Tetramethylpyrazine induces differentiation of human umbilical cord-derived mesenchymal stem cells into neuron-like cells in vitro

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Abstract. The present study evaluated the ability and optimal concentration of tetramethylpyrazine (TMP) to induce human umbilical cord-derived mesenchymal stem cells (hUMSCs) to differentiate into neuron-like cells in vitro. Human umbilical cords from full-term caesarean section patients were used to obtain hUMSCs by collagenase digestion after removal of the umbilical artery and vein. The surface antigen expression profile of cultured hUMSCs was monitored by flow cytometry. After amplification, cells of the 5th passage were divided into experimental groups A-C treated with TMP at 4.67, 2.34 and 1.17 mg/ml, respectively, in low glucose-Dulbecco’s Modified Eagle’s Medium (L-DMEM) (induction medium), while group D (control) was exposed to L-DMEM culture medium only. Differentiation of hUMSCs into neuron-like cells and morphological changes were observed every 0.5 h with an inverted phase contrast microscope for 6 h. After the 6-h induction period, proportions of cells expressing neuronal markers neuron-specific enolase (NSE), neurofilament protein (NF-H) and glial fibrillary acidic protein (GFAP) were detected by immunohistochemistry. The optimal concentration of TMP was selected on the basis of neuron-like cell positive rate. Western blotting and RT-polymerase chain reaction were applied to detect the expression of NSE, NF-H, and GFAP of the group of optimal concentration in each point-in-time. Results showed that most primary cells were adherent 12 h after seeding and first appeared as diamond or polygon shapes. Thereafter, they gradually grew into long spindle-shaped cells and finally in a radiating or swirling pattern. The cells maintained a strong proliferative capacity after continuous passage. Flow cytometry analysis of cultured hUMSCs at the 3rd, 5th and 10th passages expressed CD73, CD90 and CD105, but not CD11b, CD19, CD34, CD45 or human leukocyte antigen-DR. After 6 h of TMP treatment, typical neuron-like cells with many protrusions connected into a net-like pattern were observed in all experimental groups. These neuron-like cells were positive for NSE and NF-H, but negative for GFAP. Among the tested treatment groups, group A with TMP at 4.67 mg/ml had the highest expression of NSE and NF-H. By contrast, no change was found after induction in the control group. The mRNA expression of cells expressing neuronal markers as well as GAPDH was observed, with the relative NSE transcript levels of 0, 1.303±0.031, 1.558±0.025, 1.927±0.019 and 2.415±0.033 after 0, 1, 2, 4 and 6 h of treatment, respectively; the mRNA expression of NH-F was 0, 1.429±0.025, 1.551±0.024, 1.930±0.042 and 1.398±0.014 after 0, 1, 2, 4 and 6 h of treatment, respectively. There was no expression of GFAP before or after induction and all the groups showed high expression of GAPDH at each time point. Protein expression was also observed on cells expressing neuronal markers as well as GAPDH at each time point. The protein expression of NSE was 0, 0.717±0.097, 0.919±0.056, 1.097±0.143 and 1.157±0.055 in proper order; the protein expression of NF-H was 0, 0.780±0.103, 0.973±0.150, 1.053±0.107 and 0.753±0.094 in proper order. There was no expression of GFAP before or after induction, and all the groups showed high expression of GAPDH at each time point. Our results demonstrated that TMP can induce hUMSCs to differentiate into neuron-like cells effectively with the optimal concentration of 4.67 mg/ml. After induction, the NSE and NF-H of the neuron-like cells were positive, but the GFAP was negative.

Introduction

Mesenchymal stem cells (MSCs) derived from the mesoderm in early development are multipotential stem cells which have the properties of self-renewal and multilineage differentiation. They are widely present in all types of tissues and can differentiate in vitro into cells such as neurons, osteoblasts, chondrocytes, myocytes and adipocytes under certain conditions. Therefore, they are highly valuable in applications of cell replacement therapy and tissue engineering. In 1991, McElreavey et al isolated and cultured fibroblasts with the
cord-derived MSCs (hUMSCs) have been shown to have greater advantages than MSCs derived from bone marrow, placenta and other tissues. Firstly, ethical concerns regarding the source of the stem cells (umbilical cord) are diminished, and they can be obtained at low cost. Secondly, hUMSCs have been determined not to cause teratomas and can inhibit cancer cells. Additionally, due to their low immunogenicity and genetic stability, as well as functions in immunoregulation, stroma support, paracrine signaling and migration, hUMSCs have good clinical therapeutic potential.

There have been literature reports using antioxidants, such as thioglycerol, 2-mercaptoethanol, dimethylsulfoxide (DMSO) and butylhydroxanisole, to experimentally induce MSCs to differentiate into neuron-like cells (2). However, these chemicals cannot be used in live animals due to toxicity. Other researchers proposed traditional Chinese medicine and compound preparations with no or low cytotoxicity to induce neuron-like cells and determined the optimal inductive concentration. Possible induction mechanism is also discussed. Tetramethylpyrazine (TMP) is an active alkaloid (2,3,5,6-tetramethylpyrazine) separated and purified from a Chinese medicine called *Liguisticum walluchii*, which belongs to the family Umbelliferae. The saturation concentration of TMP in aqueous solution at 37°C was 4.67 mg/ml. TMP has been demonstrated to promote expansion of small arteries and enhance blood circulation to prevent blood stasis and antiplatelet aggregation. It has also been shown to antagonize calcium ion flow and to exhibit antiplatelet, antioxidant and antifibrotic properties (4). Although the mechanisms of action have not been clearly defined, it was suggested that the protective effects and related mechanism of TMP against central lesions may largely be attributed to inhibition of calcium channels, antioxidation, resistance of neuronal apoptosis and increase in heat shock protein expression (5). In this study, we examined various doses of TMP to induce hUMSCs to differentiate into neuron-like cells and determined the optimal inductive concentration. Possible induction mechanism is also discussed.

Materials and methods

Materials. Full-term pregnancy cesarean neonatal human umbilical cords were provided by Department Obstetrics/Gynecology, The Second Affiliated Hospital of Hebei Medical University (Hebei, China). This study was approved by the Ethics Committee of the Second Hospital of Hebei Medical University. The following reagents were obtained commercially: TMP (20 mg; purity: HPLC ≥98%; batch no. 120919 Chengdu Preferred Biological Technology Co., Ltd, Chengdu, China); low glucose Dulbecco’s Modified Eagle’s Medium (L-DMEM)/F-12, high glucose-DMEM (H-DMEM)/F-12 and fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA); trypsin (Amresco, LLC, Solon, OH, USA); EDTA and DMSO (Sigma-Aldrich, St. Louis, MO, USA); Triton X-100 (Sinopharm Chemical Reagent Co., Ltd., Beijing, China); penicillin-streptomycin (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China); paraformaldehyde (Tianjin Institute of Chemical Preparations, Tianjin, China); epidermal growth factor (EGF) (human EGF, PHG0314; Beijing Jiamei North Biological Technology Co., Ltd., Beijing, China); fluorescein isothiocyanate (FITC)-CD19, FITC-CD34, phycoerythrin (PE)-CD11b, PE-CD73, PE-CD90, PE-CD45, PE-CD105 and glial fibrillary acidic protein (GFAP) (BD Biosciences, Franklin Lakes, NJ, USA); neurofilament protein (NF-H), neuron-specific enolase (NSE) (Cell Signaling Technology, Inc., Danvers, MA, USA); anti-rabbit immunoglobulin G (IgG) (1:2,000; Affinity Biosciences, Cincinnati, OH, USA); PS immunohistochemistry kit (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China); Taq polymerase chain reaction (PCR) star mix (Beijing GenStar Biosolutions Co., Ltd., Beijing, China); EasyScript First-Strand cDNA synthesis supermix (TransGen Biotech Co., Ltd., Beijing, China); cell RIPA lysis buffer and phenylmethylsulfonyl fluoride (Beijing Solarbio Science & Technology Co., Ltd.).

Isolation and culture of hUMSCs. Umbilical cords were placed in H-DMEM/F-12 culture medium under aseptic conditions, stored at 4°C and then timely transported to a cell culture room to carry out the following steps. Each umbilical cord was rinsed thoroughly with D-Hank’s medium. After removing the blood sample, umbilical artery and umbilical vein, the umbilical cord mesenchymal tissue was cut into 1 mm³ pieces, digested with 0.2% collagenase II and placed in a culture flask containing 2 mg/ml EGF, 20% FBS, 25 mM L-Glu and 100 U/ml penicillin streptomycin/mixture at 37°C with 5% CO₂ and saturation humidity to obtain primary cells. Half of the culture medium was replaced after 24 h, and all the medium was replenished every 3 days. When the cells achieved 80-90% confluency, the medium was removed, and cells were rinsed twice with phosphate-buffered saline (PBS) and then digested with trypsin (0.25%)-EDTA (0.2 g/l) into single cells for passaging at the ratio of 1:3. The culture medium was H-DMEM/F-12 containing 100 U/ml of a penicillin-streptomycin mixture and 10% FBS.

Analysis of cellular phenotype of hUMSCs. In the logarithmic phase of growth, hUMSCs were digested with trypsin and rinsed with PBS, and then the single cell suspension was aliquotted into 10 microcentrifuge tubes at 1x10⁶/tube. Separately, mouse anti-human monoclonal antibodies CD11-PE, CD45-PE, CD73-PE, CD90-PE, CD105-PE, human leukocyte antigen (HLA)-DR-PE, CD19-FITC and CD34-FITC (each 5 µl) were added to eight of the tubes, while anti-mouse IgG1-PE and anti-mouse IgG1-FITC (each 7 µl) were added to the other two tubes as isotype controls. After mixing the contents thoroughly, the tubes were incubated at 4°C for 30 min. Thereafter, the cells were rinsed with PBS and then centrifuged. The supernatant was discarded, and the cells were resuspended with 400 µl of PBS for analysis by flow cytometry.

Differentiation of hUMSCs into neuron-like cells. Cells were obtained from the 3rd passage in logarithmic growth phase with good growth conditions, hUMSCs were rinsed twice with PBS and digested with trypsin (0.25%)-EDTA (0.2 g/l). The digestion was terminated with FBS, and then the cells were centrifuged, resuspended in culture medium and seeded at
peroxidase-conjugated streptavidin at room temperature for body at room temperature for 15 min, followed by horseradish incubated with the appropriate biotinylated secondary anti - 1:200 dilution. After incubation at 4˚C overnight, the cells were stained with freshly prepared DAB for 1 min and counterstained with hematoxylin before finally rinsed repeatedly with water.

Identification of differentiated cells. After induction for 6 h, expression levels of neuronal-specific proteins NSE, NF-H and GFAP were detected by immunocytochemistry. Briefly, after discarding the culture medium from the culture plates, the cells were rinsed once with PBS gently and fixed with 4% paraformaldehyde for 20 min at room temperature. After this step and subsequent steps, the cells were rinsed with PBS three times for 5 min each time, unless otherwise noted. The cell membranes were disrupted with 0.5% Triton X-100 in PBS for 15 min at room temperature away from light and then incubated with 3% H2O2 at room temperature for 5 min. Normal goat serum was added to the cells for blocking at room temperature for 15 min and then removed without washing. Primary antibodies to NSE, GFAP and NF-H were added at a 1:100 dilution. After rinsing with PBS for 5 min, the membranes were incubated with anti-rabbit IgG (1:2,000; Affinity Biosciences) for 2 h at room temperature and visualized by SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA) using the Image AlphaEaseFC system (Alpha Innotech Co., San Leandro, CA, USA).

Calculation of frequency of neuron-like cells. A total of 10 non-overlapping representative fields under an inverted microscope were selected in each group, in which the total number of cells and neuron-like cells were counted. The proportion of neuron-like cells was calculated and averaged from the 10 fields for each group. Results were presented as mean ± standard deviation.

Statistical analysis. Statistical analysis was performed by using SPSS 19.0 software (SPSS, Inc., Chicago, IL, USA). Comparison on the experimental data with a completely randomized design was tested by analysis of variance among groups and the Student-Newman-Keuls method (q-test). Difference in the proportion of neuron-like cells among groups was analyzed by the Chi-square test. P<0.05 was considered to indicate a statistically significant difference.

Results

Growth and morphological changes of hUMSCs. Primary cultured cells were passaged at the ratio of 1:1, and most cells were adherent after 12 h. However, the adherent cells were not outstretched, but appeared triangular or diamond in shape.

Table I. The primer sequences used for RT-polymerase chain reaction analysis in this study.

| Genes  | Primer sequences                          | Sizes (bp) | Temperature (˚C) |
|--------|------------------------------------------|------------|-----------------|
| NSE    | F: GGCACTCTACCAGGACTTTG R: GCGATGACTCACCATAACCC | 398        | 61.7            |
| NF-H   | F: TGAACACAGACGCCATGCGCTCAG R: CACCTTTATGTGAGTGGACACAGAG | 286        | 61.2            |
| GAPDH  | F: AGAAGGGCTGGGGCTCATTGG R: AGGGGCCATCCACAGTCTTC | 258        | 64.0            |

NSE, neuron-specific enolase; NF-H, neurofilament protein; F, forward; R, reverse.

1x10⁵/ml into five 6-well cell culture plates coated with polylysine. The plates were divided randomly into groups A-D and then placed in the incubator. When the cells achieved 70-80% confluency, TMP induction medium was added to cells of groups A-C at the concentrations of 4.67, 2.34 and 1.17 mg/ml, respectively. Group D was treated with L-DMEM only and served as control. All the plates were cultured in an incubator at 37˚C with 5% CO₂ and saturation humidity for 6 h. Morphological changes were observed with an inverted phase contrast microscope each 0.5 h.

RT-PCR. Total RNA was extracted by TriPure Reagent (Aidlab Biotechnologies Co., Ltd., Beijing, China) following the manufacturer's instructions and quantified with a Ultramicro-ultraviolet visible light meter (Gene Company, Ltd.). Total protein (16 mg) of each lysate was electrophoresed in a 10% SDS-PAGE gel and transferred to a polyvinylidene fluoride membrane (Beijing Solarbio Science & Technology Co., Ltd.), and protein concentrations were determined with an ultramicro-ultraviolet visible light meter (Gene Company, Ltd.). Total protein (16 mg) of each lysate was electrophoresed in a 10% SDS-PAGE gel and transferred to a polyvinylidene fluoride membrane (Beijing Solarbio Science & Technology Co., Ltd.). The membranes were blocked in 10% non-fat milk for 2 h and incubated with the GFAP, NF-H and NSE primary antibodies at 4˚C overnight. After rinsing with PBS for 5 min, the membranes were incubated with anti-rabbit IgG (1:2,000; Affinity Biosciences) for 2 h at room temperature and visualized by SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA) using the Image AlphaEaseFC system (Alpha Innotech Co., San Leandro, CA, USA).

Western blot analysis. Cell lysates were prepared with cell RIPA lysis buffer and phenylmethylsulfonyl fluoride (Beijing Solarbio Science & Technology Co., Ltd.), and protein concentrations were determined with an ultramicro-ultraviolet visible light meter (Gene Company, Ltd.). Total protein (16 mg) of each lysate was electrophoresed in a 10% SDS-PAGE gel and transferred to a polyvinylidene fluoride membrane (Beijing Solarbio Science & Technology Co., Ltd.). The membranes were blocked in 10% non-fat milk for 2 h and incubated with the GFAP, NF-H and NSE primary antibodies at 4˚C overnight. After rinsing with PBS for 5 min, the membranes were incubated with anti-rabbit IgG (1:2,000; Affinity Biosciences) for 2 h at room temperature and visualized by SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA) using the Image AlphaEaseFC system (Alpha Innotech Co., San Leandro, CA, USA).

Calculation of frequency of neuron-like cells. A total of 10 non-overlapping representative fields under an inverted microscope were selected in each group, in which the total number of cells and neuron-like cells were counted. The proportion of neuron-like cells was calculated and averaged from the 10 fields for each group. Results were presented as mean ± standard deviation.

Statistical analysis. Statistical analysis was performed by using SPSS 19.0 software (SPSS, Inc., Chicago, IL, USA). Comparison on the experimental data with a completely randomized design was tested by analysis of variance among groups and the Student-Newman-Keuls method (q-test). Difference in the proportion of neuron-like cells among groups was analyzed by the Chi-square test. P<0.05 was considered to indicate a statistically significant difference.

Results

Growth and morphological changes of hUMSCs. Primary cultured cells were passaged at the ratio of 1:1, and most cells were adherent after 12 h. However, the adherent cells were not outstretched, but appeared triangular or diamond in shape.
The cell culture medium was replaced to remove non-adherent cells after 24 h. The adherent cells proliferated rapidly and became significantly larger and more uniform, long fusiform cells after 48 h of culture. They achieved 80-90% confluency on the 7th day as a monolayer arranged in a radial or spiral shaped pattern. After digestion, the hUMSCs cells floated as spherical single cells in the culture medium and were passaged at the ratio of 1:3 after centrifugation. Most cells were adherent 24 h after passaging and achieved 80-90% confluency on the 7th day with a radial or spiral shaped arrangement (Fig. 1A). The hUMSCs of the 10th passaged maintained a strong proliferative capacity.

Cellular phenotype of hUMSCs. We detected the cellular phenotype of the 2nd, 5th and 10th passages of hUMSCs by flow cytometry and found that all generations of the cells tested co-expressed CD105, CD90 and CD73, but not CD11b, CD34, CD19, CD45 and histocompatibility antigen HLA-DR (major histocompatibility complex class II) (Fig. 2). Morphological and quantitative changes of neuron-like cells in each group. After treatment with TMP for 0.5 h, 40% of cells in group A contracted into an oval shape and extended protuberances from their cell bodies (Fig. 1B). After treatment for 1 h, the ratio of neuron-like cells reached 55%, with the cell bodies contracted and the protuberances lengthened further. At this time, the cells appeared connected in a net-like pattern. After treatment for 1.5 h, ~85% of the cells appeared as neuron-like cells. After treatment for 4 h, some of the neuron-like cells began to detach, and the ratio was reduced.
The neuron-like cells in group C required more time to reach the highest ratio, and the detaching cells could also be seen during the process of differentiation. Western blot detection of neuronal-specific markers. On the basis of neuron-like cell positive rate, we selected the TMP treatment at a concentration of 4.67 mg/ml to detect the expression of NSE, NF-H, GFAP using western blotting. Protein expression of cells expressing neuronal markers as well as GAPDG was observed after TMP treatment. The calculated relative transcript level for protein expression of NSE was 0, 0.717±0.097, 0.919±0.056, 1.097±0.143 and 1.157±0.055 before treatment, for 1, 2, 4 and 6 h, respectively; whereas, the relative level for NH-F expression was 0, 0.780±0.10, 0.973±0.150, 1.053±0.107 and 0.753±0.094, respectively. No expression of GFAP was detected before or after induction, whereas all tested samples showed high expression of GAPDH after TMP treatment (Fig. 5).

RT-PCR detection of neuronal-specific markers. RT-PCR was used to detect the expression of NSE, NF-H, and GFAP of the group C in each time point after TMP treatment. The mRNA expression of NSE was 0, 1.303±0.031, 1.558±0.025, 1.927±0.019 and 2.415±0.033 in proper order. The mRNA expression of NF-H was 0, 1.429±0.025, 1.551±0.024, 1.930±0.042 and 1.398±0.014 in proper order. There was no expression of GFAP before or after induction and all the groups showed high expression of GAPDH at each time point (Fig. 6).

Discussion

The plasticity of stem cells refers to the ability of adult stem cells to lose their phenotype of a specific tissue or germ layer and differentiate into other types of cells (6-8). Typically, MSCs exhibit cell plasticity by being able to differentiate into bone, cartilage, smooth muscle, skeletal muscle and cardiac muscle, as well as cells from other germ layers such as skin and liver (9-19). Woodbury et al (2) first reported in 2000 that BMSCs can differentiate into neuron-like cells under certain conditions, a finding that has attracted significant attention. Soon afterwards, numerous domestic and foreign laboratories carried out in vitro and in vivo studies on the neural differentiation of MSCs from different species and sources. These studies have shown that MSCs of rats, mice, humans, rabbits and other mammals can be induced to differentiate into neuron-like cells under certain conditions. In this context, newborn umbilical cords, as a reliable source of MSCs that can be obtained non-invasively and without ethical constraints, have been widely used in stem cell transplantation therapy and experiments of neural differentiation.

Some inducers, including chemical inducer, neurotrophic factor, and Chinese medicine active ingredients and their preparations, have been shown to be able to differentiate MSCs into neuron-like cells expressing surface antigen markers of neural cells. Our study confirmed that the TMP monomer (2,3,5,6-tetramethylpyrazine), an active ingredient in Chinese medicine, could effectively induce hUMSCs to differentiate into neuron-like cells in vitro and express NSE and NF-H, but not GFAP. Moreover, the optimal concentration of TMP evidenced when PBS was used to replace the primary antibody (data not shown). As shown in Table II, differences in the expression levels of NF-H and NSE among these groups were significant (P<0.01).

Table II. Comparison of antigen expression in neuron-like cells induced from hUMSCs after 6 h of TMP treatment (%). Data are presented as the mean ± standard deviation. a,b,cCompared to group A for NF-H and NSE, respectively. hUMSCs, human umbilical cord-derived mesenchymal stem cells; TMP, tetramethylpyrazine; NSE, neuron-specific enolase; NF-H, neurofilament protein.

| Group   | NF-H     | NSE     |
|---------|----------|---------|
| A-TMP 4.67 mg/ml | 80.79±4.36 | 79.76±2.46 |
| B-TMP 2.34 mg/ml | 71.30±1.94<sup>ab</sup> | 69.83±4.42<sup>ac</sup> |
| C-TMP 1.17 mg/ml | 52.01±3.66<sup>ac</sup> | 50.18±4.07<sup>ac</sup> |
| D-Control | 0        | 0       |

Figure 3. The proportion of tetramethylpyrazine-induced neuron-like cells was, from high to low, group A > group B > group C at each time point, and it declined gradually over the induction time indicating that the neuron-like cells were continually becoming detached. The neuron-like cells in group C required more time to reach the highest ratio, and the detaching cells could also be seen during the process of differentiation.

Immunocytochemical detection of neuronal-specific markers. Results from immunocytochemical staining showed that most neuron-like cells treated by different concentrations of TMP for 6 h were positive for NF-H and NSE, but negative for GFAP (Fig. 4). By contrast, no cells were found positively expressing these markers in the control group. Negative staining was observed when PBS was used to replace the primary antibody (data not shown). As shown in Table II, differences in the expression levels of NF-H and NSE among these groups were significant (P<0.01).

Figure 3. The proportion of neuron-like cells treated by different concentrations of TMP for 6 h were positive for NF-H and NSE, but negative for GFAP. By contrast, no cells were found positively expressing these markers in the control group. Negative staining was observed when PBS was used to replace the primary antibody (data not shown). As shown in Table II, differences in the expression levels of NF-H and NSE among these groups were significant (P<0.01).
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Figure 4. Different concentrations of tetramethylpyrazine (TMP) can induce differentiation of human umbilical cord-derived mesenchymal stem cells into neuron-like cells in vitro. Immunohistochemical staining of neuron-like cells for (A-C) neurofilament protein and (D-F) MAP-2 in groups treated with TMP at the concentrations of 4.67 mg/ml (group A, A and D), 2.34 mg/ml (group B, B and E), and 1.17 mg/ml (group C, C and F). The arrows in C and F indicate the neuron-like cells.

Figure 5. Detection and quantitative analysis of protein expression in tetramethylpyrazine (TMP)-inducing neuron-like cells. Protein expression of neuron-specific enolase (NSE) and neurofilament protein (NF-H) in neuron-like cells was confirmed after TMP treatment by western blotting (top panel). The lower panels show the normalized protein expression levels for NSE (lower-left panel) and NF-H (lower-right panel).
for obtaining these inductive effects was determined to be 4.67 mg/ml, which is the saturation concentration of TMP in aqueous solution at 37˚C (20).

Different inducers have different mechanisms of facilitating neuronal cell differentiation. The common feature of chemical inducers is their ability to increase the intracellular concentration of cAMP, suggesting that the second messenger is involved in the induction of MSCs to differentiate into neural precursor cells (21). Butylated hydroxyanisole, β-mercaptoethanol and other antioxidants promote an increase of intracellular cAMP in different ways and then activate the PKA pathway and phosphorylation of downstream target proteins. Moreover, PKC has an important role in the induction process to maintain cell survival. The MEK-ERK signaling pathway also plays an important role in the process of neural cell induction from MSCs. Neurotrophic factor inducers include basic fibroblast growth factor (bFGF), EGF, retinoic acid (RA), nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF). In the neuronal differentiation of mouse MSCs, the medium used by Kohyama et al (22) included a demethylation agent (5-azaC), NGF, NT-3 and BDNF, while Jin et al (23) successfully used EGF, bFGF, RA and NGF. The mechanism by which neurotrophic factors promote neural differentiation of MSCs may involve their high concentrations which can potentially simulate the microenvironment of embryonic developmental stages of neurogenesis, thereby promoting the differentiation of MSCs into neural cells. Previous studies have shown that neurotrophic factors increase the expression of MSC membrane proteins TrkA, TrkB and TrkC, which are neurotrophic receptors. The binding of neurotrophin and its receptor initiates changes in some gene expression (24). Traditional Chinese medicines may have antioxidant and anti-ischemic properties and other effects, as well as improve microcirculation. Previously, we also found that they have protective effects against nerve cell injury (25). TMP may play a role as antioxidant in promoting the increase in the intracellular second messenger cAMP, which subsequently activates the PKA pathway and the MEK-ERK signaling pathway, and thus plays a role in the neural induction process. Liu et al (26) and others have pointed out that TMP as a Ca²⁺ chelator, via the inhibition of the intracellular Ca²⁺ signal, can upregulate the expression of NSE and Nurrl, thereby accelerating the differentiation of hUMSCs into nerve cells. Zhao et al (27) indicated that sub-totipotent stem cells still express sub-totipotent genes after the embryo has developed into adulthood, but they gradually lose part of the original stem cell phenotype. If the tissue-specific gene expression programs of such cells were activated in an appropriate microenvironment, they can differentiate into various histocytes. hUMSCs are sub-totipotent stem cells, but whether the microenvironment provided by TMP can activate the specific gene expression program of nerve cells to further differentiate into neural cells will require further study.

The ultimate goal of inducing MSCs to differentiate into nerve cells is to use them in vivo as a replacement therapy. However, electrophysiological evidence of whether the induced cells can function as nerve cells is still lacking. Some researchers have claimed that the morphological change of cells was initiated by the toxicity of the inducers, and the long protuberances were caused by the reduction of cytoplasm due to destruction of the actin cytoskeleton. According to this argument, the expression of nerve cell antigen markers is more likely to be result of an abnormal combination of protein interactions rather than a series of genetic events (28). Moreover, most induced nerve cells live for only a short period of time in vitro, which creates a barrier to their effective use in transplantation therapies.
In conclusion, our study demonstrated that TMP can induce hUMSCs to effectively differentiate into neuron-like cells with the optimal concentration of 4.67 mg/ml. After induction, theNSE and NF-H of the neuron-like cells were positive but theGFAP-2 was negative. Future studies will verify whether the differentiated cells are bona fide nerve cells, and the extra-cellular environment required to maintain neuron-like cells after induction in vitro also need further investigation, which may provide additional evidence and rationale for using in vitro induced and differentiated hUMSCs in potential nerve cell transplantation.

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