The effects of seed extract of *Mucuna gigantea* on the expression of neural markers in mesenchymal stem cells

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The seed extract of *Mucuna gigantea* was evaluated, its efficacy on the differentiation of mesenchymal stem cells (MSCs) into neural lineage. The collected seeds were extracted by using 1% aqueous acetic acid. According to High-performance liquid chromatography (HPLC) analysis, the average concentration of L-3,4-dihydroxyphenylalanine (L-DOPA) was 117.5±7.6 μg/mg of crude extract. The MSCs were cultured in neural induction medium with or without the extract. The induction medium was then replaced with the serum free medium. The induction process was monitored by phase contrast microscopy, immunocytochemistry and real-time polymerase chain reaction. After 14 days of cultivation, the cells cultured in the presence of the extract showed higher degree of nestin and β-III tubulin messenger ribonucleic acid (mRNA) expression than those cultured without the extract. The maximum gene expression was observed in cells cultured with 50 μg/ml of the extract. The induced MSCs showed a change in morphology to that of neuron-like cells. Immunocytochemical staining showed the expression of neural markers such as nestin and microtubule associated protein-2 (MAP-2) on day 7 and 28, respectively. This study suggests that the extract of *M. gigantea*, which contained L-DOPA and/or the other unidentified active compounds, could induce MSCs to express neural protein and gene markers.

**Key words:** *Mucuna gigantea*, L-DOPA, mesenchymal stem cells, neuron-like cells.

**INTRODUCTION**

*Mucuna pruriens* has been described as one of the therapeutic agents for Parkinson’s disease (PD) (Manyam, 1990), a disease which is characterized as pathologically progressive degeneration of neurons (Hirsch et al., 1988; Olanow and Tatton, 1999). *Mucuna* spp. have been described to contain many bioactive substances, especially L-DOPA (Adebowale et al., 2005; Damodaran and Ramaswamy, 1937; Daxenbichler et al., 1972; Manyam et al., 2004; Modi et al., 2008; Prakash et al., 2001), which is a potent precursor of the brain neurotransmitter dopamine (Hornykiewicz, 2002; Kostrzewa, et al, 2005; Nagatsu and Sawadab, 2009). The *M. pruriens* has been shown to be more effective for PD treatment than synthetic L-DOPA (Hussain and Manyam, 1997; Manyam et al., 2004). Another plant found in the southern part of Thailand, *Mucuna gigantea*, similar to the other *Mucuna* spp., has also been reported to contain L-DOPA (Daxenbichler et al., 1972; Rajaram and Janardhanan, 1991; Wilmot-Dear, 2008). This plant,
therefore, may be an alternative source of L-DOPA for the treatment of neurodegenerative disorders including PD.

Neural stem cells (NSCs) are immature self-renewing cells that have the potential to differentiate into neurons, astrocytes and oligodendrocytes (Chu et al., 2003; McKay, 1997). However, their clinical utilities have the limitations. The human MSCs has been known about the capability to differentiate into the cells with neural phenotype at under appropriate conditions including the presence of cytokines and chemical inducers in vitro (Deng et al., 2001; Jin et al., 2003; Kabos et al., 2002; Kim et al., 2002; Lei et al., 2007; Lu et al., 2006; Tao et al., 2005; Sanchez-Ramos et al., 2000; Woodbury et al., 2000, 2002). Medicinal plants, such as Salvia miltiorrhiza, also show ability to induce the differentiation of MSCs into neuron-like cells (Ma et al., 2005). In addition, MSCs can be easily collected from several tissues. Therefore, it has been suggested as the alternative source of NSCs for studying the treatment of injury or diseases of nervous system.

Although there have been reported about the effect of L-DOPA on neural cells and neural cell lines in vitro (Liu et al., 2004; Maeda et al., 1997; Pardo et al., 1995; Pedrosa and Soares-da-Silva, 2002; Walkinshaw and Waters, 1995), the effect of the plant extract containing L-DOPA on differentiation of MSCs into neuron-like cells has never been reported. Therefore, in this study, we examined the effects of crude extract from M. gigantea seeds on directional differentiation of MSCs into neural lineage.

The expression of protein markers and genes associated to differentiated cells such as nestin (marker of neural progenitor), β-III tubulin (marker of immature neuron), or microtubule associated protein-2 (MAP-2; marker of mature neurons) (Cáceres et al., 1986; Dráberová et al., 1998; Kelly et al., 2009; Svendsen et al., 1998). The finding from this study would first suggest the potential activity of the extract for neurodegenerative disorder treatment mediated through stem cell differentiation.

MATERIALS AND METHODS

Plant material

M. gigantea (Wild.) DC., a twiner with trifoliate leaves, green flowers and pods covered with hairs (Figure 1), were collected in July 2006 from Samut Songkhram province, the southern part of Thailand. A voucher specimen (BKF 12345) was deposited at the Forest Herbarium, Department of National Parks, Wildlife and Plant Conservation, Ministry of Natural Resources and Environment, Bangkok, Thailand.

Preparation of M. gigantea seed extract

The whole seeds were ground into powder and macerated with 1% aqueous acetic acid (LabScan Asia Company Limited, Bangkok, Thailand) containing 0.1% sodium sulfite (Riedel-de Haën, Hannover, Germany) for 6 h at 60°C. Then, the filtered extract was lyophilized and stored in the light-protected container at -20°C (Behera et al., 2010).

Determination of L-DOPA content in the extract

To control the quality of the extract, its L-DOPA content was analyzed by using HPLC. Standard L-DOPA was purchased from Sigma-Aldrich (St. Louise, Missouri, USA). Standard solutions of L-DOPA were freshly prepared by dissolving dried standard in 0.1 N HCl and were diluted to the concentration of 12.5, 25, 50, 100 and 250 µg/ml. Samples and standard were analyzed by HPLC LC-20AT, SPD-M10AVP diode array detector and DGU-20A5 degasser (Shimadzu Company Limited, Kyoto, Japan). The column was a Gemini-NX 5u C18 110A, 25 cm x 4.6 mm with 5 µm particle size (Phenomenex; Torrance, California, USA). Chromatographic conditions were used as previously described (Siddhuraju and Becker, 2001) with some modifications. The running process was a gradient with two types of solvents: the eluting solution was made up of water, methanol (LabScan Asia Company Limited) and orthophosphoric acid (85%; Carlo Erba Reagents, Rodano, Milano,

![Figure 1. Parts of M. gigantea (Willd) DC: A) seeds; B) leaves; C) flowers and; D) pods.](image-url)
humidified incubator with 5% CO₂ at 37°C. Cultures were split by trypsin-ethylene diamine tetraacetic acid (EDTA) treatment (0.05% trypsin and 0.02% EDTA; Gibco, BRL) at 75% confluence.

**Induction of cell differentiation in culture**

MSCs were plated at a density of 1x10⁴ cells/cm² on poly-L-ornithine (Sigma-Aldrich) and laminin (R & D systems, Minneapolis, MN, USA)-coated tissue culture dishes and cultured in the maintenance medium (DMEM with 10% FBS and 1% Penicillin/Streptomycin). After that, the cells were cultured in neural induction medium (NM); DMEM/F12 (1:1) (Gibco, Invitrogen, Grand Island, NY, USA) supplemented with 2% B27 (Gibco, Invitrogen), 100 U/ml Penicillin/100 µg/ml Streptomycin, 20 µg/ml human recombinant basic fibroblast growth factor (bFGF; Sigma-Aldrich) and 20 ng/ml human recombinant epidermal growth factor (EGF; Sigma-Aldrich). The M. gigantea seed extract was added into the medium to study its effect at the concentration of 25, 50 or 100 µg/ml. After 7 days (for gene expression study) or 21 days (for protein expression study), the NM was replaced by DMEM/F12 (1:1) supplemented with 0.2 mM ascorbic acid, 1% N2 (Gibco, Invitrogen) and 100 U/ml Penicillin/100 µg/ml Streptomycin. Media were changed every 3 to 4 days. The concentration that gave the maximum expression of mRNA was used in the further studies. The morphological changes and protein expressions of cells in cultures were characterized by using phase contrast microscope (Te-2000u Eclipse; Nikon, Japan), and immunocytochemistry, respectively.

**Real-time polymerase chain reaction (RT-PCR)**

Total ribonucleic acid (RNA) was extracted from samples using Trizol (Invitrogen, New York, USA), and complementary deoxyribonucleic acid (cDNA) was generated with Revertaid reverse transcription (RT) (Fermentas, Burlington, Ontario, Canada) as directed by manufacturers. The cDNA was then stored at -20°C until use. The RT-PCR reaction mixtures were prepared in a 20-µl solution containing cDNA, TaqMan forward and reverse primers, probe in TaqMan Gene Expression Assay Mix and TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, California, USA). RT-PCR was performed on ABI Prism 7300 RT-PCR System (Applied Biosystems), using ABI TaqMan gene expression assays as follows: nestin Hs00707120, ß-III tubulin Hs00964962, tyrosine hydroxylase Hs01002188 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Hs99999905 (Applied Biosystems). The RT-PCR reactions were performed using thermal cycling with conditions at 50°C for 2 min and at 95°C for 10 min as initial steps followed by 45 cycles of 15 s at 95°C and 1 min at 60°C for amplification. All assays were performed in triplicate. The relative mRNA levels were calculated using the comparative Ct method, using GAPDH as the endogenous control.

**Immunocytochemistry**

Cells plated on poly-L-ornithine/laminin-coated culture dishes were washed in phosphate buffer saline (PBS) and fixed with cold methanol for 15 to 20 min. Fixed cells were washed once with PBS before staining. For staining, cells were permeabilized in 0.1% Triton X-100 in PBS for 10 min. The cells were incubated for 30 min with 3% bovine serum albumin. The primary antibodies (1:200), which were specific to neuron, were then added to the cells and incubated for 2 h in the dark at room temperature. After that, cells were washed in PBS and incubated with secondary antibody conjugated with fluorescent dye (1:200) for 1 h. After washing with PBS, the nuclei were counterstained with propidium iodide (Sigma-
Figure 3. The relationship between M. gigantea (MG) concentration and neuronal mRNA expressions in MSCs. The MSCs were cultured in neural induction medium with various concentrations (25, 50 and 100 µg/ml) of seed extract from M. gigantea for 7 days. The medium was subsequently replaced with the serum-free medium containing ascorbic acid. The expressions of: (A) nestin; and (B) β-III tubulin mRNA relative to GAPDH in MSCs at day 14 of culture were detected by using real time PCR. The maximum gene expressions of: (A) nestin; and (B) β-III tubulin were observed when the concentration of extract was 50 µg/ml. These data are expressed as mean ± SEM from 4 separate experiments.

RESULTS

HPLC analysis of M. gigantea seed extract

According to HPLC analysis, the retention time of standard L-DOPA was about 5.4 min (Figure 2). The calibration curve for L-DOPA (at five concentrations; 12.5, 25, 50, 100 and 250 µg/ml) plotted against the peak area was shown a linearity with the correlation coefficient of 1. The percentage yield of lyophilized crude extract was about 37% (w/w). The average concentration of L-DOPA found in 1 mg of lyophilized crude extract was 117.5 ± 7.6 µg.

Neural mRNA expressions in MSCs

The neural gene expression, in induce MSCs, was evaluated by using nestin, β-III tubulin and tyrosine hydroxylase gene marker. Induced MSCs could express nestin and β-III tubulin mRNA (Figure 3) at day 14 of culture, but not for tyrosine hydroxylase mRNA. The mRNA expressions were marked higher in cells cultured with the addition of 50 µg/ml extract neural induction medium with M. gigantea (NM + MG), and the expression of nestin correlated well with that of β-III tubulin. At higher concentration (100 µg/ml), M. gigantea tended to provide inhibitory effect on the differentiation of MSCs isolated from patients, resulting in the decreased expressions of gene markers.

Morphology of MSCs

After MSCs were seeded onto poly-L-ornithine/laminin-coated dish, they spread on the culture dish and developed their morphology as elongated or large flat cells in maintenance medium. The induced MSCs could develop long, thin cellular processes and refractive cell bodies, like neuron, when they were cultured in NM (Figure 4). MSCs could form the cluster from day 7. However, in the presence of NM + MG at concentration of 50 µg/ml, the clumping of cells did not appear and neuron-like cell morphology was markedly observed.

Neural protein marker expressions in MSCs

To confirm neural differentiation of MSCs, neural markers of neural progenitor (nestin) and mature neural cells (MAP-2) were used to assess cell type-specific protein expressions by immunocytochemical staining. Induced MSCs with 50 µg/ml of the extract NM + MG and without...
Figure 4. The MSCs observed by phase contrast microscope. The MSCs were cultured in the induction medium (NM) without or with the seed extract of *M. gigantea* (NM + MG) at the concentration of 50 µg/ml for 21 days. The cell culture media were subsequently changed at 3-day interval using serum-free medium containing ascorbic acid. Cells displayed long, thin cellular process and refractive cell bodies, like neurons. Clumping of cells was found in condition without the extract (NM).

the extract NM were positive staining with nestin since day 7 of induction, and much stronger expression at day 14 (Figure 5A and C). MSCs also showed positive staining with antibodies specific to MAP-2 on day 28 of culture (Figure 5B and D).

**DISCUSSION**

*M. pruriens* has been used as herbal medicines for the treatment the PD because of L-DOPA content (Manyam et al., 1990, 2004; Hussain and Manyam, 1997). L-DOPA is a potent precursor of the brain neurotransmitter dopamine in catecholaminergic neurons and is the most effective therapeutic agent for Parkinson's disease (Hornykiewicz, 2002; Kostrzewa et al., 2005; Nagatsu and Sawadab, 2009). Like *M. pruriens*, we also found L-DOPA in *M. gigantea* seed extract.

In the present study, we found that neural precursors and mature neurons could be cultured from human MSCs, and the marked morphological changes into neuron-like cells were found in case of the treatment with the extract as determination by phase contrast microscopic examination. It has been reported that L-DOPA increases the adherence of MSCs to the culture surface and also increases the proliferation and mitochondrial activity of MSCs (Kim et al., 2011). Such report supports our experimental finding that the cells cultured in neural induction medium in the presence of the extract tended to spread on poly-L-ornithine and laminin-coated dish rather than clump as sphere. This could lead to increase in MSC proliferation and differentiation. By immunocytochemistry, the difference in neural maker expression (nestin and MAP-2) between induced MSCs with and without the extract was difficult to observe as both conditions provided the expression of protein markers. However, it is interested to see that the mRNA expression of nestin, a neural precursor marker, MSCs was markedly higher in cells cultured with the extract than those without the extract.

In addition, the expression of β-III tubulin, an immature neuron marker, was also exhibited in the similar manner. It is likely that, the extract enhanced the expression of neural genes. To explain this effect, the further investigation about its mechanism on cells is needed.

In the previous study, the cytotoxicity of the L-DOPA might be prevented by the antioxidants such as ascorbic acid, glutathione, N-acetyl-L-cysteine, sodium metabisulfite (Mytilineou et al., 1993; Pardo et al., 1995;
Figure 5. The immunocytochemical characterization of neuron-like cells developed from MSCs in culture. The commercial MSCs cultured in the induction medium without (NM) or with the seed extract of Mucuna gigantea (NM + MG) at the concentration of 50 µg/ml for 21 days. The medium was subsequently replaced with the serum-free medium containing ascorbic acid. Cells with neural precursor marker (nestin; A, C) were positively stained as green color on day 14 and mature neural marker (MAP-2; B, D) on day 28. Nucleus (red) was also counterstained with propidium iodide (PI).

Pedrosa and Soares-da-Silva, 2002) or glia-conditioned medium (Mena et al., 1997) at the appropriate concentration.

From this study, M. gigantea seed extract was found to contain L-DOPA. Induced MSCs could differentiate into neuron-like cells. The addition of seed extract of M. gigantea resulted in increased neural mRNA expression in such neuron-like cells. The seed extract of M. gigantea, therefore, may be an alternative source of L-DOPA like M. pruriens for the improvement or treatment of PD symptom. However, its effect on stem cells should have the further investigation in order to clarify its mechanism on the induction of MSCs to differentiate into neural cells.

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