Brief Report

Mannotriose induced differentiation of mesenchymal stem cells into neuron-like cells

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This article demonstrates that mannotriose effectively induces the differentiation of mesenchymal stem cells into neuron-like cells *in vitro*. Rat-derived mesenchymal stem cells were investigated on their potential to differentiate into neuron-like cells induced by mannotriose purified from *Radix Rehmanniae Preparata in vitro*. The percentage of the neuron-specific enolase positive cells and the Nissl positive cells after mannotriose treatment was increased. The mRNA levels of neurofilament medium and neuron-specific enolase were upregulated in the mannotriose group compared to the control. These findings demonstrate that mannotriose purified from *Radix Rehmanniae Preparata* can effectively induce differentiation of rat-derived mesenchymal stem cells into neuron-like cells.

Keywords

Genetics; Mannotriose; Mesenchymal stem cell; Differentiation; Neuron-like cells; Herbal medicine

1. Introduction

Bone mesenchymal stem cell (BMSC) is a bone marrow-derived multipotent stem cell, which can differentiate into several somatic cells under certain circumstances, such as osteoblasts, chondrocytes, adipocytes. Mesenchymal stem cells (MSC) could also differentiate into neuron-like cells and glia-like cells [1–5]. They have abundant sources and could be isolated with high yield, which added to their ability to differentiate into non-mesodermal cell types, including neuronal lineage *in vivo* and *in vitro*. Therefore, the induction of MSCs to differentiate into neurons for cell therapy provides a new possibility for the treatment of neurological disorders and have been proposed as an alternative therapy to be applied to several pathologies of the nervous system [6–10].

Radix Rehmanniae Preparata-containing serum can induce BMSCs into neural cell differentiation [11]. Until now, many inducers for inducing neuro-differentiation of MSCs *in vitro* have been reported. The most prevalent chemical inducers were toxic to humans to varying degrees [12, 13]. Whether mannotriose is toxic for the quality control of *Radix Rehmanniae Preparata* needs further study. At least, mannotriose is natural, and *Radix Rehmanniae Preparata* could effectively treat neurologic diseases [14, 15]. Thus, it is imperative to find safer alternatives [16] validated.

In recent years, many meaningful findings related to the differentiation of MSC into neurons induced by Traditional Chinese medicines were reported, such as astragalus [17], berberine [18], tanshinone [19], acanthopanax [20]. However, no research on mannotriose of inducing the differentiation of MSCs into neurons was reported. There are two types of *Rehmanniae Radix* derived from the root of *Rehmannia glutinosa* Libosch, raw *Rehmanniae Radix* (Sheng Dihuang), and *Radix Rehmanniae Preparata* (Shu Dihuang). Two studies showed that *Radix Rehmanniae Preparata* could effectively treat neurologic diseases [14, 15], and *Radix Rehmanniae Preparata*-containing serum can induce BMSCs into neural cell differentiation [11]. However, whether mannotriose, which is the index for the quality control purified from *Radix Rehmanniae Preparata*, is the key component in promoting differentiation is not known.

Induction of MSCs to differentiate into the neuron for cell therapy could provide a new treatment for neurological diseases. Woodbury [21] first succeeded in inducing MSCs to differentiate into neuron-like cells *in vitro* with β-mercaptoethanol, dimethyl sulfoxide, and 4-hydroxyanisole. The findings showed that 80% of the induced cells showed neuron-like morphology, expressed neuron-specific enolase (NSE) and neurofilament (NF) after the induction [21]. Up to now, the most commonly used chemical agents to induce differentiation of MSCs into neuron-like cells included: (1) antioxidant agents, such as mercaptoethanol; (2) neurotrophin, such as epidermal growth factor (EGF). However, these chemical inducers are toxic to humans to varying degrees [22–26].

2. Material and methods

2.1 Experimental animal

Four-week-old Wistar rats (without gender limitations, 120 g in weight). were supplied by Shanghai Sippr-BK Experimental Animal Center [Certificate No. SCXK (Shanghai) 2013-0016].

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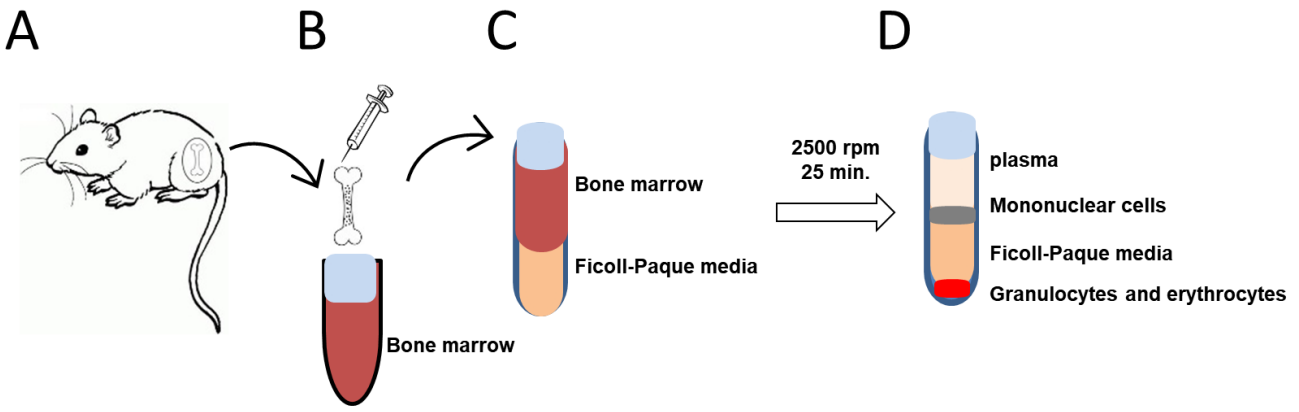

Fig. 1. Isolation of Rat MSCs. (A) Stripped the femur of Wistar rats under sterile conditions. (B) Flushed the marrow cavity with L-DMEM; (C) Equivalent Ficoll-paque lymphocyte separation medium was lightly superposed; (D) After centrifugation at 2500 rpm for 25 minutes, mononuclear cells were obtained.

2.2 Main reagent

Reagents were as follows, including Ficoll-paque lymphocyte separation medium (Sigma-Aldrich Co., St. Louis, USA), FBS, and L-DMEM (Gibco, Grand Island, NY, USA), FITC-CD34, and PE-CD44 (BD Biosciences, NJ, USA). Neuron-Specific Enolase (NSE) monoclonal antibody (FITC) (Abcam Co., Cambridge, UK.), PCR kit (TaKaRa Co., Tokyo, Japan). Mannotriose for use in research, biochemical enzyme assays, and *in vitro* diagnostic analysis was isolated and purified from Rehmannia Glutinosa in Chengdu Herb purify Co., LTD. It has a molecular formula, $C_{19}H_{34}O_{16}$.

2.3 Isolation, culture, and identification of rMSCS

Bone marrow was isolated from the femur of a normal Wistar rat under sterile conditions, flushing the marrow cavity with L-DMEM medium (including 10% FBS and 1% penicillin-streptomycin) (Fig. 1A). The medium with bone marrow was added into the centrifuge tube with 1.077 g/mL equivalent Ficoll-paque lymphocyte separation medium (Fig. 1B, C). Centrifuged for 25 minutes at 2500 rpm, mononuclear cells were collected (Fig. 1D). The cells were resuspended and cultured in an incubator at 37 °C, 5% CO_2. The non-adherent cells were removed at 48 h, and then the medium was changed every 3 days. All the animal experiments were performed in compliance with the Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine guidelines.

2.4 Flow cytometry analysis

Flow cytometry (FCM) was used to analyze the 3rd passage rMSCs, CD44, which was considered the MSC marker, and CD34, considered the hematopoietic marker progenitor cells with FCM. The 3rd generation rMSCs (~10^6) were collected. 10 μL CD44-PE or CD34-PE was added to the cells. The cells were resuspended in FCM buffer and analyzed by flow cytometry using a Becton Dickinson Accuri^{TM} C6.

2.5 Differentiation of MSCs into neuron-like cells

The 3rd generation rMSCs were passed into a 24-well cell culture plate. When cells were nearly 60%~70% confluent, the medium was removed. Drug-inducing *in vitro* was performed after pretreatment with 1 mmol/L β-mercaptoethanol for 24 hours. The cells were divided into 3 groups randomly:

(1) Control Group: Cells were pre- induced with 1 mmol/L β-mercaptoethanol for 24 hours and then cultured with L-DMEM.

(2) Mannotriose Group: Cells were pre-induced with 1 mmol/L β-mercap- to-ethanol for 24 hours and then cultured with L-DMEM containing 0.1 g/L mannotriose purified from *Radix Rehmanniae Preparata.*

(3) Positive Control Group: Cells were pre-induced with 1 mmol/L β-mercaptoethanol for 24 hours and then cultured with L-DMEM containing 5 mmol/L β-mercaptoethanol.

All the cells were incubated for 48 h at 37 °C with 5% CO_2, and the morphological changes of the cells were observed using an inverted phase-contrast microscope (Olympus Optical Company, Ltd., Tokyo, Japan).

2.6 Identification of differentiated cells by NSE immunofluorescent staining and Nissl staining

To study whether the MSCs had differentiated into neurons, neuronal markers such as NSE and Nissl were stained. Nissl staining followed the process operation: the cells were fixed with 4% polyformaldehyde, incubated with cresyl violet dye for 30 min at 37 °C, treated with 95% ethanol for 5 seconds, and finally observed and imaged under the fluorescence microscope. The expression level of NSE was detected by immunocytochemistry.

2.7 Identification of differentiated cells by PCR

Polymerase chain reaction (PCR) was used to verify the gene expressions of the markers of neurons, NSE, and neurofilament medium (NF-M). RNA extraction and reverse transcription were performed by the kit instructions (9108/9109, Takara; RR047A, Takara). The A260/280 ratio of RNA was detected. The primers were designed and synthesized by sango biotech. The GAPDH (internal control) primers were used. All primer sequences are listed. GAPDH (internal control): 5′-

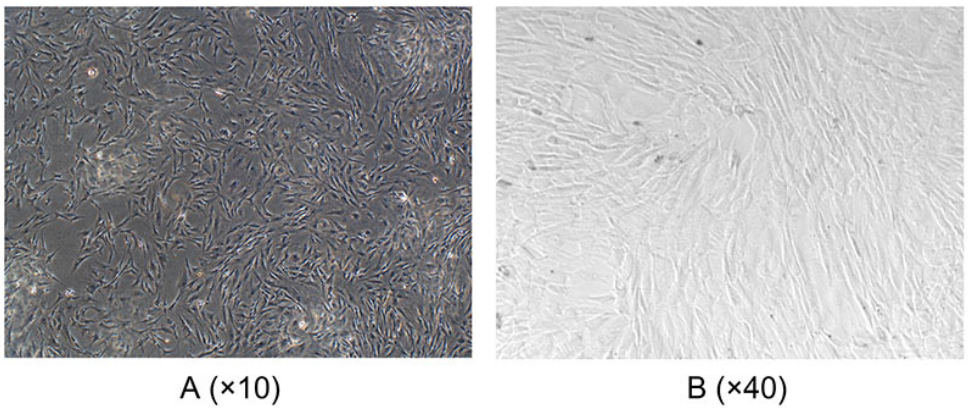

Fig. 2. The scheme illustrates the cellular morphology of MSC of P3. (A) The 3rd passage rMSCs were mainly spindle cells; (B) Cells presented typical radial, spiral, or row arrangement with uniform growth and regular arrangement.

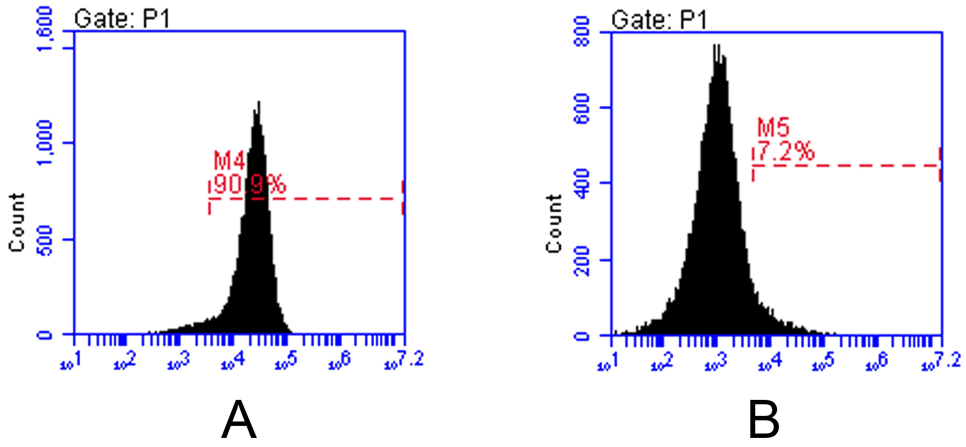

Fig. 3. Flow-cytometric analysis of cell surface markers (A) CD44 (B) CD34. The expression of a surface marker of rMSCs, CD44, was more than 90%. In contrast, the marker of hematopoietic progenitor cells, CD34, was negative, less than 10%.

AGAAGGCTGGGGCTCATTTG-3′ (forward); GAPDH (internal control): 5′-AGGGGCCATCCACAGTCTTC-3′ (reverse), product length: 258 bp; NSE: 5′-CTGGGAACTCCGACCTCATC-3′ (forward); NSE: 5′-GTACACCTCTGCCCCAAGTC-3′ (reverse), product length: 155 bp; NF-M: 5′-CGACTACAAAC TGTCCCGCT-3′ (forward); NF-M (internal control): 5′-CGGATCTCCTGGTCGTAAGC-3′ (reverse), product length: 189 bp; The amplification parameters were: 95 °C for 30 s, (95 °C for 5 s, 60 °C for 34 s) for 40 cycles. The analysis was performed with three biological replicates.

2.8 Statistical analysis

The experimental data were expressed as a mean and standard deviation. Single-factor analysis of variance and one-way ANOVA was performed using SPSS 18.0. The LSD was used to compare the differences between the two groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1 Cellular morphology of rMSCS

At 24 h, some adherent cells with irregular shapes were observed, and the cells presented good refraction. At 48 h, we found that the number of adherent cells increased significantly. They achieved 80-90% confluency on the 7th day as a monolayer arranged in a radial or spiral-shaped pattern. At this time, cells could be passaged successfully, then passaged every 2-3 days. After passage, the cells' lifespan became shorter, and the cells could adhere to the wall entirely in 24 h. As shown in Fig. 2, most of the 3rd passage MSCs were spindle cells (Fig. 2A). They presented typical radial, spiral, or row arrangement with uniform growth and regular arrangement (Fig. 2B).

In brief, MSCs were isolated from rats, purified, and cultured to the 3rd passage rMSCs in our study. Then the important cell surface markers, CD44, and CD3 were identified by FCM.

3.2 Cell surface markers

We further analyzed the expression of CD44, which was considered the marker of MSC and CD34, which was consid-

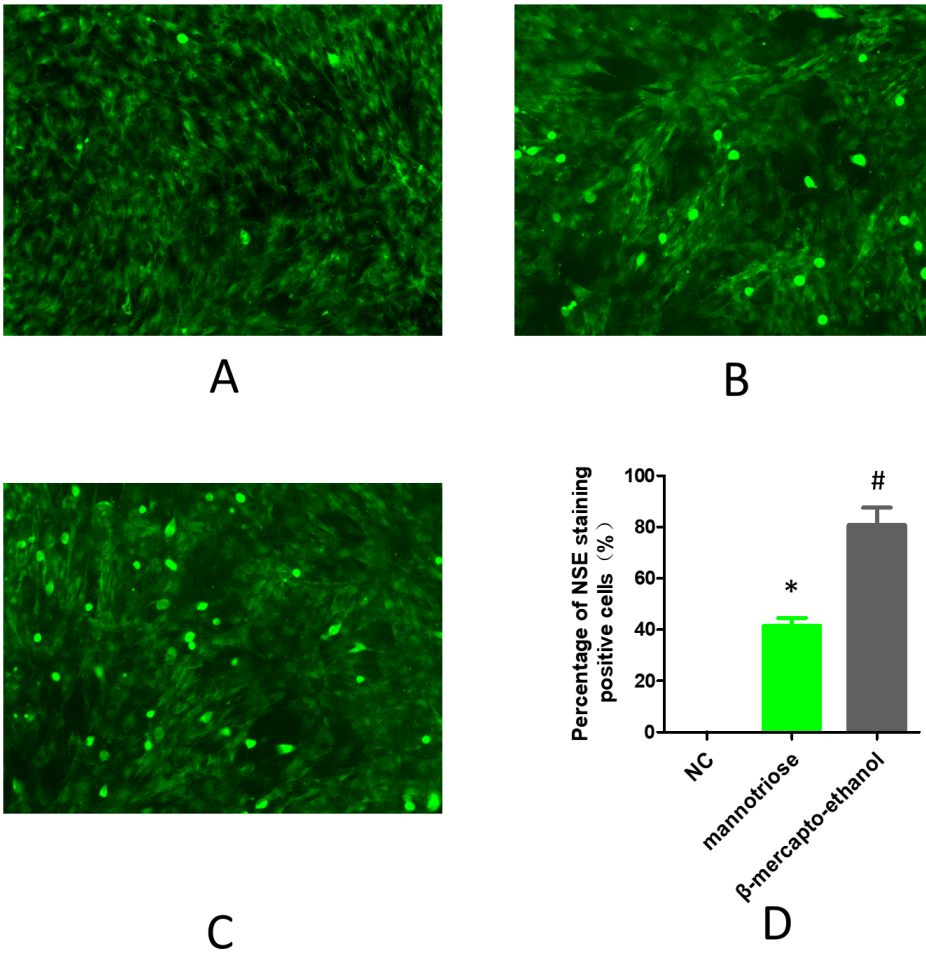

Fig. 4. NSE Immunostaining. (A) There were few positive cells in the control group; (B) Mannotriose treatment increased the number of NSE-positive cells (bright green); (C) The positive control group; (D) The percentage of NSE-positive cells. Single-factor analysis of variance and one-way ANOVA was performed using SPSS 18.0. The LSD was used to compare the differences between the two groups. $P < 0.05$ was considered statistically significant.$^{*}P < 0.05$, compared with Negative control. $^{\#}P < 0.05$, compared with Negative control.

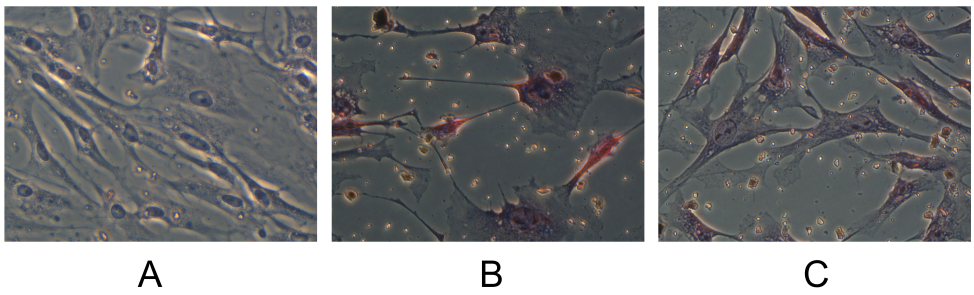

Fig. 5. Nissl Staining. (A) The control MSCs were shuttle-shaped cells; (B) Treatment with mannotriose (0.1 g/L) for 48 h changed the shape of MSCs from spindle to bipolar cells and multipolar. Some convex became thinner and longer to form filamentary pseudopod. (C) The positive control MSCs, β-mercapto Ethanol treated MSCs, showed the morphology of neuron-like cells.

ered the marker of hematopoietic progenitor cells with FCM [16]. We found that the expression of CD44 was more than 90%, while the expression of CD34 was less than 10% (Fig. 3). In our study, the expression of the surface marker of MSCs, CD44, was positive. In contrast, the marker of hematopoietic progenitor cells, CD34, was negative, consistent with the previous relevant reports.

3.3 Mannotriose increased NSE stain-positive cells

The 3rd generation rMSCs were treated with 0.1 g/L mannotriose for 48 hours. NSE was explicitly expressed on neurons and considered a marker for neurons, so we determined the induced cells' NSE expression with the immuno-

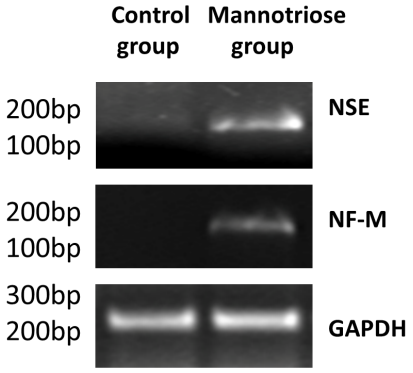

Fig. 6. Effect of mannotriose on mRNA levels of NF-M and NSE. NSE and NF-M mRNA levels increased significantly in the mannotriose (0.1 g/L) group compared with the control group.

cytochemistry method. The data showed that 0.1 g/L mannotriose treatment for 48 h increased the number of NSE stain-positive cells compared with the control group ($P < 0.01$) (Fig. 4A, B). The number of NSE stain-positive cells in the β-mercaptoethanol (the positive control) group was significantly higher than that of the control group (Fig. 4C). The numbers of NSE stain-positive cells in 10 randomly selected fields were calculated under the microscope, and the number of NSE stain-positive cells induced by mannotriose was 41.5% $\pm$ 8.7%. In comparison, the number of NSE-positive cells induced by β-mercaptoethanol was 80.9% $\pm$ 19.1% (Fig. 4D).

3.4 Nissl staining

Nissl body, one of the neuron's characteristic structures, exists in the cell body and dendrites, can be stained with basic dyes appearing as dark purple particles and plaques. Nissl staining is often used to identify neurons. We found that most neuron-like cells treated with mannotriose (0.1 g/L) for 48 h were positive for Nissl staining (Fig. 5). By contrast, there were barely positive cells by Nissl staining in the control group. Similar changes existed in the β-mercapto Ethanol group and mannotriose group. Namely, the shapes of the two groups' cells changed from spindle (Fig. 5A) to bipolar or multipolar (Fig. 5B). Some convex became thinner and longer to form filamentary pseudopod (Fig. 5B), like dendrites morphologically (Fig. 5C).

3.5 mRNA levels of NF and NSE

RT-PCR was used to detect NSE and neurofilament medium (NF-M) gene expression changes after mannotriose treatment. The data showed that NSE and NF-M mRNA levels increased significantly in the mannotriose group than in the control group (Fig. 6). Consistently with the change of NSE mRNA levels in response to mannotriose treatment, NSE's immunoreactivity had similar changes (Fig. 4).

4. Discussion

The key bioactive ingredients of *Radix Rehmanniae Preparata* were mannotriose and 5-hydroxymethyl furfural. Based on earlier studies, indicating, *Radix Rehmanniae Preparata* could effectively treat neurologic diseases [14, 15], and *Radix Rehmanniae Preparata*-containing serum can induce BMSCs into neural cell differentiation [11], and in hindsight, it would seem that mannotriose was the key bioactive ingredient of inducing the differentiation of MSCs into neuron-like cells. So, the differentiation of MSCs into neuron-like cells induced by mannotriose purified from *Radix Rehmanniae Preparata* was discussed, and mannotriose might be an alternative therapy to be applied to several pathologies of the nervous system.

Author contributions

LZ supervised the entire study, including experimental design and data analysis, wrote the manuscript. XL conducted the experiments. All authors interpreted the data and approved the final manuscript. LJ revised this manuscript. All data needed to evaluate the paper's conclusions are present in the paper and/or the materials cited here. Additional data related to this paper may be requested from the authors.

Ethics approval and consent to participate

The study was approved by the Shanghai Sippr-BK Experimental Animal Center [Certificate No. SCXK (Shanghai) 2013-0016]. All the animal experiments were performed in compliance with the guidelines of the Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine (SHUTCM).

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Conflict of interest

There are no conflicts of interest.

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