The Dual Effect of CXCR4 Antagonist AMD3100 on the Recruitment of Endogenous Endothelial Precursor Cells and Outcome of Stroke

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Research

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Abstract

Background: To examine the effect of CXCR4 antagonist AMD3100 on the recruitment of endogenous endothelial precursor cells (EPC) in ischemic boundary zone (IBZ) after permanent middle cerebral artery occlusion (pMCAO) and outcome of stroke.

Methods: Adult male SD rats underwent pMCAO. AMD3100 was injected once at 1 hour (an early phase) or on day 14 (a later phase) or for 7 consecutive days from day 1 to day 7(3 mg/kg/day) after pMCAO. Flow cytometry analyses were performed to detect endogenous EPCs in peripheral blood (PB). Endogenous EPCs in IBZ were identified by immunofluorescence staining. SDF-1 expression levels in IBZ were measured by real time PCR dynamically. Infarct volume and neurological outcome including neurological score and body weight loss were used to estimate the outcome of stroke.

Results: AMD3100-treatment could mobilize endogenous EPCs to PB of rats after pMCAO, and continuous AMD3100-treatment mobilized more EPCs to PB than single AMD3100-treatment. Single AMD3100 treatment at 1 hour after pMCAO rather than continuous AMD3100 treatment in an early phase could recruit endogenous EPCs in IBZ and improve neurological outcome after pMCAO. Single AMD3100 administration in later phase (on day 14) could not recruit endogenous EPCs to IBZ or improve neurological outcome after pMCAO. SDF-1 relative expression in IBZ increased in an early phase from day 1 to day 3, then decreased in later phase from day 7 to day 14.

Conclusions: Our findings suggested that single AMD3100 treatment in an early phase could recruit endogenous EPCs to IBZ and improve the outcome of stroke, and AMD3100 might be used for the treatment of stroke if given at proper time window.

Background

Ischemic stroke represents a major cause of disability and mortality around the world. Early opening of the blood vessels to improve the prognosis is extremely important. So far, many treatments on the vascular recanalization at acute or recovery stage after ischemic stroke are failed, and the most effective treatment is the administration of recombinant tissue plasminogen activator (rt-PA) 3-4.5 hours after stroke(1). Further studies on the recanalization and restorement are needed.

Endothelial progenitor cells (EPCs) therapy for ischemia has considered as a cell-based treatment and recently introduced into clinical practice (2, 3). EPCs are a minor population of circulating mononuclear cells that participates in adult neovascularization in pathological and physiological processes (4, 5). EPCs have been shown to account for up to 26% of all ECs in neovascularization (6). The contribution of EPCs in angiogenesis has also been documented in the recovery processes of various diseases, such as myocardial ischemia(7, 8), limb ischemia(2, 9), ischemic stroke(10, 11), and wounds(12).

However, a number of questions concerning the EPC treatment in clinical are needed to be answered. First, the safety and efficacy of EPC transplantation needs to be further improved. Second, several

technical issues have to be addressed, including dosage, route and timing of cell transplantation(13). Therefore, it is of importance to improve mobilization and recruitment of endogenous EPCs to the neovasculature of ischemic area.

Stromal cell-derived factor 1(SDF-1) and its cellular receptor CXC-chemokine receptor 4 (CXCR4) are key regulators of EPC mobilization and recruitment(14). Previous studies demonstrated that the CXCR4 antagonist AMD3100 could rapidly mobilize EPCs and enhance angiogenesis in sites of myocardial infarction. However, the role and mechanism of AMD3100 in ischemic stroke remains unclear. In the present study, we explored that, after establishment of permanent middle cerebral artery occlusion (pMCAO) models in rats, whether AMD3100 could enhance the mobilization and recruitment of EPCs to the IBZ, and if this enhancement could help improve the prognosis of pMCAO in rats.

Materials And Methods

Establishment of permanent middle cerebral artery occlusion (pMCAO) models and treatments

Adult male Sprague-Dawley rats weighing 250–280 g, 7–8 weeks' old were used in this study. Rats were obtained from and maintained in the Animal Care and Use Committee of Tongji Medical College at Huazhong University of Science and Technology, Wuhan, China. All procedures involving animal treatment were approved by the institutional committee of animal care and use. Rats were anesthetized with 10% chloral hydrate (300 mg/kg, i.p.), and surgery was done as described previously (15, 16). Briefly, the right common carotid artery, external carotid artery, and internal carotid artery were isolated via a midline incision. The right external carotid artery was ligated with a 6 − 0 nylon suture. Then, a poly-L-lysine-coated 4 − 0 monofilament nylon suture (Beijing Sunbio Biotech Co Ltd) was inserted from the right internal carotid artery and advanced for about 18 mm to occlude the origin of right MCA. Rats were maintained at 37.3 ± 0.5 °C with a feedback-regulated heating pad during surgery and killed at different times after permanent middle cerebral artery occlusion (pMCAO). Sham-operated rats underwent identical procedures but without filament insertion.

Rats were randomly assigned to sham and pMCAO groups through the use of a lottery-drawing box. AMD3100 (Abcam), a specific CXCR4 antagonist (3 mg/ml) was dissolved with normal saline solution and injected intraperitoneally after pMCAO. AMD3100 (3 mg/kg/day) was injected just once at 1 h or 14d after pMCAO named AMD-single treatment group, and was injected for 7 consecutive days after pMCAO named AMD-continuous treatment group. The normal saline solution was used in the saline-treated group.

Immunofluorescence examination

The rats were sacrificed on day 7 and 21 after pMCAO. To prepare paraffin-embedded sections, the brains were fixed by transcranial perfusion with saline, followed by perfusion and immersion in 4% paraformaldehyde before being embedded in paraffin. A tissue block for standard paraffin-embedding

was obtained from the center of the lesion (bregma − 1 mm to 11 mm). The block was serially cut into 4 μm-thick sections for immunofluorescence staining. After deparaffinization and rehydration, the sections were subjected to heat-induced antigen retrieval by using citrate buffer (10 mM, pH 6.0) in a pressure cooker for 2 min, and then washed in phosphate-buffered saline (PBS). The sections were then blocked with 10% donkey serum for half an hour and incubated with primary antibodies overnight at 4 °C. The following primary antibodies were used: mouse anti-CD34 (1:100, Novus), goat anti-VEGFR2 (1:100, Abcam), and rabbit anti-CXCR4 (1:100, Abcam). For triple immunostaining, the three primary antibodies were mixedly used. After washing in PBS, the sections were incubated with secondary antibodies for 2 hours. The secondary antibodies used were: donkey anti-mouse (Alexa Fluor 488, Invitrogen), donkey anti-goat (Alexa Fluor 647, Invitrogen), and donkey anti-rabbit (DyLight 405, Jackson Immunoresearch) antibodies. After secondary antibody incubation, the sections were washed in PBS. Sections were mounted with Fluorescence ProLong Gold antifade reagent (Beyotime), cover-slipped, and examined under a TCS SP5 multiphoton laser scanning confocal microscope (Nikon). Images were processed by using Image J (NIH Shareware) and Adobe Photoshop CS (Adobe Systems, Mountain View, CA).

The means of cells were calculated from 5 microscopic fields selected randomly in ischemic boundary zone (IBZ), and 3 consecutive sections of each brain were analyzed by a person blind to the grouping. Data were expressed as mean numbers of cells per mm^2 by following a previously report (17).

Flow cytometry analysis of circulating EPCs in PB

The rats were sacrificed on day 1, 3, 7, 14 and 21 after pMCAO. The level of circulating EPCs was determined by flow cytometry as a previous study(18). Briefly, circulating MNCs were stained with Fluorescein isothiocyanate (FITC)-conjugated anti-rat CD34 (1:100, Bioss), and phycoerythrin (PE) – conjugated anti-rat VEGFR2 (1:100, Bioss), and incubated at 4 °C for 30 minutes. The cells were washed and re-suspended in PBS buffer. The cells were then sorted on a flow cytometer (BD Biosciences), and the data were analyzed using FCS Epress 4 software (De Novo Software, Los Angeles, CA). The levels of circulating EPCs were expressed as the percentage of CD34 and VEGFR2 co-expression cells. EPC was defined by positive staining for CD34 and VEGFR2(14).

TTC staining of infarct area and determination of cerebral infarction volume

TTC staining was used to show the infarct areas after pMCAO according to a previously reported method (19). In brief, after euthanized with 3.0 ml of ethanol at the conclusion of each experiment,brains of rats were carefully removed and sectioned into 2 mm slices along the coronal plane. The brain slices were then incubated in 2% 2, 3, 5-triphenyltetrazolium chloride (TTC) at 37 °C for 20 minutes in the dark. TTC was enzymatically reduced, producing formazan (a bright red byproduct), by dehydrogenases in active mitochondria. Intensity of staining reflects functional activity of mitochondria, with unstained (white) areas being indicative of infarct. The TTC-stained sections were photographed by employing a digital camera(20) and the total infarct volume was determined by multiplying the infarct area of each slice by the thickness of that slice.

Cerebral infarction volume was determined as described previously(21). In brief, serial coronal sections were used and the infarct area of each section was measured using Image J (NIH Shareware). Infarct size was corrected for edema using the following formula: [1 − (total ipsilateral hemisphere − infarct)/total contralateral hemisphere] × 100%. Infarct volume between 2 adjacent sections was calculated by formula:

$$1/3\times h(S1+S2+\sqrt{S1*S2})$$

Infarct volume was derived from the sum of all infarct volume between each adjacent section.

Behavioral tests

Modified neurologic severity score was used for assessing neurologic function after pMCAO by an investigator who was blinded to the experimental groups as described previously. Measurements were performed from day 1 to day 21 after pMCAO. Neurologic severity score examination consists of the motor, sensory and reflex tests. According to the method, the injury severity was graded on a scale of 0 to 14 (with normal score being 0 and maximal deficit score 14). One point was awarded either for the inability to perform, or for abnormal task performance, or for the lack of a tested reflex(22).

Body weight loss

Animals were weighed before pMCAO and from day 1 to day 21 after pMCAO by an investigator who was blinded to the experimental groups. Body weight loss is presented as a loss percentage of pre-ischemic body weight.

Real-Time PCR

The levels of SDF-1a and CXCR4 of the brain tissues were determined using real-time RT-PCR methods(23). Total RNA from ischemic region was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) and suspended in 40 μl of RNase-free water according to manufacturer's protocol. RNA concentration was determined by a spectrophotometer (NanoDrop1000, Thermo, Wilmington, DE). The amplification was performed by a fast real time PCR system (7900HT, ABI, Foster, CA) using SYBR Premix Ex Taq Kit (TaKaRa, Dalian, China). A universal 2-step RT-PCR cycling condition was used: 95 °C for 30 seconds followed by 40 cycles of 95 °C for 5 seconds and 60 °C for 30 seconds. The mRNA level was normalized to the endogenous control β-actin expression in triplicate and was calculated by the 2-ΔCt method(21, 22). The primer sequences are as follows:

SDF-1 (AF189724), forward: 5'-GGTCTGGAGACTATGACTCCA-3', Reverse: 5'-GTGCTGGAACTGGAACACCA-3'; β-actin (NC_005111), forward: 5'-GAACCCTAAGGCCAACC-3', Reverse: 5'-TGTCACGCACGATTTCC-3'.

Statistical analyses

All data were reported as mean ± standard error of the mean (SEM). Statistical analyses were performed by using Statistical Package for the Social Sciences (SPSS 13.0, USA) software. The two-tailed Student's t-test or one-way ANOVA followed by post hoc Fisher's LSD multiple comparison test was used for significance assessment. $P < 0.05$ was considered statistically significant.

Results

Effect of AMD3100 in early stage on endogenous EPCs in peripheral blood (PB) after pMCAO

The biomarkers used for characterizing EPCs include both hematopoietic stem cell markers (CD34 and CD133) and EC markers, such as CD31, kinase insert domain receptor (KDR, VEGFR2), Von Willebrand factor (vWF), vascular endothelial cadherin (VE-cadherin or CD 144), Tie2, c-kit/CD117, and CD62E (E-selectin). The CD34 + KDR + antigenic combination appears to be of high sensitivity and specificity and has been used for EPC identification in our study. The counts of circulating EPC (co-expression of CD34 and VEGFR2) in peripheral blood were assessed via flow cytometry analyses. On day 3 after pMCAO, the counts of EPCs in peripheral blood were significantly greater in AMD3100-treated rats than those in saline-treated rats (Fig. 1A). Moreover, on day 7 after pMCAO, compared to the saline-treatment group, both single and continuous treatment with AMD3100 could mobilize more EPCs to PB, and the continuous administration of AMD3100 were even more than single administration of AMD3100 (Fig. 1B). Thus, these observations suggest that continuous and single AMD3100-treatment could both induce more endogenous EPCs to peripheral blood (PB) than saline-treatment in an early phase.

Duel effect of AMD3100 in early stage on endogenous EPCs recruitment in IBZ after pMCAO

Endogenous EPCs were quantified as CD34, VEGFR2 and CXCR4 triple staining positive cells, and observed in the IBZ and contralateral cortex under immunofluorescence microscopy. The data demonstrated that there were almost none endogenous EPCs in contralateral cortex (Fig. 2B1) of rats after pMCAO. Then we examined the recruitments of endogenous EPCs in IBZ among single, continuous AMD3100-treatment and saline-treatment groups after pMCAO by counting triple staining positive cells. The results exhibited that compared with the saline-treatment group after pMCAO, endogenous EPCs were increased in IBZ in single AMD3100-treatment group (Fig. 2B4 and B5); however, endogenous EPCs were decreased in IBZ in continuous AMD3100-treatment group (Fig. 2B4 and B6). These contradictory findings showed that single AMD3100 treatment rather than continuous AMD3100 treatment could recruit endogenous EPCs in IBZ after pMCAO(Fig. 2C), indicating that AMD3100 may have a duel effect on the recruitment of endogenous EPCs in IBZ after pMCAO.

Duel effect of AMD3100 administration in early stage on infarct volume and neurological outcome

The infarct volume in the single AMD3100-treated rats (17 ± 3.13 mm^3, p = 0.0085), examined by 2, 3, 5-triphenyltetrazolium chloride (TTC) staining, was decreased on day 3 after pMCAO as compared with the saline-treated rats (27.06 ± 1.72 mm^3,Fig. 3 A). In contrast, the volume in continuous AMD3100-treated rats (25.63 ± 1.44 mm^3, p = 0.204) did not show any significant differences compared with the saline-treated rats (Fig. 3A). The neurobehavioral function was evaluated by neurologic severity score, and the results revealed that single AMD3100-treatment at 1 h after pMCAO could significantly improve the performance just from day 3, while continuous AMD3100 treatment did not improve but even worsened the performance from day 10 as compared with the saline-treated rats (Fig. 3B). After pMCAO, body weight of rats began to decrease. But, after single AMD3100-treatment at 1 h after pMCAO, the loss of rat body weight decreased less than that in the saline-treated group from day 7 (Fig. 3C), while continuous AMD3100-treatment did not show any differences compared with the saline-treated rats (Fig. 3C). These findings indicate that single AMD3100 administration in early phase attenuated infarct volume and improved neurological outcome after pMCAO, while continuous AMD3100-treatment exert no effects.

AMD3100 administration in later phase did not improved neurological outcome after pMCAO

The data described above identified the effects of AMD3100 administration initiated in early stage (1 h after pMCAO), however, the effects of AMD3100 administration in later stage have not been known. We administrated single AMD3100 on day 14 after pMCAO, and body weight loss and the neurologic severity score were examined till day 21. The data showed that there was no significant difference on the loss of body weight and the neurologic severity score between the single AMD3100-treatment and saline-treatment group (Fig. 4A and B). These results suggest that single AMD3100 administration in later phase after pMCAO did not significantly improve neurological outcome.

AMD3100 administration in later phase improved mobilization of endogenous EPCs, but did not significantly alter recruitment of EPC after pMCAO

Rats were treated by single AMD3100 or saline on day 14 after pMCAO, and the peripheral blood EPCs was quantified by Flow cytometry method on day 21. The data showed the number of circulating EPCs was significantly higher in AMD3100 treatment group (0.05 ± 0.01%) than that in saline treatment group (Fig. 5A and B). However, on day 21, recruitment of EPCs in IBZ did not increase significantly in single AMD3100 treatment group compared with saline-treatment group (Fig. 5C). These results showed that AMD3100 administration in later phase could mobilize endogenous EPCs to PB, but cannot recruit endogenous EPCs in IBZ after pMCAO.

SDF-1 expression in IBZ at different time points after pMCAO

The data mentioned before raised two key points deserved to think: (1) in early stage after pMCAO, continuous AMD3100 treatment mobilized more EPCs to PB, but recruited fewer EPCs to IBZ compared with single AMD3100 treatment; (2) after pMCAO, benefits from single AMD3100 treatment in early stage did not observed in later stage. As is known, Stromal cell-derived factor-1 (SDF-1) and its receptor, CXCR4, play important roles in stem cell homing, chemotaxis and AMD3100 is a specific CXCR4 antagonist. We hypothesized that the SDF-1/CXCR4 axis was modulated differently in single and continuous AMD3100-treatment groups at different phases. Then we examined SDF-1 expression in IBZ in ischemic rats dynamically via real-time PCR. The data demonstrated that SDF-1 relative expression in IBZ peaked from day 1 to day 3, then decreased on day 7, and had a further decrease on day 14 (Fig. 6), which were all significantly greater than in sham-operate group.

Discussion

In the present study, we demonstrated that (1) AMD3100-treatment could mobilize endogenous EPCs to peripheral blood (PB) of rats after pMCAO, and continuous AMD3100-treatment mobilized more endogenous EPCs to PB than single AMD3100-treatment. (2) Single AMD3100 treatment in an early phase rather than continuous AMD3100 treatment could recruit endogenous EPCs in IBZ after pMCAO. (3) Single AMD3100 administration in an early phase attenuated infarct volume and improved neurological outcome. (4) a single AMD3100 administration in later stage (on day 14) could still mobilize a little more endogenous EPCs to the PB compared with saline-treatment, but had no effects on neurological outcome and could not recruit endogenous EPCs to IBZ after pMCAO. (5) the SDF-1/CXCR4 axis could be modulated differently in single and continuous AMD3100-treatment groups. Together, these observations highlighted the importance of endogenous EPCs mobilization and recruitment in IBZ after pMCAO and suggested that enhanced recruitment of endogenous EPCs via a single AMD3100-treatment in an early phase has potential to be an alternative for the treatment of stroke.

Formation of new blood vessels, either angiogenesis or vasculogenesis, plays an important part in neovascularization and regeneration after stroke(24). Bone marrow-derived endothelial progenitor cell (EPC) is angioblast that is believed to take part in the formation of the new blood vessels in cardiovascular diseases(25). Stromal cell-derived factor-1 (SDF-1) and its receptor, CXCR4, play important roles in stem cell homing, chemotaxis, modulating the expression of adhesion molecules, engraftment, proliferation, and cell survival(26). Intravenously transplanted hEPC was capable of homing into ischemic areas of brain, promoting angiogenesis, and improving neurobehavioral outcome in MCAO mice(27). Moreover, studies have shown that SDF-1 is expressed along the ischemic boundary zone of the brain and facilitates the migration of transplanted cells into the ischemic zone(28).

Previous studies have documented that most of EPCs expressed CXCR4 receptors(27) ,increased SDF-1 could, by combining with CXCR4 receptors, mobilize EPC from BM to PB and circulating EPC homed into ischemic area(27, 29). AMD3100 is a highly selective CXCR4 receptor antagonist and can rapidly mobilize stem cells from the BM to PM by reversibly blocking the interactions between SDF-1 and CXCR4(30). Moreover, AMD3100 can also suppress the recruitment of stem cells to the area expressing SDF-1 by blocking CXCR4(29). The plasma half-life of AMD3100 is 2–3 hours(29). Also AMD3100 is known to mobilize bone marrow derived stem cells (BMCs) in high concentrations, some low concentrations can block CXCR4 without stimulating mobilization(21).

In the current study, we determined how AMD3100, a CXCR4 antagonist could intervene endogenous EPCs in rats after pMCAO and whether AMD3100-treatment could improve prognosis of rats after pMCAO or not. The results presented here indicate that a single AMD3100-treatment in an early phase could mobilize endogenous EPCs to peripheral blood (PB) and recruit them in IBZ of brain after pMCAO, attenuating infarct volume and improving prognosis of rats after pMCAO. In addition, continuous AMD3100-treatment could mobilize more endogenous EPCs to the PB, but could not recruit endogenous EPCs to IBZ after pMCAO and had worse prognosis than a single AMD3100-treatment.

The findings presented here raise questions about the mechanism responsible for the determinant of endogenous EPCs recruitment in IBZ in rat brain after pMCAO. We hypothesized that the SDF-1/CXCR4 axis was modulated differently in single and continuous AMD3100-treatment groups. Then, we detected SDF-1 expression in IBZ in ischemic rats dynamically via real-time PCR. The data demonstrated that SDF-1 relative expression in IBZ peaked from day1 to day 3, then decreased on day 7, and had a further decrease on day 14, which were all significantly greater than in sham-operate group. Previous studies observed that on day 7, after MCAO, SDF-1 protein expression was significantly upregulated in the injured hemisphere of PBS-treated rats via Immunofluorescence staining, especially in the penumbral regions and in addition, Western blot analysis of the SDF-1 protein further confirmed this result(30).

The underlying mechanism might be that, after pMCAO, a single AMD3100 administration in an early phase first mobilized endogenous EPCs from BM to PB, then AMD3100 was degraded, and mobilized circulating EPCs were recruited to the IBZ by chemotaxis of SDF-1, which presented in a high level within 7 days. However, when AMD3100 was continuously injected, endogenous EPCs mobilized to the PB were inhibited by combining with injected AMD3100 through CXCR4 receptors of themselves before recruitment in IBZ.

The benefits associated with a single injection of AMD3100 in an early phase after pMCAO were not observed with AMD3100 treatment in later phase. Instead, the number of circulating EPCs in AMD3100 treatment group was just about 0.05% higher in AMD3100 treatment group on day 21 after pMCAO. What's more, recruited EPCs in IBZ in AMD3100 treatment group did not increase, and the prognosis of rats did not improve. There were several possible reasons. Firstly, though circulating EPCs were mobilized in AMD3100 administration group in later phase, the number of EPCs (0.11 ± 0.03%) was too few to be recruited in IBZ. Secondly, the expression of SDF-1 in ischemic area had decreased in later phase so that

the level of SDF-1 at day 21 may be not enough to function with EPCs. Thirdly, in later phase, blood brain barrier (BBB) around IBZ was almost repaired, and it was difficult for endogenous EPCs to be recruited in IBZ from the PB. Thus, in later phase after pMCAO, mobilized EPCs could not be recruited in IBZ and could not improve prognosis of rats after pMCAO.

Yang et al demonstrated that AMD3100 significantly attenuated leukocyte accumulation and infiltration into the infarct perifocal region, and effectively reduced the level of proinflammatory cytokines in the ischemic brain tissue. The dose of AMD3100 in his study was 1 mg/kg/day, which is sufficient for blocking CXCR4 without causing stem cell mobilization. Because AMD3100 is known to mobilize bone marrow derived stem cells (BMCs) in high concentrations(21). In the present study, the dose of AMD3100 we chose was 3 mg/kg/day, which could also cause stem cell mobilization. We attributed the benefits from AMD3100 treatment to the increase of endogenous EPCs mobilization and recruitment in IBZ after pMCAO, but whether inhibition inflammation effects caused by AMD3100 participated partially in the improvement after pMCAO needs further research. In addition, whether there are other mechanisms playing a role in these associated benefits, like increasing NPCs(31), caused by AMD3100 are still not known.

Conclusions

In summary, the results presented here demonstrate that a single dose of AMD3100 treatment in early stage after pMCAO enhances the preservation of neurological outcome after pMCAO. However, continuous AMD3100 treatment or single AMD3100 treatment in later stage could not get such an effect. Furthermore, these benefits are accompanied by enhancing EPCs mobilization to PB and recruitment of EPCs in IBZ after pMCAO. Together, these observations suggest that a single AMD3100 treatment in an early phase has potential to be an alternative for the treatment of stroke.

List Of Abbreviations

EPC-endothelial precursor cell

pMCAO-permanent middle cerebral artery occlusion

PB-peripheral blood

IBZ-ischemic boundary zone

rt-PA-recombinant tissue plasminogen activator

SDF-1-stromal cell-derived factor 1

CXCR4-CXC-chemokine receptor 4

TTC-2, 3, 5-triphenyltetrazolium chloride

BMCs-bone marrow derived stem cells

Declarations

Ethics approval and consent to participate

The animal experimental protocol was approved by the Human Ethics Committee of Tongji Medical College at Huazhong University of Science and Technology, Wuhan, China.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Qiao-yuan He and Ming Huang contributed to the conception of the study. Yingfeng Xia, Qiao-yuan He, Chao Li performed the cell culture and molecular biology experiments. Chao Li and Nanliang Fu performed the animal behavioral tests. Yingfeng Xia and Ming Huang contributed significantly to analysis and manuscript preparation. Ming Huang wrote the manuscript; Nanliang Fu, Ruyi Yuan, Jing Mou, Huiyao Xian helped perform the analysis with constructive discussions. All authors read and approved the final manuscript.

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Figures

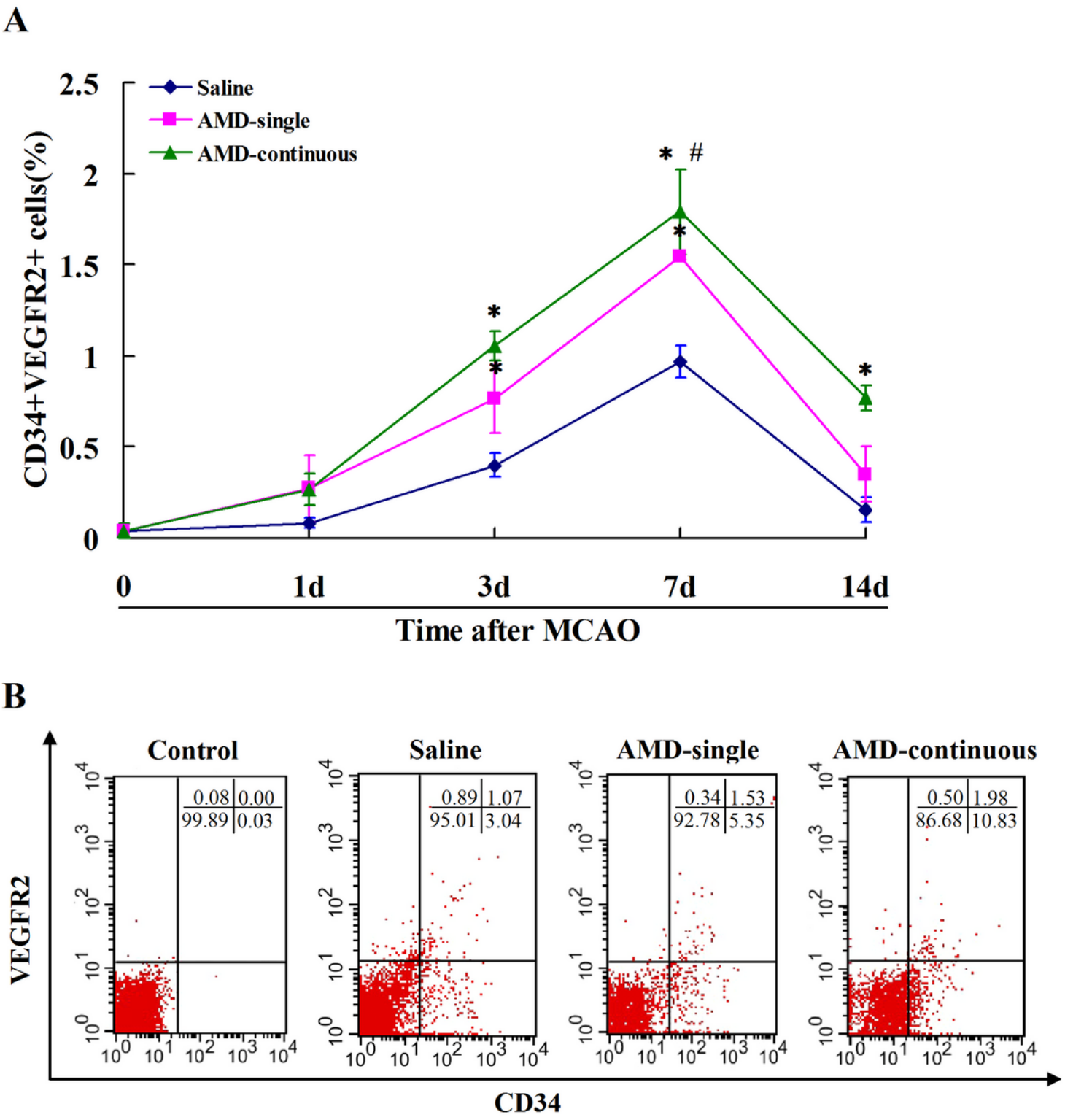

Figure 1

Continuous and single AMD3100-treatment could both induce more endogenous EPCs to peripheral blood (PB) in an early phase. (A) Percent of CD34 and vascular endothelial growth factor receptor (VEGFR2) staining positive cells in peripheral blood (PB) of saline (blue), single (red) and continuous (green) AMD3100-treated rats on day1, day 3, day 7 and day 14 after pMCAO. Data are presented as mean±SEM, n=4 per group. *P<0.05, AMD-single or AMD-continuous vs. Saline, #P<0.05, AMD-continuous vs. AMD-

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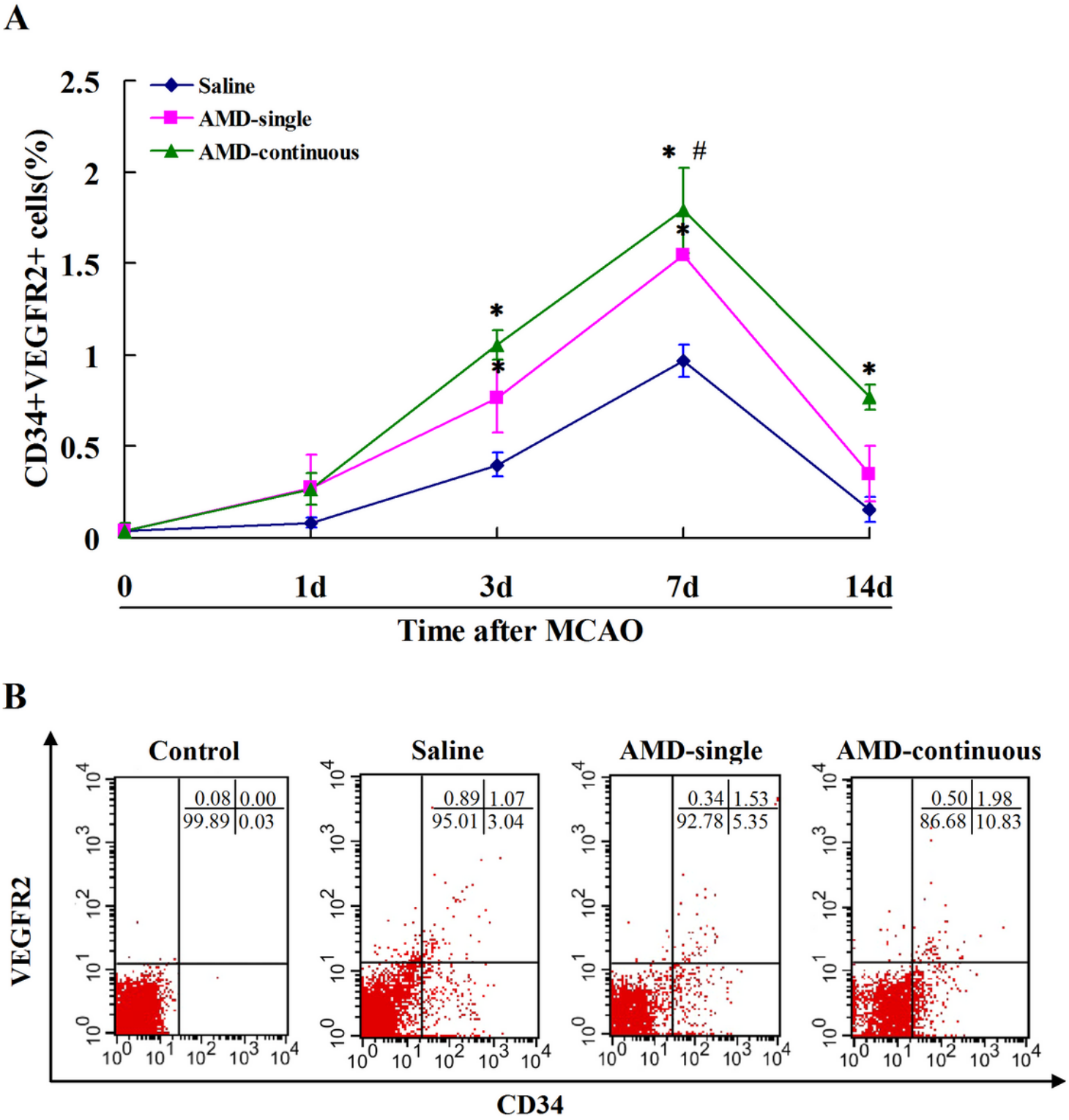

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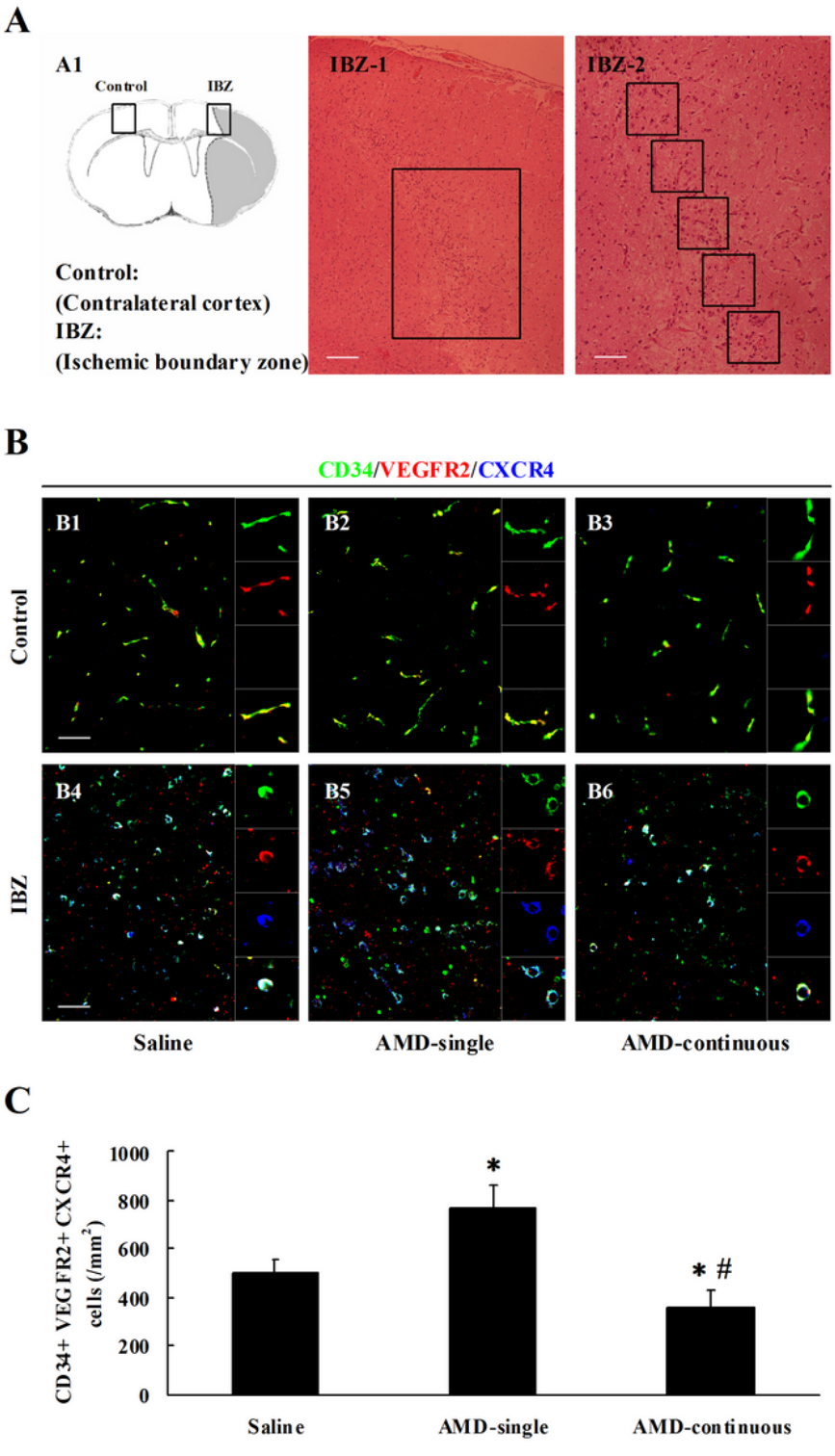
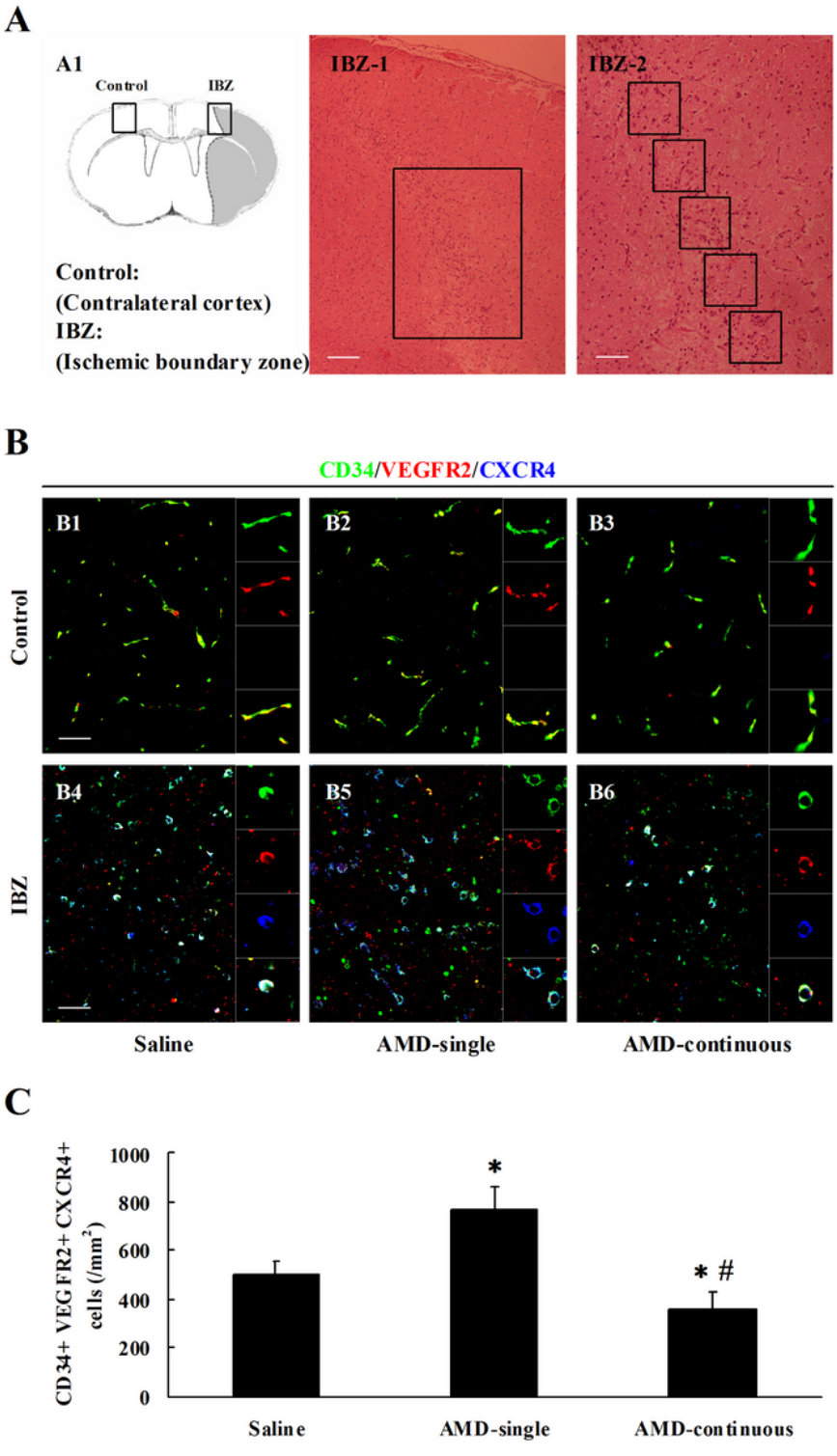


Figure 2

Single AMD3100 treatment rather than continuous AMD3100 treatment could recruit endogenous EPCs in IBZ after pMCAO. (A) (A1) Coronal section illustrates the infarct region (gray) and boxes represent contralateral cortex (Control) and ischemic boundary zone (IBZ) illustrating areas that we display in IBZ-1 (Scale bar, 200μm) and IBZ-2 (Scale bar, 100μm) with Haematoxylin-Eosin (HE) staining. IBZ-2 is high magnification of the box area in IBZ-1. And the five boxes in IBZ-2 shows the detail fields that pictures in B1-6 get from. (B) Immunofluorescent staining showing the CD34 (green), VEGFR2 (red) and CXCR4 (blue) triple positive cells (white) in the control (B1-3) and IBZ (B4-6) in saline, single and continuous AMD3100-treated rats respectively. Scale bar, 20μm. n=4 or 5 pre group. Cells in the right boxes (B1-6) are high magnifications of representatives located by the white arrows. (C) The mean triple positive cells in the five box areas in IBZ-2 (B4-6). Bar graph shows the quantification of triple positive cells in B4-6. Data are mean±SEM, n=3 per group. *P<0.05, AMD-single vs. saline in IBZ. *#P<0.01, AMD-continuous vs. AMD-single in IBZ.



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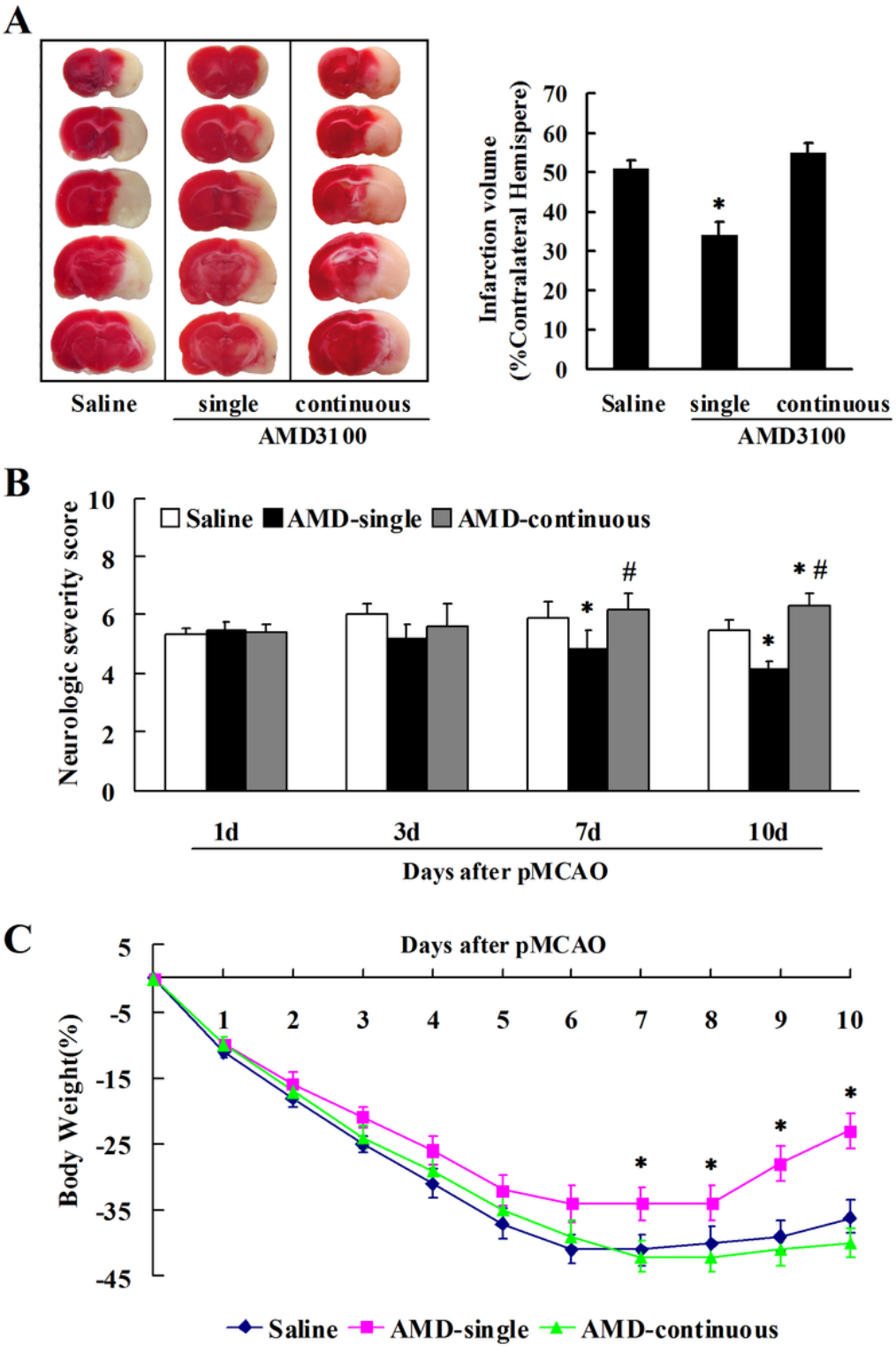
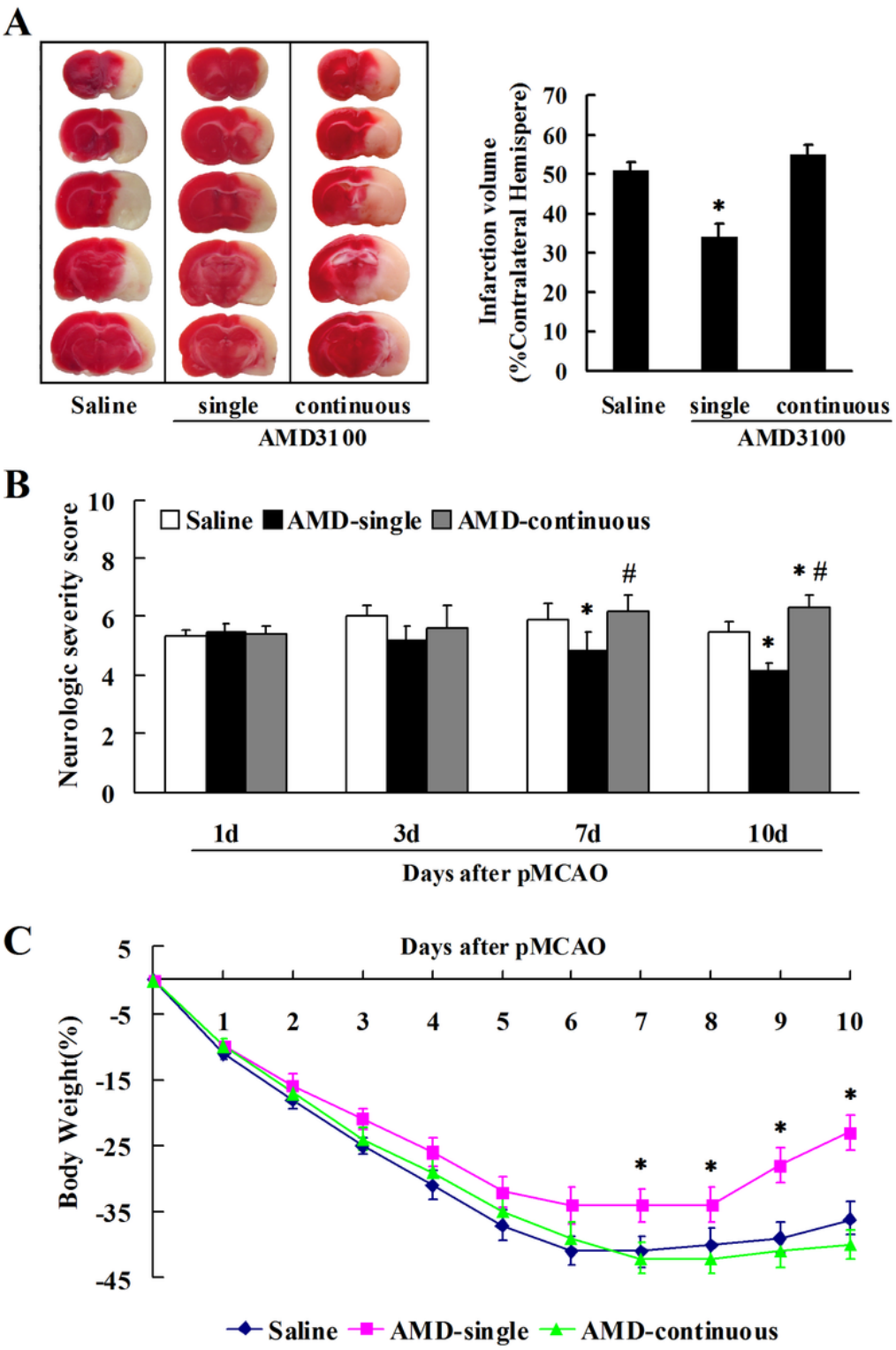


Figure 3

Single AMD3100 administration in an early phase attenuated infarct volume and improved neurological outcome. (A) TTC staining. (B) Bar graph shows quantitative analysis of infarction volume in TTC staining among saline, single and continuous AMD3100-treated rats. Data are mean ± SEM, n=3 to 5 per group. *P<0.05, AMD-single vs. saline. (C) Bar graph shows neurologic severity score on day 1, day 3, day 7 and day 10 in saline (white), single (black) and continuous (gray) AMD3100-treated rats respectively. Data are mean ± SEM, n=3 to 5 per group. *P<0.05, AMD-single vs. saline. #P<0.05, AMD-continuous vs. AMD-single. (D) Curve graph shows body weight loss from day 1 to day 10 after pMCAO in saline (blue), single (red) and continuous (green) AMD3100-treated rats respectively. Data are mean ± SEM, n=3 to 5 per group. *P<0.05, AMD-single vs. saline.



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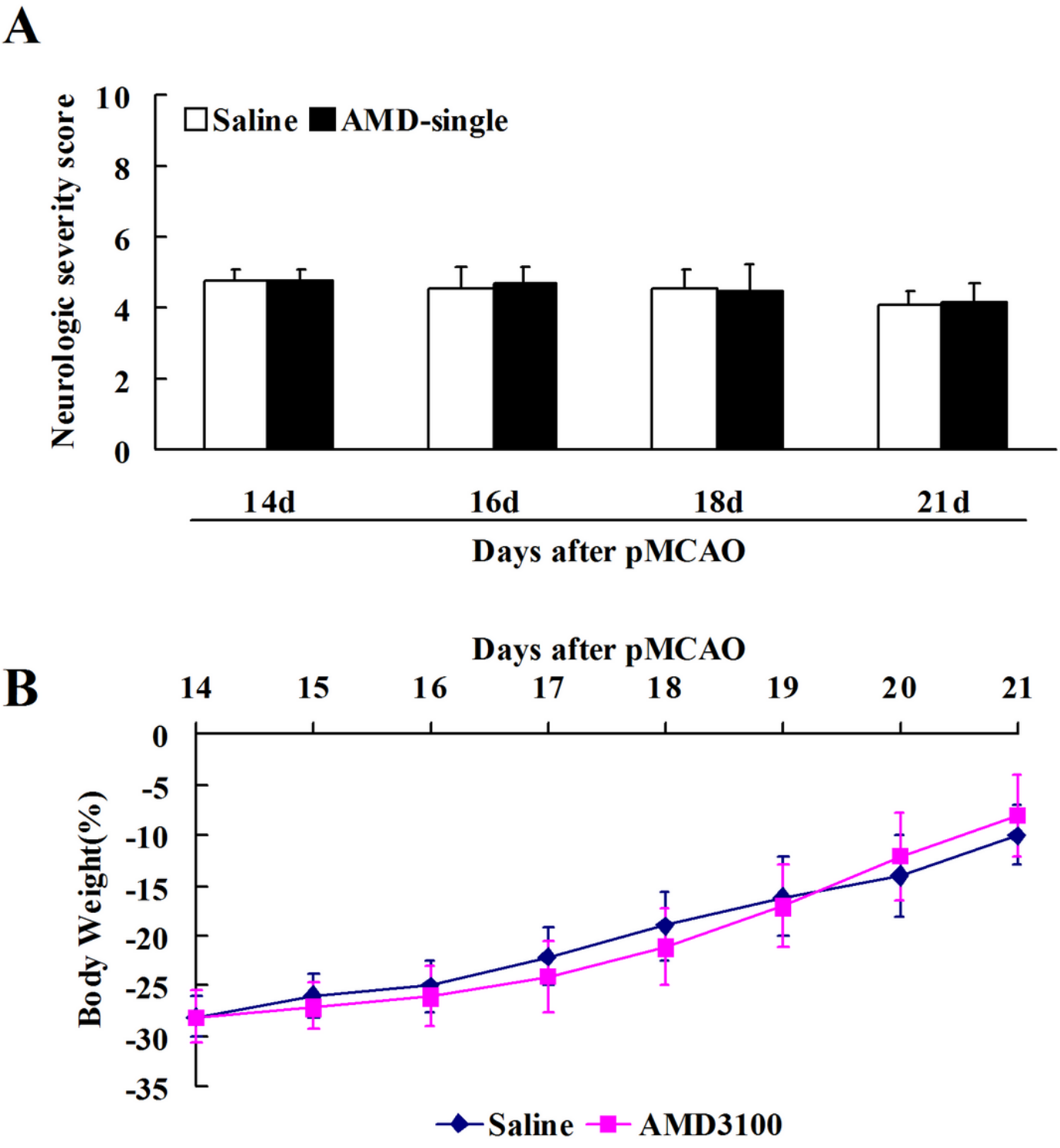
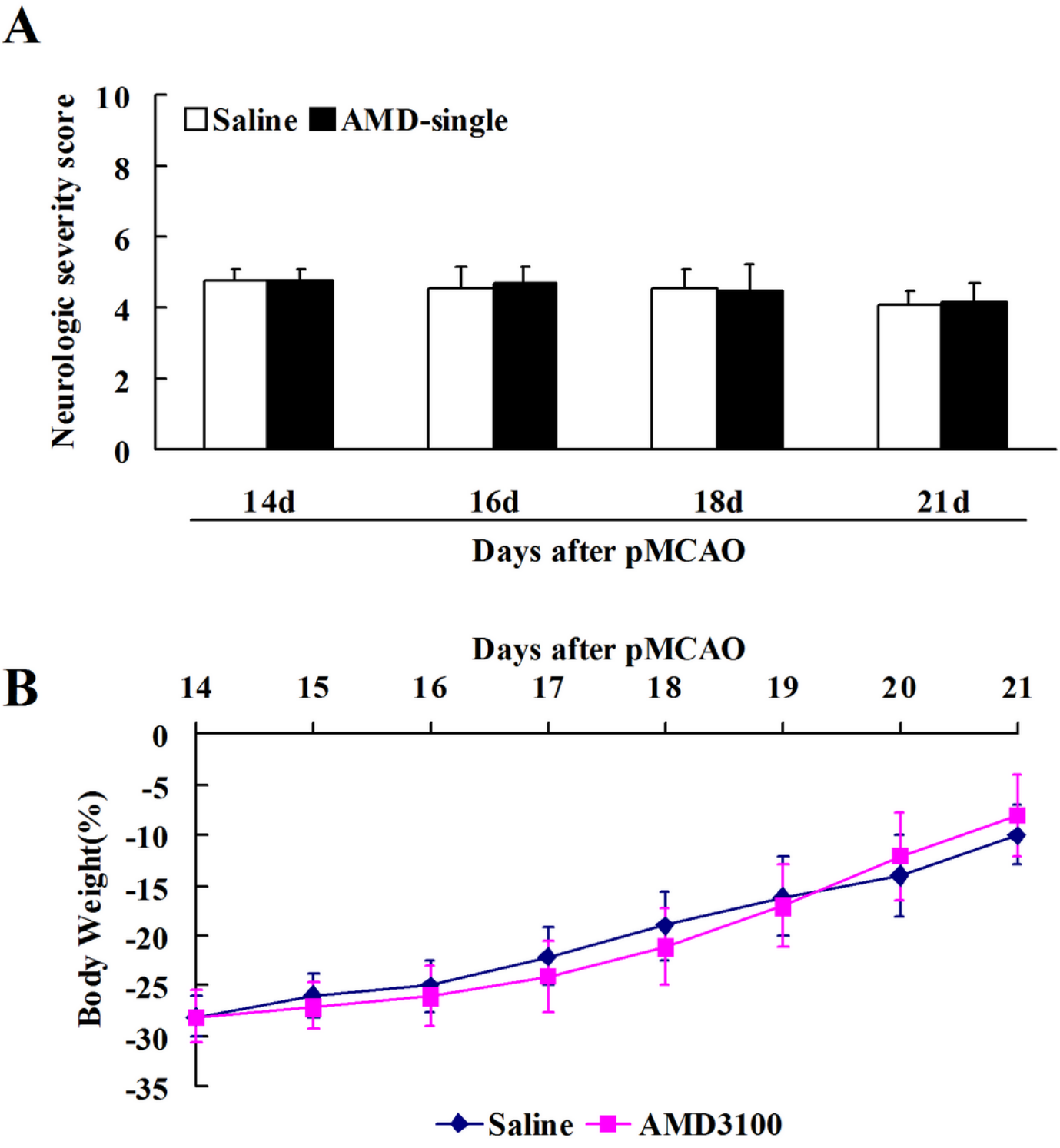


Figure 4

Single AMD3100 administration in later phase did not improved neurological outcome after pMCAO. From day 14 after pMCAO, the neurologic severity score and body weight loss of rats treated with saline (blue) or single AMD3100 (red) were evaluated from day 14 to day 21. Bar graph (A) shows neurologic severity score and curve graph (B) shows body weight loss. Data are mean±SEM, n=3 to 5 per group.



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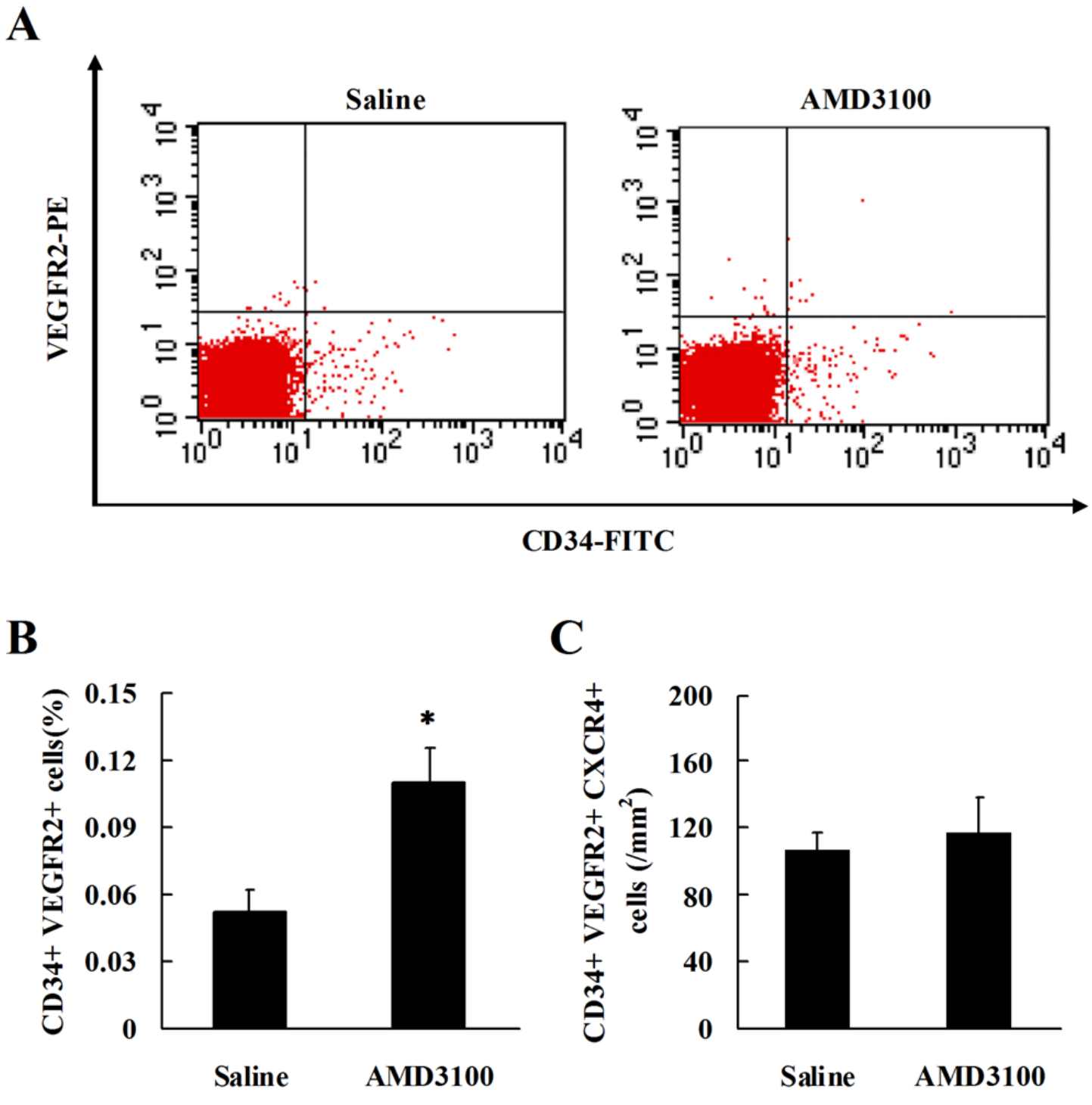
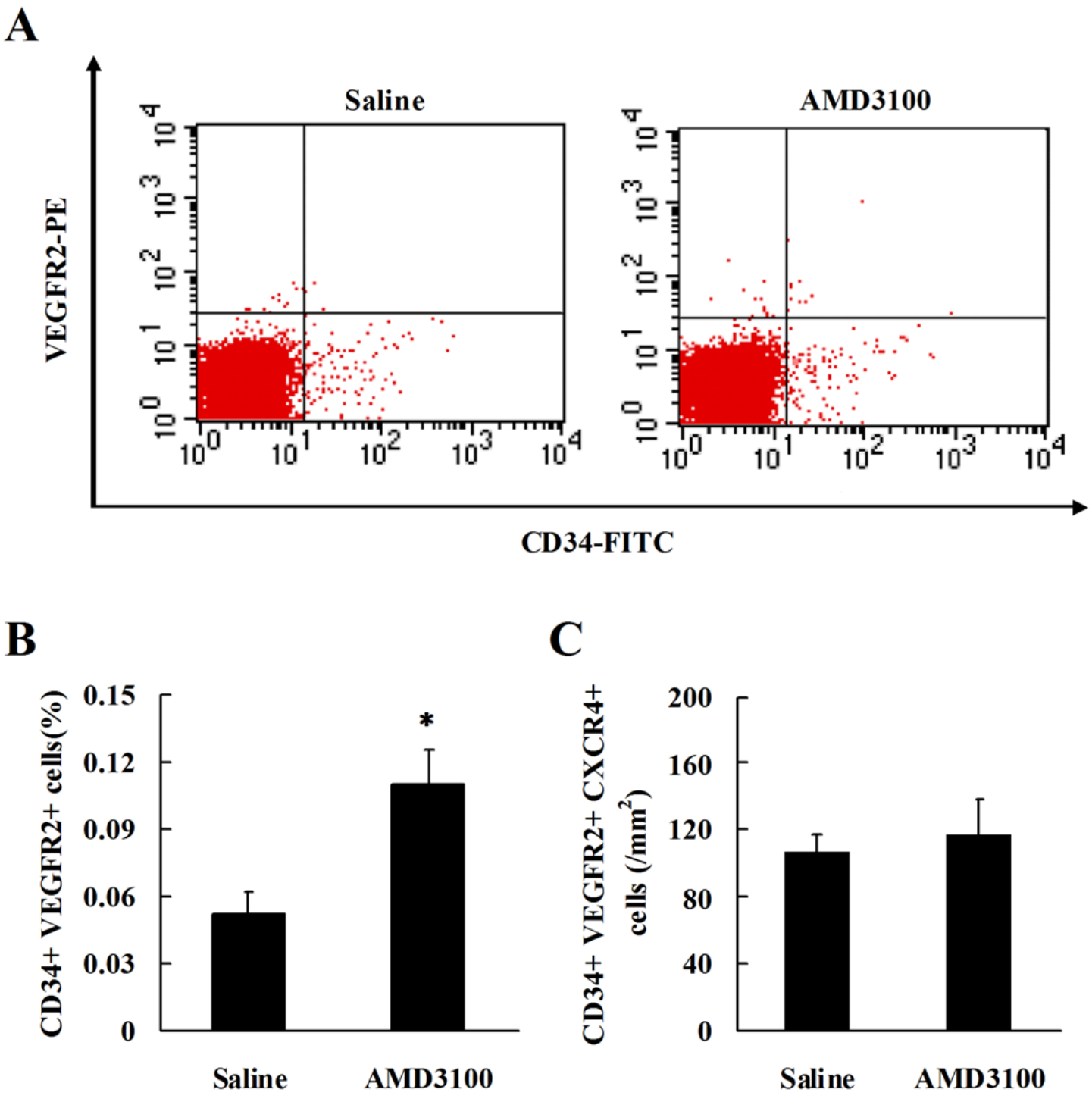


Figure 5

AMD3100 administration in later phase improved mobilization of endogenous EPCs, but did not significantly alter recruitment of EPC after pMCAO. (A) The peripheral blood (PB) EPCs (labeled by CD34

and VEGFR2) were confirmed by flow cytometry analysis on day 21 after AMD3100 administration on day 14. (B) Bar graph shows the percent of circulating EPCs on day 21 after AMD3100 administration. Data are mean±SEM, n=3 to 5 per group.□p□0.05, AMD3100 vs. saline. (C) Endogenous EPC recruitment in IBZ was examined by immunofluorescence staining with CD34, VEGFR2 and CXCR4 on day 21 after single AMD3100 treatment on day 14. The bar graph shows that there was no difference of endogenous EPCs recruitment between single AMD3100 and saline treatment.



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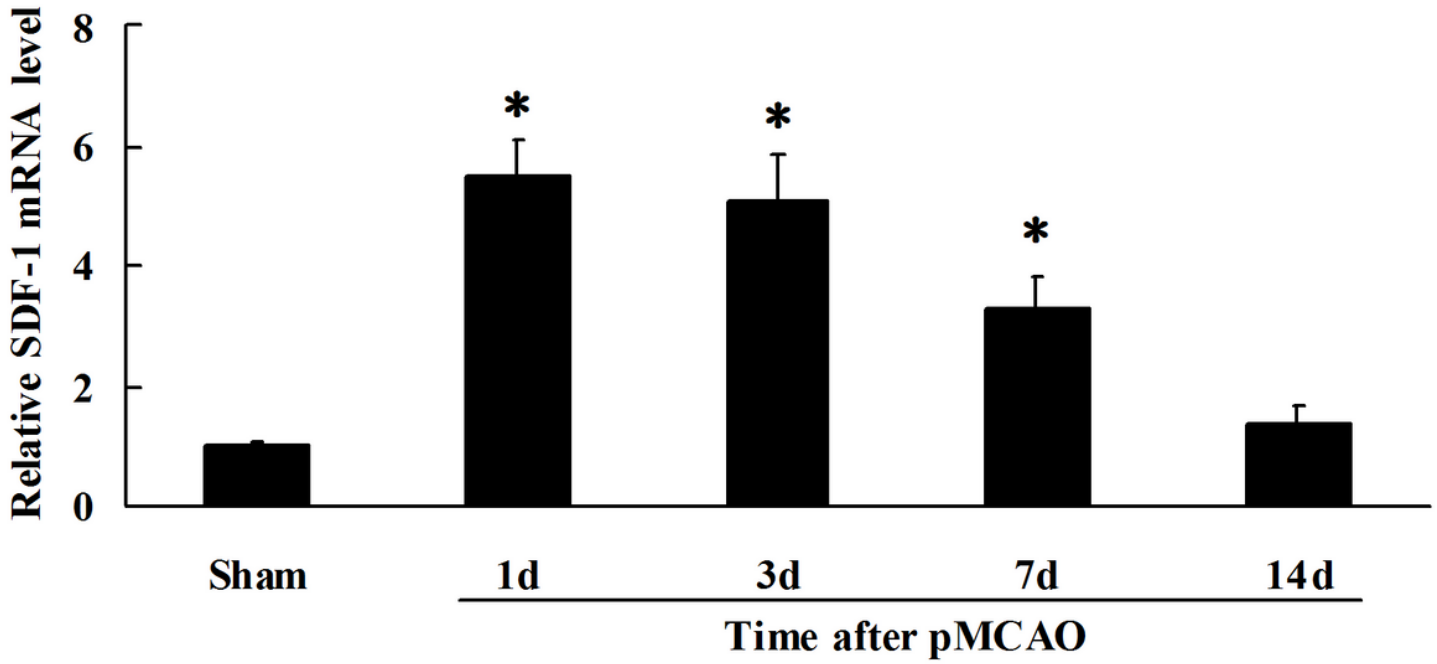
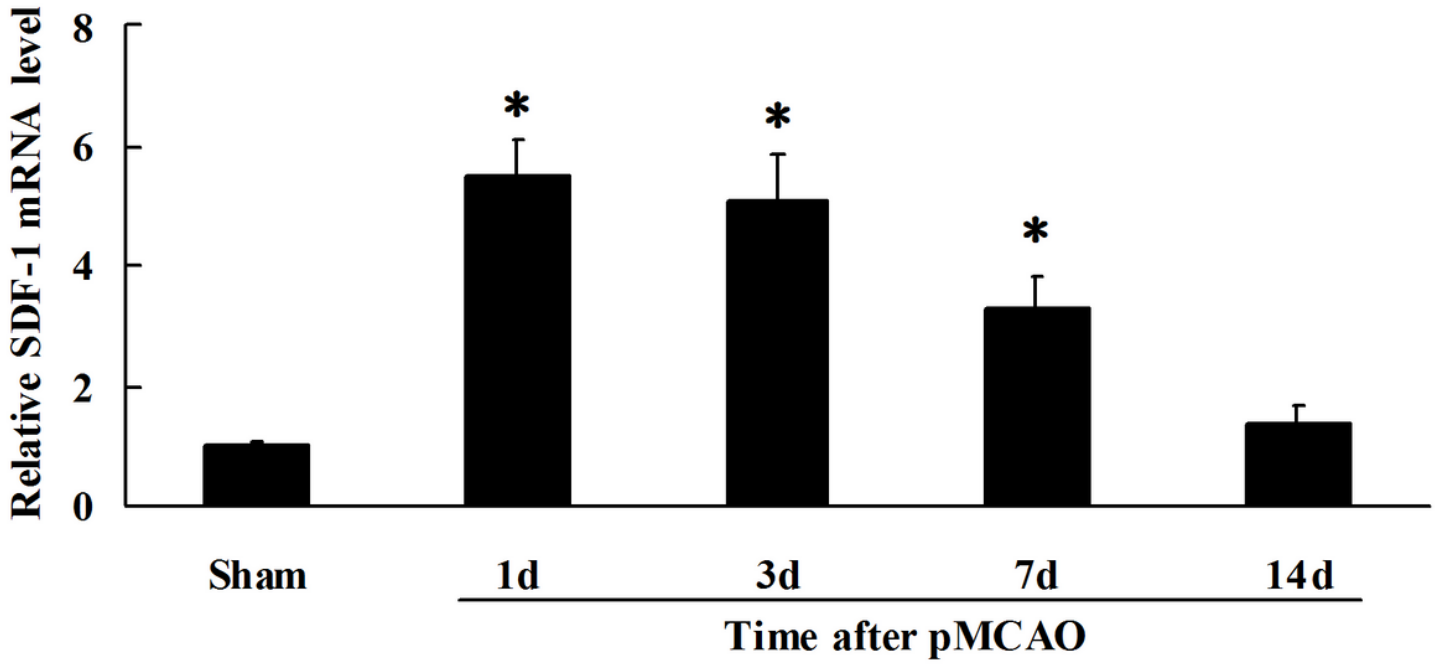


Figure 6

The relative expression of SDF-1 in IBZ at different days after pMCAO. The relative expression of SDF-1 in IBZ on day 1, day 3, day 7 and day 14 were determined by real-time PCR as well as in tissue of sham-operated rats.□p□0.05, vs. Sham group.



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