

## Original Article

# Regulation of curcumin on proliferation and migration of endogenous neural stem cells and its effect on SDF-1/CXCR4 pathway

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**Abstract:** Purpose: The goal of this study was to examine regulation of curcumin on the proliferation and migration of endogenous neural stem cells and its effect on SDF-1/CXCR4 pathway. Methods: The endogenous neural stem cells (NSCs) of rats were isolated and cultured *in vitro*, and treated with curcumin or CXCR4 inhibitor AMD3100. NSCs were divided into a normal control group, a curcumin group, a curcumin + AMD3100 group and a AMD3100 group. MTT test, Transwell test and scratch test were used to detect the proliferation, invasion and migration of NSCs. The qRT-PCR and Western Blot were used to detect the expression levels of CXCR4 mRNA and CXCR4 protein in NSCs respectively. Results: Curcumin promoted proliferation of NSC which can be inhibited by AMD3100. The number of cell penetration and migration distance in a normal control group, a curcumin group and a curcumin + AMD3100 group were higher than those in the AMD3100 group ( $P < 0.05$ ). The relative expression levels of CXCR4 mRNA and CXCR4 in the normal control group, the curcumin group, the curcumin + AMD3100 group were higher than those in the AMD3100 group ( $P < 0.05$ ). The relative expression levels of SDF-1 mRNA in the curcumin group, the curcumin + AMD3100 group were higher than those in the normal control group and the AMD3100 group ( $P < 0.05$ ). Conclusion: Curcumin can enhance proliferation, migration and invasion of NSCs by regulating SDF-1/CXCR4 pathway, which provides experimental evidence for the application of curcumin in the clinical treatment of CIRI.

**Keywords:** Curcumin, endogenous neural stem cells, proliferation, migration, SDF-1/CXCR4

## Introduction

Ischemic Cerebral Vascular Disease (ICVD) often leads to cerebral ischemia-reperfusion injury (CIRI), leading to irreversible degeneration and necrosis of nerve cells [1, 2] and the incidence of ICVD accounts for 70% to 80% of all cerebrovascular diseases [3]. After ischemia, hypoxia and inflammation of the central nervous system, stromal cell derived factor (SDF-1) could be highly expressed in ischemic tissue and smooth muscle of injured vessels. The concentration gradient of SDF-1 was formed. The related cells promoting the high expression of specific receptor CXCR4 migrated to the injured regions in reverse concentration gradient and exerted corresponding biological effects [4, 5]. Some studies [6] have found that the level of SDF-1 in brain tissue and the expression of

CXCR4 on the surface of mesenchymal stem cells significantly increased after cerebral infarction, resulting in the migration of mesenchymal stem cells to infarcted brain tissue. It has been suggested that the SDF-1/CXCR4 pathway may play an important role in the directional migration of stem cells to ischemic-hypoxic brain injury. Recent studies have found that ischemia and hypoxia of brain can induce chemokines in damaged brain tissue. Chemokines can induce the proliferation and directional migration of neural stem cells (NSCs) to the damaged regions. Then the NSCs differentiated into neurons, astrocytes or oligodendrocytes, thereby replacing necrotic nerve cells to repair damaged brain tissue [7, 8]. However, the number of NSCs involved in migration and repair of brain tissue was small, which was not enough to achieve the ideal remediation effects. Therefore,

studies on the mechanism of directional migration of NSCs to repair brain tissue have important clinical significance.

Curcumin is a phenolic pigment extracted from the rhizome of *curcuma longa*. Studies have shown that curcumin can reduce the volume of cerebral infarction and the degree of cerebral edema after focal CIRI in rats, and improve the behavioral score of rats [9, 10]. After spinal cord injury in rats, curcumin was injected intraperitoneally. Nestin positive cells were found to be increased near the injured regions one week after spinal cord injury. Furthermore, curcumin promoted functional recovery by activating Wnt/beta-catenin signaling pathway of endogenous NSCs. Therefore, SDF-1/CXCR4 pathway may be one of the mechanisms of curcumin. In this study, regulation of curcumin on proliferation [11] and migration of endogenous neural stem cells and its effect on SDF-1/CXCR4 pathway were validated at the cellular level.

### Material and methods

#### *Study subjects*

Six healthy male SD rats were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. with strain code 101 and production license No. SCXK (Beijing) 2012-0001. The rats were fed with common nutritive feed (Beijing Zhecheng Technology Co., Ltd.). Drinking water was acidified water with pH value between 2.5 and 3 after high pressure sterilization. The average age of rats was (56.3 ± 2.4) days and average body weight was (280-350) g. The rats were fed separately in feeding boxes with feeding temperature 18-22°C and relative humidity 40%-70%. The bedding was changed regularly every morning and evening. The ambient noise was lower than 85 dB, and the ammonia concentration was not more than 20 ppm. The ventilation was carried out 8-12 times per hour. The nest was changed 1-2 times per week, and the nest was cleaned and disinfected. The noise was lower than 60 dB, and the ammonia concentration was not more than 14 ppm. The ventilation was carried out 15 times per hour, and the cycle light was carried out every 12 hours by fluorescent lamp.

#### *Establishment of middle cerebral artery occlusion (MCAO) model in rats*

The MCAO model was established by Zea Longa method [12]. SD rats were fasting for 12 hours

and drank water freely before operation. Before the experiment, 10% chloral hydrate (350 mg/kg) was injected intraperitoneally to anesthetize. Distal external carotid artery of rats was ligated and disconnected. The artery sheath was separated. The distal external carotid artery was exposed and ligated. The arterioles were clamped at the proximal cranial end of the internal carotid artery and the proximal central end of the common carotid artery. The tail end of the proximal filament was lifted. The CCA was slightly elevated from its original position and aligned with the internal carotid artery. At the proximal end of the external carotid artery, a small cut had a small amount of arterial blood. The prepared suture was inserted via this small cut and the internal carotid artery (the clamp was loosened) into the anterior cerebral artery to block the blood flow of the middle cerebral artery. The average insertion depth of suture was (18.5 ± 0.5) mm. At the same time, the breathing of rats was accelerated briefly, accompanied by twitching. There was no blood seepage at the cut of the blood vessel, suggesting that the middle cerebral artery was completely embolized and the model was successfully prepared. After 120 minutes of ischemia, the suture was retreated to the internal carotid artery to restore blood flow. During this process, the surgical site was covered with normal saline gauze to prevent drying and contamination. The suture was pulled out, and the stump of external carotid artery was ligated. The skin was sutured and disinfected with alcohol. After the operation, the rats were fed in a single cage with clean and ventilated at about 25°C.

#### *Isolation and culture of NSCs*

The model rats were sterilized in 0.5% iodophor. The brain was taken, the hippocampus was separated, and the meningovascular was stripped. The hippocampus tissue was transplanted into the high sugar DMEM/F12 medium containing 2% B27. The tissue was cut and lightly blown into cell suspension. The cell suspension was filtered with the 400-mesh screen, stained with trypan blue and counted. The cell suspension was inoculated into 6-well culture plate with EGF (concentration of 20 µg/L) and bFGF (concentration of 20 µg/L). The culture plate was placed in a 37% and 5% CO<sub>2</sub> balanced humidity incubator. The medium was replaced half-volume every 2-3 days. The cells were sub-cultured once every 7-10 days, three times in total.

### *Intervention methods*

Normal control group: NSCs were not treated with any treatment factors. Curcumin group: The NSCs ( $1 \times 10^4$ ) were added to the basal medium containing 10 ng/mL bFGF/EGF. Then 1  $\mu$ M/L curcumin was added. The NSCs were cultured at 37°C for 48 hours. AMD3100 group: The NSCs ( $1 \times 10^4$ ) were added to the basal medium containing 10 ng/mL bFGF/EGF. Then 1.5 g/mL AMD3100 was added. The NSCs were cultured at 37°C for 48 hours. Curcumin + AMD3100 group: The NSCs ( $1 \times 10^4$ ) were added to the basal medium containing 10 ng/mL bFGF/EGF. Then 1  $\mu$ M/L curcumin was added. The NSCs were cultured at 37°C for 48 hours. Afterwards, 1.5 g/mL AMD3100 was added and the NSCs were cultured at 37°C for 48 hours. Curcumin and AMD3100 were purchased from Shanghai Hengfei Biotechnology Co., Ltd., and the serial numbers were C140600 and S8030-1 respectively.

### *Proliferation of MTT in vitro*

The cells were made into  $4 \times 10^6$ /mL single arranged cell suspension. The cells were routinely inoculated and cultured on 96-well cell culture plate. Four collecting time points were set for 24, 48, 72, and 96 hours, and three parallel wells were set at each time point. When the cells were cultured to each time point, 20  $\mu$ L MTT (5 mg/mL) solution was added and the cells were cultured for 4 hours at 37°C. The supernatant containing impurities were removed and dimethyl sulfoxide (DMSO) was added. The culture plate was placed on the horizontal shaking table for 10 minutes. Finally, the absorbance at 570 nm was measured on the microplate reader VersaMax (Molecular Devices (Shanghai) Co., Ltd.). MTT test kit was purchased from Sigma-Aldrich Company (USA).

### *Transwell migration experiment*

The NSCs were grouped and adjusted the cell concentration to  $1 \times 10^5$  cells/ml. The 200  $\mu$ L cell suspension was gently added to the upper chamber of Transwell. The medium containing EGF/bFGF was added to the lower chamber of Transwell. After cultured in the incubator for 12 hours, the chamber was taken out. The medium in the upper chamber were discarded. The cells were fixed with 4% paraformaldehyde for 10 minutes. After washing with PBS, the cells were stained with 1% crystal violet dye for ab-

out 3 minutes. The cells not passed through the upper surface of the chamber membrane were gently wiped off with medical cotton swabs. The chamber was washed with PBS. The cells were photographed under inverted microscope. Five high power fields were randomly selected and the number of cell penetration was counted. Transwell was purchased from Shanghai Yanhua Biological Technology Co., Ltd. with the Article Number 354480.

### *Cell scratch test*

The cells were routinely inoculated into 6-well plate, and three groups of repetitive wells were set up. When the cell fusion reached about 90%, the tip of 20  $\mu$ L pipette tip was perpendicular to the 6-well culture plate for drawing according to the prepared horizontal line. PBS was used for cleaning (3 times) and DMEM medium with 1% FBS was used for further culture.

### *qRT-PCR*

After cell lysis, the total RNA was extracted by TRIzol. The DNA was synthesized by reverse transcription system. The upstream and downstream primers were added. The genes were amplified by 7900HT high-speed RT-PCR system using the cDNA as template. The circular threshold and response efficiency were determined by the system software. The reverse transcription reaction system was 1.0  $\mu$ L oligo dt-adaptor primer (500  $\mu$ g/mL), 5.0  $\mu$ L dNTP mixture (2.5 mM), 2  $\mu$ g total RNA, and 12  $\mu$ L Rnase Free ddH<sub>2</sub>O. The incubation was carried out for 5 minutes at 65°C. After the reverse transcription reaction, the PCR amplification system was 2  $\mu$ L cDNA template, 10  $\mu$ L SYBR Green Real-time PCR Master Mix, 1  $\mu$ L upstream primer, 1  $\mu$ L downstream primer, and 20  $\mu$ L double distilled water. Pre-denaturation at 95°C for 30 seconds, denaturation at 95°C for 5 seconds, annealing at 60°C for 15 seconds, and extension at 72°C for 45 seconds (a total of 40 cycles). The dissolution curve was analyzed after the experiment. GAPDH was used as the results were analyzed by 2- $\Delta$ Ct method. TRIzol Reagent was purchased from Chengdu East Creative Technology Co., Ltd. with the Article Number 1559026. SYBER GREEN Real-time Fluorescence Quantitative PCR Kit was purchased from Nanjing CoBioer Biotechnology Co., Ltd. with the Article Number 430151. The primer sequence was designed and synthe-

**Table 1.** Primer sequences

	Upstream	Downstream
CXCR4	5'-GTGCCTTAGCCCACTACTTCAGA-3'	5'-GGACATTCACCTCCAATTCAGCAA-3'
SDF-1	5'-GCATTGACCCGAAGCTAAG-3'	5'-ATGGCAAAGTGTCAAAACA-3'
GAPDH	5'-CGGAGTCAACGGATTGGTCGTAT-3'	5'-AGCCTTCTCCATGGTGGTGAAGAC-3'

sized by Hepeng (Shanghai) Biotechnology Co., Ltd. (**Table 1**).

#### Western blot

After cell lysis, the supernatant was centrifuged for 40 minutes at 12,000 r/min at 4°C. The supernatant was extracted and the protein content of the supernatant was quantified by Bradford method based on BSA. The 20 µg protein sample was analyzed by 10% SDS-PAGE electrophoresis. The protein was transferred to cellulose nitrate film at 100 V for 1 hour and sealed in sealing solution at 37°C for 1 hour. The first antibody (anti-rat CXCR4 and SDF-1) were added into different groups and incubated overnight at 4°C. At the same time, one film was incubated with TBS-T solution without antibody as negative control. After repeated washing, the films were incubated with anti-IgG antibody labeled with alkaline phosphatase (AP) and shaken at room temperature for 1 hour. After washing, Western Blotting was used to determine the absorbance (A) of each band for quantitative analysis. The Western blot test kit was purchased from Shanghai Youduo Biotechnology Co., Ltd. with the Article Number JC-445.

#### Statistical methods

SPSS19.0 (Asia Analytics Formerly Spss China) was used to analyze the data. The enumeration data are expressed as [n (%)], and the comparison of the rates was performed by  $\chi^2$  test. The measurement data are expressed as  $\bar{x} \pm sd$ , and the comparison between the two groups was performed by independent sample t test. The repeated measurements analysis of variance was used for the comparison at different time in the groups.  $P < 0.05$  indicated there was statistical difference.

## Results

#### MTT experimental results

The results of cell proliferation test have shown that there was no significant difference in cell

absorbance between the four groups at 24 hours ( $P > 0.05$ ). There was significant difference in cell absorbance between the four groups at 48 h, 72 h and 96 h ( $P < 0.05$ ). The absorbance value of the normal control group, the curcumin group, and the curcumin + AMD3100 group were higher than that of the AMD3100 group at 48 and 72 hours ( $P < 0.05$ ). The absorbance value of the normal control group and the curcumin group were higher than that of the curcumin + AMD3100 group at 48 and 72 hours ( $P < 0.05$ ). The absorbance value of the curcumin group was higher than that of the normal control group at 48 and 72 hours ( $P < 0.05$ ). There was no significant difference in the absorbance between the curcumin + AMD3100 group and the AMD3100 group at 96 hours ( $P > 0.05$ ). The absorbance of the curcumin + AMD3100 group and the AMD3100 group were lower than that of the normal control group and the curcumin group at 96 hours ( $P < 0.05$ ). The absorbance of curcumin group was higher than that of the normal control group at 96 hours ( $P < 0.05$ ) (**Table 2** and **Figure 1**).

#### Transwell experimental results

The results of cell invasion test have shown that the number of cell penetration in the normal control group, the curcumin group and the curcumin + AMD3100 group were higher than those in the AMD3100 group. The number of cell penetration in the normal control group and the curcumin group were higher than those in the curcumin + AMD3100 group ( $P < 0.05$ ). The number of cell penetration in the curcumin group was higher than those in the normal control group ( $P < 0.05$ ) (**Figure 2**).

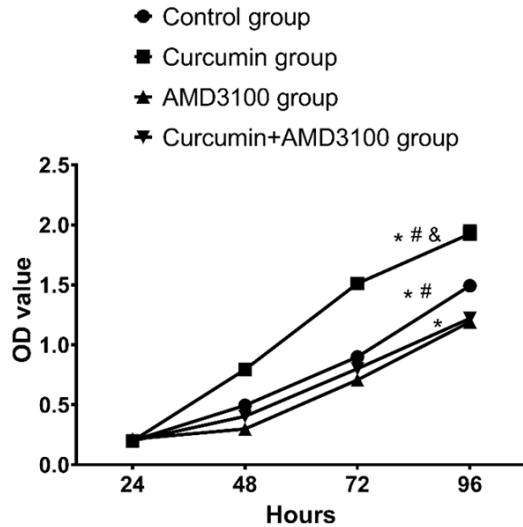
#### Results of cells scratch test

The results of cell migration test have shown that the migration distance in the normal control group, the curcumin group and the curcumin + AMD3100 group were higher than those in the AMD3100 group. The migration distance in the normal control group and the curcumin

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**Table 2.** Comparison of absorbance of four groups

	Normal control group	Curcumin group	AMD3100 group	Curcumin + AMD3100 group	F	P
24 h	0.201 ± 0.011	0.198 ± 0.007	0.215 ± 0.020	0.200 ± 0.010	2.161	0.245
48 h	0.496 ± 0.014	0.794 ± 0.016	0.299 ± 0.013	0.405 ± 0.019	1106.876	< 0.001
72 h	0.900 ± 0.013	1.512 ± 0.042	0.708 ± 0.020	0.801 ± 0.028	1014.947	< 0.001
96 h	1.494 ± 0.037	1.926 ± 0.059	1.192 ± 0.020	1.221 ± 0.023	480.699	< 0.001

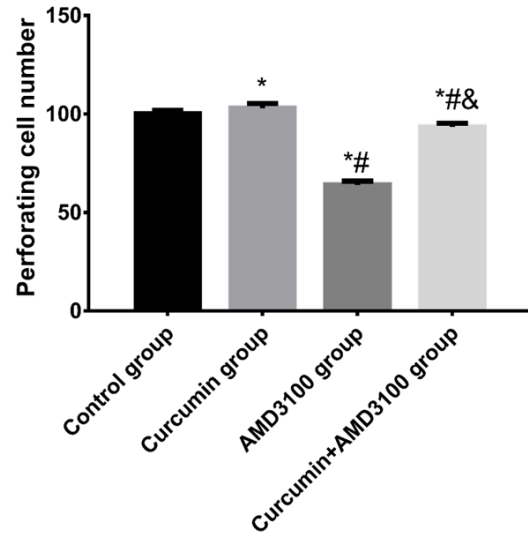


**Figure 1.** Cell Proliferation of Four Groups by MTT Test. Cell proliferation of the normal control group, the curcumin group and curcumin + AMD3100 group were higher than that of the AMD3100 group ( $P < 0.05$ ). The cell proliferation of the normal control group and the curcumin group were higher than that of the curcumin + AMD3100 group ( $P < 0.05$ ). The cell proliferation of the curcumin group was higher than that of the normal control group ( $P < 0.05$ ). \*indicated  $P < 0.05$  compared with the AMD3100 group; #indicated  $P < 0.05$  compared with the curcumin + AMD3100 group; and &indicated  $P < 0.05$  compared with the curcumin group.

group were higher than those in the curcumin + AMD3100 group ( $P < 0.05$ ). The migration distance in the curcumin group was higher than those in the normal control group ( $P < 0.05$ ) (Figure 3).

### Results of qRT-PCR

The relative expression levels of CXCR4 mRNA and SDF-1 mRNA detected by qRT-PCR have shown that the relative expression levels of CXCR4 mRNA in the normal control group, the curcumin group, the curcumin + AMD3100 group were higher than those in the AMD3100 group ( $P < 0.05$ ). The relative expression levels of CXCR4 mRNA in the normal control group



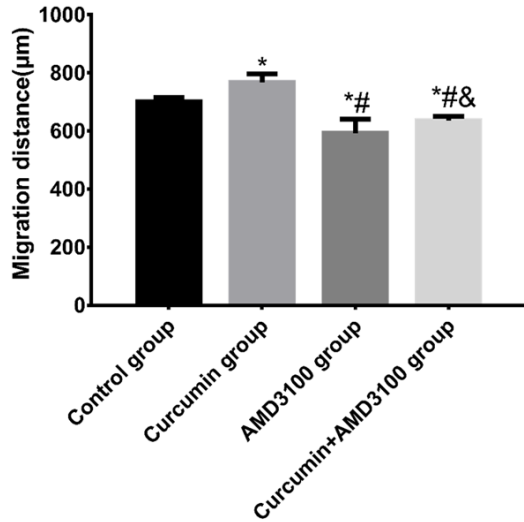
**Figure 2.** Cell Invasion of Four Groups by Transwell Experiment. \*Indicated that  $P < 0.05$  compared with the normal control group, #indicated that  $P < 0.05$  compared with the curcumin group, and &indicated that  $P < 0.05$  compared with the AMD3100 group.

and the curcumin group were higher than those in the curcumin + AMD3100 group ( $P < 0.05$ ). The relative expression level of CXCR4 mRNA in the curcumin group was higher than those in the normal control group ( $P < 0.05$ ). The relative expression levels of SDF-1 mRNA in the curcumin group, the curcumin + AMD3100 group were higher than those in the normal control group and the AMD3100 group ( $P < 0.05$ ). However, there was no significant difference in the relative expression levels of SDF-1 mRNA between the curcumin group and the curcumin + AMD3100 group ( $P < 0.05$ ), nor between the normal control group and the AMD3100 group ( $P < 0.05$ ) (Figure 4A, 4B).

### Results of western blot

The relative expression levels of CXCR4 and SDF-1 detected by Western Blot have shown that the relative expression levels of CXCR4 and SDF-1 in the normal control group, the curcumin group, the curcumin + AMD3100 group

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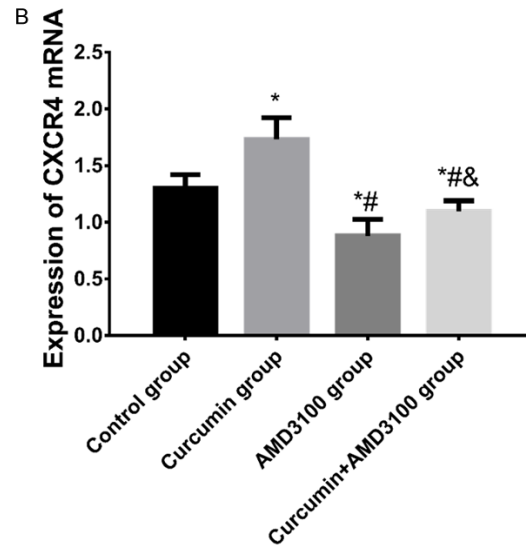
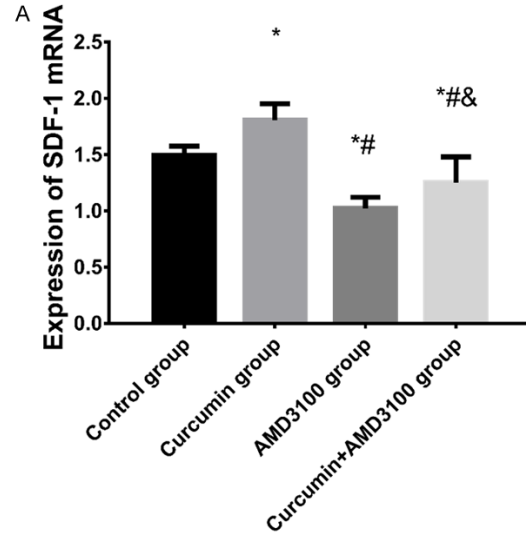


**Figure 3.** Cell Migration of Four Groups by Cell Scratch Test. \*indicated that  $P < 0.05$  compared with the normal control group, #indicated that  $P < 0.05$  compared with the curcumin group, and &indicated that  $P < 0.05$  compared with the AMD3100 group.

were higher than those in the AMD3100 group ( $P < 0.05$ ). The relative expression levels of CXCR4 and SDF-1 in the normal control group and the curcumin group were higher than those in the curcumin + AMD3100 group ( $P < 0.05$ ). The relative expression levels of CXCR4 and SDF-1 in the curcumin group were higher than those in the normal control group ( $P < 0.05$ ). The relative expression levels of SDF-1 in the curcumin group, the curcumin + AMD3100 group were higher than those in the normal control group and the AMD3100 group ( $P < 0.05$ ). However, there was no significant difference in the relative expression levels of SDF-1 between the curcumin group and the curcumin + AMD3100 group ( $P < 0.05$ ), nor between the normal control group and the AMD3100 group ( $P < 0.05$ ) (Figure 5A-C).

### Discussion

Curcumin is an ancient drug. Modern medicine has found that curcumin has anti-inflammatory, antioxidant, anticoagulant, antiviral and anti-tumor effects. Curcumin can be used to treat cancer, diabetes, coronary heart disease, arthritis, Alzheimer's disease and other chronic diseases [13, 14]. Its toxicity and side effects are low. Studies have shown that long-term oral administration of high doses has no obvious toxicity and side effects on the body. Curcumin

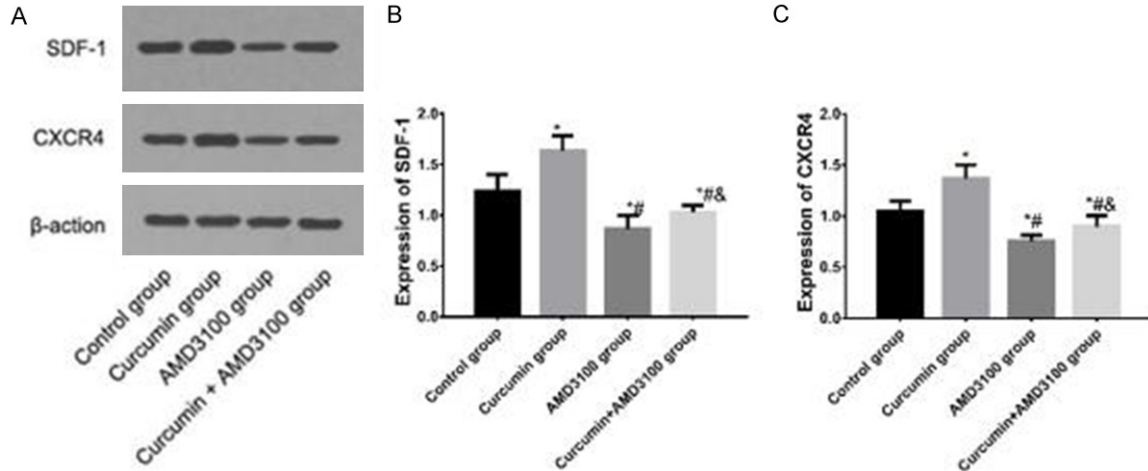


**Figure 4.** The Expression of CXCR4 mRNA and SDF-1 mRNA in Four Groups by QRT-PCR. \*indicated that  $P < 0.05$  compared with the normal control group, #indicated that  $P < 0.05$  compared with the curcumin group, and &indicated that  $P < 0.05$  compared with the AMD3100 group.

is easy to obtain, cheap and has good potential for clinical application. Due to the above advantages, curcumin has become a research hotspot in various fields. Currently, there is no research on the mechanism of curcumin promoting NSCs migration to repair CIRI by regulating signaling pathways.

The results in this study have shown that after AMD3100 intervention, the expression of CXCR4 in NSCs decreased significantly. The expression of SDF-1 in NSCs had no significant change. The proliferation, invasion and migra-

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**Figure 5.** The Expression of CXCR4 and SDF-1 in Four Groups by Western Blot. \*indicated that  $P < 0.05$  compared with the normal control group, #indicated that  $P < 0.05$  compared with the curcumin group, and &indicated that  $P < 0.05$  compared with the AMD3100 group.

tion of NSCs decreased significantly. However, after curcumin intervention, the expression of SDF-1 and CXCR4 in NSCs increased significantly. Proliferation, invasion and migration of NSCs increased significantly. It has been suggested that reducing curcumin may affect the proliferation, invasion and migration of NSCs by regulating SDF-1/CXCR4 signaling pathway. Analysis of curcumin + AMD3100 group also showed this point [15]. Compared with the curcumin group, expression of SDF-1 in the curcumin + AMD3100 group had no significant change. But expression of CXCR4 decreased significantly. Proliferation, invasion and migration of NSCs decreased significantly. AMD3100 is an inhibitor [16], which can specifically inhibit the expression of CXCR4 and the signal transduction of SDF-1/CXCR4 pathway, thus inhibiting the effect of curcumin on NSCs.

In the study of Liu et al. [17], it was found that curcumin could increase mitochondrial biogenesis, improve neurological deficits, reduce infarct volume and increase the number of neurons in CIRI rats. Li et al. [9] also reported that curcumin had protective effects on CIRI in rats by activating JAK2/STAT3 signaling pathway to alleviate neurological impairment, and reduce cerebral infarction area and cerebral water content. Recent studies have also found that curcumin had a regulatory relationship with SDF-1/CXCR4 pathway. Curcumin could inhibit cell migration by the down-regulation of CXCR4, SDF-1 and MMP2/MMP9 in endometrial carcinoma

cells mediated by Slit-2 [18]. The absorption of tumor stem cells to curcumin was much more than NSCs, about 3-8 times. It was one of the reasons why curcumin had different toxicities and side effects on the two kinds of stem cells [19]. In the study of curcumin and NSCs, curcumin could protect neuron stem cells by the classical WNT signaling pathway [20]. NSCs are a group of cells with self-renewal and multiple differentiation potential, which can differentiate into neurons, oligodendrocytes and astrocytes under appropriate conditions [7, 21]. Endogenous NSCs can proliferate, migrate and differentiate into neurons and glial cells under ischemic and hypoxic conditions [22]. It has also been suggested that the central nervous system can be repaired by its own endogenous stem cells.

At present, there are few reports about curcumin used in clinical treatment of CIRI. In the study of Song et al. [23], human NSCs were isolated, cultured *in vitro* and labeled with superparamagnetic iron oxide (SPIO). Then the NSCs were transfused back via tail vein to MCAO model in rats. The results showed that NSCs in the cell infusion group migrated and accumulated in the cerebral infarction site. Compared with the control group, the cerebral infarction area decreased. In the study of Nakatomi et al. [24], the epidermal growth factors were pumped into the lateral ventricle in cerebral ischemia injury of gerbils for 7 days. Compared with the group without epidermal growth factor injection, the

number of BrdU-labeled neurons in the ischemic regions increased 10 times after 2 weeks and 100 times after 13 weeks. The water maze test showed that the dysfunction caused by cerebral ischemic injury in rats had been significantly improved. Therefore, *in situ* activation, proliferation, directional migration and specific differentiation of endogenous NSCs could replace the nerve cells lost by cerebral ischemia injury and play a role of self-repairing. Therefore, the following hypothesis was proposed that curcumin can mediate SDF-1/CXCR4 pathway to regulate proliferation, migration and differentiation of endogenous NSCs to repair CIRI. This also provided more experimental evidence for curcumin clinical treatment of CIRI. In the future, the clinical effect of curcumin in CIRI will be analyzed.

In conclusion, curcumin can enhance proliferation, migration, and invasion of NSCs by regulating SDF-1/CXCR4 pathway, which provides experimental evidence for the application of curcumin in the clinical treatment of CIRI.

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### Disclosure of conflict of interest

None.

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