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The active principle region of *Buyang Huanwu* decoction induced differentiation of bone marrow-derived mesenchymal stem cells into neural-like cells

Superior effects over original formula of Buyang Huanwu decoction☆

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Abstract
The present study induced *in vitro*-cultured passage 4 bone marrow-derived mesenchymal stem cells to differentiate into neural-like cells with a mixture of alkaloid, polysaccharide, aglycone, glycoside, essential oils, and effective components of *Buyang Huanwu* decoction (active principle region of decoction for invigorating *yang* for recuperation). After 28 days, nestin and neuron-specific enolase were expressed in the cytoplasm. Reverse transcription-PCR and western blot analyses showed that nestin and neuron-specific enolase mRNA and protein expression was greater in the active principle region group compared with the original formula group. Results demonstrated that the active principle region of *Buyang Huanwu* decoction induced greater differentiation of rat bone marrow-derived mesenchymal stem cells into neural-like cells *in vitro* than the original *Buyang Huanwu* decoction formula.
Key Words: active principle region; bone marrow-derived mesenchymal stem cells; *Buyang Huanwu* decoction; differentiation; nerve cells

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INTRODUCTION

As seed cells, bone marrow-derived mesenchymal stem cells (BMSCs) can differentiate into neural cells in the presence of appropriate induction factors[1-2]. Previous results have shown that antioxidants β-mercaptoethanol, dimethyl sulfoxide, and 4-hydroxyanisole can be used to induce BMSCs to differentiate *in vitro* into neural cells[3]. Studies have utilized neurotrophic factor, epidermal growth factor[4], basic fibroblast growth factor[5], brain-derived neurotrophic factor, dimethyl sulfoxide, mercaptoethanol, and poly (3-hydroxyalkanoates) as inducers[6-9], but the induced neural-like cells exhibited short survival *in vitro* with toxicity and degenerating trends[10].

Buyang Huanwu decoction (BYHWD), a Chinese medical formula, has been clinically used to treat sequela of ischemic stroke. Modern pharmacological studies have shown that BYHWD regulates immune functions, provides anti-inflammatory action[11], dilates cerebral vessels[12], improves microcirculation and hemorheology[13], serves as an anti-coagulant, inhibits thrombosis, protects against free radicals, and provides neuroprotection[14]. *In vitro* MSC transplantation, in combination with BYHWD, has been shown to improve MSC survival, homing rates, and differentiation rates in animal models[15-16]. However, due to the complexity of Chinese medicine components, it is necessary to determine the effective components that promote BMSC differentiation into neural cells. In the present study, a mixture of five active components of BYHWD (active principle region) was used to induce BMSC differentiation.

RESULTS

Morphology of primary and passage BMSCs

In primary BMSC cultures, the cells were rounded, irregularly sized, dense, and floating. The cells began to adhere at 4–6 hours after culture, and were almost confluent at 24 hours. Cell culture medium was replaced at 48 hours, and the floating cells were discarded. Cells grew in colonies

at 3–4 days and gradually fused into lamella, with short-fusiform or needle tip shapes. Nucleoli were observed, and the cells reached confluency at 7–10 days. Passaged cells adhered within 24 hours. Cells from passage three exhibited a larger cell body than the primary culture. The majority of cells were fusiform-shaped or aligned in a fibriform-order with transparent cytoplasm. Nucleoli were also observed (Figure 1).

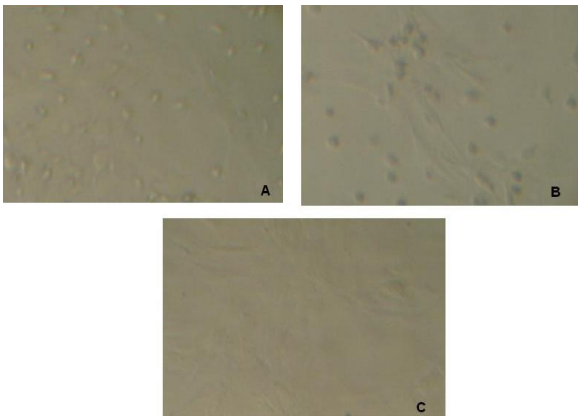

Figure 1 Morphology of primary and passaged bone marrow-derived mesenchymal stem cells (× 100).

(A) Primary culture.

(B) Cell colonies of passage 2.

(C) Fusiform-shaped or fibriform-arranged cells from passage 3.

Identification of BMSC surface antigens

Flow cytometric results showed that the CD90 positive rate was > 98%, and the CD11b and CD45 positive rates were < 2% (Figure 2), indicating high homogeneity and purity.

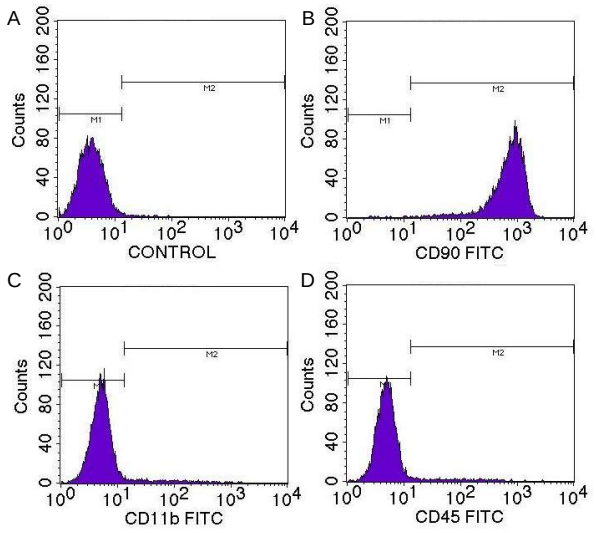

Figure 2 CD11b, CD45, and CD90 expression in rat bone marrow-derived mesenchymal stem cells (flow cytometry).

(A) Blank control cells; fluorescein isothiocyanate (FITC)-labeled CD90 (B), CD11 (C), and CD45 (D)-positive cells.

Maximum cytotoxic concentration of BYHWD and active principle region for BMSCs

Absorbance of the original BYHWD formula (drug concentration < 0.78%) was similar to cells cultured in drug-free media ($P > 0.05$), which suggested that the original BYHWD formula was not cytotoxic within this concentration range. With decreasing drug concentrations, absorbance increased and cell viability was enhanced. Therefore, 0.39% of the original BYHWD formula was used in the experiment (Table 1).

Table 1 Cytotoxicity of original *Buyang Huanwu* decoction formula

Group	Absorbance (570 nm)	Injury rate (%)
Concentration of original *Buyang Huanwu* decoction formula (w/v, %)		
50	0.588 6±0.083 6[a]	48.87
25	0.625 1±0.077 3[a]	44.82
12.5	0.693 1±0.107 4[a]	37.27
6.25	0.827 7±0.019 3[a]	22.33
3.12	0.856 6±0.032 8[a]	19.12
1.56	0.876 2±0.013 6[a]	16.94
0.78	0.915 1±0.016 6[b]	12.62
0.39	0.992 2±0.063 9	4.06
0.19	1.028 8±0.003 9	0.00
0.1	1.029 3±0.003 2	−0.06
Cell control	1.028 8±0.004 5	
Blank control	0.128 1±0.005 5	

[a]$P < 0.01$, [b]$P < 0.05$, *vs.* cell control group. Absorbance (A) values are expressed as mean ± SD.

Paired comparison was performed using the least significant difference *t*-test. Injury rate (%) = $[(A_{\text{cell control group}}-A_{\text{original formula group}})/(A_{\text{cell control group}}-A_{\text{blank control group}})] \times 100\%$.

Bone marrow-derived mesenchymal stem cells in the cell control group were cultured with medium-free *Buyang Huanwu* decoction.

Absorbance of the BYHWD active principle region (drug concentration < 0.078 g/L) was similar to cells cultured in drug-free media ($P > 0.05$), which suggested that the BYHWD active principle region was not cytotoxic within this concentration range. With decreasing drug concentrations, absorbance increased and cell viability was enhanced. Therefore, 0.039 g/L of the BYHWD active principle region was used in the present study (Table 2).

Nestin and neuron-specific enolase (NSE) protein expression in BMSCs treated with the BYHWD active principle region

At 4 weeks after induction, nestin or NSE expression was not detected in the normal control group (Dulbecco's-modified Eagle medium, DMEM) or negative control group (complete medium), but was observed in the cytoplasm of BMSCs from the positive control group (induction with β-mercaptoethanol), as well as the original formula and active principle region groups, with colony growth and scattered distribution. In addition, nestin- and NSE-positive cells were fusiform- or polygon-shaped (Figure 3).

These results were consistent with results from western blot analyses. Nestin and NSE were not expressed in the normal control or negative control groups.

Table 2 Cytotoxicity of active principle region of *Buyang Huanwu* decoction

Group	Absorbance (570 nm)	Injury rate (%)
Concentration of active principle region of *Buyang Huanwu* decoction (g/L)		
10	0.624 1±0.069 4[a]	44.40
5	0.660 3±0.058 0[a]	40.43
2.5	0.742 0±0.080 9[a]	31.50
1.25	0.822 1±0.022 6[a]	22.72
0.625	0.843 8±0.027 9[a]	20.35
0.312	0.870 8±0.011 9[a]	17.40
0.156	0.906 0±0.011 8[a]	13.54
0.078	0.979 1±0.035 9[b]	5.55
0.039	1.028 4±0.004 2	0.16
0.019	1.028 5±0.003 9	0.14
0.01	1.028 1±0.005 2	0.19
Cell control	1.029 8±0.005 7	
Blank control	0.115 9±0.012 6	

[a]$P < 0.01$, [b]$P < 0.05$, *vs*. cell control group. Absorbance (*A*) values are expressed as mean ± SD. Paired comparisons were performed using the least significant difference *t*-test.

Injury rate (%) = [($A_{\text{cell control group}}$−$A_{\text{active principle region group}}$)/($A_{\text{cell control group}}$−$A_{\text{blank control group}}$)] × 100%. Bone marrow-derived mesenchymal stem cells in cell control group were cultured with medium-free *Buyang Huanwu* decoction.

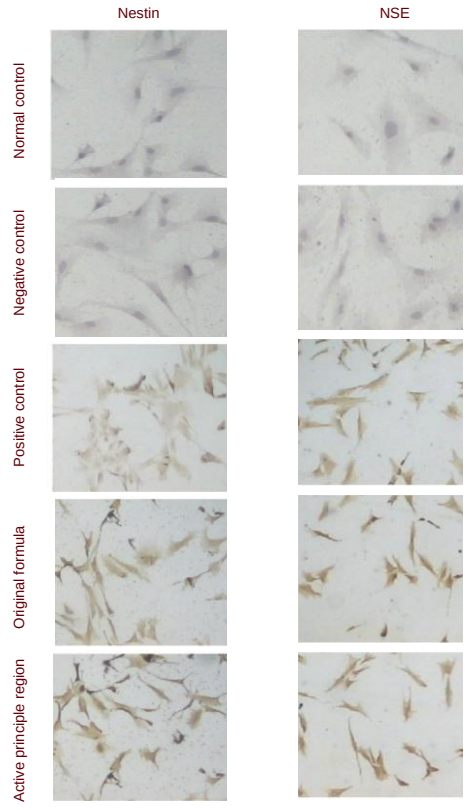

Figure 3 Nestin and neuron-specific enolase (NSE) expression in bone marrow-derived mesenchymal stem cells treated with active principle region of *Buyang Huanwu* decoction (BYHWD; immunohistochemical staining, × 200).

Inverted microscope showed no nestin or NSE expression in normal control and negative control groups, but expression was observed in positive control, original formula, and active principle region groups. Nestin and NSE positive expressions appear as a brown staining.

Nestin expression in the original formula and active principle region groups was < the positive control group, and NSE expression in the active principle region group was > the original formula and positive control groups ($P < 0.05$; Figure 4, Table 3).

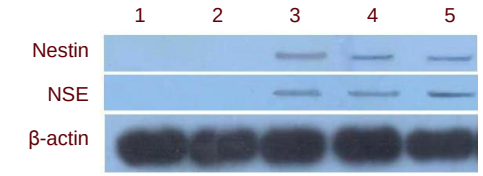

Figure 4 Nestin and neuron-specific enolase (NSE) protein expression in bone marrow-derived mesenchymal stem cells treated with active principle region of *Buyang Huanwu* decoction.

1: Normal control group; 2: negative control group; 3: positive control group; 4: original formula group; 5: active principle region group.

Table 3 Nestin and neuron-specific enolase protein expression (absorbance of target protein/β-actin; western blot) in bone marrow-derived mesenchymal stem cells treated with *Buyang Huanwu* decoction

Group	Nestin	Neuron-specific enolase
Normal control	0	0
Negative control	0	0
Positive control	0.145±0.023	0.190±0.014
Original formula	0.090±0.012[a]	0.183±0.040
Active principle region	0.087±0.008[a]	0.206±0.024[ab]

[a]$P < 0.05$, *vs*. positive control group; [b]$P < 0.05$, *vs*. original formula group. Data are expressed as mean ± SD from eight wells per group. Paired comparison was performed using the least significant difference *t*-test.

Nestin and NSE mRNA expression in BMSCs treated with the BYHWD active principle region

NSE and nestin mRNA expression was not detected in normal control or negative control groups, but was observed in the positive control, original formula, and active principle region groups (Figure 5).

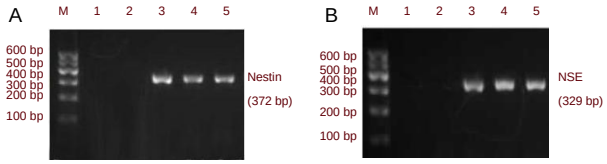

Figure 5 Gel electrophoresis of nestin (A) and neuron-specific enolase (NSE; B) mRNA expression.

1: Normal control group; 2: negative control group; 3: positive control group; 4: original formula group; 5: active principle region group; M: marker.

Semi-quantitative analysis of NSE and nestin mRNA expression showed that nestin mRNA expression was significantly less in the original formula and active principle region groups compared with the normal, positive, and negative control groups. However, NSE mRNA expression was greater than the normal, positive, and negative control groups ($P < 0.05$). In addition, NSE

and nestin mRNA expression was greater in the active principle region group compared with the original formula group ($P < 0.05$; Table 4).

Table 4 Nestin and neuron-specific enolase mRNA expression (absorbance of target gene/β-actin mRNA; reverse transcription-PCR) in bone marrow-derived mesenchymal stem cells

Group	Nestin	Neuron-specific enolase
Normal control	0	0
Negative control	0	0
Positive control	0.388±0.043	0.231±0.015
Original formula	0.284±0.044[a]	0.312±0.039[a]
Active principle region	0.319±0.038[ab]	0.358±0.124[ab]

[a]$P < 0.05$, *vs.* positive control group; [b]$P < 0.05$, *vs.* original formula group. Data are expressed as mean ± SD from eight wells per group. Paired comparisons were performed using the least significant difference *t*-test.

DISCUSSION

Previous results have shown that BMSCs do not express CD14, CD34, or CD45, but do express CD29, CD44, CD90, CD120, and CD124[17]. Cultured cells in the present study were identified as a high-purity cell population with properties resembling BMSCs.

Nestin is an early protein expressed in neural stem cells and it also serves as a marker for neural precursor cells. Nestin expression in BMSCs suggests neural cell differentiation[18]. NSE is a neuronal-specific marker, and NSE expression in BMSC cell cultures could suggest neuronal protein expression, as well as neuronal function. β-mercaptoethanol served as a positive control inducer, and BMSCs expressed NSE and nestin, which was consistent with previous results[19]. In addition, nestin and NSE immunohistochemical staining revealed expression throughout all cells from the original formula and active principle region groups, suggesting that induced BMSCs expressed nestin and NSE. Moreover, western blot detection supported these results. Nestin protein and mRNA expression was less in the original formula and active principle region groups compared with the positive control group, indicating that BMSCs might differentiate into fully mature cells. NSE protein and mRNA expression was greater in the active principle region group compared with the original formula and positive control groups, indicating that the BYHWD active principle region induced greater NSE expression compared with the original formula and β-mercaptoethanol.

MATERIALS AND METHODS

Design

An *in vitro,* cell-induced, differentiation study.

Time and setting

The experiment was performed at the Central Laboratory, Institute for Acupuncture and Moxibustion, Anhui University of Traditional Chinese Medicine, China from March 2009 to March 2010.

Materials

Animals

A total of 10 male, Sprague Dawley rats, aged 6–8 weeks and weighing 130 ± 20 g, were purchased from Shanghai Animal Experimental Center, license No. SCXK (Hu) 2009-0004. The rats were housed at 25°C with 60% humidity. Experimental procedures were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China[20].

Drugs

The original BYHWD formula comprised 60 g *Radix Astragali*, 6 g *Radix Paeoniae Rubra*, 6 g *Rhizoma Chuanxiong*, 9 g *Radix Angelicae Sinensis*, 9 g ground dragon, 9 g *Semen Persicae*, and 9 g *Flos Carthami* (Department of Pharmacy, First Hospital of Anhui University of Traditional Chinese Medicine, China) and the formula was prepared by the College of Pharmacy, Anhui University of Traditional Chinese Medicine using water-extraction and alcohol-precipitation methods[21]. BYHWD active components were extracted using acid-base precipitation and resin-bed methods[22]. The active components were separately isolated with a > 70% purity. The active component content was determined using high-pressure liquid chromatography and chemical analysis[23]: 9.76 mg ligustrazine in 1 g alkaloid; 2.39 mg astragaloside IV in 1 g glycoside, and 17.9 mg amygdaloside in 1 g glycoside. In addition, essential oil was emulsified with tween. According to methods from a previous study[24], the active principle region was prepared according to the following ratio: 0.58 g alkaloid, 11.44 g polysaccharide, 8.74 g aglycone, 14.4 g glycoside, and 2.5 g essential oil in 1 kg crude drug. The required concentration was prepared with serum-free L-DMEM culture medium.

Methods

BMSCs isolation, culture, and identification

The rats were sacrificed and immersed in 75% alcohol for 10 minutes. The tibia and femur were harvested under sterile conditions, and both ends were removed. The medullary cavity was washed with DMEM solution and cells were collected. The cells were added to DMEM (Gibco, Los Angeles, CA, USA) containing 10% fetal bovine serum (Hyclone, Logan, UT, USA). Cells at a density of 1×10^9/mL were seeded in a culture flask with a base area of 50 cm^2, and were incubated in 5% CO_2 saturated humidity at 37°C. The suspension was discarded after 48 hours, and the culture medium was replaced, followed by replacement every two days. The floating hematopoietic cells were discarded, and attached BMSCs were retained. When cells reached 80% confluency, they were digested with 0.25% trypsin (Sigma, St. Louis, MO, USA) and collected. These procedures were repeated until passage three (P3). P3 cells were digested with 0.25% trypsin, prepared into suspension, blocked with 1% bovine serum albumin for

10 minutes, washed with phosphate buffered saline (PBS) three times, incubated with fluorescein isothiocyanate-labeled anti-CD11b, CD45, and CD90 antibodies (1: 1 000; 0.5 mL; Seratec, Goettingen, Germany; control group was treated with PBS) at 4°C for 30 minutes, centrifuged at 1 500 r/min for 5 minutes, washed with PBS three times, and fixed with 1% paraformaldehyde. Cell surface antigens were detected using FACScan flow cytometry (BD, Franklin Lakes, NJ, USA). BMSCs had a CD45 and CD11b positive rate < 5%, and a CD90 positive rate > 95% was considered high purity[25].

Maximum cytotoxic concentration of original formula and active principle region for BMSCs

BMSCs from P4 in the logarithmic phase were digested with 0.25% trypsin and the suspension was concentrated to 1 × 10^4/mL. Cells were then added to 96-well culture plates, 0.2 mL/well, and the cells were incubated in 5% CO_2 saturated humidity at 37°C for 24 hours. The culture solution and detached cells were then discarded. BYHWD (2.4 g/mL crude drug)[26] was filtered through a membrane and cultured in medium. Cell proliferation rates in different BYHWD concentrations were determined using the 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) method[27]. According to the 2^{-n} multiple proportion dilution (n = 1–9), BYHWD was diluted with cell culture solution to concentrations of 50% (2^{-1}), 25% (2^{-2}), 12.5% (2^{-3}), 6.25% (2^{-4}), 3.12% (2^{-5}), 1.56% (2^{-6}), 0.78% (2^{-7}), 0.39% (2^{-8}), and 0.19% (2^{-9}). The pH value ranged between 7.2 and 7.4.

Cells were respectively treated with 20 μL induction solution containing active principle region at final concentration of 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039, 0.019, and 0.01 g/L, followed by 180 μL culture solution containing 10% fetal bovine serum. The cell control group was cultured in drug-free solution (200 μL). The blank control group was cultured in 200 μL ultrapure water. Each concentration was incubated in four adjacent wells. All groups were incubated in 5% CO_2 saturated humidity at 37°C for 72 hours, followed by incubation with 20 μL MTT (5 mg/mL) at 37°C for 4 hours. The supernatant was discarded, and 150 μL dimethyl sulfoxide (Sigma) was added to each well. Absorbance (A) at 570 nm was determined using MK3 microplate reader (Thermo Fisher, Waltham, MA, USA). The mean of four adjacent wells was used to calculate cell injury percent: injury rate (%) = $[(A_{\text{cell control group}}-A_{\text{medication group}})/(A_{\text{cell control group}}-A_{\text{blank control group}})] \times 100\%$[28]. Drug concentrations with an injury rate < 5% were selected as the atoxic concentration.

Induction of BMSC differentiation

BMSCs from P4 were randomly assigned to active principle region, original formula, positive control, negative control, and normal control groups, respectively, and were treated with 5 mL 0.39% active principle region, 0.039% BYHWD, β-mercaptoethanol (5 mM; Sigma), DMEM and complete medium (DMEM containing 10% fetal bovine serum) for 24 hours. The medium was discarded, the cells were washed with DMEM twice, and then incubated in the above-mentioned culture conditions. The medium was replaced every 3 days. After induction for 28 days, the cells were identified.

Nestin and NSE immunohistochemistry

Cells were induced for 4 weeks and were digested with 0.25% trypsin, prepared into cell suspension, washed with PBS, and seeded in coverslip-coated 6-well culture plates, with 2 × 10^5 cells/well. After 48 hours, the culture solution was discarded, and the cultures were fixed with formalin, followed by antigen retrieval. Cells were incubated with rabbit anti-rat nestin and NSE monoclonal antibodies (1: 300; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C, followed by horseradish peroxidase-labeled goat anti-rabbit IgG (Santa Cruz Biotechnology) at room temperature. Staining visualized with diaminobenzidine. Negative control was treated with PBS rather than primary antibody. Cells with brown particles in the cytoplasm were regarded as positive. Nestin- and NSE-positive cells were observed by inverted microscope (Nikon, Tokyo, Japan).

RT-PCR for nestin and NSE mRNA expression

Cells were induced for 4 weeks and then digested with 0.25% trypsin, prepared into cell suspensions of 1 × 10^7/mL, and digested with Trizol (Invitrogen, Burlington, Canada). Total RNA was extracted. Following identification by electrophoresis, the ratio of $A_{260\ nm}$ to $A_{280\ nm}$ was determined using a DU640 nucleic acid protein analyzer (Backman, Salt Lake City, UT, USA) and concentrations were calculated. β-actin mRNA was used as the internal reference. According to sequences from NCBI Genbank (http://www.ncbi.nlm.nih.gov/gene), primers were designed using Primer Premier 5.0 (Premier Biosoft, Palo Alto, USA), were verified by Blast, and were synthesized by Invitrogen.

PCR primer sequence and amplified target fragments

Primer	Primer sequence (5'–3')	Product size (bp)
NSE[29]	Sense: TCT CCT GCT GAC CCT TCC	329
	Antisense: CGA TGA CTC ACC ATA ACC C	
Nestin[30]	Sense: GAG CCA TTG TGG TCT ACT GA	372
	Antisense: GAT GCA ACT CTG CCT TAT CC	
β-actin[31]	Sense: AGA CCT TCA ACA CCC CAG C	657
	Antisense: AGC CAC CAA TCC ACA CAG A	

RT-PCR was performed using a two-step method[32]. RNA was reverse-transcribed using a gradient PCR system (Bio-rad, Hercules, USA) at 65°C for 5 minutes, 42°C for 60 minutes to synthesize cDNA, and 70°C denaturation for 5 minutes, followed by 35 cycles of PCR amplification (nestin: 94°C for 30 seconds, 54°C for 1 minute, 72°C for 1 minute; NSE: 94°C for 30 seconds, 56°C for 1 minute and 72°C for 1 minute), and a final extension step at 72°C for 10 minutes. PCR products

were separated on a 2% agarose gel and photographed and quantified using a gel imaging system (UVItec, London, UK). The absorbance ratios of nestin or NSE mRNA to β-actin mRNA were calculated. All experiments were performed in triplicate.

Western blot analysis of nestin and NSE protein expression

Total protein was extracted from BMSCs induced for 4 weeks, and protein concentrations were determined using the bicinchoninic acid method[33]. Following sodium dodecyl sulfate-polyacrylamide gel and membrane transfer, the membranes were incubated with monoclonal rabbit anti-rat nestin, NSE, and β-actin antibodies (1: 300; Santa Cruz Biotechnology) overnight, followed by horseradish peroxidase-labeled goat anti-rabbit IgG (1: 3 000; Santa Cruz Biotechnology) at room temperature for 2 hours. Following enhanced chemiluminescence for 1–10 minutes, results were detected[34]. Absorbance was analyzed using Image-Pro Plus 6.0 (Media Cybernetics, Bethesda, USA). Target protein expression was represented by absorbance ratio of target protein to β-actin protein (Santa Cruz Biotechnology).

Statistical analysis

Data were analyzed using SPSS version 17.0 (SPSS, Chicago, IL, USA). Continuous variables were expressed as mean ± SD. Data were compared using analysis of variance. Paired comparisons were performed using the least significant difference *t*-test. $P < 0.05$ was considered statistically significant.

Author contributions: Jinghui Zheng conceived and designed the study, analyzed data, and wrote the manuscript. Yi Wan revised the manuscript and contributed to statistical analysis. Jianhuai Chi and Dekai Shen were responsible for data analysis. Tingting Wu, Weimin Li, and Pengcheng Du conducted the experiments.

Conflicts of interest: None declared.

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Ethical approval: This study received permission from the Animal Ethics Committee of Anhui University of Traditional Chinese Medicine, China.

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