Neuronal-like differentiation of bone marrow-derived mesenchymal stem cells induced by striatal extracts from a rat model of Parkinson's disease☆

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Abstract

A rat model of Parkinson's disease was established by 6-hydroxydopamine injection into the medial forebrain bundle. Bone marrow-derived mesenchymal stem cells (BMSCs) were isolated from the femur and tibia, and were co-cultured with 10% and 60% lesioned or intact striatal extracts. The results showed that when exposed to lesioned striatal extracts, BMSCs developed bipolar or multi-polar morphologies, and there was an increase in the percentage of cells that expressed glial fibrillary acidic protein (GFAP), nestin and neuron-specific enolase (NSE). Moreover, the percentage of NSE-positive cells increased with increasing concentrations of lesioned striatal extracts. However, intact striatal extracts only increased the percentage of GFAP-positive cells. The findings suggest that striatal extracts from Parkinson's disease rats induce BMSCs to differentiate into neuronal-like cells in vitro.

Key Words

bone marrow-derived mesenchymal stem cell; Parkinson's disease; striatal extract; induced differentiation; nerve cell; glial fibrillary acidic protein; nestin; neuron-specific enolase; neural stem cell; regeneration; neural regeneration

Research Highlights

(1) After exposure to lesioned striatal extracts, bone marrow-derived mesenchymal stem cells developed bipolar or multi-polar morphologies.
(2) Induction by lesioned striatal extracts increased the percentage of cells that expressed glial fibrillary acidic protein, nestin and neuron-specific enolase.
(3) Intact striatal extracts only increased the percentage of glial fibrillary acidic protein-positive cells.

Abbreviations

BMSCs, bone marrow-derived mesenchymal stem cells; I-SM, intact striatal extract-containing medium; L-SM, lesioned striatal extract-containing medium; GFAP, glial fibrillary acidic protein; NSE, neuron-specific enolase

INTRODUCTION

Parkinson's disease is a neurodegenerative pathology that specifically targets the basal ganglia. The most important neuropathological alteration is the loss of dopaminergic neurons in the substantia nigra, which causes severe depletion of striatal dopamine. Pharmacological substitution of dopamine produces remarkable benefits for some years

but is hampered by complications as the disease progresses. A more ambitious approach aims to restore brain function by replacement of the dopaminergic neurons and their connections by fetal or embryonic stem cell transplantation[1-2].

Bone marrow-derived mesenchymal stem cells (BMSCs), a heterogeneous population of adherent cells with multipotency, are considered to be a source for cell transplantation therapy and have gained increasing attention because there is no source limitation, there are fewer ethical concerns, and there is less immune rejection[3-6] compared with the use of fetal tissue, which can directly provide dopaminergic neurons[7]. Many studies have confirmed that BMSCs can differentiate into nerve cells *in vitro* under various conditions[8-9].

In fact, cell transplantation strategies require the acquisition of BMSCs in both high purity and large numbers. It is necessary to harvest high quantity and high quality differentiated nerve cells, including dopaminergic neurons *in vitro* for cell transplantation therapy.

It has been suggested that BMSCs injected into the striatum can survive, express tyrosine hydroxylase and promote functional recovery in Parkinson's disease models[10-12]. Some researchers have proposed that striatal extracts can promote cell differentiation *in vitro* [13-15]. Therefore, we hypothesized that striatal extracts may induce BMSCs to differentiate into nerve cells *in vitro*. The present study investigated whether *in vitro* treatment with striatal extracts can promote neuronal differentiation of BMSCs at the levels of morphology and protein expression.

RESULTS

Morphology of cultured BMSCs
BMSCs were isolated by their adherence to the culture flask. The BMSCs became relatively homogeneous in appearance as the passages progressed. After two passages, the cells were flat, spindle or polygonal-shaped. Some cells with processes were observed (Figure 1).

Effect of striatal extracts on BMSCs
BMSCs remained unchanged when co-cultured with intact striatal extract-containing medium (I-SM) and lesioned striatal extract-containing medium (L-SM) for 6 hours. At 12–24 hours, some cells detached from the

flask, while the remaining adherent cells began to retract and became compacted, some of which developed bipolar or multi-polar morphologies. After 48 hours, the number of bipolar and multi-polar cells increased and some became interconnected with each other, but there were still some cells that maintained their original morphology (Figures 2C–F).
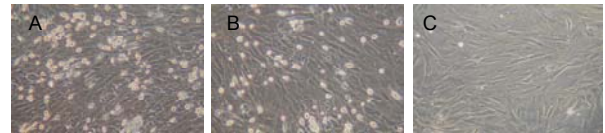

Figure 1　Morphology of bone marrow-derived mesenchymal stem cells under a normal culture condition (optical microscope, × 100).

(A–C) Primary, first and second passages of cells exhibit a simple morphology with a flat-, spindle- or polygonal-shaped appearance.
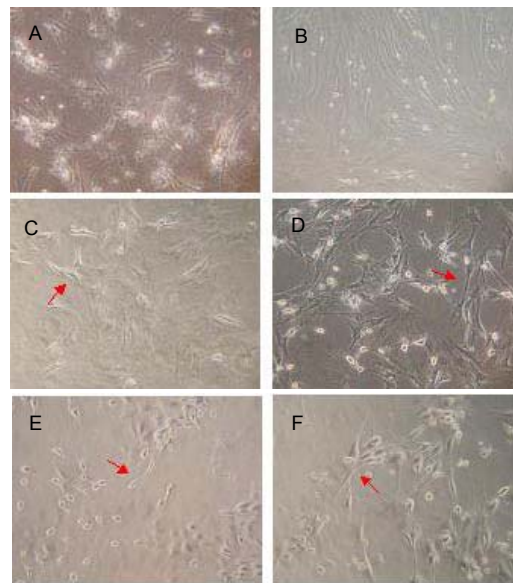

Figure 2　Morphology of bone marrow-derived mesenchymal stem cells (BMSCs) at 48 hours (phase contrast microscope, ×100)

(A) Serum-free medium group: Most BMSCs cultured in serum-free medium detached from the culture flask.

(B) Serum-containing medium group: BMSCs cultured in serum-containing medium proliferated quickly and demonstrated a flat-, spindle- or polygonal-shaped appearance.

(C–F) 10% I-SM, 60% I-SM, 10% L-SM and 60% L-SM groups: BMSCs cultured in 10% or 60% I-SM or L-SM showed a spherical cell body with bipolar or multi-polar processes, some of which appeared to connect with each other. Arrows: BMSCs with processes.

I-SM: Intact striatal extract-containing medium; L-SM: lesioned striatal extract-containing medium.

The percentages of cells that expressed nestin, glial fibrillary acidic protein (GFAP) and neuron-specific enolase (NSE) were all higher in L-SM than those in the serum-containing medium group. The percentage of NSE-positive cells was higher in 60% L-SM than that in 10% L-SM. The proportions of GFAP-positive cells were similar between 60% and 10% L-SM groups. The percentages of GFAP-positive cells were higher in I-SM than that in the serum-containing medium group, but there was no difference between 60% and 10% I-SM groups. Tyrosine hydroxylase was not expressed by cells cultured in various concentrations of L-SM or I-SM (Figures 3 and 4).

BMSCs cultured in serum-free medium did not adhere to the culture flask (Figure 2A), and thus immunohistochemistry could not be carried out. BMSCs cultured in serum-containing medium proliferated quickly and remained flat-, spinal- or polygonal-shaped (Figure 2B). Approximately $15.05 \pm 3.92\%$ of cells expressed GFAP, but no cells expressed NSE, nestin or tyrosine hydroxylase (Figures 3 and 4).

Our results suggested that the number of GFAP-, nestin-, and NSE-positive cells was increased after the BMSCs were cultured in medium containing striatal extracts.
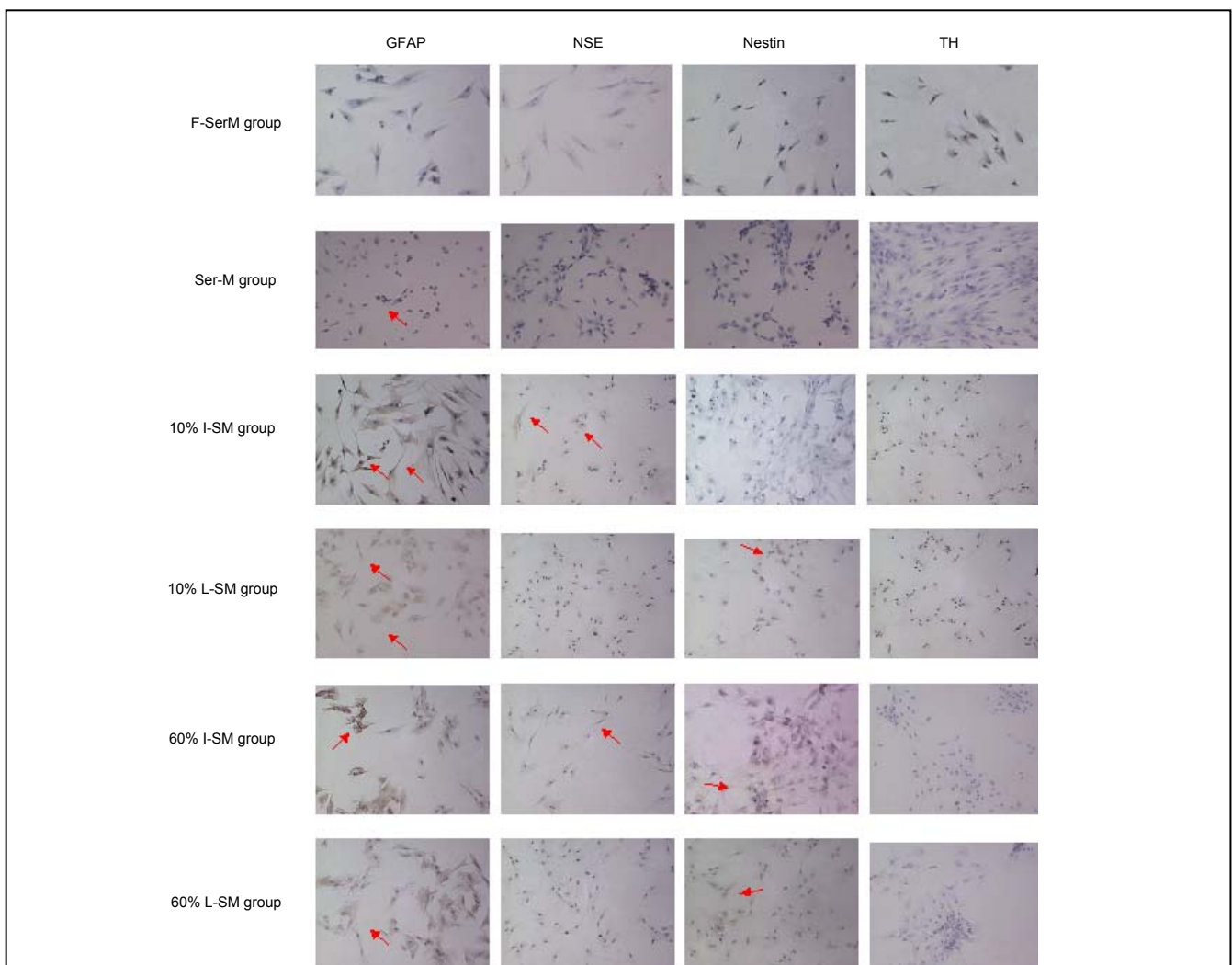

Figure 3   Expression of GFAP, NSE, nestin and TH after BMSC co-culture with striatal extracts for 48 hours (immunohistochemistry, × 100).

The percentage of GFAP- and NSE-positive cells increased after BMSCs were co-cultured with lesioned striatal extracts, and the number of NSE-positive cells increased with increasing concentrations of extracts. The percentage of GFAP-positive cells increased after BMSCs were co-cultured with intact striatal extracts. A small number of GFAP-positive cells were present when BMSCs were co-cultured with serum-containing medium.

Red arrows: Immunopositive BMSCs. BMSCs: Bone marrow-derived mesenchymal stem cells; F-SerM group: BMSCs cultured in serum-free medium; Ser-M group: BMSCs cultured in serum-containing medium; 10% I-SM, 60% I-SM, 10% L-SM and 60% L-SM groups: BMSCs cultured in 10% or 60% intact or lesioned striatal extracts; GFAP: glial fibrillary acidic protein; NSE: neuron-specific enolase; TH: tyrosine hydroxylase.

Figure 4   Percentage of GFAP-, NSE-, nestin- and TH-positive cells after BMSC co-culture with striatal extracts for 48 hours.

Data are expressed as mean ± SD and compared between two groups by one-way analysis of variance. $^{a}P < 0.05$, *vs*. Ser-M group; $^{b}P < 0.05$, $^{c}P < 0.01$, *vs*. 10% I-SM group; $^{d}P < 0.05$, *vs*. 60% I-SM group.

BMSCs: Bone marrow-derived mesenchymal stem cells; F-SerM group: BMSCs cultured in serum-free medium; Ser-M group: BMSCs cultured in serum-containing medium; 10% I-SM, 60% I-SM, 10% L-SM and 60% L-SM groups: BMSCs cultured in 10% or 60% intact or lesioned striatal extracts; GFAP: glial fibrillary acidic protein; NSE: neuron-specific enolase; TH: tyrosine hydroxylase.
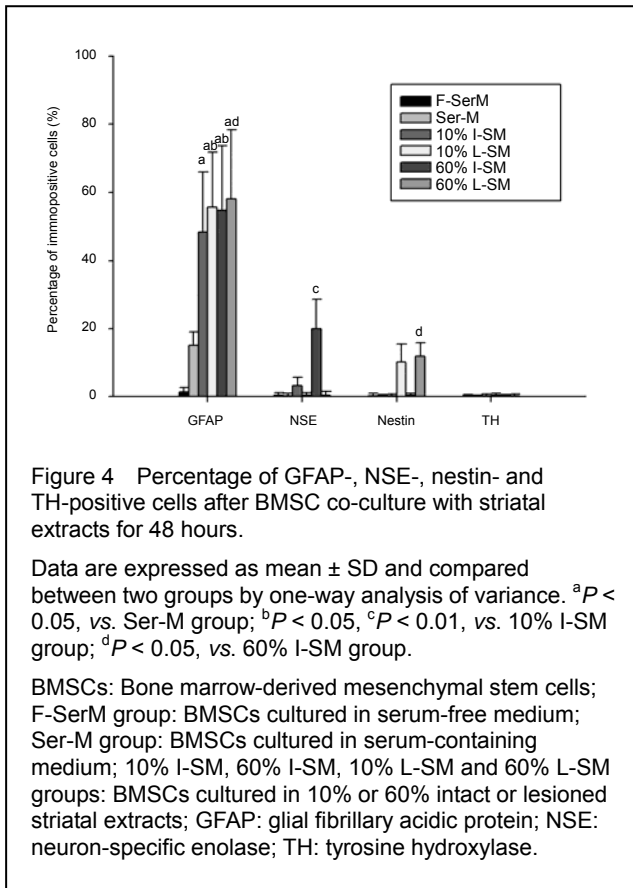
DISCUSSION

Both *in vitro* and *in vivo* studies have demonstrated that many factors such as the microenvironment, cell-cell contact and the extracellular matrix play key roles in determining the function and differentiated fate of stem cells[16-20]. Woodbury *et al* [16] found that the environment can apparently evoke the pluripotentiality that far exceeds the traditional fate restrictions of cells derived from the classical embryonic germ layers. The addition of antioxidants to the medium of adult rat BMSC cultures leads to neuronal-like morphological changes of BMSCs, and some cells express nestin and NSE. Nervous tissue and the cell microenvironment may enhance the ability of BMSCs to differentiate into nerve cells. Researchers have concluded that direct contact with host brain tissue is essential for BMSCs to differentiate into neurons[21]. After injection into the lateral ventricle of neonatal rats, some BMSCs within the striatum express GFAP[21]. This phenomenon suggests that these multipotent mesenchymal progenitors from bone marrow can adopt nerve cell fates when exposed to the brain microenvironment.

The nigrostriatal dopamine pathway is a key regulator of

motor function in the mammalian brain[22]. Parkinson's disease is characterized by a progressive degeneration of dopaminergic cells in the midbrain, which leads to dopamine depletion and neurochemical alteration in the striatum[22]. The striatum is the main projecting site for nigra dopaminergic neurons, and can secrete soluble factors that in turn promote the survival, growth and differentiation of protecting neurons. Among studies on the trophic action of dopaminergic neurons, striatal extracts have constantly received close attention. Early in 1986, striatal extracts were shown to promote the growth of dopaminergic neurons[15]. Researchers have found that denervation of the striatum results in production of neurotrophic factors including glial-derived neurotrophic factor, brain-derived neurotrophic factor and as-yet-unidentified trophic substances, which may be responsible for increased survival and the phenotype of dopaminergic neurons[13, 23].

In this study, we found that most cells of the serum-free medium group lost their adherent characteristic and detached from coverslips, reflecting a condition of malnutrition. Most BMSCs of the serum-containing medium group maintained their original appearance, while some cells had small processes and expressed GFAP. However, NSE-, nestin- and tyrosine hydroxylase-positive cells were few. Similar results were obtained in a study by Bossolasco *et al* [24]. This spontaneous differentiation might depend on the fact that BMSCs themselves can secrete various kinds of neurotrophic factors and cytokines[25].

In all four induction groups, the majority of BMSCs changed morphologically with striking neurite extensions. Compared with the serum-containing medium group, more cells treated with intact striatal extracts expressed GFAP. The percentage of GFAP positive cells in 10% L-SM group was slightly higher than that of the 10% I-SM group, and some cells expressed NSE. Nestin- and tyrosine hydroxylase-positive cells appeared infrequently in these groups. In addition, compared with the serum-containing medium group, significantly more cells treated with lesioned striatal extracts expressed GFAP, and some cells expressed nestin. There was no difference between the two groups in terms of GFAP and nestin expression. NSE- and tyrosine hydroxylase- positive cells were also infrequently found in these groups.

These data suggest that when exposed to Parkinson's disease rat striatal extracts, BMSCs change morphologically with neurite extensions. Some of the cells expressed neural-specific proteins such as GFAP,

NSE and nestin. Therefore, we concluded that striatal extracts might induce BMSCs to differentiate into neuroglial cells or even neurons. We failed to detect the dopaminergic phenotype with this approach, because tyrosine hydroxylase was nearly negative. The high expression ratio of GFAP indicated a glial differentiation tendency of BMSCs under this kind of condition, which is consistent with *in vivo* studies[26-28]. Neuronal-like differentiation might depend on the neurotrophic factors in the extracts, and the neurotrophic and other factors secreted by the BMSCs themselves.

In 10% and 60% groups, we found that GFAP expression in L-SM groups was statistically higher than that in I-SM groups, and that NSE and nestin expression showed a significant difference between L-SM and I-SM groups. This result suggests that intact striatal extracts and lesioned striatal extracts may be different in terms of their trophic activities, which might lead to different cell functions[29]. The lesioned striatal extracts appeared to exhibit a stronger trophic activity for the neural differentiation of BMSCs than that of the intact extract. This finding has not been reported previously.

The fact that the striatal trophic activity is elevated with increasing age suggests a potential compensatory reaction against degenerative changes[30], which might explain the above phenomenon. Moreover, brain self-protection mechanisms indicate that when the brain is injured, the release of neural protection factors would be dramatically increased[31-32]. On the other hand, *in vivo* and *in vitro* studies have shown that the lesional microenvironment might cause BMSCs to responsively secrete more neurotrophic and growth factors than those under a normal microenvironment[33-35], and a disease state itself might generate a more favorable condition for neuronal differentiation of this kind of stem cell[27, 29, 31-32]. Some researchers have considered that the acquisition of a glial phenotype by grafted BMSCs might lead to the release of prosurvival cytokines within the lesioned striatum, which might be another explanation for the results we obtained[36].

In the intact striatal extract groups, we noticed that GFAP and NSE expression in the 60% group was significantly higher than that in the 10% group, indicating that intact striatal extracts have a concentration-dependent effect on BMSC differentiation. However, a similar phenomenon was not observed in lesioned striatal extract groups. We failed to delineate the underlying reason because of the limitation of the current experimental condition.

Concerned that the preparative method of striatal extracts was imperfect, we addressed the hypothesis that the absence of tyrosine hydroxylase-positive cells was likely due to the low levels of trophic factors in the extracts, although we did not measure the content of different trophic factors in the striatal extracts. Further studies should be carried out to resolve this issue and to understand the mechanism of BMSC differentiation into neurons induced by striatal extracts from Parkinson's disease rats, which is the foundation for searching for a feasible approach to obtain sufficient dopaminergic neurons *in vitro* for cell transplantation therapy of Parkinson's disease.

In summary, striatal extracts can induce BMSCs to differentiate into cells with neuronal and glial phenotypes *in vitro*. We also found that lesioned striatal extracts exhibit a stronger trophic activity for neuronal differentiation of BMSCs than that of intact extracts, and intact striatal extracts show a concentration-dependent effect on BMSC differentiation. Although the underlying mechanisms were not determined in the present study, our results provide an interesting prospect and insight into possible candidate cells to replace the cells that degenerate in Parkinson's disease, and into the application of neuronal differentiation of adult stem cells.

MATERIALS AND METHODS

Design
An *in vitro* cytology study.

Time and setting
The experiment was performed at the Central Laboratory of the Second Affiliated Hospital, Soochow University, China from March 2006 to May 2007.

Materials
Ten healthy Sprague-Dawley rats of a clean grade, female or male, aged 2–3 weeks and weighing approximately 60–90 g, were used in this study. Another 40 male rats, aged 2–3 months and weighing 220–240 g, were used to establish Parkinson's disease models. All rats were provided by the Animal Center of the Medical College, Soochow University (License No. SYXK (Su) 2007-0035), housed in a temperature-controlled environment with free access to food and water in a 12-hour light/12-hour dark cycle, and handled in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[37].

Methods

Unilateral 6-hydroxydopamine injection for establishment of Parkinson's disease models

Rats were anesthetized with 3.6% chloral hydrate (1 mL/100 g, supplemented as necessary) by intraperitoneal injection, and then fixed in a stereotaxic frame (KOPF Company, Hamburg, Germany). A 10 µL Hamilton syringe was lowered into the right medial fore-brain bundle at two coordinates (from the bregma: right 2.5 mm; posterior 1.8 mm; depth 7.5 mm, and right 2.5 mm; posterior 1.8 mm; depth 8.0 mm; according to *The Stereotaxic Atlas of Rat Brain* [38]). Four microliters of 6-hydroxydopamine (Sigma, St. Louis, MO, USA) were infused at 0.5 µL/min into each point. The microsyringe remained in place for 5 minutes prior to slow withdrawal[39].

Apomorphine-induced rotational behavior for assessment of models

Apomorphine-induced rotational behavior was observed to determine whether the creation of the 6-hydroxydopamine-induced lesion had been successful[39]. At 4 weeks post-lesion, rats were placed in a quiet environment. Counting of rotations began 5 minutes after subcutaneous injections of apomorphine (0.5 mg/kg diluted in 0.9% saline), and rotational asymmetry was monitored over 30 minutes. Rats with at least 210 rotational turns in 30 minutes were regarded as successfully modeled and selected for subsequent use.

Preparation of striatal extracts

We prepared intact striatal extracts (on the left side) and lesioned striatal extracts (on the right side). Under deep anesthesia, the selected model rats were sacrificed by rapid decapitation after the rotational behavior test. Intact and lesioned striata were dissected on dry ice. Striata were weighed and homogenized with specific volumes of PBS in a proportion of 50 mL PBS (0.1 mM) per 100 mg wet weight. Tissue homogenates were centrifuged at 10 000 × g at 4 °C for 1 hour. After filter sterilization, the resulting supernatants were stored at –70 °C until use.

After rapid warming, intact and lesioned striatal extracts were respectively mixed with serum-free culture medium (50% L-DMEM and 50% F12) to prepare 10% and 60% I-SM and L-SM, according to the concentration ratio of the volumes of striatal extracts and total solution.

Isolation and culture of BMSCs

Rats were sacrificed and bone marrow was harvested under sterile conditions by aspiration from femurs and tibias in a rinse solution consisting of 10% fetal bovine serum (Sijiqing Biological Engineering Material Co., Ltd., Hangzhou, China), 45% L-DMEM (Gibco, Carlsbad, CA, USA), 45% F12 (Gibco), 100 U/mL penicillin, and 100 µg/mL streptomycin. After standing for 2–3 minutes, the supernatant containing the cells was recovered and centrifuged at 800 r/min for 5 minutes. The supernatant was discarded, and complete culture medium consisting of 15% fetal bovine serum, 42.5% L-DMEM, 42.5% F12, 100 U/mL penicillin, and 100 µg/mL streptomycin was added. Cells were resuspended and seeded in 75 mL culture flasks at a density of 1×10^6 cells/mL at 37°C in a humidified atmosphere with 5% CO_2. Culture medium was changed every 3–4 days.

Induced differentiation of BMSCs using striatal extracts

Second passage BMSCs at 80% confluence were seeded at a density of 2×10^5/mL on coverslips in six-well plates. Culture medium was changed after 3 days. In experimental groups, BMSCs were cultured with 10% I-SM (10% I-SM group), 10% L-SM (10% L-SM group), 60% I-SM (60% I-SM group), or 60% L-SM (60% L-SM group). In control groups, cells were cultured in medium consisting of 15% fetal bovine serum, 42.5% L-DMEM, and 42.5% F12, or serum-free medium (50% L-DMEM and 50% F12). Cells and their morphological changes were observed under an inverted phase contrast microscope (Olympus, Tokyo, Japan) at 48 hours.

Immunohistochemistry for detection of nestin, GFAP, NSE and tyrosine hydroxylase

BMSCs were washed with PBS, blocked with serum in 0.3% Triton X-100/PBS, and incubated for 1 hour at room temperature with mouse anti-rat nestin (1:200; Chemicon, Santa Cruz, CA, USA), mouse anti-rat GFAP (1:600; DAKO, Glostrup,Denmark), rabbit anti-rat NSE (1:600; NeoMarkers, Fremont, CA, USA) and mouse anti-rat tyrosine hydroxylase (1:200; Chemicon) monoclonal antibodies. After three rinses with PBS, cells were incubated with biotinylated goat anti-mouse/rat IgG (1:200; Chemicon) at 37°C for 15 minutes. The cells were incubated with avidin biotinylated horseradish peroxidase for another 15 minutes, and then diaminobenzidine for 2 minutes followed by rinsing under tap water to terminate the reaction. For imaging, 10 non-overlapped fields of view were randomly selected from each sample under a light microscope (Olympus) and photos were taken using a medicine image analysis system (Motic Med 6.0, Motic, Xiamen, China). Brown or deep-brown cells were considered positive cells, and the number of deep-blue

cell nuclei was regarded as the total cell number. Percentages of nestin-, GFAP-, NSE- and tyrosine hydroxylase-positive cells = nestin-, GFAP-, NSE-, and tyrosine hydroxylase-positive cells/total cell number × 100%, respectively.

Statistical analysis

Data were expressed as mean ± SD, and compared between two groups by one-way analysis of variance. All analyses were performed using SPSS 11.5 software (SPSS, Chicago, IL, USA). *P*-values less than 0.05 were considered statistically significant.

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