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Chinese Herbs for Inducing Rat Bone Marrow Mesenchymal Stem Cells Differentiated into Neuron-Like Cells

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Abstract

The purpose of this study was to observe the influence of two Chinese herbs of ligustrazine and Xuefuzhuyu injection on neuron-like cells differentiation from rat bone marrow mesenchymal stem cells (BMSCs) respectively. Most primarily cultured BMSCs adhered to the wall at 3 days after cultured, which proliferated faster after passaged, and the 5th passage of cells were mostly purified into BMSCs. In the present study, the 5th passage of BMSCs were (97.51±1.22) % CD44 positive, but negative for CD45, which were identified by immunocytochemical technique. Serum-free low-sugar Dulbecco's modified Eagle's medium (L-DMEM) contains 1.25 g/L ligustrazine and 3.0 g/L Xuefuzhuyu injection were used to induce the 5th passage of BMSCs respectively in vitro. Neuron-like cells with prominence and bifurcation could be detected after induction under an inverted phase contrast microscope. The immunocytochemical method showed that nestin and neuron-specific enolase (NSE) were positive in neuron-like cells, but without glial fibrillary acidic protein (GFAP) expression. Both ligustrazine and Xuefuzhuyu injection have the potential for inducing neuron-like cells differentiation from rat bone marrow mesenchymal stem cells respectively. Compared with Xuefuzhuyu injection-induced cells, neuron-like cells induced with ligustrazine might be better in the level of morphological changes, as well as nestin and NSE expression.

Keywords: bone marrow mesenchymal stem cells, neuron-like cells, ligustrazine, xuefuzhuyu injection, induce, differentiation, traditional Chinese medicines

1. Introduction

Brain tissue is frail, anatomically deep, and is prone to liquefactive necrosis after injury, and also it has a low renewable capacity for self-repair and generation of new functional neurons in treatment of central nervous system diseases including trauma, degenerative and hereditary diseases and cerebral vascular diseases, which hamper recovery(Engert et al., 2023; Najafi et al., 2023; L. W. Wang et al., 2024; Xue et al., 2015). However, there are some current treatment methods like surgery, medication and physiotherapy for the therapy of neurological diseases, but unfortunately, which are limited in effectiveness with poor prognosis

(Hernandez et al., 2020; Willing, Das, Howell, Mohapatra, & Mohapatra, 2020). Stem cell transplantation for treating refractory diseases of the central nervous system (CNS) is presently considered a promising therapeutic strategy. Bone marrow mesenchymal stem cells (BMSCs) are non-hematopoietic stem cells of the bone marrow, which can differentiate into neurons and astrocytes under appropriate conditions and have capacity to cross the blood brain barrier and migrate into injured tissues systematically (Feng, Liao, Xu, Zhang, & Zhang, 2022; Muniswami, Kanthakumar, Kanakasabapathy, & Tharion, 2019; Tsai et al., 2014). Nevertheless, when BMSCs were directly transplanted into the CNS and placed in a damaged environment, they tend to differentiate into glial cells (Damianakis et al., 2022; Oraee-Yazdani et al., 2021). Some previous studies indicated that if the transplanted cells were pre-cultured, the survival rate of them were high and the development of mature neurons were significantly increased, meanwhile the induction of stem cells to differentiate into neural precursor cells before transplantation may help to control the differentiation of transplanted cells in a damaged environment (Elgamal et al., 2019; Li et al., 2024; Xia, Yuan, Wang, Xiong, & Xin, 2021; Zayed et al., 2022). Regarding repair of CNS damage, controlling the differentiation process of transplanted cells is a key problem that needs to be solved.

Numerous studies suggest that extracts of traditional Chinese medicines (TCMs), such as ligustrazine, Radix Angelicae Sinensis, Radix Ginseng, Radix Astragali, lycium barbarum polysaccharide, and Radix Salviae Miltiorrhizae, have been used to induce differentiation of BMSCs towards neuron-like cells (Feng et al., 2022; Si et al., 2014; J. Wang et al., 2021; Zhang et al., 2018). Xuefuzhuyu injection (name: Xuebijing) is an extraction of TCMs compounds, whose effective components are extracted from safflower, red peony root, Rhizoma Chuanxiong, Radix Salviae Miltiorrhizae, and Radix Angelicae Sinensis. And the clinical application of Xuefuzhuyu injection is to antagonize endotoxins, improve microcirculation, regulate immune functions and protect vascular endothelial cells. Furthermore, Xuefuzhuyu injection has been shown to be an effective inducer for BMSCs differentiated into neuron-like cells in our previous study (Yin et al., 2011). In the theory of TCMs, both ligustrazine and Xuefuzhuyu injection are promoting blood circulation for removing blood stasis and regulating qi-flowing for relieving pain. And thus, the present study attempted to compare the effects of the single Chinese herbal of ligustrazine and Chinese herbal compound of Xuefuzhuyu injection on BMSCs differentiation towards neuron-like cells in vitro in equivalent environment with uniform conditions at the same time, and to determine which inducer is more effective.

2. Materials and Methods

2.1 Materials

Specific pathogen-free, male, four-week-old, Sprague Dawley (SD) rats, weighing 88–105g, were provided by Guangdong medical college of China for this study. All protocols were conducted in accordance with the *Guidance Suggestions For The Care And Use Of Laboratory Animals*, published by the Ministry of Science and Technology of the People's Republic of China. Ligustrazine hydrochloride injection was purchased from Tianjin Pharmaceutical Group Xinzheng Co., Ltd. And the specification of this injection was 2 mL: 40 mg as well as the batch number was 0706091. Xuefuzhuyu injection was purchased from Tianjin Chase Sun Pharmaceutical Co., Ltd. (batch number was 080418), the active ingredients of which were safflower yellow-A, paeoniflorin, ligustrazine, ferulic acid, tanshinone, and protocatechuic aldehyde.

2.2 BMSCs Isolation and Culture

According to previously described BMSCs isolation and culture method (Aghajani et al., 2016; Dennie, Louboutin, & Strayer, 2016; Nakano et al., 2016), rats were intraperitoneally anesthetized with pentobarbital (1.0 mL/kg, 3 g/L). Femurs and tibias were dissected from the attached muscle and connective tissue. The bone ends were removed, and marrow was extruded with a 5-mL syringe was inserted into the bone shaft and flushed 3 times with 2 mL low-sugar Dulbecco's modified Eagle's medium (L-DMEM, Hangzhou Genome Biomedical Technology, Hangzhou, China), supplemented with 15% fetal bovine serum (FBS, HyClone, Logan City, Utah, USA). Cell suspensions were collected and mixed, and subsequently transferred to a centrifuge tube for centrifugation at 1000 r/min× g for 10 minutes. Next, supernatant was removed, and the cell precipitant was resuspended in 4 mL L-DMEM containing 15%

FBS, 100 U/mL penicillin, and 100 U/mL streptomycin, followed by inoculation into 25-mL culture flasks at 2×10^7 cells/L. The cells were incubated at 37℃ with 5% CO_2 and 95% humidity. Half of the culture medium was replaced after the first 24 hours, and non-adherent cells were removed. Thereafter, total exchange of culture medium was performed every 3 days. Cell growth was monitored under an inverted phase contrast microscope every day. At near-confluency to 80%~90%, cells were passaged, following digestion with 0.25% trypsin (Sigma, St. Louis, MO, USA) at 37℃.

2.3 Immunocytochemistry of BMSCs

The fifth passage cells were cultured in a 24-well plate, fixed with 4% paraformaldehyde for 25 minutes, thoroughly washed with phosphate-buffered saline (PBS, 0.01 mol/L, pH 7.2–7.4), respectively incubated with rabbit-anti-rat CD44 antibody and rabbit-anti-rat CD45 antibody for 2 hours (1: 100, Biosynthesis Biotechnology, Beijing, China), washed with PBS, incubated with secondary antibody (goat anti-rabbit secondary antibody, BOSTER, Wuhan, China) for 20 minutes, and incubated with streptavidin-biotin-enzyme complex (1: 200; ABC detection kit, Zhongshan Golden Bridge Biotechnology, Beijing, China) for 20 minutes. DAB served as a chromagen. Finally, cells were observed under a microscope to detect CD44 and CD45 expression. BMSCs were randomly selected from 10 non-overlapping fields of vision under a microscope, and the number of positive cells was calculated as a proportion of total cells number. For the negative control, primary antibody was replaced with PBS.

2.4 Cell Differentiation

According to a previously described method (Yin et al., 2011), prior to differentiation induction, the fifth passage cells were cultured in 24-well plates with pre-induction medium (1 mL) consisting of L-DMEM/15% FBS/10 μg/L basic fibroblast growth factor (bFGF, Prime Gene Bio-Tech, Shanghai, China) for 24 hours. The preinduction medium was then removed, cells were washed 3 times with PBS, and then the cells were transferred to differentiation medium (1 mL) containing serum-free L-DMEM with 1.25g/L ligustrazine and 3.0 g/L Xuefuzhuyu injection, respectively, for 6 hours. Cells in the control group were cultured in medium containing L-DMEM and 15% FBS without any differentiation inducer. Morphological changes of BMSCs were observed under an inverted phase contrast microscope every 30 minutes, and observation ended at 6 hours after induction.

2.5 Immunocytochemistry

Neuron-like cells were identified with rabbit anti-rat nestin antibody, rabbit anti-rat neuron-specific enolase (NSE) antibody, and rabbit anti-rat glial fibrillary acidic protein (GFAP) antibody (1: 100, Biosynthesis Biotechnology, Beijing, China). For the negative control, primary antibody was replaced with PBS.

Cells were observed under an inverted phase contrast microscope (100 × magnification), and cells expressing nestin, NSE, or GFAP were quantified in 10 non-overlapping, randomly selected fields of vision under a microscope.

2.6 Statistical Analysis

All data were statistically processed using SAS 8.1 software (SAS, Cary, North Carolina, USA) and were expressed as Mean ± standard deviation. So, Student-Newman-Keuls for completely randomized design analysis of variance was used to compare groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1 Primary and Passage Culture of BMSCs

Three days after primary culture, lots of cells were adherence, which were shaped as fusiform or polygon. And cell colony was formed, which was closed to each other. The non-adherent cells were removed by exchanging of culture medium. The adherent cells proliferation was rapid. 7 days after primary culture, 80%~90% cells confluence were observed, and suspension cells in culture medium were mostly removed. Following digestion with 0.25% trypsin at 37℃, the passaged cells shaped round. Most passage BMSCs were adherence in 24 hours, and were uniformly distributed like fusiform shape afresh, which proliferated more rapidly than primary cultured cells. 80%~90% cells confluence were observed on the 5 days after passaged. The fifth passage cells were mostly purified into BMSCs, which were arranged like bostrychoid or whirlpool (Figure 1). The boundary of cell body was more definite, and the morphological features were uniform.

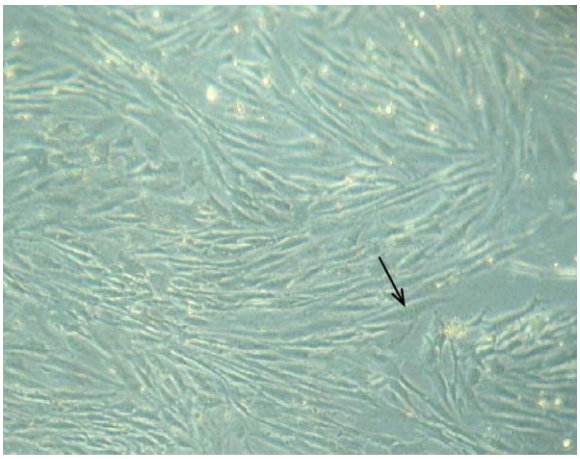

Figure 1. Morphology of passage 5 bone marrow mesenchymal stem cells (arrows) under an inverted phase microscope (magnification, × 100)

3.2 Identification of BMSCs Surface Markers

Immunocytochemistry revealed that the majority of fifth passage cells were CD44-positive and CD45-negative. And the number of CD44 positive cells was (97.51±1.22） % of the total cell population.

3.3 Morphological Changes of BMSCs Towards Neuron-Like Cells Differentiation

Morphological changes were not obvious following pre-induction with L-DMEM/FBS/ bFGF for 24 hours (Figure 2). After the cells were placed in induction medium composed of serum-free L-DMEM with 1.25g/L ligustrazine or 3.0 g/L Xuefuzhuyu injection, for 1 hour, morphological changes occurred. Initially, the cytoplasm had retraction towards the nucleus, and cells bodies became increasingly refractile or transparent, exhibiting a typical neuronal perikaryon appearance. After 4 hours, most cells formed a bipolar, triangular or multipolar shape, which is similar to neuron-like cells. The processes continued to elongate, displaying primary and secondary branches, as well as growth cone-like terminal expansions and putative filopodia extensions. Cells in the control group remained fusiform.

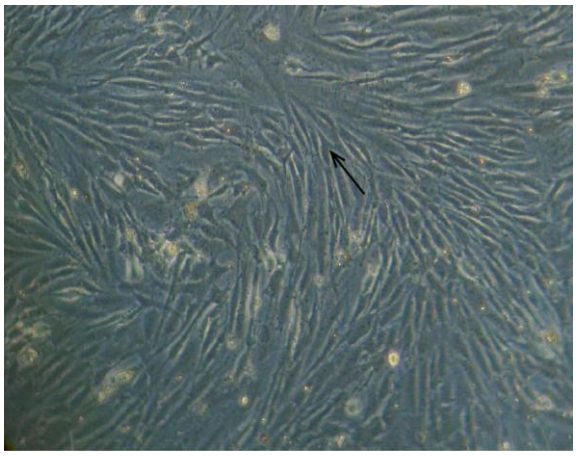

Figure 2. Morphology of bone marrow mesenchymal stem cells (arrows) following pre-induction with L-DMEM/FBS/ bFGF for 24 h (magnification, × 100)

3.4 Identification of Neuron-Like Cells

Following treatment with ligustrazine and Xuefuzhuyu injection for 6 hours, the majority of neuron-like cells presented cell bodies and processes stained brown-yellow for nestin and NSE expression (Figures 3, Figures 4), but no GFAP expression. Cells in the control group did not express nestin, NSE, or GFAP, which was similar to cells in the negative control group, for which primary antibody was replaced with PBS. Expression changes of BMSCs induced with ligustrazine and Xuefuzhuyu injection are shown in Table 1.

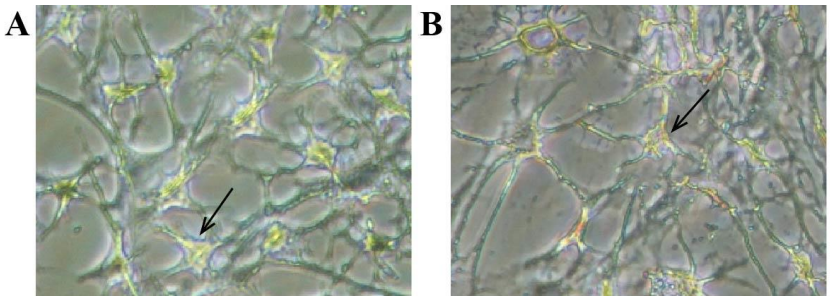

Figure 3. (A) Positive expression of nestin in neuron-like cells formed bipolar shape or multipolar shape (arrows) induced with ligustrazine (magnification, × 200); (B) Positive expression of NSE in neuron-like cells formed bipolar shape or multipolar shape (arrows) induced with ligustrazine (magnification, × 200)

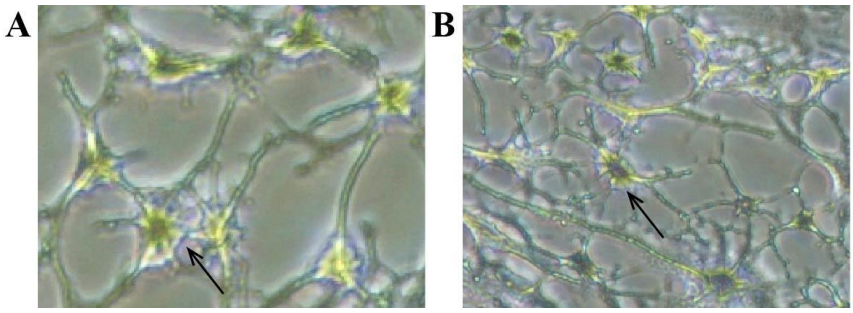

Figure 4. (A) Positive expression of nestin in neuron-like cells formed bipolar shape or multipolar shape (arrows) induced with Xuefuzhuyu injection (magnification, × 200); (B) Positive expression of NSE in neuron-like cells formed bipolar shape or multipolar shape (arrows) induced with Xuefuzhuyu injection (magnification, × 200)

Table 1. Expression of neuronal-like cells antigen of BMSCs induced by ligustrazine and Xuefuzhuyu injection ($\overline{x} \pm s$, n=5 per group, %)

Group	Nestin	Neuron-specific enolase	Glial fibrillary acid protein
BMSCs induced by ligustrazine	75.17±4.02	70.20±4.26[a]	0
BMSCs induced by Xuefuzhuyu injection	69.02±5.68	58.79±6.25	0
Control	0	0	0

Data are presented as Mean ± standard deviation. [a]P< 0.05, vs Xuefuzhuyu injection group. n=5 represents each inducer group occupying five wells in a 24-well plate.

4. Discussion

BMSCs are abundantly sourced, easily isolated, cultured and self-renewable, which also possess the potential for multilineage differentiation, and their autologous transplantation is without any ethical issue (He, Ye, Zhou, & Tan, 2018; Iijima et al., 2018; Kiang & Gorbunov, 2014). And therefore, BMSCs transplantation are regarded as an effective therapeutic approach for treating damaged nerves and improving the functional recovery of CNS diseases (L. J. Wang, Zhang, & Li, 2014; Xu et al., 2022).

In the present study, the majority of fifth passage cells were CD44-positive and with CD45 negative expression, which suggested that they were BMSCs but not hematopoietic cells from bone marrow (Barberini et al., 2014; Han, Huang, & Wang, 2015; Sierra-Sanchez, Ordonez-Luque, Espinosa-Ibanez, Ruiz-Garcia, & Arias-Santiago, 2018). bFGF is an effective growth factor, which promotes BMSCs proliferation and maintains cells in an immature state. Following incubation of BMSCs with bFGF 24 hours, there were no significant morphological changes (Yuan, Qin, Wei, Chen, & Li, 2022; Zhou et al., 2023). In addition, there were no obvious morphological changes observed following incubation with L-DMEM/FBS/bFGF for 24 hours in the current study. Nestin is a distinct sixth class of intermediate filament proteins; it is specifically expressed in neuroepithelial stem cells and is used to distinguish neural stem cells from other differentiated cells in the neural tube (Qin et al., 2017). NSE is a soluble protein in neurons and neuroendocrine cells and has served as a specific marker for mature neurons (Du et al., 2018; Isgro, Bottoni, & Scatena, 2015). GFAP is an intermediate filament protein expressed primarily in astrocytes (Ganne, Balasubramaniam, Griffin, Shmookler Reis, & Ayyadevara, 2022; Kim, Lee, Lim, & Kim, 2022). Immunocytochemistry results verified that the differentiated cells exhibited a morphology similar to neuron-like cells, which suggested that ligustrazine and Xuefuzhuyu injection induced neural differentiation of BMSCs in vitro. Due to the complexity of TCMs prescriptions, we defined the optimal inductive concentration for effective differentiation of

1.25g/L ligustrazine and 3.0 g/L Xuefuzhuyu injection in the preliminary study (Yin et al., 2011).

To date, the majority of Chinese traditional herbal drugs used to induce BMSCs neural differentiation have been shown to exhibit anti-oxidative effects, and many of these effects are dose-dependent. However, the differentiation effects induced by ligustrazine and Xuefuzhuyu injection were not dose-dependent in the previous study (Yin et al., 2011), which suggested mechanisms other than antioxidation.

5. Conclusion

These results illustrate that both ligustrazine and Xuefuzhuyu injection have the potential of inducing neuron-like cells differentiation from rat bone marrow mesenchymal stem cells respectively. Our findings revealed that morphological changes with a typical neuronal perikaryon appearance were visible in cells at 1hour post-induction of ligustrazine and Xuefuzhuyu injection. After 4 hours, most cells formed a bipolar or multipolar shape, which is similar to neuron-like cells. Compared with Xuefuzhuyu injection-induced cells in vitro in equivalent environment with uniform conditions at the same time, those induced with ligustrazine might be better in the level of morphological changes, as well as expression of nestin and NSE in the induction process of differentiation into neuron-like cells, which suggested that Xuefuzhuyu injection might be insufficient for differentiation of BMSCs into neuron-like cells.

TCMs play an important role in health care systems and obtain more focus on clinical practice throughout the world. Previous studies have shown that natural single or compounds from the medicinal plants used in TCMs are a good source for drug discovery. Furthermore, whether single or compound Chinese herbal act as inducer for BMSCs differentiated into neuron-like cells are without appreciable toxicity. And however, further studies are needed to illustrate whether the in vitro effects of these TCM single or compounds can be reproduced in vivo, and whether differentiated neuron-like cells can mature into functional neurons and integrate into the CNS network.

Author Contributions

DHM responsible for the experimental design. YQY and CCL implemented the experiments. YQY provided experimental data. CCL analyzed experimental data. YQY drafted the manuscript. CCL was responsible for statistical analysis. DHM supervised the manuscript. All authors approved the final version of the manuscript.

Conflicts of Interest

None declared.

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