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Original Article

Protective effects of AMD3100 on ischemic stroke by inhibiting inflammation and promoting angiogenesis and neuranagenesis

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Abstract: Ischemic stroke is one of common neurological diseases with high morbidity rate. CXCL12/CXCR4 plays an important role in development of ischemic stroke. AMD3100 is a selective antagonist of CXCR4. This study aims to investigate the protective effects of AMD3100 in ischemic stroke and its possible mechanisms. Firstly, we establish Middle Cerebral Artery Occlusion (MCAO) model and treated with AMD3100 in the acute stage. Then, the volume of cerebral infarction and the levels of IL-6, TNF-α and IFN-γ were detected in the acute stage of ischemic stroke, the diameter and area of microvascular, the neural progenitor cells proliferation and the neuranagenesis ability were detected in the recovery stage. The results showed that, after treated with AMD3100, the infarct volume, the level of IL-6, TNF-α and IFN-γ were significantly reduced in the acute stage of ischemic stroke. And the cerebral angiogenesis, neural progenitor cells proliferation and neuranagenesis ability were significantly increased in the recovery stage of ischemic stroke. Therefore, we concluded that AMD3100 plays an protective effect in ischemic stroke by inhibiting inflammation and promoting angiogenesis, neural progenitor cells proliferation and neuranagenesis.

Keywords: Ischemic stroke, CXCL12/CXCR4, AMD3100, inflammation, angiogenesis, neuranagenesis

Introduction

Ischemic stroke is caused by clogged blood vessels, thereby putting target organs at risk of cellular death, Ischemic strokes often result in death or severe disability [1]. In the early stage of cerebral ischemia, a large number of chemokines may be induced, causing the production of oxygen free radicals and inflammatory factors, leading to inflammatory reactions and causing necrosis and apoptosis of neurons. Further development of cerebral ischemia can inhibits brain angiogenesis, proliferation of neural stem cells, and regeneration of nerve cells, resulting in permanent neurological damage and neurological deficits [2]. Recent studies have shown that inhibition of ischemic chemokine expression is considered to be a potential target for ischemic stroke [3, 4].

CXC chemokine ligand 12 (CXCL12) and its receptor CXC ligand receptor 4 (CXCR4) plays an important role in all stages of ischemic stroke. It involves the regulation of inflammatory cells, and mobilization, migration and proliferation of endothelial progenitor cells (EPCs) and neural progenitor cells (NPCs) [5, 6]. It is an important targets for ischemic stroke. AMD-3100 is a selective antagonist of CXCR4. In the acute stage of ischemic stroke, AMD3100 can antagonize the migration and infiltration of inflammatory cells [7]. In the recovery stage of ischemic stroke, AMD3100 can mobilize bone marrow or peripheral blood stem cells, and recruit EPCs and NPCs that from Subventricular zone (SVZ) to reach ischemic site, and regulate the proliferation or apoptosis, thereby promoting angiogenesis and neuranagenesis [8].

Our previous study showed that in the acute stage of stroke, AMD3100 can antagonize the migration and infiltration of inflammatory factor of IL-6, TNF-α and IFN-γ in rat ischemic stroke model, thus inhibit the development of inflammation, and reduce the acute injury of brain tissue. This article will continue to investigate the effects of AMD3100 on cerebral angiogenesis,

neural progenitor cells proliferation and neuranagenesis in the recovery period of ischemic stroke.

Materials and methods

Experimental animal and reagents

Experimental animals: Seventy-two clean grade healthy male Sprague Dawley (SD) rats, weighing 2800-3200 g, were provided and fed by the experimental center of Meihekou Central Hospital (animal certification number was Mhkh-201701-553). All animals were adaptive feeding 7d before experiment, environmental temperature was controlled between 22 to 26°C, the humidity was controlled between 40 to 70%, eat and drink freely. All the animals, reagents and treatment methods used in the experiments were approved by the animal experiment ethics committee of Meihekou Central Hospital. AMD3100 (Plerixafor) were purchased from Abcam Co., Ltd (USA), EINECS numbers was S803110, specification was 100 uM, purity was 99.31%. 2, 3, 5-triphenyltetrazolium chloride (TTC), FITC-dextran, Hoechst and Thymidine analog 5-bromo-2-deoxyuridine (BrdU) were purchased from Sigma Co. Ltd (USA). Rat anti BrdU IgG, Goat anti mouse DCX polyclonal antibody, Goat anti mouse NeuN polyclonal antibody and mouse anti-human β-actin polyclonal antibody were purchased from Abcam Co., Ltd (USA). Rabbit anti IL-6, TNF-α and IFN-γ antibodies were purchased from Abcam Co., Ltd (USA).

Methods

Establishment of ischemic stroke model of rat: Establishment of Middle Cerebral Artery Occlusion (MCAO) model: Fasting for 12 h before the operation and then intraperitoneal injection of 3% pentobarbital sodium (40 mg/kg) for anesthesia. Rats were fixed on the operating table with right arm reclining. Exposing the left temporal, cut the hair off, and then the operation area was disinfected with iodine complex and covered with sterile hole sheets. Split the skin, isolate the temporalis muscle, and expose the temporal wing plates, drill to duramater with tooth drill, then expose the proximal end of the middle cerebral artery, block middle cerebral artery between olfactory bundle and venae cerebri inferiores with electric coagulating apparatus, at last, suture the temporalis muscle and the skin sequentially. Prevention of infection by intraperitoneal injection of gentamycin sulfate.

Animal grouping: Seventy-two SD rats were randomly divided into three groups with twenty-four rats per group. The Sham operation group was subjected to craniotomy but the middle cerebral artery was not blocked. MCAO model group and AMD3100 treatment group were established into models of MCAO. AMD3100 treatment group were injected intraperitoneally with AMD3100 of 0.5 mg/kg/24 h, Sham operation group and MCAO model group were injected intraperitoneally with corresponding volume of 0.9% normal saline. Continuous administration for 3 d.

The volume of cerebral infarction was measured by TTC staining: Four days later, TTC staining was used to measure the volume of cerebral infarction. Four rats in each group were killed, and the brain tissue was carefully removed and placed in the brain slice mold. Then, 4 coronal slices of 1 mm thick were cut, and placed into an isotonic phosphate buffer solution containing 2% TTC rapidly. A few minutes later, the normal tissue showed rosy red, and the infarcted tissue was pale. The cerebral infarction volume of each animal were calculated and analyzed by Image J image system. The percentage of infarct volume to the contralateral hemisphere was used as the final result, in order to eliminate the influence of cerebral edema.

The levels of IL-6, TNF-α and IFN-γ were detected by Immunohistochemistry: Four days later, four rats in each group were killed, and the brain tissue was carefully removed and placed in the brain slice mold. Then, coronal slices of 1 mm thick were cutted. All the slices had gone through the conventional dewaxing and hydration process and were handled with 3% H_2O_2 solution. After sealing solution, rabbit anti-rat IL-6, TNF-α and IFN-γ polyclonal antibody were respectively added (the effective concentration was 1:100). Then all the slices were placed at 4°C overnight and were added with goat anti-rabbit IgG in the next day with incubation at 37°C for 30 min. Visualization was done by DAB solution and redye with hematoxylin. The negative control group was dealt with PBS buffer instead of the primary antibody. 5 fields of view under high power magnification were randomly selected from each sample and the cell in which

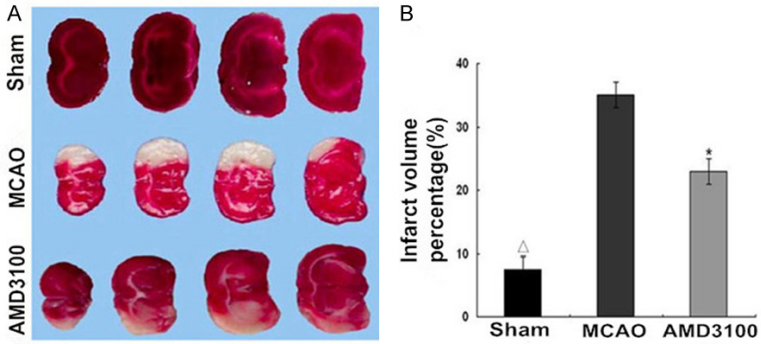

Figure 1. The volume of cerebral infarction was measured by TTC staining (n = 4). A. TTC staining. B. The percentage of infarct volume. $^{\Delta}P < 0.05$ versus MCAO model group and AMD3100 treatment group. $*P < 0.05$ versus MCAO model group.

the cytoplasm was stained brown or yellow was considered as expression-positive cell. Then the percentage of positive cells in total cells was calculated. Percentage of positive cells = (number of positive cells/total cells) * 100%.

The diameter and area of three-dimensional microvascular: Fourteen days later, four rats in each group were injected 1 mL FITC-dextran with 50 mg/mL from femoral vein, then the rats were decapitated and brain was removed. After 48 h fixation with 4% paraformaldehyde, a coronal oscillatory section was performed on the infarct site. Lamina thickness was 100 μm. Eight interest areas were selected by laser scanning confocal microscopy, and the sequence was scanned with 0.5 μm step size for 100 layers. After three-dimensional reconstruction of the image, two experienced researchers who did not know the experiment were designed to analyze the diameter and area of the microvasculature using 3D Doctor 3.5 edition software.

Neural progenitor cells proliferation was detected by BrdU immunofluorescence staining: Fourteen days later, four rats in each group were killed, Then, coronal slices of 1 mm thick were cut. Clean three times by 0.01 M PBS. Subsequently slices were immersed in 2 mmol/L HCl, incubated for 30 min, and immersed in 0.01 mol/L boric acid solution with PH = 8.5, neutralized for 10 min. Then, serum dilution containing rat anti BrdU antibody at concentration of 1:300 were added, and temperature incubation, 4°C for the night. Afterwards, drop biotinylated Goat anti rat IgG at concentration of 1:300 and fluorescein linked streptavidin in turn, and incubate at room temperature for at least 2 h. Lastly, dehydration, mount, and observed under microscopic.

Neuranagenesis ability was detected by DCX/NeuN labed immunofluorescence staining: Fourteen days later, four rats in each group were killed, Then, coronal slices of 1 mm thick were cutt. All the slices had gone through the conventional dewaxing and hydration process and were handled with 3% H_2O_2 solution. After sealing solution, rabbit anti-rat DCX and NeuN polyclonal antibody were respectively added (the effective concentration was 1:100). Then all the slices were placed at 4°C overnight and were added with goat anti-rabbit BrdU IgG in the next day with incubation at 37°C for 30 min. Visualization was done by DAB solution and redyeing was finished with hematoxylin. The negative control group was dealt with PBS buffer instead of the primary antibody. 5 fields of view under high power magnification were randomly selected from each sample and the cell in which the cytoplasm was stained brown or yellow was considered as expression-positive cell. Then the percentage of positive cells in total cells was calculated. Percentage of positive cells = (number of positive cells/total cells) * 100%.

Statistical analysis

SPSS17.0 statistics software was employed to undergo statistical analysis. The measure data are represented by x ± s, t the Student's t-test of two factors is used in the comparison of averages between various groups, whereas for comparing two groups, LSD is applied. A two-tailed value of $P < 0.05$ was considered statistically significant. Thep values were designated as $^{\Delta}P < 0.05$, $*P < 0.05$ and $**P < 0.01$.

Results

TTC staining

After TTC staining, the normal tissue showed rosy red, and the infarcted tissue was pale, mainly located in frontoparietal cortex and cau-

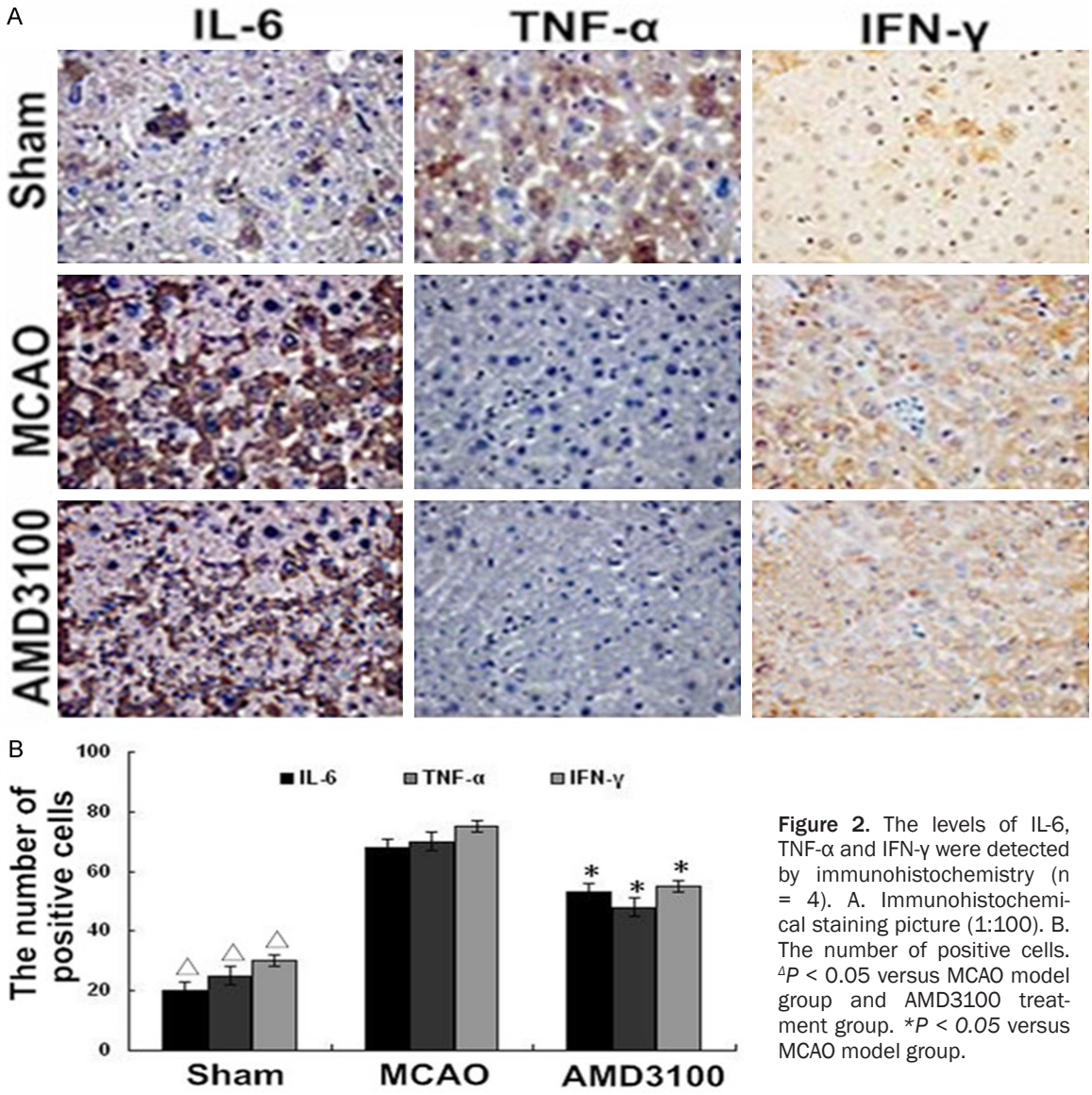

Figure 2. The levels of IL-6, TNF-α and IFN-γ were detected by immunohistochemistry (n = 4). A. Immunohistochemical staining picture (1:100). B. The number of positive cells. $^{\Delta}P < 0.05$ versus MCAO model group and AMD3100 treatment group. *$P < 0.05$ versus MCAO model group.

doputamen, some tissues showed semi dark. The TTC staining results demonstrated that there was no obvious pale infarct area in the sham operation group, the MCAO model group showed a significantly larger range of pale infarcts, the AMD3100 treatment group also showed a significantly larger range of pale infarcts,but infarct volume percentage was significantly lower than that in the MCAO model group ($P < 0.05$, **Figure 1**). It was suggested that AMD3100 showed protective effects in ischemic stroke.

AMD3100 can inhibit the production of IL-6, TNF-α and IFN-γ

Compared with sham operation group, the levels of IL-6, TNF-α and IFN-γ in MCAO model group and AMD3100 treatment group were increased significantly ($P < 0.05$, **Figure 2**). But, compared with MCAO model group, the levels of IL-6, TNF-α and IFN-γ in AMD3100 treatment group were decreased significantly ($P < 0.05$, **Figure 2**). It was demonstrated that AMD3100 can antagonize the the migration and infiltration of inflammatory factor IL-6, TNF-α and IFN-γ in the acute stage of ischemic stroke.

AMD3100 promote angiogenesis

The results showed that the diameter of microvessels in MCAO model and AMD3100 treatment group was obviously smaller than that of sham operation group, but the area significantly higher. While, the diameter and area

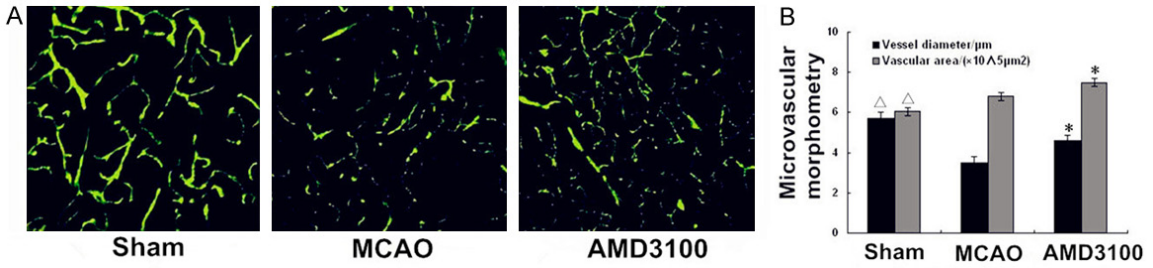

Figure 3. The diameter and area of three-dimensional microvascular were measured by FITC-dextran imaging (n = 4). A. FITC-dextran imaging. B The diameter and area of microvascular. $^{\Delta}P < 0.05$ versus MCAO model group and AMD3100 treatment group. *$P < 0.05$ versus MCAO model group.

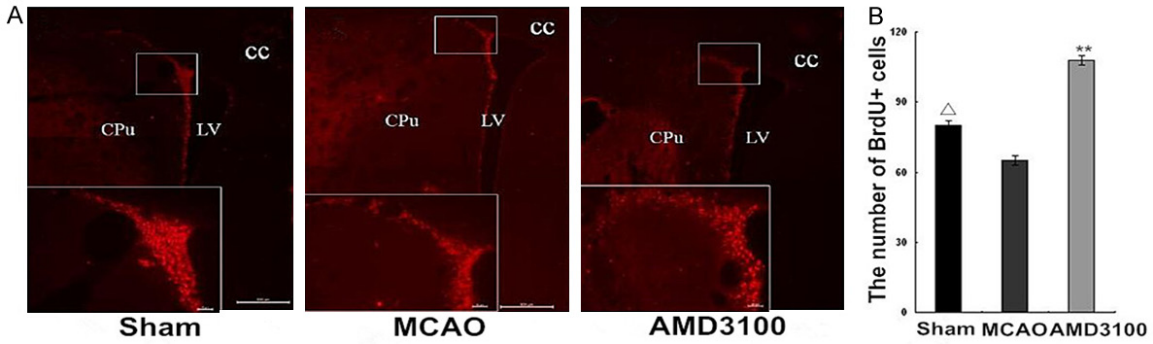

Figure 4. Neural progenitor cells proliferation was detected by BrdU immunofluorescence staining (n = 4). A. BrdU staining imaging. B. The number of BrdU staining cells. $^{\Delta}P < 0.05$ versus MCAO model group and AMD3100 treatment group .*$P < 0.05$ versus MCAO model group.

of microvessels in AMD3100 treatment group were significantly higher than those in the model group ($P < 0.05$, **Figure 3**). It was indicated that AMD3100 can promote angiogenesis in the recovery stage of ischemic stroke.

AMD3100 promote NPCs proliferation

The BrdU immunofluorescence staining results demonstrated that the Brd U+ cells of ischemic side in AMD3100 treatment group was significantly more than those in the model group, and even higher than sham operation group ($P < 0.05$, **Figure 4**). It was suggested that AMD3100 can promote NPCs proliferation in the recovery stage of ischemic stroke.

AMD3100 promote neuranagenesis

The Immunohistochemistry results showed that the positive cells in AMD3100 treatment group was significantly more than those in the model group and sham operation group ($P < 0.05$, **Figure 5**). It was suggested that AMD3100 can promote neuranagenesis in the recovery stage of ischemic stroke.

Discussion

Cerebrovascular Diseases (CVD) is the most common and multiple neurological disease. In recent years, it has become the leading cause of death and disability of the global population, and a serious threat to human health and safety. While acute ischemic stroke (cerebral infarction) accounting for about 60% to 80% of all cases is the most common type of stroke. Even in the survival of patients with stroke, still there are varying degrees of neurological dysfunction, lead to varying degrees of disability, carry a heavy burden to families and society [2, 3].

After ischemic stroke, there is a close correlation between the inflammatory response, angiogenesis and neurogenesis in the injured area, which together affect the recovery of neurological function. Among them, the immune inflammatory reaction is involved in every stage of the ischemic cascade. Therefore, the prognosis of ischemic stroke may be improved by adjusting the immune inflammatory response to achieve a balance between the three

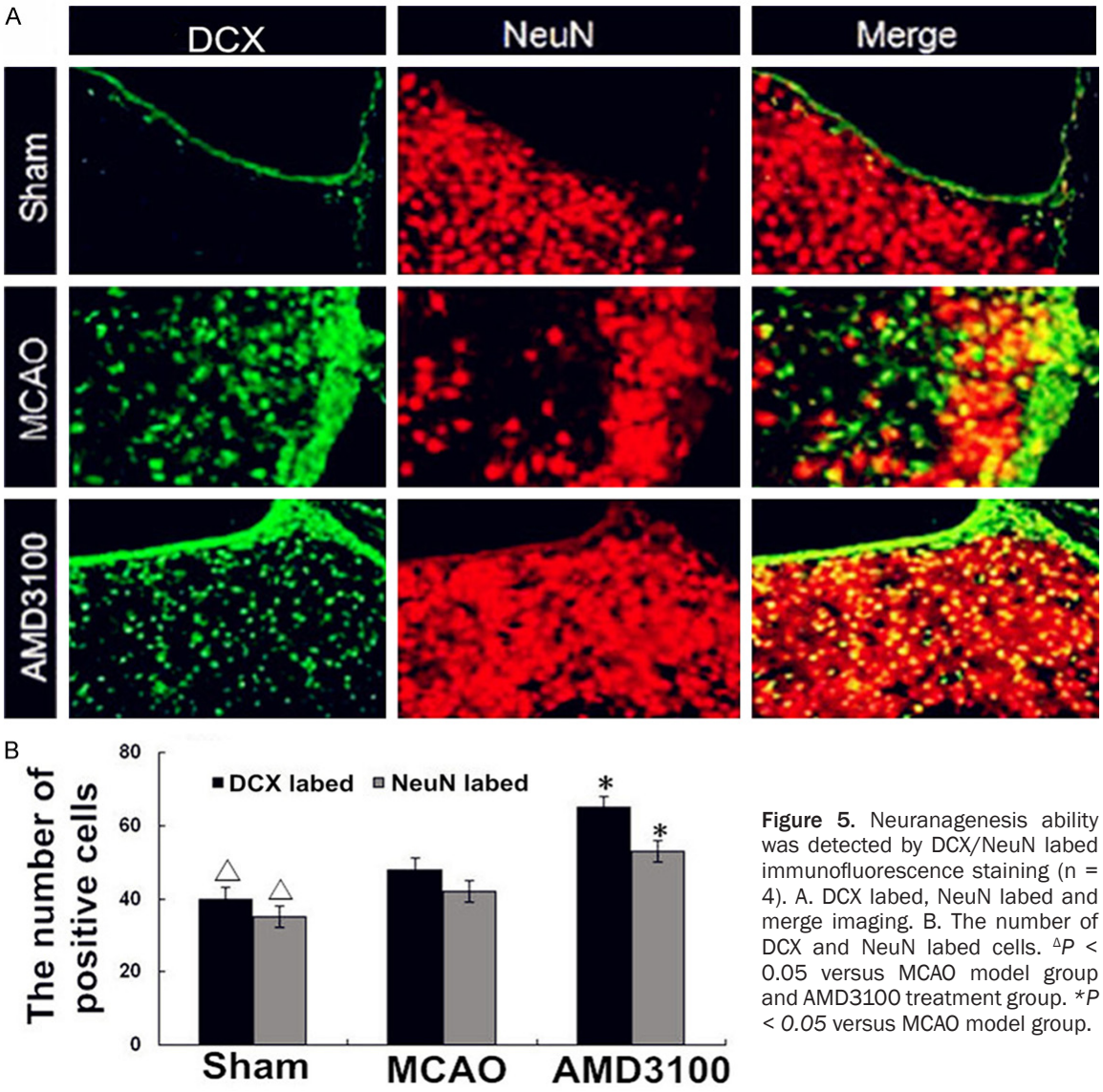

Figure 5. Neuranagenesis ability was detected by DCX/NeuN labed immunofluorescence staining (n = 4). A. DCX labed, NeuN labed and merge imaging. B. The number of DCX and NeuN labed cells. $^{\Delta}P < 0.05$ versus MCAO model group and AMD3100 treatment group. $^{*}P < 0.05$ versus MCAO model group.

aspects at different stage of ischemic stroke [9]. Previous studies have also shown that the potential thrombolytic therapeutic time window may be prolonged by inhibiting the inflammatory response. And it also can protect the blood-brain barrier in the early stage and prevent the reperfusion injury. However, the interaction between the immune system and the central nervous system is very complex [10]. Therefore,there is no specific target for the treatment of immune inflammatory response of ischemic stroke.

CXC chemokine ligand 12 (CXCL12) was a powerful inflammatory chemokine known as stromal cell derived factor-1 (SDF-1) and pre-B cell stimulatory factor (PBSF) [11]. CXCL12 has two receptors, they are CXCR4 and CXCR7 respectively, the expression of CXCR7 is beneficial to cell growth, survival and adhesion, and the expression of activated CXCR4 is beneficial to cell proliferation and migration [12]. CXCL12/CXCR4 was participate in the inflammatory reaction and the process of cell migration and homing after ischemia and reperfusion, which are closely related to Neuronal apoptosis, degeneration and neurogenesis, and also angiogenesis. It is an important target in the course of ischemic stroke [13].

AMD3100 is a selective antagonist of CXCR4. AMD3100 can inhibits CXCL12-mediated chemotaxis with a potency lightly better than its affinity for CXCR4. AMD3100 also antagonizes SDF-1/CXCL12 ligand binding with an IC50 of 651 nM. But it does not inhibit calcium flux

against cells expressing CXCR3, CCR1, CCR2b, CCR4, CCR5 or CCR7 when stimulated with their cognate ligands, nor does inhibit receptor binding of LTB4. It can promote angiogenesis and neuranagenesis and antagonize the infiltration of inflammatory cells [14]. Currently, AMD3100 has been approved by the FDA as a mobilizing agent for hematopoietic stem cells for clinical use. In the acute stage of ischemic stroke, AMD3100 can antagonize the migration and infiltration of inflammatory cells, such as L-6, TNF-α and IFN-γ, and also nitricoxide synthase (NOS), reactive oxygen species (ROS), matrix metalloproteinase (MMPs), therfore, inhibiting the occurrence of inflammation and reducing the acute injury of brain tissue [15]. In the recovery stage of ischemic stroke, AMD3100 can mobilize bone marrow or peripheral blood stem cells, and recruit EPCs and NPCs that from Subventricular zone (SVZ) to reach ischemic site, and regulate the proliferation or apoptosis, thereby promoting angiogenesis and neuranagenesis. Moreover, EPCs and NPCs, which are collected at the ischemic site, can stimulate angiogenesis and neurogenesis through paracrine mechanisms [16].

In our study, we successfully established Middle Cerebral Artery Occlusion (MCAO) model and treated it with AMD3100 during the acute phase of ischemic stroke. Our results showed that, after treated with AMD3100, the infarct volume, inflammatory factors such as IL-6, TNF-α and IFN-γ of rat brain tissues were significantly reduced in the acute stage of ischemic stroke. Fourteen days later, we further studied brain tissue in rats. We found that the cerebral angiogenesis, neural progenitor cells proliferation and neuranagenesis were significantly increased.

In conlusion, AMD3100 can inhibit inflammation, which prevent brain from acute injury in the acute stage of ischemic stroke, and promote angiogenesis, neural progenitor cells proliferation and neuranagenesis in the recovery stage of ischemic stroke. It plays an obvious protective effect in the cerebral injury of ischemic stroke. Worthy of further research and exploration.

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Disclosure of conflict of interest

None.

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