

## ORIGINAL ARTICLES

# CXC-chemokine receptor 4 antagonist prevents acute kidney injury potentially through recruiting bone marrow-derived stem cells

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## Abstract

**Background:** Existing literature suggests that stromal-cell derived factor 1 (SDF-1) interacts with CXC-chemokine receptor 4 (CXCR4) in regulating the homing of stem cells derived from bone marrow. The CXCR4 antagonist, AMD3100, disrupts this SDF-1/CXCR4 interaction and triggers stem cell mobilization. We investigated whether AMD3100 could ameliorate renal ischemia reperfusion (I/R) injury via recruiting circulating stem cells to injured kidneys.

**Methods:** We divided Sprague-Dawley rats into four groups, Sham, Sham + AMD3100, I/R, and I/R + AMD3100. All groups were treated with single subcutaneous injections of AMD3100 (5 mg/kg) or saline after sham surgery or I/R injury. Serum and renal tissues were harvested 12 hours and 3 days after treatment. We assessed survival, renal function changes, and histopathological alterations. TUNEL staining and caspase-3 expression levels were harnessed to measure tubular cell apoptosis, and circulating CXCR4 and CD34 positive mononuclear cells were identified by flow cytometry.

**Results:** The I/R + AMD3100 group displayed significantly higher survival, lower serum creatinine, less prominent renal damage upon histopathological examination, and a lower degree of apoptosis than the I/R group. In addition, the AMD3100 treated group showed a significantly higher degree of CXCR4 and CD34 positive cell mobilization in the circulation and increased recruitment of these cells into the injured kidneys.

**Conclusions:** AMD3100 promotes bone marrow stem cell mobilization and improves the recovery of renal function after I/R injury, and this effect may offer a promising therapeutic approach for acute kidney injury.

## Key words

Acute kidney injury, AMD3100, Bone marrow-derived stem cells, CXCR4, Apoptosis

## 1 Introduction

Acute kidney injury (AKI) from ischemia and reperfusion (I/R) occurs in 5% of in-patients, and the incidence increases further to nearly 50% in those admitted for sepsis<sup>[1]</sup>. The mortality rate of patients with AKI reaches as high as 60% in

those admitted to intensive care units. Thus, AKI constitutes a significant concern in modern medicine. Furthermore, evidence suggests that dialysis-requiring AKI correlates with adverse patient outcomes, while even a small increase in serum creatinine is associated with higher risk of long-term mortality and worse renal survival [2]. In line with these findings, AKI carries a high morbidity and mortality for those affected. However, very few therapeutic options for AKI exist other than supportive management. The urgent need for effective AKI treatment stimulates research focusing on the therapeutic feasibility of potential molecules.

During the last ten years, a better understanding of the plasticity of stem cells from adults has led to the hope that stem cells could be a promising therapeutic modality for many illnesses. Stem cell-based therapy has been potentially effective in treating immune disorders and promoting organ regeneration, and there are encouraging results in the treatment of renal failure with different types of stem cells [3-6]. Li *et al.* cultured mesenchymal stem cells (MSCs) and transplanted these MSCs in an I/R injury mouse model. They found that exogenous MSC infusion promoted renal recovery through the enhancement of peritubular capillaries and tubular epithelial cell repair, as well as anti-inflammatory and anti-apoptotic effects [7]. Although the use of exogenous MSCs can ameliorate renal I/R injury, the underlying mechanism remains unclear. However, there are several prerequisites for using MSCs: the sources of MSCs should be rich, the nature of MSCs needs to be well defined and homogeneous, and the culture condition should be pathogen-free. These inherent concerns limit the application of exogenous stem cells.

To address these concerns of utilizing exogenous MSCs, researchers turn to the endogenous stem cells for improving renal function during AKI. Earlier studies have demonstrated that, in chimeric mice, bone marrow-derived stem cells (BMSCs) might be recruited to the injured kidneys and develop into tubular epithelia, the main sites sustaining injury during AKI [8]. Others reported that bone marrow hematopoietic stem cells (HSCs) can be mobilized into circulation and ameliorate renal dysfunction following I/R injury. HSCs can be recruited to the kidneys, attenuating peritubular capillary loss, promoting tubular epithelial regeneration, and prolonging animal survival [9, 10]. Our previous study also showed that host bone marrow cells could replace the entire allogeneic liver in a model of allogeneic liver transplantation [11]. Our recent data further demonstrated that post-conditioning (POC) prompted recruitment of CXCR4+/CD34+ stem cells to the kidneys sustaining I/R injury, through regulating the hypoxia-inducible factor (HIF) / stromal cell-derived factor (SDF) / CXCR4-chemokine receptor 4 (CXCR4) pathway, which is associated with regulated reactive oxygen species (ROS) generation [12].

The HIF/SDF/CXCR4 axis has been proposed to be responsible for mediating stem cell mobilization and migration into injured renal tissues. AMD3100 (AMD3100, Mozobil), a small-molecule antagonizing CXCR4, can modulate the immune response of asthma and rheumatoid arthritis in animal models and also mobilize HSCs into the circulation [13-15]. Based on the above findings, we questioned whether stem cells of endogenous origin, induced by AMD3100 through the SDF/CXCR4 pathway, could be mobilized and recruited to the injured kidneys after I/R injury and attenuate AKI severity. Our current study suggests that AMD3100 may be a potential option for modestly attenuating renal IR injury. In the current study, a subcutaneous injection of AMD3100 mobilized HSCs to repair renal injury in the rat IR model. Our results provide novel insight into the mechanisms responsible for endogenous HSCs recruitment to the ischemic injured kidneys, and may improve the efficiency of HSCs therapy in the future.

## 2 Materials and methods

### 2.1 Reagents and antibodies

Pentobarbital sodium and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St Louis, MO, USA). AMD3100 was obtained from Sigma (St Louis, MO, USA). Antibodies against c-kit, CXCR4, and caspase-3 were bought from Abcam (Cambridge, UK), while all other antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

## 2.2 Animals

Male Sprague-Dawley (SD) rats (8-10 weeks old; Beijing, China) were used in all experiments. Animals were housed in a pathogen-free facility at Capital Medical University, in compliance with the guide for Care and Use of Laboratory Animals (U.S, National Institutes of Health DHEW Publication, 2001). The environment was kept at constant temperature with a 12-hour light-dark cycle, and animals had unrestricted access to tap water and standard diet. The Institutional Animal Care and Use Committee of Capital Medical University approved all studies.

## 2.3 In vivo model of I/R

The procedures for renal I/R injury were modified from methods described previously<sup>[16]</sup>. In brief, rats were randomly allocated into four groups: Sham, Sham + AMD3100, I/R, and I/R + AMD3100. Renal injury for the in vivo study was introduced by 45 minutes of left renal pedicle clamping after right nephrectomy with pentobarbital sodium (25 mg/kg) assisted general anesthesia. After ischemia for 45 minutes, we removed the clamps and kidneys were reperfused. In the Sham group, animals received abdominal incision without clamping of renal pedicle. In the I/R + AMD3100 group, rats were subjected to a subcutaneous injection of 5 mg/kg AMD3100 during renal ischemia<sup>[17]</sup>. Serum was collected at 12 hours and 3 days following I/R injury. Serum creatinine (Cr) was assayed in the hospital laboratory, while renal tissues were harvested and preserved at -80°C until subsequent treatment.

## 2.4 Renal histopathology

On day 3 following I/R injury, rats were sacrificed and their left kidneys were fixed in 10% neutral buffered formalin. The 4- $\mu$ m sections from the above paraffin-embedded tissues were deparaffinized, hydrated gradually using decreasing concentrations of ethanol, and examined with hematoxylin eosin (H&E) staining. Four micrometer renal tissue sections underwent antigen retrieval (microwave heating in a 0.1 M sodium citrate, pH 6, for 10 minutes) and were then incubated with anti-CXCR4 antibody (1:200), anti-c-kit antibody (1:500), and anti-cleaved-caspase 3 antibody (1:200) at 4°C overnight, followed by the streptavidin peroxidase-conjugated secondary anti-rabbit IgG antibody incubation at a 1:300 dilution for 30 minutes, stained with 3, 3-Diaminobenzidine (DAB), and then counterstained with hematoxylin for immunohistochemical analysis.

## 2.5 Western blot analysis

Frozen renal tissue samples (30 mg) were homogenized in 2 ml of 5% SDS, 5 mM EDTA, 20 mM Tris-HCl (Final pH 7.8) first, and then centrifuged at 12,000 rpm for 15 minutes at 20°C. The Mirco BCA protein assay kit (piece) was used to measure total protein, Western blotting was done as previously described<sup>[12]</sup>. In summary, 50  $\mu$ g of total protein was separated by 10% sodium dodecyl sulfate (SDS) polyacrylamid fluoride (PVDF) membranes (Meck, Millipore, Billerica, MA, USA) through a transblot system (Bio-Rad, Hercules, CA, USA). Five percent non-fat milk in tris-buffered saline-Tween 20 (TBST) was used to block the PVDF membranes for 1 hour at room temperature, and then we incubated at 4°C overnight the membranes with specific anti-bodies against c-kit, CXCR4, caspase-3, and  $\beta$ -actin. After washing, we incubated the membranes with horseradish peroxidase-conjugated secondary antibodies. Chemiluminescence (Bio-Rad) was used to visualize the protein bands, and band density was quantified by densitometry.

## 2.6 Fluorescence-activated cell sorting (FACS)

We collected 5 ml of whole blood in tubes containing 0.2 ml heparin (1,000 U/ml). Samples were immediately mixed to prevent clotting, and then kept on ice until further use. Erythrocytes were removed by a 10% buffered ammonium chloride solution. FACS buffer (PBS supplemented with 1% BSA, containing 0.05% NaN<sub>3</sub>) was used to wash the remaining cells, and these cells were subsequently incubated at 4°C with antibodies against c-kit, CXCR4, and CD34 for 60 minutes. Later, these cells were washed with FACS buffer three times, and incubated with secondary antibody labeled with Alexa Fluor 488 (Cell Signaling Technology; Beverly, USA) at 4°C for 30 minutes. Samples were analyzed with a FACScan flow cytometer (BD FACS caliber flow cytometer, San Diego, CA, USA) to detect light scattering and fluorescence emission patterns by analyzing  $1 \times 10^5$  events per sample.

## 2.7 TUNEL staining

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was done through a MEBSTAIN Apoptosis Kit Direct (MBL) in accordance with the manufacturer's protocol. TUNEL stain can detect nucleosome-sized DNA fragments by tailing their 3'-OH ends with digoxigenin nucleotides using terminal deoxynucleotidyl transferase (TdT). TUNEL-positive cells were counted using light microscopy.

## 2.8 Statistical analyses

SPSS12.0 software was used for data analyses. Continuous values are expressed as mean  $\pm$  standard deviation (SD). Statistical significance was determined by two-sided ANOVA.  $P < .05$  was set as statistically significance. For the survival plot, statistical significance was determined by Chi-Square, and a  $P < .05$  was also set as the level of statistically significance.

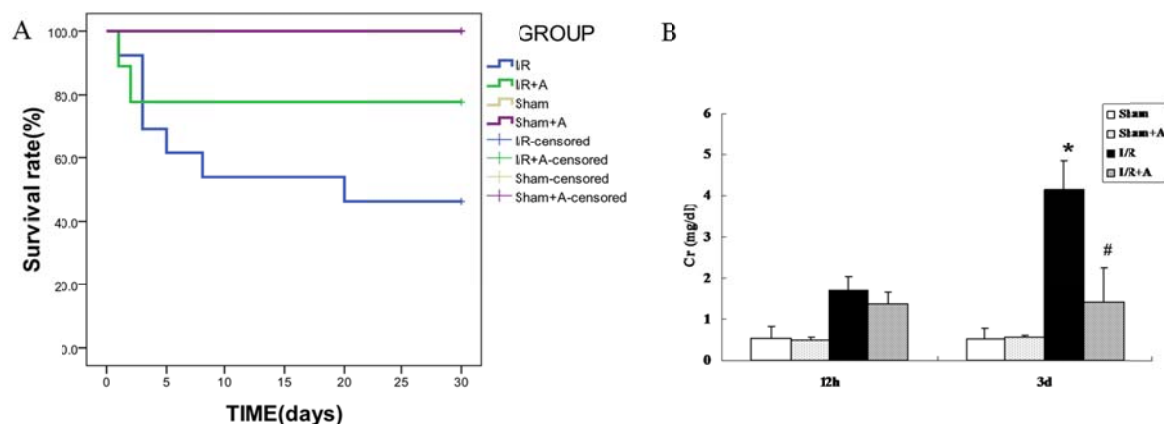
## 3 Results

### 3.1 AMD3100 increases survival of rats with I/R injury

SD rats were allocated into four groups, and ten rats from each group were utilized for analysis. In the I/R group, the survival rate was only 50%, while in the I/R + AMD3100 group the survival rate was 80%, with rats surviving 30 days after insult ( $P < .05$ ). There was no mortality in rats from the Sham group and the Sham + AMD3100 groups (see Figure 1A).

### 3.2 AMD3100 improves renal function after I/R injury

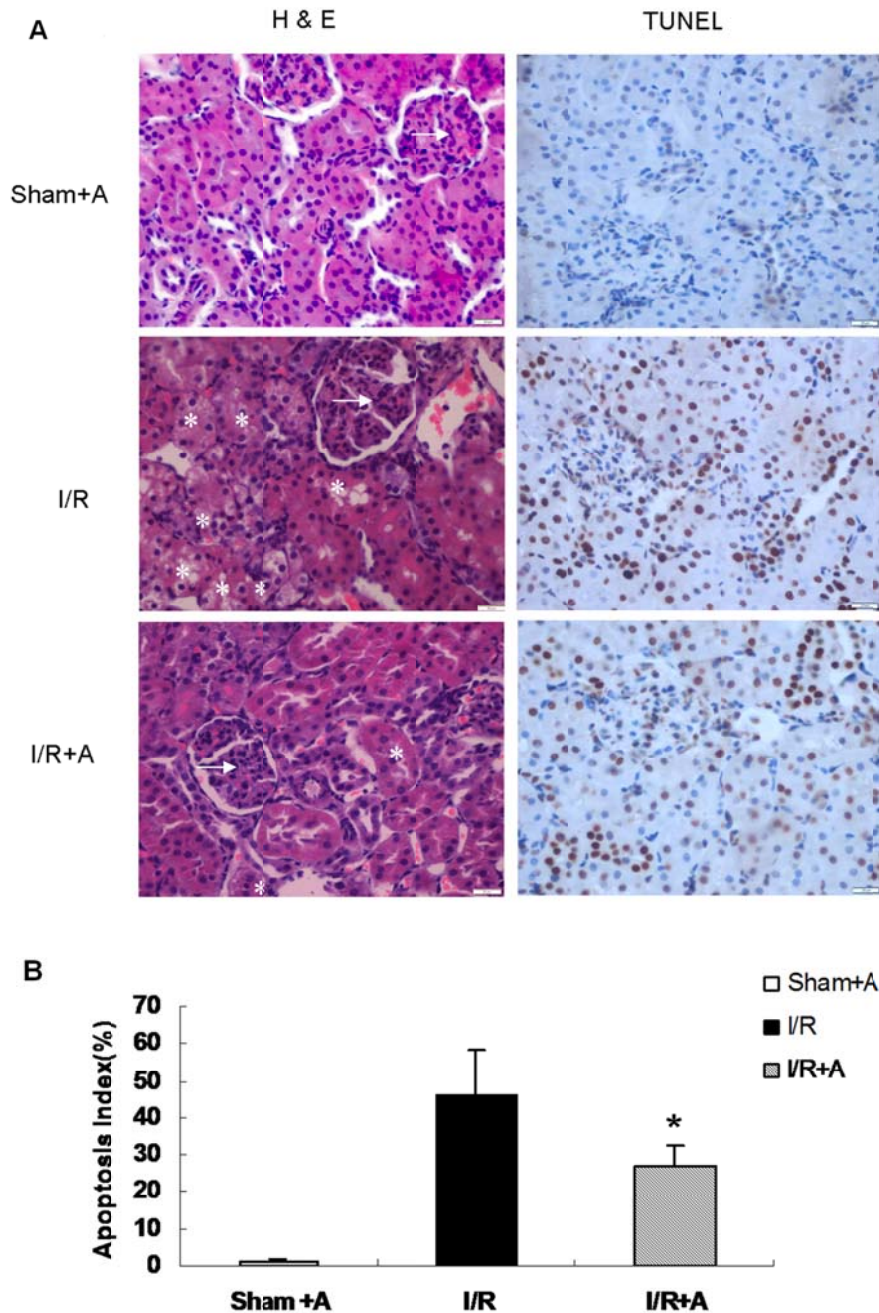
Serum Cr levels were measured after I/R and AMD3100 treatment. Serum Cr levels in the I/R group increased on day 3 compared to the Sham group. AMD3100 significantly reduced serum Cr levels in the I/R + AMD3100 group compared to those in the I/R group ( $P < .05$ ) (see Figure 1B).



**Figure 1.** AMD3100 improved survival and renal function after I/R injury. (A) The effect of AMD3100 on survival of the I/R injured rats. AMD3100 increased the survival rate in the I/R + AMD3100 group compared to the I/R group ( $n = 10$  in each group). (B) AMD3100 significantly decreased serum Cr levels in the I/R + AMD3100 group compared to the I/R group. The results are presented as mean  $\pm$  standard deviation (SD) ( $n = 5$ / group/ time point). \* $P < .05$ , compared to Sham; # $P < .05$ , compared to I/R.

### 3.3 AMD3100 markedly ameliorates renal histopathological changes

H&E staining was used to examine the renal histopathological changes at day 3 after I/R injury (see Figure 2). Rats in the I/R group developed AKI characterized by tubular epithelial swelling, necrosis, neutrophil infiltration, tubular cell sloughing, and had an increased amount of tubular protein casts. In contrast, AMD3100 significantly improved most of the renal histopathological changes in the I/R + AMD3100 group compared to the I/R group. No significant injury was observed in the kidneys from rats in the Sham + AMD3100 group.

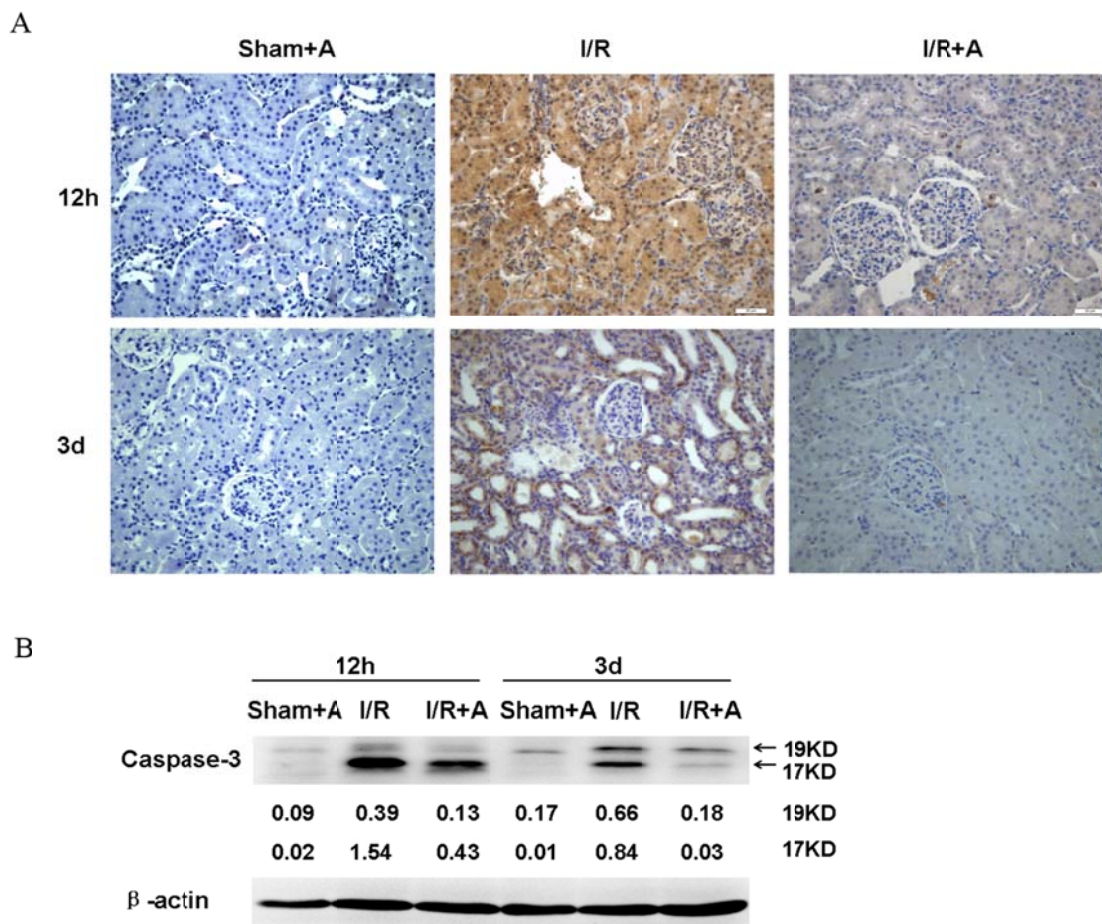


**Figure 2.** Histopathological changes and TUNEL stain results. (A) H&E staining of renal tissues at day 3 after I/R injury. (H&E  $\times 400$ , scale bar = 20  $\mu\text{m}$ ). The injured renal tubules were marked by asterisks while the glomeruli were marked by arrows. (B) More TUNEL positive cells were observed in renal tissues from the I/R group compared to the I/R + AMD3100 group,  $*P < .05$ .



### 3.4 AMD3100 inhibits apoptosis after I/R injury of kidneys *via* reducing caspase-3 expression

TUNEL assay was employed to assess the apoptotic changes of renal tissues from all groups. As shown in Figure 2, more cells with positive TUNEL staining were observed in renal tissues from the I/R group, while AMD3100 inhibited apoptosis in injured renal tissues from the I/R + AMD3100 group. No TUNEL positive cells were found in renal tissues from the Sham + AMD3100 group (see Figure 2). To clarify the mechanism through which AMD3100 reduces apoptosis, we assessed the expression of caspase-3, an apoptotic marker, by immunohistochemical staining and Western blotting. We found that caspase-3 expression was increased in renal sections from the I/R group compared to the I/R + AMD3100 group after 12 hours and 3 days, respectively (see Figure 3). In addition, fewer number of tunnel positive and caspase-3 positive cells in the glomerulus among the treated and the untreated groups were found; these findings indicate that the I/R mainly damages the renal tubules in the kidney.

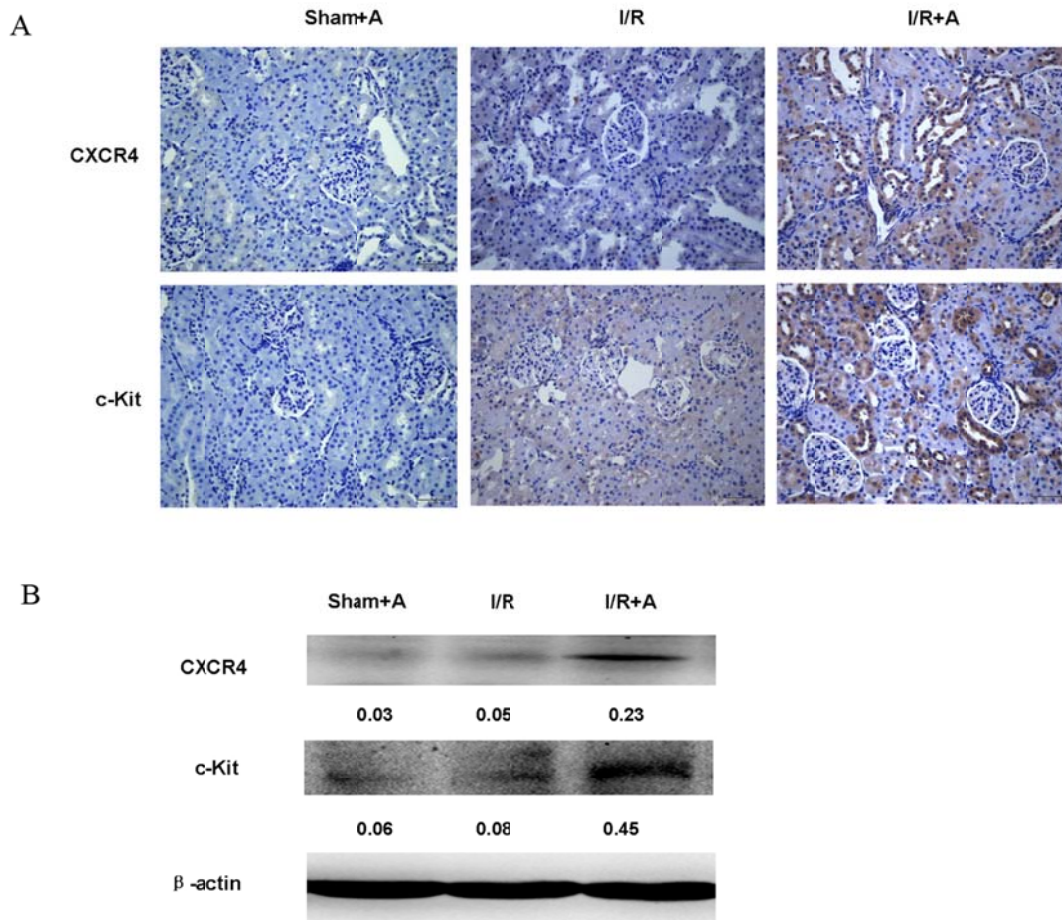


**Figure 3.** Caspase-3 expression. (A) Immunohistochemical staining showed that renal caspase-3 expressions increased at 12 hours and 3 days after I/R injury, while AMD3100 lowered caspase-3 expression. (B) Western blotting for caspase-3 in the kidneys of rats was performed at 12 hours and on day 3 after I/R injury. AMD3100 decreased caspase-3 expression at both time points.

### 3.5 AMD3100 mediates recruitment of hematopoietic cells in the kidney

HSCs are the putative sources of progenitor cells. We utilized CXCR4 and c-kit as markers to determine if HSCs facilitate renal repair on day 3 after I/R injury. CXCR4 and c-kit positive cells were detected in the I/R + AMD3100 group. These

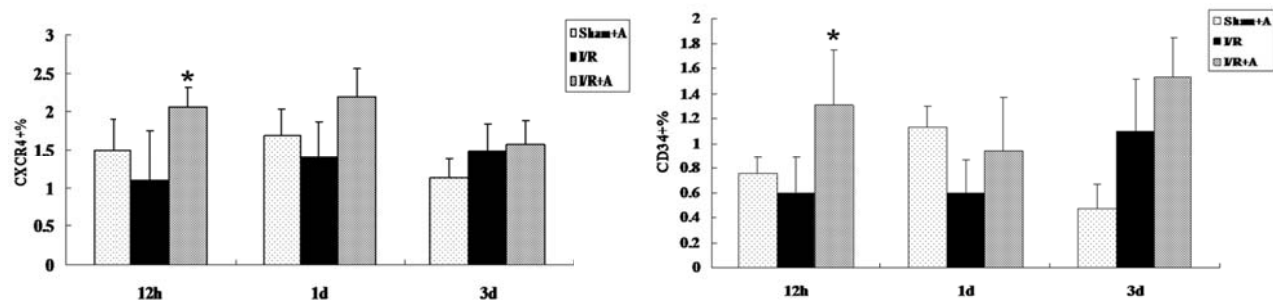
cells were similar in morphology to tubular cells as determined by immunohistochemistry staining (see Figure 4A). In contrast, few CXCR4 and c-kit positive cells were detected on day 3 after injury in the I/R group. Similar findings were obtained using Western blotting, as higher levels of CXCR4 and c-kit expressions were observed in renal tissues from the I/R + AMD3100 group compared to those from the I/R group (see Figure 4B). Since the I/R mainly damages the renal tubules in the kidney<sup>[12]</sup>, more CXCR4 and c-kit positive cells were detected in renal tubules rather than glomeruli. This could be the reason why the CXCR4 staining in the glomeruli of the treated group remains unstained.



**Figure 4.** AMD3100 increased the expression of CXCR4 and c-kit in renal tissues following I/R injury. (A) CXCR4 and c-kit expression in renal tissues were determined by immunohistochemical staining on day 3 after injury. More CXCR4 and c-kit positive cells were observed in the I/R + AMD3100 group compared to the I/R group. (B) Western blot of renal tissues on day 3 also found that the renal expression of CXCR4 and c-kit increased in the I/R + AMD3100 group compared to the I/R group.

### 3.6 AMD3100 mobilizes CXCR4/CD34 positive HSCs to the injured kidneys

The lymphocyte compartment of peripheral blood, which contains the majority of mobilized HSCs, was analyzed using FACS. We found that the percentage of CXCR4/CD34 positive cells in the circulation was markedly higher in the I/R + AMD3100 group compared to the I/R group at 12 hours after reperfusion (see Figure 5). Although many c-kit positive cells were detected in renal tissues from the I/R + AMD3100 group on day 3 after I/R injury, few circulating c-kit positive cells were detected by FACS (data not shown).



**Figure 5.** Hematopoietic stem cell mobilization in response to AMD3100. CXCR4 and CD34 positive cells were determined by flow cytometry. Significantly more CXCR4 and CD34 positive cells in peripheral blood were mobilized by AMD3100 at 12 hours. The results are presented as means  $\pm$  SD. \* $P < .05$ , compared to the I/R group.

## 4 Discussion

Ischemia-related AKI is a major contributor of morbidity and mortality for patients admitted to intensive care units<sup>[18]</sup>. Emerging evidence suggests that AKI correlates closely with subsequent development of chronic kidney disease if the renal repair process is maladaptive<sup>[19, 20]</sup>. Stem cell-based therapy recently emerged as a promising therapeutic option for patients developing AKI. With their plasticity and capability to trans-differentiate into diverse cell types, stem cells from bone marrow and umbilical cord blood have been extensively investigated for their potential to enhance renal tubular recovery after injury. BMSCs could be an attractive therapeutic option for renal regeneration due to the ease of isolation. Utilization of these cells will avoid the ethical dilemma involving embryonic stem cells<sup>[21, 22]</sup>.

Currently, the issue of whether stem cells of intrarenal or extrarenal origin are involved in renal repair after I/R injury is still controversial. Previously, BMSCs have been deemed responsible for this action, and injection of BMSCs after introduction of renal injury leads to significant amelioration of AKI, both functionally and histopathologically<sup>[23, 24]</sup>. Other studies identified that BMSC injection does not contribute significantly to restoring epithelial integrity after renal ischemia; rather, it is the proliferation of local tubular cells that might be responsible for the replenishment of tubular epithelia after ischemia<sup>[25]</sup>.

Apart from the exogenous injection of stem cells, studies also showed that endogenous stem cells were beneficial for improving renal function and repair of AKI after injury. Our previous work found that circulating CD34 and CXCR4 positive cells, presumably with a bone marrow origin, migrated to injured renal tissues after reperfusion, while POC further promoted the recruitment of CD34 and CXCR4 positive stem cell. We propose that POC facilitated the mobilization and recruitment of CD34 and CXCR4 positive stem cells into injured kidneys via the regulation of the HIF/SDF/CXCR4 pathway, which is associated with regulated ROS generation.

The chemokine receptor has recently attracted growing interest as a novel therapeutic agent, and its role in multiple diseases has been reported. AMD3100 is a CXCR4 reversible inhibitor that has been approved by the United States Food and Drug Administration for HSC mobilization. Recently, AMD3100 has also been found to modulate immunity in rodent models of asthma and rheumatoid arthritis<sup>[13-15]</sup>. In light of the above findings, we aimed to investigate whether AMD3100 can ameliorate renal injury following I/R in this study. We discovered that AMD3100 increased the survival of rats following renal I/R injury, attenuated serum Cr levels (see Figure 1), and improved renal histopathological changes. Furthermore, AMD3100 also inhibited apoptosis in injured renal tissues through decreasing caspase-3 expression, suggesting that AMD3100 can enhance the repair of injured kidneys in rats (see Figures 2 and 3). Finally, we also showed that CXCR4, CD34 and c-kit positive stem cells could be mobilized and recruited into injured kidneys after AMD3100 treatment (see Figures 4 and 5). Due to the fact that immature B cells also express CD34, more surface markers could be utilized, such as CD38 and CD10, to better characterize the nature of the bone marrow cells in further work<sup>[26, 27]</sup>. Zuk *et al.*



also examined whether CXCR4 receptor antagonism ameliorates the loss of renal function following I/R [28]. However, there are some differences between this study and ours. Firstly, different rat models of renal I/R injury were used. Zuk *et al.* used a rat model of bilateral renal I/R injury, which induces more severe kidney injury and inflammation response. In our study, kidney injury was induced by clamping only the unilateral renal pedicle for 45 minutes following right nephrectomy. Secondly, in our study, we used a different time and dose of the injected AMD3100. Zuk *et al.* used a single dose of plerixafor at 1 mg/kg administered 15 minutes before ischemic clamping, as well as two doses of 0.1 mg/kg administered 15 minutes before ischemia and 3 hours later. In contrast we induced, I/R rats using a subcutaneous injection of a large dose of AMD3100 (5 mg/kg) during renal ischemia, which has the capacity to mobilize HSCs and facilitate recruitment of the cells into the kidney to repair the renal injury. Finally, the mechanisms of prevention of renal I/R injury by AMD3100 in the two studies is also different. In Zuk's research, the mechanism by which plerixafor inhibition ameliorates AKI is via modulation of leukocyte infiltration and inhibition of release of proinflammatory chemokines/cytokines, rather than a HSC-mediated effect. In contrast, in our study the mechanism of prevention of renal I/R injury by AMD3100 is attributed to HSC mobilization and migration to the injured foci to repair renal injury. Thus, the discrepancies between Zuk's study and ours can perhaps be explained by variations in the animal models themselves and /or time and dose of AMD3100.

In addition, the mechanism of recruitment and mobilization of BMSCs by AMD3100 could depend on the SDF/CXCR4 pathway [29]. It was reported that treatment with AMD3100 and chemotherapy influences the surface expression of CXCR4, very late antigen-4 (VLA-4), and CXCR7 in surviving acute lymphoblastic leukemia (ALL) blasts [30]. Moreover, prolonged exposure of ALL blasts to AMD3100 led to a persistent increase in surface CXCR4 expression, modulating the surface expression of adhesion molecules, and enhancing SDF-1 $\alpha$ -induced chemotaxis, all of which may have implications for therapeutic resistance [30].

In conclusion, our results showed that AMD3100, a CXCR4 antagonist, could improve renal function after renal ischemia and reperfusion, potentially through the mobilization and recruitment of CXCR4 and c-kit positive cells into injured kidneys. This therapeutic potential of AMD3100 in AKI also supports the concept that organ repair by stem cells originating in organs other than kidney could be feasible, and the utility of AMD3100 in other causes of AKI warrants further investigation.

## Acknowledgments

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