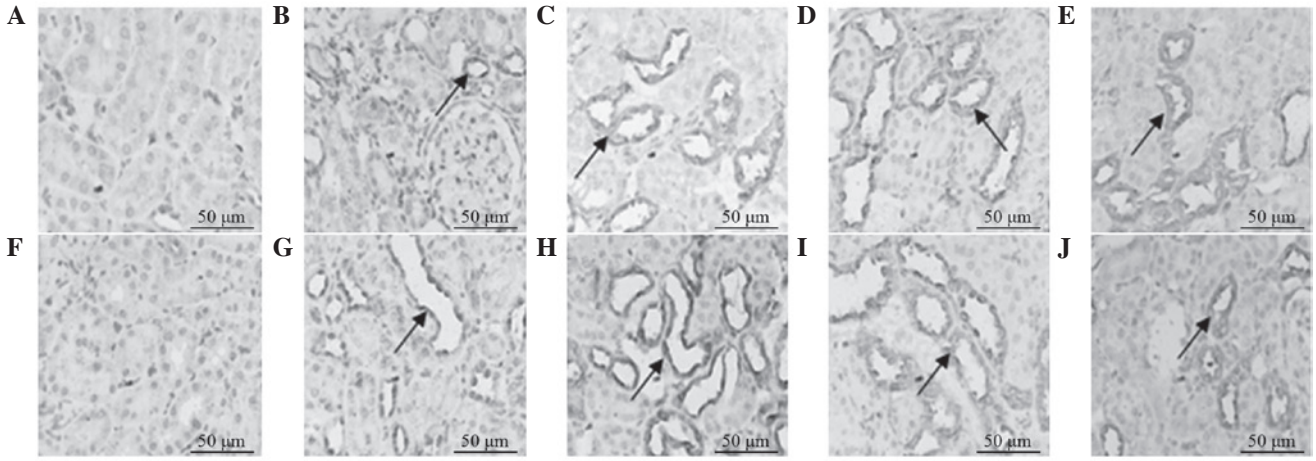

Figure 3. Expression of hepatocyte growth factor (HGF; arrows, immunohistochemistry staining; magnification, x200) in the kidneys of the (A) control group; model group on days (B) 5, (C) 10, (D) 17 and (E) 24; (F) treatment control group; and treatment group on days (G) 5, (H) 10, (I) 17 and (J) 24. The protein expression levels of HGF in the model and treatment groups increased on postoperative day five, and peaked on postoperative day 10 and 17 in the treatment and model groups, respectively. HGF protein expression was significantly higher in the treatment group compared with the model group.

treatment control groups. Furthermore, the ratio for the treatment control group was significantly higher compared with that for the model group (P<0.05). The percentage of $CD34^+$ cells gradually decreased over time. On postoperative days 10 and 17, the percentages of $CD34^+$ cells in the treatment group were significantly higher compared with the percentages for the other groups (Table I).

The percentages of $CD34^+$ cells in the renal tissues of the model and treatment groups increased significantly on postoperative day five. The differences were statistically significant (P<0.05) compared with the percentages for the control and treatment control groups. At all the indicated time points, the percentages of $CD34^+$ cells in the treatment group were higher compared with those in the model group, and the differences on postoperative day 5 and 10 were statistically significant (P<0.05). For the two groups, the expression of CD34 gradually decreased with time (Fig. 1).

Expression levels of HGF and EGF mRNA. The mRNA expression levels of HGF and EGF in the renal tissues of the model and treatment groups increased on postoperative day five, and peaked on postoperative day 10 and 17 in the treatment and model groups, respectively. The mRNA expression levels of HGF in the treatment group on days 5 and 10 were significantly higher compared with those in the model group. In addition, the mRNA expression levels of HGF and EGF in the treatment group on days 5, 10 and 17 were significantly higher compared with those in the model group (P<0.05). Following the peak mRNA expression of HGF and EGF, the levels gradually decreased to a normal level over time. The mRNA expression levels of HGF and EGF were low in the control and treatment control groups (Fig. 2).

Protein expression levels of HGF and EGF. The protein expression levels of HGF and EGF in the model and treatment groups increased on postoperative day five, and peaked on postoperative day 10 and 17 in the treatment and model groups, respectively. The protein expression levels of HGF and EGF in the treatment group on days 5 and 10 were significantly higher compared with those in the model group (P<0.05). Following peak expression of HGF and EGF protein, the levels gradually decreased to a normal level over time. The protein expression

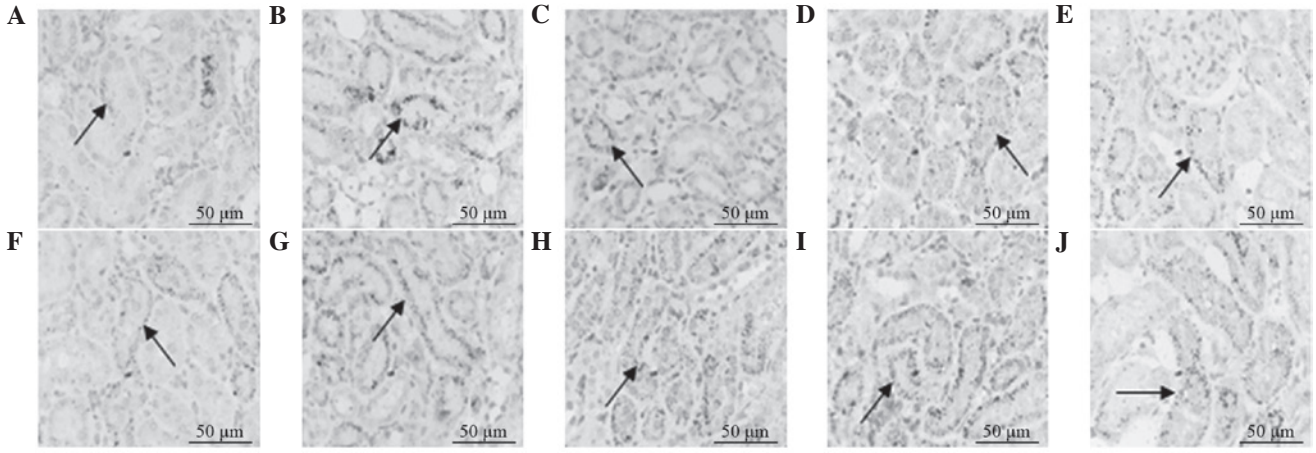

Figure 4. Expression of epidermal growth factor (EGF; arrows, immunohistochemistry staining; magnification, x200) in the kidneys of the (A) control group; model group on days (B) 5, (C) 10, (D) 17 and (E) 24; (F) treatment control group; and treatment group on days (G) 5, (H) 10, (I) 17 and (J) 24. The protein expression levels of EGF in the model and treatment groups increased on postoperative day five, and peaked on postoperative day 10 and 17 in the treatment and model groups, respectively. EGF protein expression was significantly higher in the treatment group compared with the model group.

levels of HGF and EGF were low in the control and treatment control groups (Figs. 3 and 4).

Discussion

Herrera *et al* (16) established a mouse ARF model using intramuscularly-injected glycerol, and the results demonstrated that BMSCs were able to migrate into the damaged kidney and differentiate into the tubular epithelium, thereby promoting the morphological and functional recovery of the injured kidney. In addition, Morigi *et al* (17) established a cisplatin-induced ATN model, and the results revealed that the infusion of BMSCs was able to improve renal function. The *in situ* hybridisation method revealed that BMSCs may be directly involved in renal reconstruction through differentiation into renal tubular cells.

Under normal conditions, the BMSC count in the peripheral blood is extremely low; however, when the body is under stress, ischaemia or injury, the number of BMSCs in the peripheral circulation can increase significantly. Kale *et al* (1) demonstrated that renal ischaemic injury can increase the BMSC count in the blood circulation significantly, although the number of BMSCs was not sufficient for repair following a serious injury. Orlic *et al* (18) combined SCF and G-CSF for the mobilisation of BMSCs to treat mice with myocardial infarction, and found that the number of BMSCs in the peripheral blood was as much as 250 times more compared with the normal amount. In clinical studies, using a combination of SCF and G-CSF has resulted in a two- to three-fold increase in $CD34^+$ cells compared with that for the single application of G-CSF (19).

The majority of studies have considered $CD34^+$ cells as BMSCs (20); thus, $CD34^+$ cells have been treated as the source cell marker of biological activities and clinical applications in BMSC mobilisation.

The percentage of $CD34^+$ cells in the peripheral blood of the control group was ~0.1%, indicating that under physiological conditions, only a small number of BMSCs are circulating. At day five following treatment, the percentage of $CD34^+$ mononuclear cells in the peripheral blood of the model group was higher compared with the control group, which was consistent with previous observations (21). The results indicate that stress factors, such as ischaemia and injury, are able to mobilise the autologous BMSCs. Therefore, if the body has a self-healing mechanism that allows the regeneration of ischaemic tissues, the mechanism may involve certain cytokines, including G-CSF and endothelial cell growth factor, which are locally secreted following ischaemia (22,23). The percentage of $CD34^+$ cells in the treatment control group was significantly higher compared with that in the model group, indicating that SCF and G-CSF have a strong ability to mobilise BMSCs. The ratio of $CD34^+$ cells in the treatment group reached the highest level on postoperative day five; thus, the effect of BMSC mobilisation was the strongest at this time point and may be the synergic result of kidney damage and mobilisation agents.

In the present study, the combination of SCF and G-CSF was demonstrated to mobilise a large number of BMSCs into circulation; however, the mobilised BMSCs had to directionally migrate to the kidney to complete the damage repair. A number of studies (24-26) have demonstrated that BMSCs exhibit a 'homing' characteristic, whereby they migrate to the ischaemia-damaged tissues. Inflammatory damage has been hypothesised to be an initiating factor of BMSC homing (26). Helmuth (27) described this process as follows: 'BMSC heard the call of the damaged tissues'.

On postoperative day five, the expression levels of CD34 on the cells in the renal tissues of the model group increased. The level of CD34 expression exhibited a statistically significant difference when compared with that for the treatment control group, which confirms the hypothesis that the microenvironment formed by the tissue damage is one of the essential conditions to induce BMSC homing. On days 5, 10 and 17, the CD34 expression levels in the renal tissue cells of the treatment group were significantly higher compared with those of the model group, indicating that under simple injury factors, the number of BMSCs that undergo differentiation and homing to the damaged tissue may be smaller. By contrast, the combination of SCF and G-CSF significantly increased the number of BMSCs in the peripheral blood over a relatively short time period. These conditions may cause various factors

in the bone marrow to migrate back to the necrotic lesions and participate in the regeneration of the necrotic tissues and blood vessels. Therefore, a sufficient number of BMSCs and the microenvironment around the injury are two key factors required for BMSC homing and differentiation.

Having undergone mobilisation and migration to the renal necrotic areas, the autologous BMSCs may undergo differentiation into 'environment-dependent' cells. These cells may differentiate from the renal tubular epithelial cells to participate in the regeneration of tissues and blood vessels. Therefore, BMSC mobilisation agents may be used to mobilise BMSCs into the blood circulation, increasing the cell count in the peripheral blood in order to promote the repair of renal tissues (28).

A number of scholars have hypothesised that during the repair process of ATN, the surviving cells are dependent on an 'atavistic' process to enter the cell cycle and proliferate as the new epithelial cells (29). Subsequently, the cells would stretch, migrate and recover the basement membrane. A number of studies have hypothesised that certain growth factors or specific hormones, including HGF, EGF and IGF-1, are able to promote this transformation process, and accelerate the differentiation of nascent epithelial cells (30-32). In addition, several scholars have demonstrated that BMSCs are able to secrete a number of cytokines, thereby promoting the proliferation and repair of the endogenous renal tubular epithelial cells (33,34).

In the present study, HGF and EGF expression levels in the model group were shown to increase slowly, reaching a peak on postoperative day 17 ($P<0.05$). A possible explanation for this observation may be that when ATN occurs, the body mobilises BMSCs in a compensatory response, which affects the secretion and synthesis of growth factors, thereby reducing the renal tubular necrosis and promoting tubular regeneration and proliferation. This condition may be a self-protective mechanism of the body following ATN. In the treatment group, the expression levels of HGF and EGF were significantly increased following the application of SCF and G-CSF. Thus, it was hypothesised that this condition may be associated with an increased number of BMSCs, as a result of the application of mobilisation agents. When the expression levels of HGF and EGF genes and proteins are upregulated, the renal tubular damage is reduced and the repair of renal tissues is accelerated.

In conclusion, following the occurrence of ATN, the repair mechanisms of the renal tubular epithelial cells can mobilise the autologous BMSCs to migrate into the injured area of renal tubule necrosis and differentiate into epithelial cells or fuse with the residual cells, directly contributing to the repair of the renal tissues. On the other hand, the functions of autocrine and paracrine secretion can increase the gene and protein expression levels of HGF, EGF, IGF-1 and other growth factors in the kidney, thereby regulating the microenvironment of the kidney, as well as stimulating DNA synthesis, cell proliferation and division. In addition, various growth factors are able to provide a good microenvironment to promote the self-repair of the kidney tissues (35). By contrast, the protective mechanism of the body, which mobilises the autologous BMSCs following kidney damage, is limited. Therefore, the results of the present study have demonstrated that a combination of BMSC mobilisation agents, namely G-CSF and SCF, can fully mobilise the BMSCs and increase their cell count in the peripheral blood, which accelerates and promotes the regeneration and repair of renal tubular epithelial cells. However, the experimental period was short and future studies should allow a longer time period to observe any side-effects of BMSCs, SCF and G-CSF. In addition, further experiments are required to observe the differentiation of BMSCs in ATN.

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