

## Original Article

# Granulocyte-colony stimulating factor in combination with AMD3100 confers greater neuroprotection after hypoxic-ischemic brain damage than a solitary treatment in mice

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**Abstract:** Objective: Hypoxic-ischemic brain damage (HIBD) remains a significant cause of acute deaths and chronic nervous system damage. Granulocyte-colony stimulating factor (G-CSF) has been shown to have neuroprotective activity via inhibition of apoptosis and inflammation in various stroke models. AMD3100, a novel small molecule that acts as a specific CXCR4 antagonist, is shown with an efficient ability for suppressing inflammatory response. In this study, we aim to determine the protective effects of G-CSF in combination with AMD3100 treatment after experimental hypoxia-ischemia (HI). Methods: ICR mice were subjected to unilateral carotid artery ligation followed by 2.5 hour of hypoxia. Animals were randomly assigned to five groups: Sham, Vehicle, HI with G-CSF treatment, HI with AMD3100 treatment, and HI with G-CSF+AMD3100 treatment. The memory and cognitive function were tested by Morris water maze and neural behavior was tested by specific behavioral rating scale. Neurological outcome was also evaluated by tissue histology and TUNEL staining. Results: The results indicated that G-CSF combined with AMD3100 improved memory and cognitive functions and improved tissue damage after HI. In addition, G-CSF combined with AMD3100 improved the number of NSE and BCL-2 positive cells; however, decreased the number of GFAP positive cells of HI model; and decreased the number of apoptotic cells potentially. Conclusions: Our data suggest that administration of G-CSF in combination with AMD3100 significantly improved neurological function after HIBD.

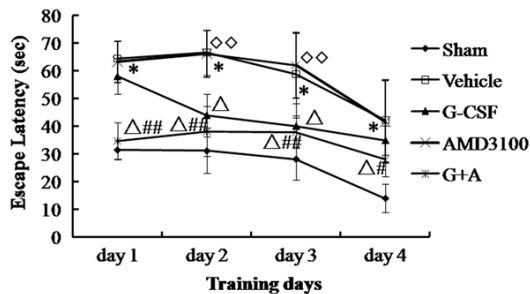
**Keywords:** Granulocyte-colony stimulating factor, AMD3100, neuroprotection, hypoxia-ischemia, brain damage

## Introduction

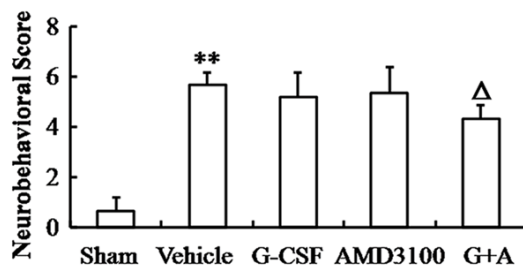
Hypoxic-ischemic brain damage (HIBD) is a clinical syndrome with high mortality and morbidity. Among survivors, 20-40% develop significant neurological impairments such as cerebral palsy, mental retardation, and epilepsy associated with life-long medical, social, emotional, and economic difficulties [1-3]. Presently, the only available treatment, hypothermia, has limited beneficial effects and is only effective in mildly-affected children. There is an urgent need to develop more effective therapeutic strategies.

Granulocyte-colony stimulating factor (G-CSF), a neurotropic factor involved in proliferation,

differentiation, and functional integration of neural cells, is a neuroprotective agent in a wide spectrum of experimental models of neurological disease [4]. Protection ranges from reductions in infarct size during the acute phase to attenuation of long-term functional neurological deficits. Experimentally, neuroprotection occurs by reducing apoptosis in the ischemic penumbra and attenuating the inflammatory cascade [5, 6]. It also appears to be neuroreparative through potentiating angiogenesis and neurogenesis, in part by activating brain endothelial cells and mobilizing hematopoietic stem cells (HSCs) to migrate to the ischemic lesion [7]. More contentiously, bone marrow-derived stem cells have been shown to differentiate



**Figure 1.** Performance of mice in the Morris water maze. \* $P < 0.05$  vs. sham group;  $\Delta P < 0.05$  vs. vehicle group;  $\diamond P < 0.01$  vs. G-CSF group;  $\# P < 0.05$  vs. AMD3100 group. G+A: G-CSF+AMD3100 group.



**Figure 2.** Neurological outcome of mice in behavioral rating scale. \*\* $P < 0.01$  vs. sham group;  $\Delta P < 0.05$  vs. vehicle group. G+A: G-CSF+AMD3100 group.

into neurons and glia, and experimental transplants of HSCs have been observed to improve outcome of HIBD [8].

AMD3100, a novel small molecule that acts as a specific CXCR4 antagonist, is shown with an efficient ability for bone marrow HSC mobilization with clinical therapeutic potential [9]. In 2008, it was approved as a HSC mobilizing drug by US Food and Drug Administration for autologous stem cell transplantation. Stem cells were mobilized to replace damaged tissues via their paracrine factors after they arrive at the injured region [10]. A recent study indicated that G-CSF combined with AMD3100 promoted angiogenesis in a hindlimb ischemic model. These suggested that AMD3100 would be a potential therapy of HIBD via mobilization of stem cells. Another report showed that CXCR4 antagonist AMD3100 significantly suppressed inflammatory response and reduced blood-brain barrier disruption [11].

We hypothesized that G-CSF in combination with AMD3100 will not only have neuroprotective activity but will also show significant improvement in the neurological outcome of mice with HIBD.

## Materials and methods

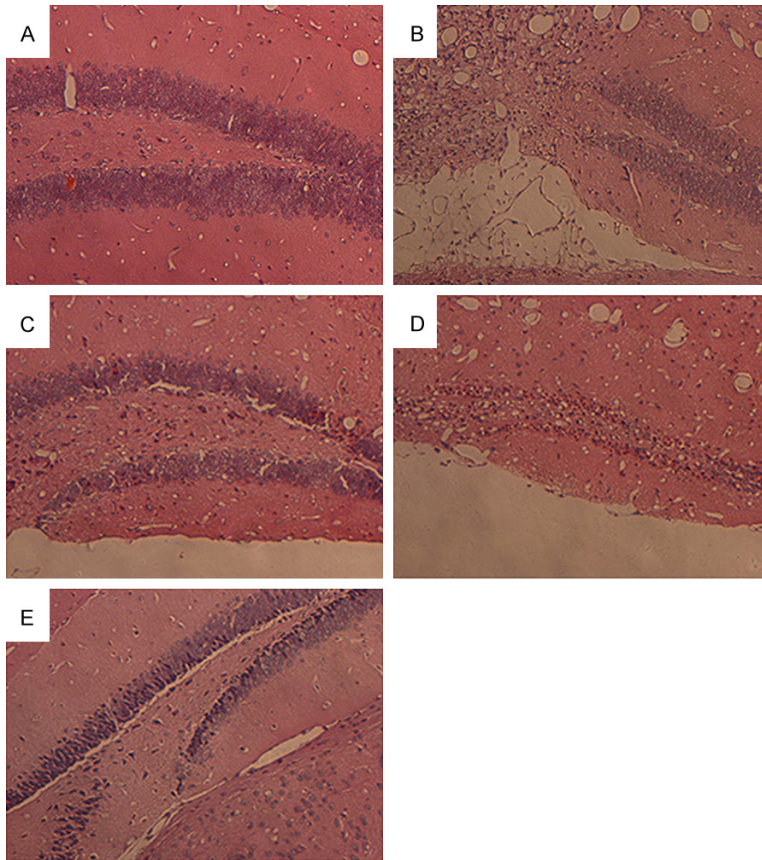
### Animal and HIBD model

All animal investigations were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by NIH and approved by the institutional animal care committee of Zhejiang Chinese medicinal University. Male Institute of Cancer Research (ICR) mice (18-22 g) were purchased from Zhejiang Chinese medicinal University Animal Center (Laboratory Animal Certificate: SCXK 2013-0184). All animals were allowed free access to food and water.

As HIBD model was induced according to the protocol of our previous study [12]. The mice were anesthetized with 4% chloral hydrate solution (400 mg/kg), and the left carotid artery was isolated and ligated doubly. After 2-3 h, the waking mice were put into a transparent container which was ventilated with a constant flow of mixed gas containing 8% oxygen and 92% nitrogen for 2.5 h.

### Experimental grouping and treatment

The animals were randomly assigned into five experimental groups ( $n=18$  per group): sham-operated mice (sham), mice subjected to the hypoxic-ischemic (HI) model administered with vehicle (PBS), HI mice administered with G-CSF (G-CSF), HI mice administered with AMD3100 (AMD3100), HI mice administered with G-CSF and AMD3100 (G-CSF+AMD3100). In G-CSF group, HI mice were pretreated with G-CSF once daily for 5 days (200  $\mu\text{g/kg}$  i.p.) (Jiuyuan Gene Engineering Co. LTD., Hangzhou, China). In AMD3100 group, HI mice were pretreated with vehicle (PBS) once daily for 5 days. Twenty-four hours after the last injection, mice were administered a CXCR4 antagonist (AMD3100 5 mg/kg i.p.) (Sigma-Aldrich, St. Louis, MO, USA). In G-CSF+AMD3100 group, HI mice were pretreated with G-CSF once daily for 5 days (200  $\mu\text{g/kg}$  i.p.) followed by one AMD3100 injection (5 mg/kg i.p.). Morris water maze learning task and Neurobehavioral Tests ( $n=6$  per group) was performed at 14 days after HI. Meanwhile, the mice were euthanized at 14 days after HI for obtaining brain tissue samples, histological and immunohistochemical detection ( $n=6$  per group), terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining ( $n=6$  per group), respectively.



**Figure 3.** Pathological changes of hippocampal tissues in different groups. A. Pathological changes of sham group (magnification, 200×); B. Pathological changes of vehicle group (magnification, 200×); C. Pathological changes of G-CSF group (magnification, 200×); D. Pathological changes of AMD3100 group (magnification, 200×); E. Pathological changes of G-CSF+AMD3100 group (magnification, 200×).

#### *Spatial version of the Morris water maze learning task*

The animals were tested in the spatial version of the Morris water maze (MWM) learning task beginning at 14 day after HI. The MWM was undertaken to investigate the HI-induced spatial memory impairment as described previously [13]. Briefly, the spaced training protocol was performed for 4 successive days. On each day, the mice received 4 consecutive training trials during which the hidden platform was kept in a constant location. A different starting location was used for each trial, which consisted of a swim followed by a 30 s platform sit. Any mouse that did not find the platform within 90 s was guided to it by the experimenter. Memory retention was evaluated in a 60 s probe trial performed in the absence of the escape platform 24 h after the last training session.

#### *Neurobehavioral tests*

A behavioral rating scale to grade the extent of injury was followed as described previously [14]. Three behaviors were tested: ipsilateral circling, bilateral grasp, and beam walking. The extent of circling to the side of the infusion was graded from 0 (no circling) to 4 (always circled). Grasp was tested by placing the rat's paws on the edge of a box 14 inches high; strength of the hemiparetic paw was graded from 0 (grasped well) to 4 (unable to grasp with forepaw). Beam walking was graded by placing each rat on a beam; a grade of 0 indicated that it easily traversed the beam, while a grade of 4 was given those unable to walk on the beam. A total injury score was calculated as the sum of the grades on the three tests.

#### *Histology*

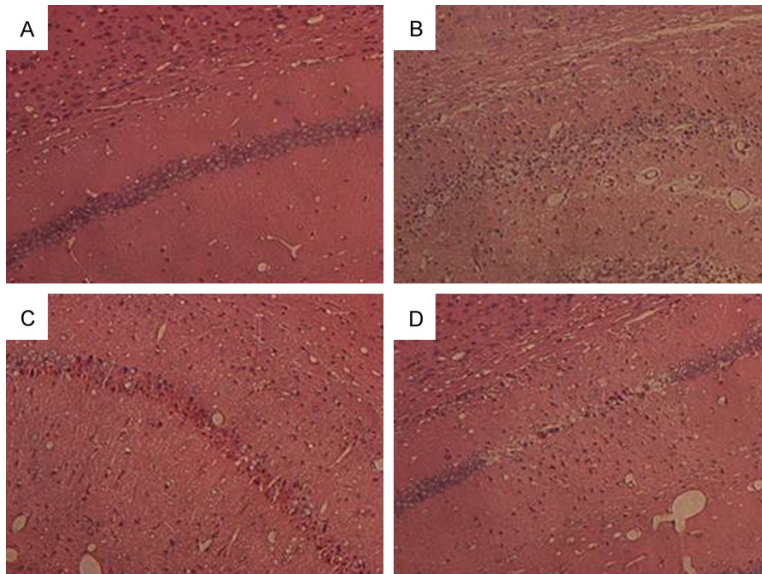
All histopathological analyses described below were performed by a histologist and a pathologist blinded to mice'

treatment. We evaluated all hippocampus tissues.

Brains were removed from the skull, submerged in a 10% formaldehyde solution, was subjected to routine follow-up procedures. Coronal paraffin sections (5  $\mu$ m) of paraformaldehyde (PFA)-fixed brains were stained with H&E or incubated with NSE or BCL-2 (Abcam Technology, Cambridge, MA, USA) or GFAP (Cell Signaling Technology, CST, USA) followed by biotinylated horse-anti-mouse antibody (Vector Laboratories, Burlingame, CA). Binding was visualized with Vectastain ABC kit (Vector Laboratories, Burlingame, CA). NSE, GFAP and BCL-2 staining were quantified using Image J software.

#### *TUNEL staining*

Neuronal apoptosis was measured by TUNEL staining (Roche, Mannheim, Germany), a meth-



**Figure 4.** Pathological changes of hippocampal CA1 region in the ischemic side of brain tissue. A. Pathological changes of sham group (magnification, 200×); B. Pathological changes of vehicle group (magnification, 200×); C. Pathological changes of G-CSF group (magnification, 200×); D. Pathological changes of G-CSF+AMD3100 group (magnification, 200×).

od used to observe DNA strand breaks in nuclei. Briefly, the sections were postfixed in ethanol-acetic acid (2:1) and rinsed. They were then incubated with proteinase K (100 µg/mL), rinsed, in cubated in 3% H<sub>2</sub>O<sub>2</sub>, permeabilized with 0.5% Triton X-100, rinsed again, and incubated in the TUNEL reaction mixture. Next, the sections were rinsed and visualized using Converter POD. 3,3'-Diaminobenzi-dine (DAB) was used for the coloration of apoptotic cells.

#### Statistical analysis

All data were expressed as mean ± standard deviation (SD). Differences between group means were assessed by analysis of variance for multiple comparisons using SPSS 19.0. A *p* value of <0.05 was considered statistically significant.

#### Results

##### *Treatment with G-CSF combined with AMD3100 improved memory and cognitive function after HI*

In the hidden platform version of the water maze test, mean escape latency times differed significantly between the HI mice and sh-

am ones. The G-CSF administration group showed shorter escape latencies than the PBS control group at day 2 and day 3 ( $P<0.05$ ). However, the escape latency for the AMD3100 group did not differ significantly from that of the PBS group. Interestingly, the G-CSF combined with AMD3100 administration group showed shorter escape latencies than the G-CSF solitary treatment group at day 1 ( $P<0.05$ ). The group differences in escape latencies were not due to differences in swimming ability, since the three groups did not differ significantly in swimming speed. Taken together, these results demonstrate superior cognitive function for G-CSF combined with AMD3100 administration and suggest that G-

CSF and AMD3100 protected the animals from behavioral deficits induced by hypoxic-ischemic exposure (**Figure 1**).

##### *G-CSF combined with AMD3100 improved neurological outcome at 2 weeks post HI*

In order to test the effects of G-CSF, AMD3100 and G-CSF+AMD3100 treatment on the neurobehavioral impairments induced by HI, neurological outcome was assessed using behavioral rating scale at 14 days after HI. In both behavioral tests, animals in the vehicle group performed significantly worse than sham operated mice ( $P<0.05$ ). Mice administered with G-CSF or AMD3100 showed a tendency to improve neurological outcome however no significance was reached ( $P>0.05$ ). Nonetheless, the combinational treatment of G-CSF+AMD3100 showed to significantly improve neurological outcome compared to vehicle treated mice ( $P<0.05$ ,  $4.333\pm0.5164$  vs.  $5.667\pm0.516$ ) (**Figure 2**).

##### *G-CSF combined with AMD3100 improved tissue damage at 2 weeks post HI*

As shown in **Figures 3 and 4**, most H&E-positive cells in the vehicle-treated hypoxia-ischemia group were shrunken with eosinophilic

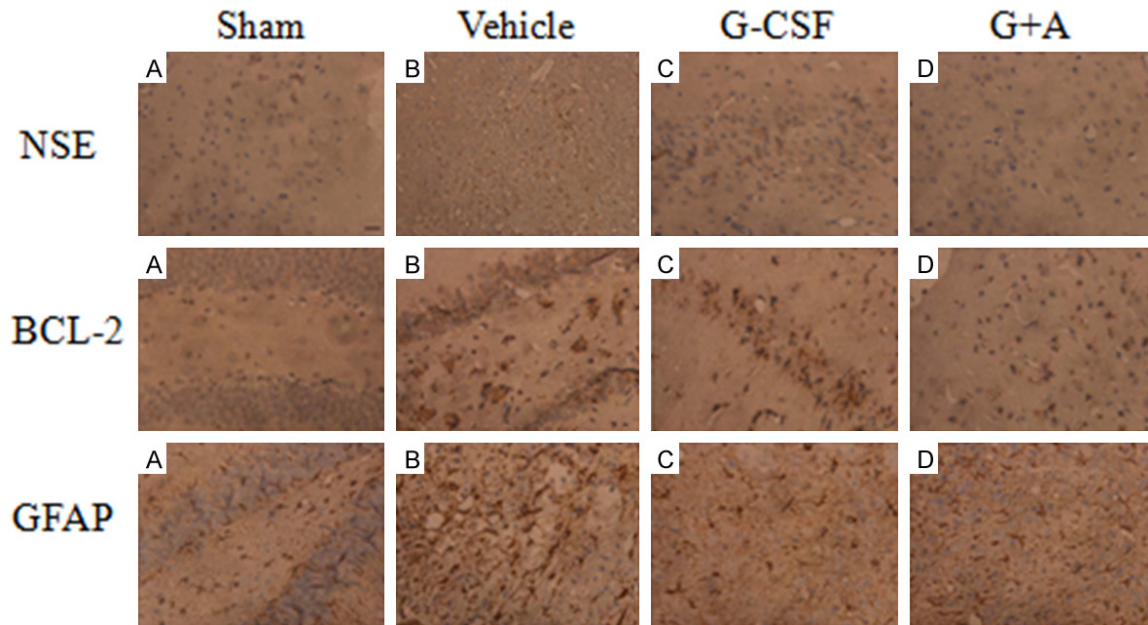


Figure 5. Immunohistochemical detection in different groups of hippocampal tissues.

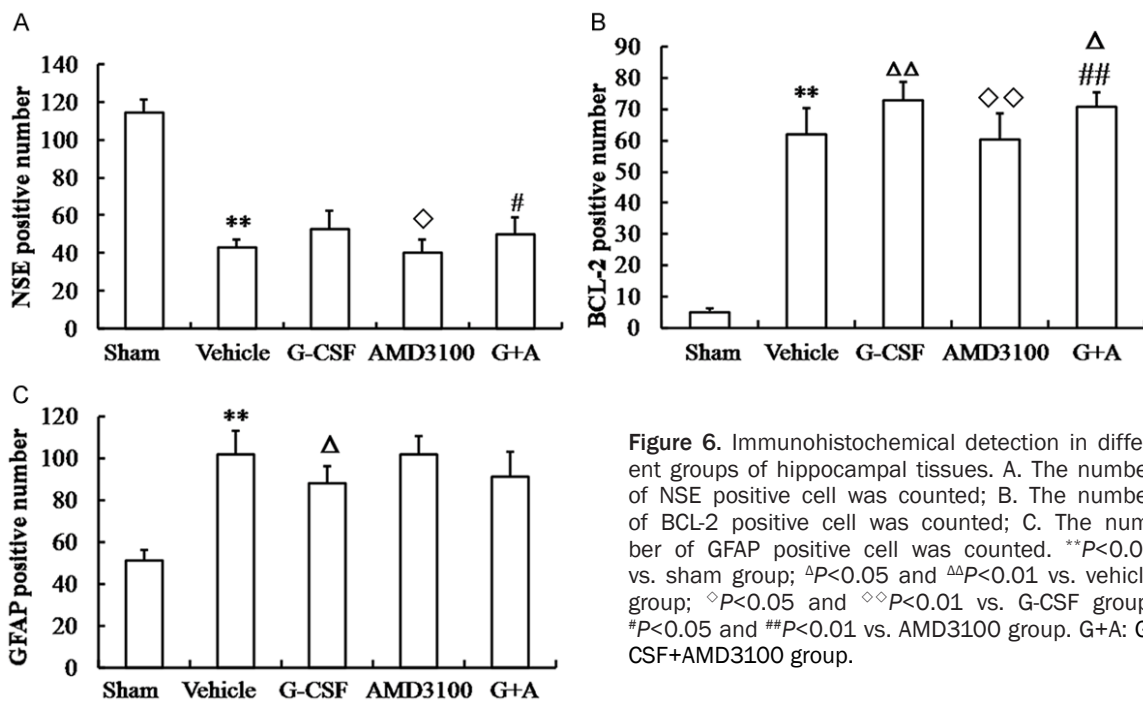
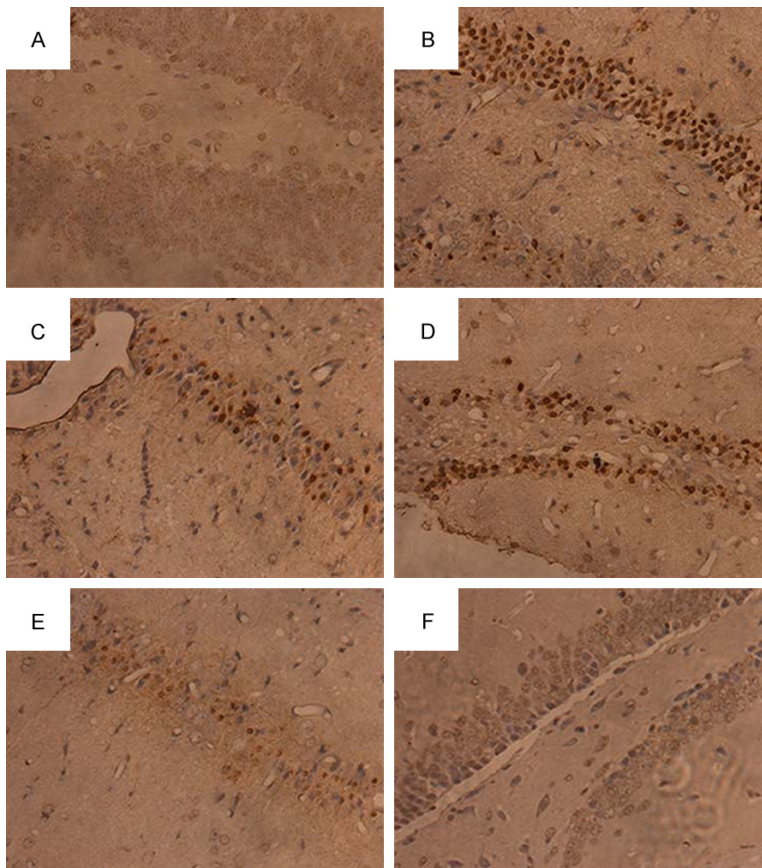


Figure 6. Immunohistochemical detection in different groups of hippocampal tissues. A. The number of NSE positive cell was counted; B. The number of BCL-2 positive cell was counted; C. The number of GFAP positive cell was counted. \*\* $P < 0.01$  vs. sham group;  $\Delta P < 0.05$  and  $\Delta\Delta P < 0.01$  vs. vehicle group;  $\diamond P < 0.05$  and  $\diamond\diamond P < 0.01$  vs. G-CSF group;  $\# P < 0.05$  and  $\#\# P < 0.01$  vs. AMD3100 group. G+A: G-CSF+AMD3100 group.

cytoplasm and triangulated pyknotic nuclei, indicating irreversible neuronal injury. The number of H&E-positive cells in this group was also lower than that seen in the sham group. However, in the G-CSF treated hypoxia-ischemia group, H&E-stained cells were similar to those in the sham group, and degenerating cells were

slightly observed in the damaged area; the number of H&E-positive cells was much higher than that in the vehicle-treated hypoxia-ischemia group. In G-CSF+AMD3100 group, H&E-stained cells were also similar to those in the sham group, and degenerating cells were slightly observed in the damaged area.



**Figure 7.** Neuronal apoptosis of different groups in TUNEL staining. A. The number of TUNEL positive cells of HI mice in sham group (magnification, 400×); B and C. The number of TUNEL positive cells of HI mice in vehicle group (magnification, 400×); D. The number of TUNEL positive cells of HI mice in G-CSF group (magnification, 400×); E. The number of TUNEL positive cells of HI mice in AMD3100 group (magnification, 400×); F. The number of TUNEL positive cells of HI mice in G-CSF+AMD3100 group (magnification, 400×).

*G-CSF combined with AMD3100 improved the number of NSE and BCL-2 positive cells, decreased the number of GFAP positive cells of HI mice*

The number of NSE, BCL-2, GFAP positive cells in hippocampal tissues was revealed in **Figures 5 and 6**. As shown in **Figure 6A**, the number of NSE-positive cells in the vehicle-treated hypoxia-ischemia group was much lower than that seen in the sham group ( $P<0.01$ ). In the G-CSF treated hypoxia-ischemia group, NSE-positive cells were increased significantly than the PBS vehicle group. However, in AMD3100 group, NSE-positive cells were similar to those in the PBS group. In G-CSF+AMD3100 group, the number of NSE-positive cells was enhanced significantly than the PBS vehicle group ( $P<0.05$ ). The trends of change of number of BCL-2

positive cells in each group were similar with NSE-positive cells, shown in **Figure 6B**.

As shown in **Figure 6C**, the number of GFAP-positive cells in the vehicle-treated hypoxia-ischemia group was much higher than that seen in the sham group ( $P<0.05$ ). In the G-CSF treated hypoxia-ischemia group, GFAP-positive cells were decreased significantly than the PBS vehicle group. However, in AMD3100 group, GFAP-positive cells were similar to those in the PBS group. In G-CSF+AMD3100 group, the number of GFAP-positive cells was reduced significantly than the PBS vehicle group.

*G-CSF combined with AMD3100 decreased the number of TUNEL positive cells of HI mice*

Photomicrographs of TUNEL-positive cells in the hippocampal dentate gyrus are presented in **Figure 7**. Compared with the sham group, increased apoptotic index was detected in the PBS vehicle group. Treatment with AMD3100 or G-CSF did not alter the number of TUNEL-positive cells

compared with the vehicle group. Remarkably, treatment with G-CSF combined with AMD3100 induced a significant decrease in TUNEL-positive cells in the brain compared with the PBS vehicle group.

## Discussion

Hypoxia-ischemia is a devastating condition resulting in neuronal cell death and often culminates in neurological deficits. To date, effective treatment avenues are still lacking hence the need for future research in this field. The current study focuses on two agents G-CSF and AMD3100 which might have a promising mobilization effects on stem cells. Both G-CSF and AMD3100 are known to stimulate the proliferation and migration of bone marrow cells, such as the hematopoietic cells. While G-CSF is im-

portant for stimulating the production and recruitment of cells from the bone marrow into the circulation, AMD3100 might be responsible for the improvement the efficiency of stem cell mobilization [15-17].

G-CSF is one of the most studied growth factors in the setting of stroke. Previous studies have reported that G-CSF can pass the blood brain barrier and has shown to be neuroprotective in a rat model of focal cerebral ischemia [18, 19]. Clinically, increasing evidences have shown the therapeutic potential of G-CSF and AMD3100 in patients with ischemic stroke. Previous findings have also reported G-CSF's ability to reduce lesion volumes and improve neurological outcome after cerebral ischemia in rodents [20, 21]. Based on the above findings, it is of particular interest to us to study the effects of the combinational treatment of G-CSF with AMD3100 on neurological outcome. We tested this hypothesis by administering G-CSF, AMD3100, and a combination of G-CSF+AMD3100 post HI and consecutively for 5 days. To our knowledge, the neuroprotective effect of the combinational treatment has not been studied to date.

AMD3100 has been shown to result in a significant mobilization of progenitor cells via disrupting SDF-1 $\alpha$ /CXCR4 bond, the primary progenitor cell anchor in the bone marrow. Meanwhile, as a selective antagonist of CXCR4, it might impair the homing of progenitor cells to injured tissue. Previous report indicated that pre-treatment with the AMD3100 significantly prevented the recruitment of MSCs to the injured brain [22]. Application of AMD3100 at a dosage of 1.25 mg/kg/d leads to reduced homing of circulating stem cells to the injured tissue [23]. Abundant evidence suggested that higher dose AMD3100 (5 mg/kg) functions as a stem cell mobilizing agent. In this study, we use AMD3100 for 5 mg/kg.

The hippocampus and the sensorimotor cortex are critical for regulation of memory and cognitive function and are highly affected by HI [24]. As we already known, damage to those regions causes severe deterioration in functional performance. According to water maze test, G-CSF and AMD3100 treatment groups alone did not show any significant improvement in neurological score; however the combinational treatment group did show significant improvements.

Our results are consistent with previous work, where G-CSF given for 5 days improved neurological outcome. From the present study and previous studies we can conclude that G-CSF shows significant neurological improvement as early as 2 weeks, and G-CSF+AMD3100 showed even further improvement when assessed at 2 weeks post HI.

Overall, the above findings from this study are clinically relevant and provide foundation for exploring clinical translation. In conclusion, our study indicated that G-CSF in Combination with AMD3100 confers greater neuroprotection after HIBD than a solitary treatment.

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## Disclosure of conflict of interest

None.

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