Original article

Human umbilical cord Wharton's Jelly-derived mesenchymal stem cells differentiation into nerve-like cells

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Keywords: *Wharton's Jelly* · *mesenchymal stem cells* · *differentiation* · *nerve cells* · *Salvia miltiorrhiza*

Background The two most basic properties of mesenchymal stem cells (MSCs) are the capacities to self-renew indefinitely and differentiate into multiple cells and tissue types. The cells from human umbilical cord Wharton's Jelly have properties of MSCs and represent a rich source of primitive cells. This study was conducted to explore the possibility of inducing human umbilical cord Wharton's Jelly-derived MSCs to differentiate into nerve-like cells.

Methods MSCs were cultured from the Wharton's Jelly taken from human umbilical cord of babies delivered after full-term normal labor. Salvia miltiorrhiza and β-mercaptoethanol were used to induce the human umbilical cord-derived MSCs to differentiate. The expression of neural protein markers was shown by immunocytochemistry. The induction process was monitored by phase contrast microscopy, electron microscopy (EM), and laser scanning confocal microscopy (LSCM). The pleiotrophin and nestin genes were measured by reverse transcription-polymerase chain reaction (RT-PCR).

Results MSCs in the Wharton's Jelly were easily attainable and could be maintained and expanded in culture. They were positive for markers of MSCs, but negative for markers of hematopoietic cells and graft-versus-host disease (GVHD)-related cells. Treatment with Salvia miltiorrhiza caused Wharton's Jelly cells to undergo profound morphological changes. The induced MSCs developed rounded cell bodies with multiple neurite-like extensions. Eventually they developed processes that formed networks reminiscent of primary cultures of neurons. Salvia miltiorrhiza and β-mercaptoethanol also induced MSCs to express nestin, β-tubulin Ⅲ, neurofilament (NF) and glial fibrillary acidic protein (GFAP). It was confirmed by RT-PCR that MSCs could express pleiotrophin both before and after induction by Salvia miltiorrhiza. The expression was markedly enhanced after induction and the nestin gene was also expressed.

Conclusions MSCs could be isolated from human umbilical cord Wharton's Jelly. They were capable of differentiating into nerve-like cells using Salvia miltiorrhiza or β-mercaptoethanol. The induced MSCs not only underwent morphologic changes, but also expressed the neuron-related genes and neuronal cell markers. They may represent an alternative source of stem cells for central nervous system cell transplantation.

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Mesenchymal stem cells (MSCs) in human umbilical cord blood are multipotent stem cells that differ from hematopoietic stem cells.[1,2] Recent reports about trans-differentiation of mononuclear cells derived from human umbilical cord blood into neural cells aroused great interest

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among investigators for their clinical implication and significance in cell transplantation of the central nervous system (CNS).[1,3] In the present study, we induced Wharton's Jelly-derived MSCs to differentiate into neural cells after isolation and expansion of MSCs from human umbilical cord. Therefore, the hypothesis that human umbilical cord Wharton's Jelly contained multipotent progenitor cells that could proliferate and differentiate into neural cells (neuronal and/or glial cells) could be tested.[4,5]

METHODS

Cell culture and main reagents

Human umbilical cord Wharton's Jelly for this study was aseptically collected from infants delivered by full-term normal labor at Second Affiliated Hospital of Shantou University Medical College (SUMC). Salvia miltiorrhizza was supplied by Shanghai Chinese & Western Pharmaceutical Co., China. Dulbecco's modified eagle's medium (DMEM), N2 medium, fetal bovine serum (FBS) and 1% glutamine were bought from Gibco Co., USA. Epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), β-mercaptoethanol, β-tubulin Ⅲ, neurofilament (NF), glial fibrillary acidic protein (GFAP) and PI were purchased from Sigma Co, USA. Nestin, streptavidin-biotin-avidin complex (SABC) and fluorescein isothiocyanate (FITC)-coupled anti-mouse immunoglobulin G (IgG) antibodies were bought from Boster Co, China. Ancell Co. France supplied the following: CD_{29}, CD_{44}, CD_{59}, CD_{14}, CD_{33}, CD_{34}, CD_{45}, CD_{38}, CD_{117}, CD_{80}, CD_{86}, CD_{40}, CD_{40L}. Trizol was from Invitrogen, USA, and materials for reverse transcriptase-polymerase chain reaction (RT-PCR) were supplied by TaKaRa, Japan.

Wharton's Jelly was processed within 24 hours of collection and cut into pieces of about 1.5 –2.5 mm for culture. These pieces were placed in a 6-well plate and cultured in DMEM, supplemented with 10% FBS, EGF 5ng/ml, bFGF 5ng/ml, penicillin-streptomycin (100 U/ml and 100 mg/ml, respectively) and amphoterin B (1 μg/ml). The culture plate was put in the incubator with saturated humidity at about 37℃, containing 5% (v/v) of CO_2. The medium was changed every 3 days during the culture. Passaging began when the cells reached 70% confluence. After digestion with 0.25% trypsin for 3 to 5 minutes, cells were then centrifuged.

Cell identification

After the cultured cells from Wharton's Jelly reached the 3rd and 8th passages, the surface antigens of the cells were identified by FACScan cytometry, and their immunophenotypes were determined.

Induction of Wharton's Jelly cells

When the cells reached the 3rd and 8th passages, induction of Wharton's Jelly primary culture was performed. With the cells at 60%-70% confluence, pre-induction was performed with 20 ng/ml of bFGF added to the medium for 24 hours. The medium was then changed and the inducing agent, either Salvia miltiorrhiza or β-mercaptoethanol, was added. For the former, 15 mg of Salvia miltiorrhiza was added to 100 ml medium; 4–6 hours later, the medium was removed and replaced by 4% formaldehyde for cell fixation. On the other hand, some cells were induced for 12–24 hours and continued to be cultured in N2 medium for further 12–24 hours before fixing with 4% formaldehyde.[6] The second inducing agent, β-mercaptoethanol was added into the DMEM (with 2% FBS) at a concentration of 1 to 2 mmol/L for 24 hours. The medium was then removed and replaced by DMEM with the addition of β-mercaptoethanol at a final concentration of 2 to 4 mmol/L for 1 to 5 hours. After cultured in N2 medium for further 12–24 hours, the cells were fixed with 4% formaldehyde.[7] The whole induction process was carefully monitored using the phase contrast microscope. The cell morphology before and after induction was compared using electron microscope.

Immunohistochemistry

After induction, the Wharton's Jelly primary culture was analyzed by immunohistochemistry using the SABC method. Nestin (the marker of neuronal stem cells at early stage of differentiation), NF, β-tubulin Ⅲ (the neuron marker), and GFAP (the glial cells markers) were used as primary antibodies. The experiments were repeated at least three times.

Semi-quantitative RT-PCR

Total RNA of the cells was extracted by Trizol for semi-quantitative RT-PCR, which was performed under the following conditions: nestin, forward primer: 5'-AGAGGGGAATTCCTGGAG-3' and reverse primer: 5'-CTGAGGACCAGGACTCTCTA-3' (495 bp), denaturation at 53℃. For neurite outgrowth-promoting protein (pleiotrophin), forward

primer 5'-CCAAAGCCAAGAAAGGGAAG-3' and reverse primer 5'-TTTATTGGGGGGAAAAA GTCAG-3' (342 bp) were used followed by denaturation at 52℃. β-actin was used as an internal reference in each reaction, with forward primer: 5'-TGGCACCACACCTTCTACAATGAGC-3' and reverse primer: 5'-GCACAGCTTCTC-CTTAATGTCACGC-3' (396 bp).

RESULTS

MSCs culture

Wharton's Jelly was cut into pieces and cultured in DMEM. Five to seven days later, cells grew as a flat single layer, when the cells reached confluence they resembled fibroblasts (Fig. 1A), with the formation of spherical cellular masses of different sizes. The duration of primary culture was 10 – 14 days. After passaging, the cells could proliferate 4 to 5 times in 3 to 5 days, but this proliferation rate somewhat decreased after 9 times of passaging.

Cell identification

The MSCs of the 3rd and 8th passages were examined by FACScan cytometry. We found that the positive ratios of the MSCs surface-marker CD_{29}, CD_{44}, CD_{59} were 35.7%, 76.7%, and 95.4%, respectively, from the 3rd passage, and 39.9%, 52.1%, and 90.2%, respectively, from the 8th passage. However, these cells did not express hematopoietic lineage markers CD_{14}, CD_{33}, CD_{34}, CD_{45}, CD_{38} and CD_{117}. Nor did they exhibit the GVHD-related markers CD_{80}, CD_{86}, CD_{40} and CD_{40L}.

Morphological change

The MSCs of the 3rd and 8th passages were cultured in DMEM, and induction was performed. After Salvia miltiorrhiza was added into the medium, the morphology of these cells markedly changed after 1 to 2 hours. Four to eight hours later the cells became contracted and smaller, and formed bipolar or multipolar prominences. As a result, these cells appeared spherical, star-like or elongated. Furthermore, 24 hours later, these morphological changes were most evident. Fig. 1A demonstrated that through the electron microscope, the un-induced MSCs displayed an elongated, flat fibroblast-like morphology with granules on the surfaces. No obvious protrusions from the cell bodies were found, nor were there networks between the cells. After induction by Salvia miltiorrhiza for 24 hours, some MSCs developed multiple neurites extending from the cell body, single long axon-like processes also developed (Fig. 1B). The induced MSCs resembled bipolar or multipolar neurons, with processes that formed networks reminiscent of primary cultures of neurons (Figs. 1C and 1D).

As to the induction by β-mercaptoethanol, changes in the cellular morphology were not evident at the low concentration of 1 – 2 mmol/L at 24 hours. However, cell morphology changed greatly at the higher concentration of 2 – 4 mmol/L, even after 1 to 5 hours, similar to that induced by Salvia miltiorrhiza. In the N2 medium, the cultured MSCs remained stable.

Immunohistochemistry

After bFGF was added into DMEM culture for the pre-induction of Wharton's Jelly MSCs, no obvious morphological changes occurred. The immunohistochemical results demonstrated that MSCs were weakly positive for nestin, β-tubulin Ⅲ, NF, and GFAP expressions (Fig. 2A showed weakly positive expression of NF). By contrast after induction by Salvia miltiorrhiza, these MSCs not only demonstrated morphological changes (Fig. 2B), but also showed strong positive staining for nestin, β-tubulin Ⅲ, NF and GFAP. Fig. 3A showed positive nestin staining in MSCs induced by Salvia miltiorrhiza for 4 hours. Figs. 3B, 3C and 3D

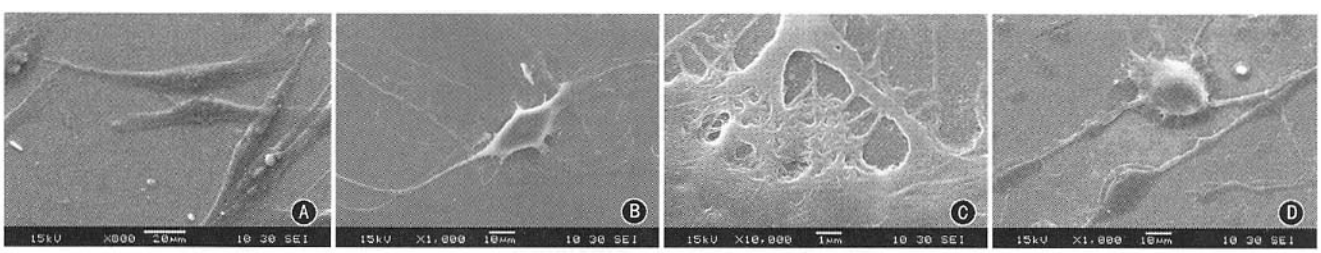

Fig. 1. MSCs morphology by electron microscope. **A**: Un-induced MSCs display an elongated, flat fibroblast-like (original magnification ×800); **B**, **C** and **D**: after induction by Salvia miltiorrhiza (**B**, **C**, **D**: original magnification ×1000, ×10 000, ×1000, respectively).

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demonstrated positive staining in MSCs, induced by 24 hours of Salvia miltiorrliza, for β-tubulin Ⅲ, NF and GFAP expressions, respectively. Fig. 4 showed positive staining in MSCs, induced by 5 hours of 4 mmol/L β-mercaptoethanol, for nestin, β-tubulin Ⅲ, NF, and GFAP, respectively.

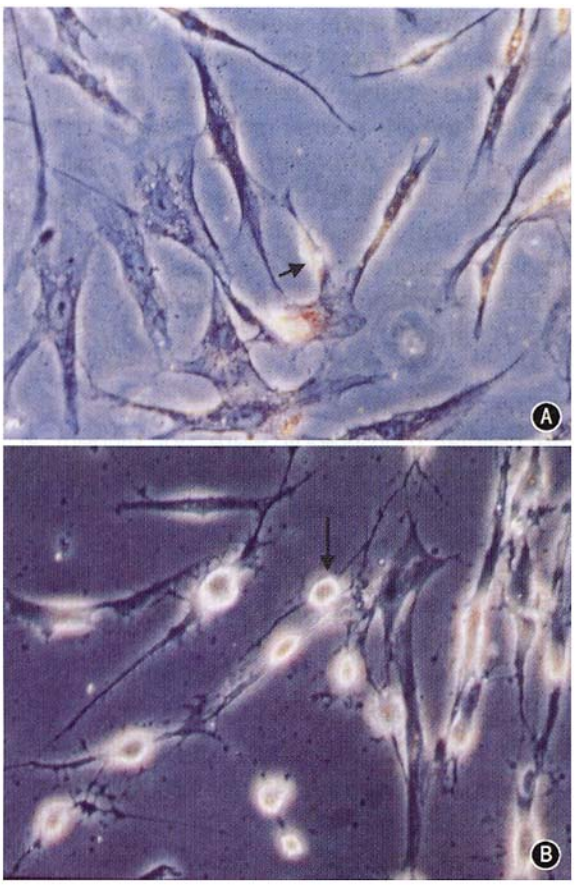

Fig. 2. **A**: Pre-induction NF has been shown weakly positive (SABC, original magnification × 200); **B**: MSCs morphology change after induction by salvia miltiorrhiza (SABC, original magnification ×200).

Confocal microscopy revealed that the expression of neuronal protein β-tubulin III was different between the untreated and induced MSCs. The latter cells showed much stronger expression than the untreated ones (Figs. 5A and 5B).

Results of RT-PCR

Fig. 6 demonstrated that there was no nestin band before the induction. However, the nestin band appeared after induction by Salvia miltiorrhiza for 4 hours.

The marker of neural cell pleiotrophin (Neurite outgrowth-promoting protein) was positive both before and after the induction by Salvia miltiorrhiza for 24 hours, but the expression was markedly increased after induction, as revealed in Fig. 7.

DISCUSSION

MSCs is a group of multipotent cells that can expand, self-replicate, and differentiate into many cell types under appropriate conditions.[8-11] Furthermore, Kobayashi et al[12] revealed that matrix cells from the Wharton's Jelly showed similar characteristics with the MSCs from bone marrow. Mitchell et al[5] reported that these matrix cells could proliferate over 80 times and express the markers of stem cells such as CD_{117} and telomerase; and under the conditions of bFGF, low concentration of serum and dimethyl sulfoxide, they also expressed neural markers.

The present study found that the Wharton's Jelly MSCs could proliferate rapidly in DMEM with FBS, EGF and bFGF added, and after passaging could proliferate 4 to 5 times within 3 to 5 days. Moreover, cell proliferation could be maintained for 10 passages. These proliferated cells were mainly elongated fibroblast-like cells with or without branching, very similar to the MSCs from the bone marrow. Our results were consistent with previous reports showing that Wharton's Jelly contained some stem cells that could expand, self-replicate, and differentiate into multiple cell types under appropriate induction conditions.[13] With FACScan

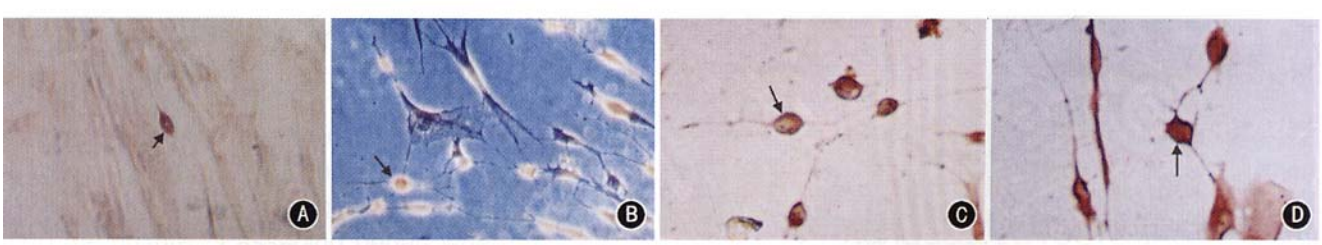

Fig. 3. The MSCs express neural markers arrow after induction with Salvia miltiorrhiza. **A-D**: Immunocytochemical detection of nestin, NF, β-tubulin Ⅲ and GFAP respectively [SABC, original magnification ×200 (**A, B**), ×400 (**C, D**)].

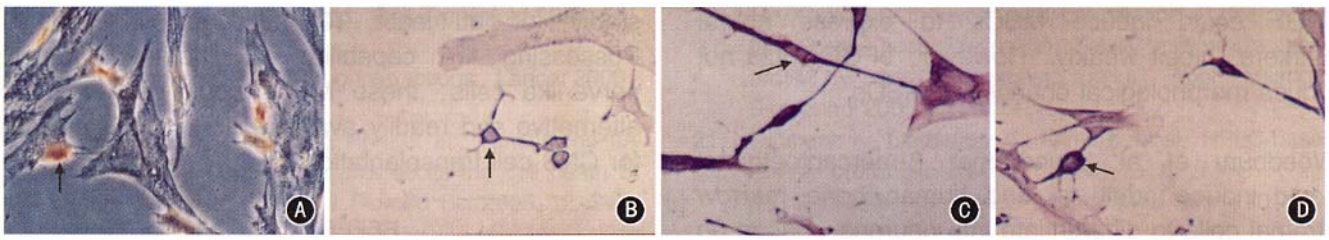

Fig. 4. The MSCs express nestin (**A**), NF (**B**), β-tubulin Ⅲ (**C**), and GFAP (**D**) arrow after induction treatment with β-mercaptoethanol [SABC, original magnification ×200 (**A**), ×400 (**B, C, D**)].

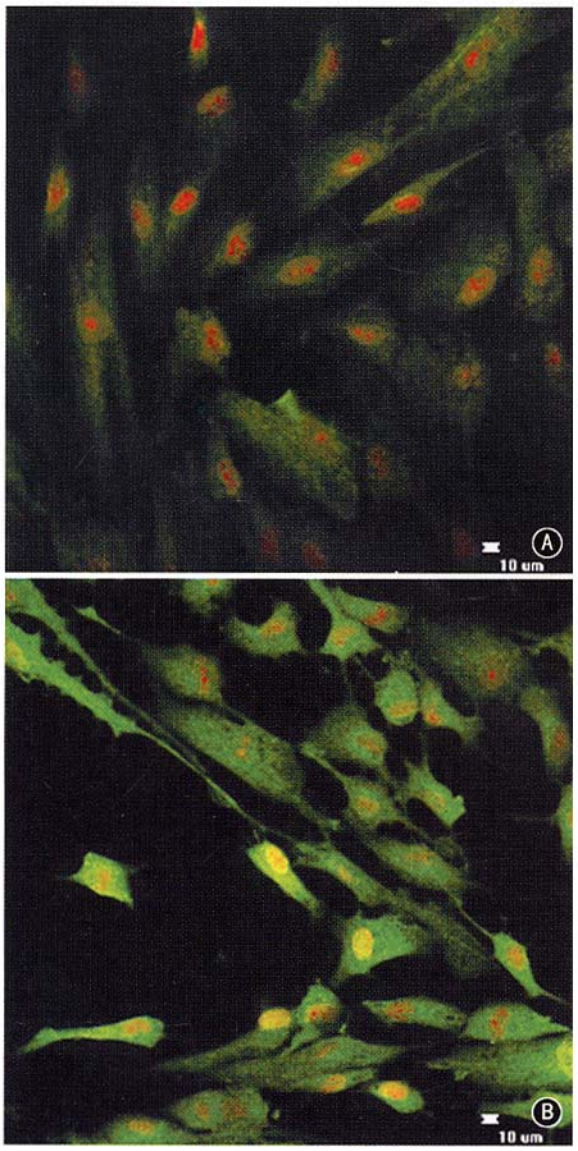

Fig. 5. Confocal microscopy showing the expression of β-tubulin III. The induced cells (**B**) show much stronger expression (green fluorescence) than the untreated ones (**A**).

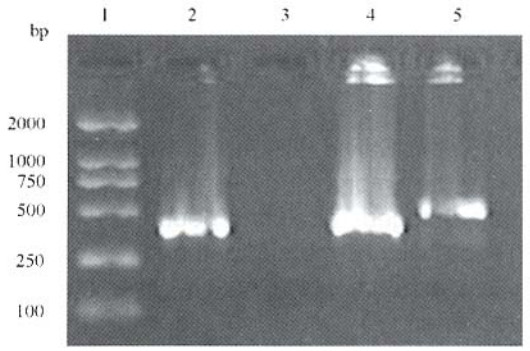

Fig. 6. RT-PCR analysis of nestin in neural stem cells. Lane **1**, DL2000 marker; lane **2**: β-actin (396 bp), before induction; lane **3**: nestin (495 bp), before induction; lane **4**: β-actin (396 bp), after induction; lane 5: nestin (495 bp), after induction.

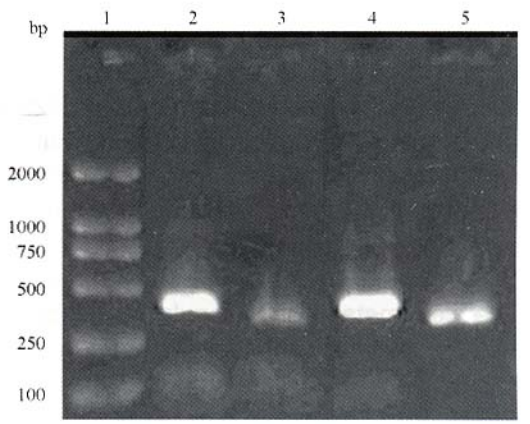

Fig. 7. RT-PCR analysis of the pleiotrophin in neural cells. Lane **1**: DL2000 marker; lane **2**: β-actin (396 bp), before induction; lane **3**: pleiotrophin (342 bp), before induction; lane **4**: β-actin (396 bp), after induction; lane 5: pleiotrophin (342 bp), after induction.

cytometry, we showed that there were no markers of hematopoietic stem cells and cells responsible for graft rejection in cultured MSCs. We concurred with recent studies that Wharton's Jelly could be more convenient and feasible for cell transplantation, and also could represent a more economical source of MSCs, compared with bone marrow. [14,15]

MSCs in human umbilical cord cells could proliferate stably in DMEM medium supplemented with bFGF, and expressed weak positive staining for nestin, β-tubulin Ⅲ, NF and GFAP. This confirmed that

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bFGF could induce MSCs to express neural markers, albeit weakly. However, bFGF could not induce morphological changes in MSCs.

Woodbury et al[7] found that β-mercaptoethanol could induce adult rat and human bone marrow stromal cells to differentiate into neurons. Based on this, we used β-mercaptoethanol as positive control for neural cell differentiation, and had similar results. β-mercaptoethanol could induce these MSCs to not only demonstrate morphological changes, but also show strong positive staining for nestin, β-tubulin Ⅲ, NF and GFAP.

Similarly, after Salvia miltiorrhiza was added into the medium, the morphology of these MSCs cells markedly changed, acquiring neural cell markers as those seen with β-mercaptoethanol. According to our pilot experiments, nestin staining became positive after 4 hours of Salvia miltiorrhizza induction. However the other neural markers needed longer induction. The reason for this discrepancy remains uncertain. The induction effects showed no significant variation among different MSCs passages. This suggests that Wharton Jelly-derived MSCs, in addition to being stable, could maintain the potential and ability to differentiate after at least 8 passages.

The induction mechanism of Salvia miltiorrhiza is still unclear. Some studies demonstrated that Salvia miltiorrhiza not only could act as an inducing agent, but its extract, tanshinone could even induce apoptosis and death in tumor cells.[16-19] Inferring from the induction process, Salvia miltiorrhiza could influence cell metabolism, regulate molecular and enzymatic activities, and finally change the cell morphology.[20-22] Our study with Wharton Jelly-derived MSCs showed that Salvia miltiorrhiza, besides transforming the cell morphology, also up-regulated the expression of neuron-related genes. RT-PCR showed that pleiotrophin was positive both before and after induction with Salvia miltiorrhiza, but its expression and that of nestin were markedly increased in the latter case. This finding suggested that this could be one of the mechanisms by which Salvia miltiorrhiza exerts its neuronal induction effects.

In summary, our present study confirmed morphologically, immunohistochemically and at molecular level that the matrix in Wharton 's Jelly was also a source of MSCs. These cells replicate stably for at least 10 passages in culture. Possessing the capability of differentiating into nerve-like cells, these MSCs could represent an alternative and readily available source of stem cell for CNS cell transplantation.

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Children's health and the environment

Investing in children's health is essential to ensure human and economic development. Healthy children have the best chances for healthy, productive lives. At the Fourth Ministerial Conference on Environment and Health in 2004, the countries in the WHO European Region committed themselves to building a healthy future for the Region's children by adopting the Children's Environment and Health Action Plan for Europe. It provides a framework for action by the 52 diverse countries in the Region. This book was written to provide the guidance and tools that countries need to carry out the Action Plan at the local and national levels, and Region-wide. The aim is to transform the framework document into national action plans suited to each country's circumstances, priorities and resources.

The book has three parts. Part I provides the scientific evidence on children's susceptibility to environmental risk factors, and an overview of environmental risk factors and their effects on children's health. Part II is the core of the publication: tables proposing child-specific actions and therefore concrete ways in which a country can work to reduce children's exposure to environmental risk factors and improve their health. This gives countries the opportunity to act on their own national priorities, while still addressing Region-wide environmental risk factors. Part III focuses on the tools required to ensure implementation of national action plans: setting priorities; building partnerships; taking a precautionary approach to uncertain risks; carrying out strategies for advocacy and information, education and communication; and using indicators to monitor progress at the national and the Region-wide levels. This publication is intended to act as a handbook for countries to use in building a safe and health future for all of Europe's children.

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