Possible Mechanism of Therapeutic Effect of 3-Methyl-1-phenyl-2-pyrazolin-5-one and Bone Marrow Stromal Cells Combination Treatment in Rat Ischemic Stroke Model

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Abstract

Background: The functional improvement following bone marrow stromal cells (BMSCs) transplantation after stroke is directly related to the number of engrafted cells and neurogenesis in the injured brain. Here, we tried to evaluate whether 3-methyl-1-phenyl-2-pyrazolin-5-one (MCI-186), a free radical scavenger, might influence BMSCs migration to ischemic brain, which could promote neurogenesis and thereby enhance treatment effects after stroke.

Methods: Rat transient middle cerebral artery occlusion (MCAO) model was established. Two separate MCAO groups were administered with either MCI-186 or phosphate-buffered saline (PBS) solution to evaluate the expression of stromal cell-derived factor-1 (SDF-1) in ischemic brain, and compared to that in sham group (n = 5/group/time point [at 1, 3, and 7 days after operation]). The content of chemokine receptor-4 (CXCR4, a main receptor of SDF-1) at 7 days after operation was also observed on cultured BMSCs. Another four MCAO groups were intravenously administered with either PBS, MCI-186, BMSCs (2 × 106), or a combination of MCI-186 and BMSCs (n = 10/group). 5-bromo-2-deoxyuridine (BrdU) and Nestin double-immunofluorescence staining was performed to identify the engrafted BMSCs and neuronal differentiation. Adhesive-removal test and foot-fault evaluation were used to test the neurological outcome.

Results: MCI-186 upregulated the expression of SDF-1 in ischemic brain and CXCR4 content in BMSCs was enhanced after hypoxic stimulation. When MCAO rats were treated with either MCI-186, BMSCs, or a combination of MCI-186 and BMSCs, the neurologic function was obviously recovered as compared to PBS control group (P < 0.01 or 0.05, respectively). Combination therapy represented a further restoration, increased the number of BMSCs and Nestin+ cells in ischemic brain as compared with BMSCs monotherapy (P < 0.01). The number of engrafted-BMSCs was correlated with the density of neuronal cells in ischemic brain (r = 0.72, P < 0.01) and the improvement of foot-fault (r = 0.70, P < 0.01).

Conclusion: MCI-186 might promote BMSCs migration to the ischemic brain, amplify the neurogenesis, and improve the effects of cell therapy.

Key words: 3-Methyl-1-phenyl-2-pyrazolin-5-one; Bone Marrow Stromal Cells; Ischemic Stroke; Neurogenesis; Stromal Cell-derived Factor-1

Introduction

Stem cell therapy is a promising method to remodel the injured brain after ischemic stroke.[1] Bone marrow stromal cells (BMSCs) can be easily obtained from patients themselves without ethical or immunological problems. Interaction of BMSCs and ischemic brain might lead the stem cells to produce trophic factors and promote neurogenesis, those might contribute to function recover.[2,3] Therefore, BMSCs have emerged as a potential candidate for cell therapy after stroke. Stromal cell-derived factor-1/chemokine receptor-4 (SDF-1/CXCR4) axis has been
demonstrated to play an important role in BMSCs migration to injury tissue.\textsuperscript{[45]} SDF-1 is presented around damaged central nervous system (CNS)\textsuperscript{[67]} and its receptor CXCR4 is widely expressed on BMSCs. Therefore, the transplanted BMSCs have the potential to be guided toward ischemic brain, around which SDF-1 is expressed.\textsuperscript{[9]}

3-Methyl-1-phenyl-2-pyrazolin-5-one (MCI-186), a free radical scavenger, can enhance the number of endogenous neural stem cells (NSCs) in the ischemic brain after a stroke.\textsuperscript{[9]} However, there are no reports to evaluate whether MCI-186 can influence the migration of transplanted BMSCs to ischemic brain and thus amplify the neurogenesis and improve the effects of cell therapy. In the present study, we aimed to examine the hypothesis. In vitro, CXCR4 expression on normal and hypoxic BMSCs was examined using immunostaining and enzyme-linked immunosorbent assay (ELISA). In vivo, MCI-186 was injected into the right middle cerebral artery occlusion (MCAO) rats to monitor the level of SDF-1 in ischemic brain. Furthermore, we treated MCAO rats with BMSCs, MCI-186, or a combination of MCI-186 and BMSCs to determine the donor BMSCs migration toward ischemic brain and neurogenesis and to assess whether this combination treatment could induce additional functional improvement after a stroke.

**Methods**

All experimental procedures were approved by the Nantong University Administrative Panel on Laboratory Animal Care (No: NTU200805-14) and conformed to international guidelines on the ethical use of animals.

**Isolation and characterization of bone marrow stromal cells**

The BMSCs were harvested from male Sprague-Dawley rats (3–4 weeks old). The procedures were reported previously.\textsuperscript{[10]} The adherent cells were passed 4 times. At passage 4 (P4), cells were harvested for experiments. Some BMSCs were incubated at 37°C in flasks to identify the localization of CXCR4 on BMSCs. A rabbit polyclonal antibody CXCR4 (1:200, Abcam, Hong Kong, China) was used for cellular fluorescence labeling. Hoechst 33342 (2 μg/ml, Sigma, USA) was used for staining cell nuclei. The rest cells were labeled with bromodeoxyuridine (5-bromo-2-deoxyuridine [BrdU], 30 μg/ml, Sigma, St. Louis, MO, USA) for 3 days before intravenous administration to identify the cell migration in the ischemic brain, and then the labeled cells were washed and digested for transplantation.

**Enzyme-linked immunosorbent assay for chemokine receptor-4 in cultured bone marrow stromal cells**

Cultured BMSCs (P4) were incubated under normoxic (21% O\textsubscript{2}) or hypoxic (1% O\textsubscript{2}) conditions for 24 h, then the culture mediums were withdrawn and the protein extract was obtained from BMSCs. CXCR4 was measured in these samples with an ELISA kit (Abcam, Hong Kong, China) for rat CXCR4 according to the manufacturer’s instructions.

**Fluorescent staining in cultured bone marrow stromal cells**

Some normoxic (21% O\textsubscript{2}) and hypoxic (1% O\textsubscript{2}) BMSCs grown on coverslips were fixed with 4% formaldehyde for 30 min, subsequently treated with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 5 min and blocked with PBS containing 3% normal goat serum for 1 h. Then, the cells were incubated overnight at 4°C with anti-CXCR4 antibodies, washed 3 times with PBS, and incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (1:100, Sigma, USA) for 1 h. After additional washes, the slides were incubated with Hoechst 33342 for 3 min and mounted with an antifade solution. Cells were examined under a Leica fluorescence microscope (Leica DM400B, Wetzlar, Germany).

**Animal transient middle cerebral artery occlusion model and experimental groups**

Adult male Sprague-Dawley rats weighing 240–270 g served as subjects, the animals were randomly allocated into different groups. All surgical procedures were conducted under aseptic conditions. MCAO rats were subjected to 2 h right MCAO via the intraluminal filament technique followed by reperfusion.\textsuperscript{[11]} The sham group received the same surgical procedures except that the right-sided MCA was not occluded. During the operation, rectal temperature was monitored in all animals and temperature was maintained at constant (37.0 ± 0.5°C) with a heating pad and an overhead lamp. Rats in vehicle-treated MCAO control group and the sham-operated group received an injection of PBS via the tail vein 1 time. Rats in MCI-186-treated group received intraperitoneal injections of MCI-186 (3 mg/kg, Promega, Madison, WI, USA) immediately after MCAO. Because of the short half-life of MCI-186, we applied the free radical scavenger twice a day until day 7. Rats in BMSCs-treated group received an intravenous injection of BMSCs (2 × 10\textsuperscript{5}) via the tail vein 24 h after MCAO. Rats in combination therapy group received MCI-186 and BMSCs after MCAO. First, we observed the effects of MCI-186 on the expression of SDF-1 in ischemic brain. Animals from the sham-operated group, MCAO control group, and MCI-186 treated group were sacrificed at 1, 3, and 7 days after operation for reverse transcriptase-polymerase chain reaction (RT-PCR) \((n = 5/\text{group/time point})\), and immunofluorescence staining was performed at 7 days after operation for SDF-1 \((n = 5/\text{group})\). Second, animals from MCAO control group, MCI-186-treated group, BMSCs treated group, and combination therapy group \((n = 10/\text{group})\) were sacrificed at 7 days after operation for immunohistochemical staining to identify engrafted-BMSCs and evaluate neurogenesis. Neurological function tests were performed at each time point.

**Reverse transcriptase-polymerase chain reaction assay of stromal cell-derived factor-1**

Total RNA was isolated from the ischemic boundary zone (IBZ, between the infarct core area and noninfarct area) using TRIzol reagent. RT-PCR was performed using Promega Revert Aid First Strand cDNA Synthesis Kit (Madison, WI, USA) according to
the manufacturer’s instructions. cDNA was synthesized with oligo-dT primers, and PCR was performed with designed primers using a master mix purchased from Promega. PCR products (5 μl) were analyzed in 2% agarose electrophoretic gels stained with ethidium bromide and visualized by ultraviolet transillumination. The density of each band was quantified with the Image 1.59 program (Vilber Lourmat, France). All results represented the average density of positive bands obtained from five independent experiments using different reaction cycles. The PCR primers used in this study were designed using online software Applied Biosystems, USA. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. The sequences of the primers were as follows:

- **SDF-1:** Forward: 5'-GGTCTGGAGACTATGACCTCCA-3; Reverse: 5'-GTGCTGGAACTGGAACACCA-3
- **GAPDH:** Forward: 5'-GTTCCAGTATGACTCTACCC-3; Reverse: 5'-AGTCTTGAGTGGTGATGGC-3.

For quantitative results, all samples were normalized against the GAPDH control band, and the results were expressed as the relative fold change of SDF-1/GAPDH.

### Immunofluorescence staining of brain sections of rats

The brains were fixed in 4% paraformaldehyde and embedded in paraffin, and a series of 5 μm thick coronal sections were cut from a standard paraffin block obtained from the center of the lesion (Bregma −1 to +1 mm) for immunostaining. Some sections were treated with the primary antibody of rabbit polyclonal anti-SDF-1 (1:100, Chemicon, USA) at room temperature for 2 h. After incubation, sections were washed in PBS and placed for 2 h in FITC-conjugated goat anti-rabbit IgG (1:100, Sigma, USA) for SDF-1 staining.

Double-immunofluorescence staining was employed to visualize the cellular colocalization of BrdU and Nestin for engrafted BMSCs and neuronal stem cells. Sections were incubated in 2 mol/L HCl at 37°C for 30 min to denature DNA and exposed BrdU, rinsed thoroughly in PBS and incubated in a mixture of mouse anti-BrdU (1:100, Sigma, USA) and rabbit anti-Nestin (1:100, Sigma, USA) at room temperature for 2 h. After incubation, sections were washed in PBS and placed for 2 h in tetramethyl rhodamine isothiocyanate-conjugated goat anti-rabbit IgG (1:100, Sigma, USA) for BrdU and FITC-conjugated goat anti-rabbit IgG (1:100, Sigma, USA) for Nestin. All sections were visualized with a fluorescence microscope (Leica DM4000B, Wetzlar, Germany). Immunoreactive cells were photographed using a 3-CCD color video camera (DXC-970MD, Sony, Japan) interfaced with Micro Computer Imaging Device software (Imaging Research, Leica, Germany).

For quantitative measurements of BrdU and Nestin positive cells, five sections from each block, each containing eight fields consisting of cortex and stratum from the IBZ[12] was digitized. Cell counts were averaged for each animal and expressed as cells/mm². All analyses were performed by observers who were blinded to the experimental groups.

### Neurological behavioral tests

BMSCs treated, MCI-186 treated, and combination treated MCAO rats (n = 10/group) received an adhesive-removal test and foot-fault evaluation[13,14] and were compared to the MCAO control group. Before MCAO and at 1, 3, and 7 days after MCAO, two investigators who were blinded to the experimental groups performed the assessment.

### Statistical analysis

All measurements were performed blinded. Results were presented as a mean ± standard deviation (SD). Statistical analyses were performed using SPSS 16.0 software (IBM, Chicago, IL, USA). The statistical differences between different groups were evaluated with one-way analysis of variance (ANOVA). A value of P < 0.05 was considered statistically significant. Correlation analysis was used to discuss the relationship.

### Results

#### Chemokine receptor-4 expression on bone marrow stromal cells

CXCR4 expression on normoxic and hypoxic BMSCs was evaluated using immunostaining [Figure 1a] and ELISA [Figure 1b], respectively. Immunostaining showed
the cultured BMSCs under normoxic condition just expressed a marginal CXCR4, but when cells were stimulated by hypoxia, the expression of CXCR4 was obviously upregulated. ELISA also revealed that the content of CXCR4 protein in hypoxic BMSCs was higher than that in normoxic culture cells \( (P < 0.05) \).

**3-Methyl-1-phenyl-2-pyrazolin-5-one upregulated stromal cell-derived factor-1 expression in ischemic brain**

The expression of SDF-1 in ischemic brain was monitored using immunostaining and RT-PCR. Immunofluorescent staining revealed that MCAO substantially increased the density of SDF-1 positive cells in the IBZ [Figure 2a]. RT-PCR also found that SDF-1 mRNA [Figure 2b and 2c] began to increase on day 1, peaked on day 3, and remained high on day 7 in the IBZ \( (P < 0.01, \text{respectively}) \) after MCAO as compared to sham. MCI-186 upregulated the expression of SDF-1 after ischemic stroke and postponed their decline \( (P < 0.05 \text{ vs. MCAO control group, } P < 0.01 \text{ vs. MCAO control group, respectively}) \).

**Combination therapy significantly improved neurological outcome**

Figure 3 shows that either MCI-186 or BMSCs monotherapy caused an improvement on adhesive-removal [Figure 3a] and foot-fault [Figure 3b] tests at 7 days after MCAO \( (P < 0.01 \text{ vs. MCAO control group, } P < 0.01 \text{ vs. MCAO control group, respectively}) \).

![Figure 2: Effects of MCI-186 on SDF-1 expression in ischemic brain.](image)

**Discussion**

Transplanted BMSCs can migrate selectively into ischemic brain area and present a promising recovery from stroke\[^{[24-26]}\] SDF-1/CXCR4 axis has been identified as a critical mediator for stem cell migration.\[^{[19]}\] SDF-1 is a small molecule with alternatively spliced forms, belonging to the CXC chemokine family.\[^{[17]}\] In CNS, SDF-1 is triggered in the impaired tissue subsequent to ischemic attack,\[^{[18]}\] presents a significant role in orchestrating cells and promotes axon outgrowth and neurogenesis.\[^{[19]}\] CXCR4 is widely expressed on BMSCs.\[^{[4]}\] The interaction of CXCR4 and SDF-1 directs BMSCs migrating toward ischemic brain, around which SDF-1 is presented abundantly,\[^{[20]}\] and inhibition of CXCR4 expression decreases BMSCs movement.\[^{[21]}\]

In the present study, SDF-1 expression was upregulated in IBZ from day 1, and peaked at day 3 after stroke, correlating well with previous reports.\[^{[22]}\] MCI-186 amplified the effects and kept the high level to day 7. MCI-186 has shown a strong inhibition of DNA damage after brain injury.\[^{[22]}\] Both human and animal experiments revealed that MCI-186 could protect NSCs from irradiation and increase their proliferation around the brain lesion.\[^{[22-24]}\] In the previous study, we also observed that MCI-186 significantly reduced the consumption of antioxidant superoxide dismutase and the oxidative product of malondialdehyde,\[^{[25]}\] the mechanism of its improving SDF-1 expression needs further explore.

In parallel, *in vitro* experiments showed that CXCR4 content in BMSCs was elevated under hypoxia as compared to normal cultured condition, so it might be possible that the hypoxia brain environment might also stimulate the
expression of CXCR4 on BMSCs and contribute to cell migration.

When transplantation of BMSCs in MCAO rats, our study showed the combination therapy of BMSCs and MCI-186 obviously increased the number of engrafted-BMSCs in ischemic brain and induced additional functional improvement, there was a tight correlation between them. Our data suggested that MCI-186 promoting the trafficking of BMSCs to ischemic brain might be connected with the upregulated expression of SDF-1 in IBZ, and the efficiency of the cell therapy might be correlated with the number of BMSC retained in ischemic brain.

The neuroprotective effects of BMSCs arise not only from their ability to provide trophic support to neurons at risk near the infarct area but also from their differentiation to neurotization when they are engrafted into damaged CNS. The intermediate filament protein Nestin is a widely accepted molecular marker for NSCs and has the potential to develop into neuroectodermal, endodermal, and mesodermal lineages. It is reported in adult, the maximal level of Nestin expression after brain ischemia was on day 7 and continued to 4 weeks. However, the number of endogenous NSCs is too small to meet the need of damage. Here, we found some transplanted BMSCs (BrdU+ cells) co-located with Nestin+ cells and revealed that the engrafted-BMSCs could differentiate into neural lines and improve neurogenesis. Thus, the increased number of engrafted-BMSCs and NSCs in modified ischemic brain mediated by combination therapy might improve the efficiency of cell transplantation.

There are several limitations in this study. First, the mechanism of MCI-186 up-regulating the expression of SDF-1 in ischemic brain is not very clear. Second, it is better to add a group of using hypoxic BMSCs to treat ischemic rats to see whether the therapeutic effect is better. Third, it should be better to use whole brain coronal sections for immunostaining and to add a dynamic observation, such as days 1, 3, and 7.

In summary, our data demonstrated that MCI-186 could promote transplanted BMSCs migrating to the ischemic brain by stimulating SDF-1/CXCR4 axis, thereby enhance

Figure 3: Combination therapy significantly improved restoration. Combination therapy and BMSCs or MCI-186 monotherapy improved functional outcome (adhesive-removal and foot-fault evaluation tests) in MCAO rats (a and b). *P < 0.01 versus MCAO control group; †P < 0.05 versus MCAO control group; ‡P < 0.01 versus MCI-186 group; §P < 0.05 versus MCI-186 group. BMSCs: Bone marrow stromal cells; MCI-186: 3-methyl-1-phenyl-2-pyrazolin-5-on; MCAO: Middle cerebral artery occlusion; SD: Standard deviation.

Figure 4: Combination therapy improved the transplantation and differentiation of BMSCs in ischemic brain. (a) Immunofluorescence staining for BrdU and Nestin positive cells at day 7 after MCAO. BrdU (red, 1) and Nestin (green, 2) in BMSCs monotherapy group, BrdU (red, 4) and Nestin (green, 5) in the combination group. Panels 3 and 6 are the merged images, respectively. Arrows represent the double-immunofluorescence cells. Scale bar indicates 200 µm in BMSC and combination group. (b) The number of engrafted-BMSCs (i) and Nestin positive cells (ii) in BMSCs monotherapy group and combination group. *P < 0.01 versus BMSCs monotherapy group. BMSCs: Bone marrow stromal cells; BrdU: 5-bromo-2-deoxyuridine; MCAO: Middle cerebral artery occlusion.
neurotization and produce additive effects on BMSCs transplantation in rat ischemic stroke model.

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**Conflicts of interest**

There are no conflicts of interest.

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