Original Article

Dopaminergic Induction of Umbilical Cord Mesenchymal Stem Cells by Conditioned Medium of Choroid Plexus Epithelial Cells Reduces Apomorphine-Induced Rotation in Parkinsonian Rats

Abbas Aliaghaei PhD[1,2,3], Mossa Gardaneh PhD•[1], Nader Maghsoudi PhD•[2], Parvin Salehinejad PhD[4], Ehsan Gharib MSc[1]

Abstract

Background/Objective: Degeneration of dopaminergic neurons in Parkinson's disease (PD) implies cell replacement using potentially differentiable sources as a promising therapeutic solution. We tested the capacity of conditioned medium from choroid plexus epithelial cells (CPECs-CM) to induce the dopaminergic potential of umbilical cord matrix mesenchymal stem cells (UCMSCs).

Methods: We isolated UCMSCs from human umbilical cord and CPECs from rat brain. Following expansion and characterization, CPECs-CM were collected, tested for expression of various growth factors, and applied to UCMSCs. Differentiation was examined and UCMSCs were injected into 6-OHDA-leasioned striatum to test their survival and function.

Result: RT-PCR and immuno-staining demonstrated neuronal/dopaminergic signaling in UCMSCs induced by CPECs-CM and accelerated by addition of retinoic acid (RA) and fibroblast growth factor-2. Expression of β–tubulin-3, Nestin and MAP2 confirmed neuronal differentiation whereas tyrosine hydroxylase, aromatic acid decarboxylase and dopamine transporter were expressed as signs of dopaminergic differentiation. Post-transplantation, the UCMSCs survived, showed reduced rate of apoptosis and led to animals' recovery from apomorphine-induced rotations.

Conclusion: The combination of neurotrophic factors present in CPECs-CM and RA can synergize to maximize dopaminergic differentiation of potential cell sources including UCMSCs. Our study may have implications for PD cell replacement therapy.

Keywords: Choroid plexus, dopaminergic, mesenchymal stem cells, Parkinson, umbilical cord

Cite this article as: Aliaghaei A, Gardaneh M, Maghsoudi N, Salehinejad P, Gharib E. Dopaminergic Induction of Umbilical Cord Mesenchymal Stem Cells by Conditioned Medium of Choroid Plexus Epithelial Cells Reduces. *Arch Iran Med*. 2016; **19(8):** 561 – 570.

Introduction

Parkinson's disease (PD) is a well-characterized neurodegenerative disorder hallmarked by the loss of a specific set of dopaminergic (DAergic) neurons called A9 neurons that reside within the midbrain subregion of substantia nigra pars compacta (SNpc).[1] A range of cell types from embryonic stem cells to fetal and adult mesencephalic tissues have been used as precursors to generate DAergic neurons for cell replacement therapy (CRT) purposes in PD.[2]

Neuronal differentiation of mesenchymal stem cells (MSCs) is part of a global effort toward CRT in PD with the paracrine effect of the cells considered a critical force.[3] This paracrine induction could improve survival as well as regeneration of host neurons and ultimately increase the efficacy of repairing target neural tissues. In support of this notion, MSCs transplanted into the brain reduce apoptosis and regulate neuro-inflammation.[4,5] They further induce endogenous neuronal growth and promote functional recovery of nerve injury.[6,7] Relevant to PD, both protection of endogenous DAergic neurons and repair of DAergic pathways upon application of MSCs to 6-OHDA-lesioned brain have been reported.[8,9]

Umbilical cord MSCs (UCMSCs) originate from umbilical cord matrix or Wharton's jelly, and express MSC markers and have the potential to differentiate to neural lineages.[10,11] The UCMSCs can be differentiated to DAergic neurons with the ability to restore behavioral deficits upon injection to lesioned animals.[12] They can also be engineered to overexpress neurotrophic factors involved in functional recovery of injured neural tissues, similar to $CD34^+$ cells of umbilical cord blood[13] Moreover, genetic manipulations of UCMSCs can be planned to facilitate their *in vivo* tracking, expand their migratory capabilities and support their tissue-recovering potential.[14,15]

Choroid plexus epithelial cells (CPECs) have a neuroepithelial origin. The most important function of these cells is to biosynthesize cerebro-spinal fluid or CSF.[16] Besides, they secrete growth factors including neurotrophic factors and proteins with unknown function.[17] Studies indicate that CPECs secrete sonic hedgehog in the dorsal region of an embryonic brain.[18] Culture of embryo's mesencephalic pieces in the presence of CSF induces expression of DAergic transcription factor Otx2 by precursor cells.[19]

Due to the capacity of CPECs in secreting a cocktail of growth factors and mitogens, we hypothesized that CPECs-conditioned medium (CEPC-CM) might function as a potential inducer of neuronal differentiation. Therefore, we used this medium either alone or in combination with retinoic acid (RA) to examine

Authors' affiliations: [1]National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran. [2]Neuroscience Research Center (NRC), Shahid Beheshti University of Medical Sciences, Tehran, Iran. 3 Anatomy and Cell Biology Department, Shahid Beheshti University of Medical Sciences, Tehran, Iran. [4]Department of Anatomy, School of Medicine, Kerman University of Medical Sciences, Kerman, Iran.

·**Corresponding authors and reprints:** Mossa Gardaneh PhD, National Institute of Genetic Engineering and Biotechnology, Pazhoohesh Blvd, Tehran-Karaj HWY Kilometer 15, P.O. Box: 14965/161, Tehran, Iran. Tel: +98-21-4458-0344, Fax: +98-21-4458-0395, E-mail: Mossa65@nigeb.ac.ir, mossa65@yahoo.com
Nader Maghsoudi PhD, Neuroscience Research Center (NRC), Shahid Beheshti University of Medical Sciences, Evin, Tehran, Iran. Tel: +98-21-2242-9768, Fax: +98-21-2243-2047, E-mail: nmaghsoudi@sbmu.ac.ir
Accepted for publication: 22 May 2016

Table 1. Animal groups and time frame of their injections and tests.

	Sham	Control	Test
Day 0	Saline	6-OHDA	6-OHDA
Day 7	Apomorphine injection, Rotational Test	Apomorphine injection, Rotational Test	Apomorphine injection, Rotational Test
Day 9	—	Saline	UCMSCs
Week 3 (Day 14)	TUNEL Assay	TUNEL Assay	TUNEL Assay
Week 3 (Day 14)	IHCH Analysis	IHCH Analysis	IHCH Analysis
Weeks 3,4	Rotational Test	Rotational Test	Rotational Test

the potential of those inducing factors in driving neuronal and DAergic differentiation of UCMSCs, applicable for neurogenesis and neuronal repair in PD brain.

Materials and Methods

Isolation and culture of UCMSCs

The umbilical cord was obtained from Taleghani Hospital in Tehran, Iran upon consent of its donor. The cord was rinsed with PBS and isolated of amniotic membrane. Then, the jelly fraction of the cords was cut into pieces and cultured in DME/F12 medium supplemented with 10% FBS and antibiotics. Two weeks later, the tissues were discarded and the isolated growing cells (UCMSCs) were fed with the same medium. The cells were grown to 60% confluence and passaged by trypsinization. We used the third passage of UCMSCs for characterization and differentiation steps.

Flow cytometry

A number of 1×10^5 cultured UCMSCs were washed, fixed and incubated for 15 min at 4 °C with a 1:9 dilution of normal goat serum in PBS. They were then labelled with the following antibodies for 1 h: FITC-conjugated anti-CD34, FITC-conjugated anti-CD44 (Chemicon; USA), FITC-conjugated anti-CD45 (Ediscience; USA), PE-conjugated anti-CD90 (Dako, Denmark) and PE-conjugated anti-CD105 (R&D; USA). The cells were washed with 2% FBS in PBS and analyzed using a FACSCalibur machine (Becton Dickenson, USA). The control population was stained with matched isotype antibodies (FITC-conjugated and PE-conjugated mouse IgG monoclonal isotype standards) and confirmed by positive fluorescence of the limbal samples. At least 10,000 events were recorded for each sample and data were analyzed using WinMDI software (USA).

Adipogenic and osteogenic differentiation of UCMSCs

We applied previously-reported methods of differentiation.[19] For adipogenic differentiation, cell growth medium was supplemented with 50 µg/mL ascorbate-1 phosphate, 10^{-7} M dexamethasone and 50 µg/mL indomethacin (Sigma). For osteogenic differentiation, we added 50 µg/mL ascorbacte phosphate, 10^{-8} M dexamethasone and 10 mM β-glycerophosphate (Sigma). The medium for each differentiation setting was changed every three days. Oil red and alizarin red (Sigma) were used, respectively, for detection of adipogenic and osteogenic differentiation.

Isolation and culture of CPECs

CPECs were isolated from rat brain according to a previous study with some modifications.[20] From coronal sections of the brain, choroid plexus tissue was separated from lateral ventricles and trypsinized at 37°C for 20 min. The samples containing CPECs were fed with serum, centrifuged and grown in DMEM/F12. To prepare CPECs-CM, serum free medium was used for cell growth, and the medium was collected and filtered forty hours later and stored at -20°C until use.

Dopaminergic differentiation

The cultured UCMSCs were grown in 24-well plates to 60–70% confluence. As a pre-differentiation step, UCMSCs were cultured in DMEM/F12 supplemented with 0.5 µM retinoic acid (RA) (Sigma) and 5 ng/mL fibroblast growth factor 2 (FGF2) (Royan Institute, Tehran) for three days. DAergic differentiation was followed by incubation of the cells with CPECs-CM for 14 days. We also cultured UCMSCs with CPECs-CM alone to monitor the effect of CPECs-CM on DAergic differentiation.

Reverse transcription (RT)-PCR analysis

Total RNA was extracted using High Pure RNA Isolation Kit (Roche), DNase treated and tested for its integrity by agarose gel electrophoresis, as we have reported.[21] From each RNA sample, 2 µg was used for cDNA synthesis in a 60-min reaction at 42°C using MuLV reverse transcriptase (Fermentas, Lithuania) in the presence of random hexamers and RNase inhibitor.

Table 2 lists primer pairs used for amplification of gene candidates. As an internal control, we also amplified a 301-bp fragment of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene. An equal amount of each cDNA sample was subjected to PCR. The samples were first denatured at 95°C for 2'. PCR reactions were then carried out for 30 cycles consisting of denaturation at 94°C for 45", annealing at 59°C for 1' and extension at 72°C for 45". The PCR products were run on a 1.2% agarose gel electrophoresis and the images were analyzed for the density of their bands as previously reported.[21] The RT-PCR analysis was repeated three times as three independent experiments.

Generation of parkinsonian rat models and cell transplantation

Twelve adult Sprague-Dawley rats (250–300 g) were equally split to three experimental groups of *Sham*, *Control* and *Test*, anesthetized and placed in a stereotaxic frame. The animals received either saline (*Sham* group) or 6-OHDA injection (*Control* and *Test* groups; 12.5 µg/5 µL in saline containing 0.2 mg/mL ascorbic acid; Sigma) in MFB [target coordinates: antero-posterior (AP), -3.60; medio-lateral (ML), 1.8; dorso-ventral (DV), 8.2 mm from Bregma's atlas] using a Hamilton syringe.[22] One week after toxin injection, the animals were subjected to the first round of rotational test and 48 h later to cell transplantation. Seventy two hours before injection, cell samples were stained with the DiI fluorescent dye (Invitrogen) according to the manufacturer's instructions and further grown in DME/F12 supplemented with 10% FBS. The cells were trypsinized, resuspended in saline and

Table 2. Primer sequences for RT-PCR.

Gene Candidate	Primer Sequence (Forward, FW; Reverse, RV)	Product Length (bp)	Sequence Accession Number
hTH	FW: CTGATTGCTGAGATCGCCTTCC RV: ATATTGTCTTCCCGGTAGCCG	194	NM_199292
hAADC	FW: CGCCAGGATCCCCGCTTTGAAATCTG RV: TCGGCCGCCAGCTCTTTGATGTGTTC	241	NM_00079
rTTR1	FW: GGCTCACCACAGATGAGAAGTTC RV: ACAAATGGGAGCTACTGCTTTGGC	269	NM_012681
hDAT	FW: ATTGTCACCACCTCCATCAAC RV: ACTGCCCAACACCATAGAACC	471	NM_001044
hGFAP	FW: GAAGACCGTGGAGATGCGGGA RV: TCATGAGACGGGGCAGAGGC	107	NM_002055
hNestin	FW: GGCCCGTACCCCTACCTTGG RV: TGTACTGGCCTCTTGCCGCT	240	NM_006617
hMAP2	FW: GACTCCCTCTACAGCTGAGCCT RV: TCTTCCGTGCCTTGCATGTCC	152	NM_002374
hKCNH1	FW: ACCACTGGAGGGGAGAGAGAGTC RV: GGTCCAGCTTACGGGCCACT	100	NM_172362
hKCNH5	FW: GATCCTGGCCGCTGCTCTCC RV: ACTGGAGCGCCTGACGATGT	100	NM_139318
rGDNF	FW: CTGCCCGCCGGTAAGAGG RV: CGTCATCAAACTGGTCAGGATAATC	126	NM_019139
hGAPDH	FW: GTGGTCTCCTCTGACTTCAACA RV: TTGCTGTAGCCAAATTCGTTGT	126	NM_002046
rBDNF	FW: AGGCACTGGAACTCGCAATG RV: AAGGGCCCGAACATACGATT	50	NM_007540
rNGF	FW: TGTGGACCCCAAACTGTTTAAGA RV: GTCTAAATCCAGAGTGTCCGAAGAG	100	NM_001277055
rFGF2	FW: ACCCGGCCACTTCAAGG RV: GATGCGCAGGAAGAAGCC	47	NM_019305
rVEGF	FW: GAGGAAAGGGAAAGGGTCGGG RV: CACAGTGAACGCTCCAGGATT	158	NM_001287107

injected to the striatum (target coordinate: AP, 0.84; ML, 3.5; DV, 5.4 mm Bregma's atlas). We injected 1×10^5 cells per *Test* animal and injections last 5 min. The *Control* group of animals received saline only.

Behavioral testing

The animals were tested for rotational behavior by apomorphine hydrochloride (0.5 mg/kg, i.p.) at time points of 1, 3, and 4 weeks after injection of 6-OHDA. The basis of the rotational tests is that a denervated caudate/putamen becomes supersensitive to dopamine and dopamine receptor,[23] the injured animal turns contralateral to the denervated side after administration of a direct dopamine receptor agonist, such as apomorphine, and the number of rotations correlates with the extent of the lesion.

The rotations were measured according to a method described previously.[24] Briefly, the animals were allowed to habituate for 10 min, full rotations were counted in a cylindrical container (a diameter of 33 cm and a height of 35 cm) at 10-min intervals for 60 min in a quiet isolated room. The net number of rotations was defined as the positive scores minus the negative scores.

TUNEL assay

Animals were sacrificed and striatal sections were prepared after brain dissection. An apoptosis kit (Millipore) was applied to the sections in order to determine the rate of apoptosis among the grafted cell populations. Four photographs of each slide were prepared and used to count TUNEL-positive cells. The collected data were used to determine the average of number of apoptotic cells.

Immunocyto- and immunohistochemistry

Two weeks after induction of cell differentiation, the cell samples were fixed using 4% paraformaldehyde and treated with 0.3% Triton-X briefly, and then with blocking serum for 1 hour. Primary antibodies against beta-tubulin (AB15568; Abcham), TH (Lnc-1; Millipore) and GFAP (AB4648; Abcham) were applied at a 1:1000 working concentration overnight at 4°C. The cells were then rinsed with PBS and treated with a secondary antibody conjugated to a fluorescent dye at a dilution of 1:300.

For immunohistochemical analysis, brain sections were prepared, treated with Triton-X and blocked using blocking serum for half an hour. The tissues were incubated with an anti-TH primary antibody for three hours. After incubation with a secondary antibody, the samples were examined using fluorescent microscope.

Statistical analyses

Data in the figures of *in vitro* studies are represented as the mean ± SEM (mean of standard error) of three or more separate experiments carried out in triplicate. Data in the figures of *in*

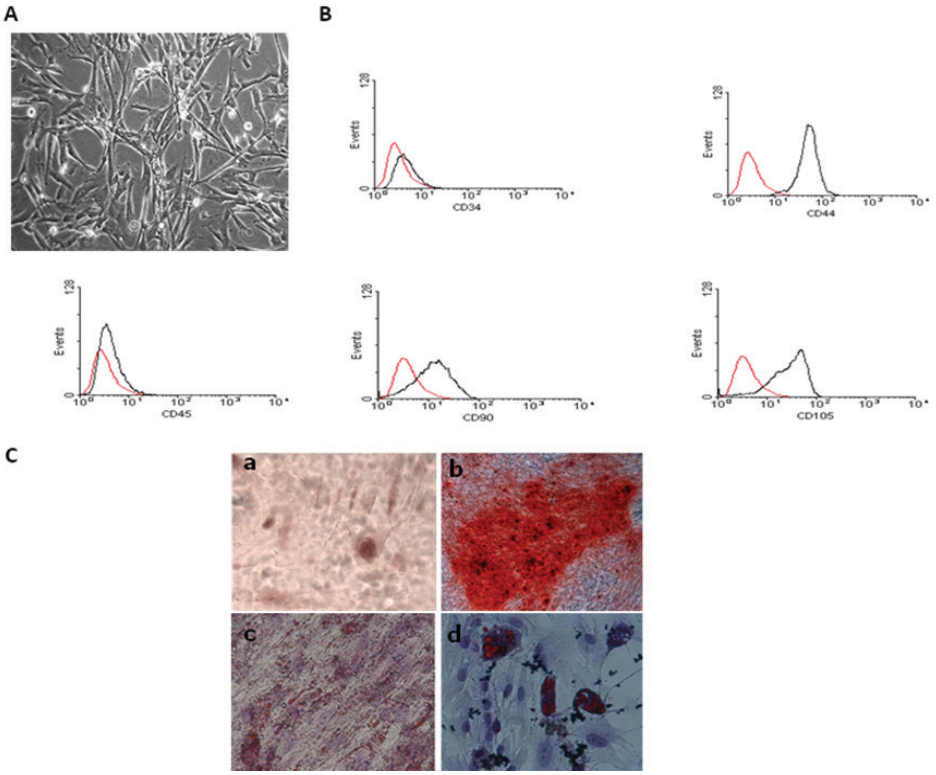

Figure 1. Isolated and cultured human UCMSCs. **(A)** Microscopic image of cultured cells after isolation. Magnification: 100 micrometer (μM). **(B)** Flow cytometric analysis of UCMSCs. Expression of CD34, CD44, CD45, CD90 and CD105 was tested. **(C).** Differentiation of UCMSCs to (a to b) osteogenic cells, that show mineral precipitates as red plaques after being stained with alizarin red and (b to c) adipogenic cells stained with oil red and show lipid-containing vacuoles. Magnification: 50 μM.

vivo studies are the mean ± SEM of experiments on minimum three rats in each experimental group. All statistical analyses were performed using SPSS version 17.0. We used Independent Student's T-Test to analyze differences between two groups. Differences between three groups were analyzed by one-way analysis of variance (ANOVA), followed by *post-hoc* Duncan Multiple-comparisons Test. We considered a value of $P<0.05$ as statistically significant, and $P < 0.01$ or $P < 0.001$ as highly significant.

Results

UCMSCs show fibroblast-like phenotype and express mesenchymal stem cell markers

Umbilical cord matrix tissue was cultured using an explant method. A few days later, UCMSCs migrated away from tissues. Two weeks after we cultured umbilical cord matrix tissue, fibroblast-like cells appeared in culture dishes (Figure 1A). The cells grew with high speed and rapidly covered the surface. Flow cytometric analyses indicated that the cultured UCMSCs do not express hematopoietic markers CD34 and CD45; however, they express mesenchymal stem cell markers CD90, CD44 and CD105 (Figure 1B).

UCMSCs can differentiate to adipocytes and osteoblasts.

The UCMSCs were exposed to an osteogenic medium for a period of two weeks that induced them to form osteoblasts. This induction was shown by precipitation of minerals when the cells were stained with alizarin red (Figure 1C.a and b). On the other hand, when the UCMSCs were grown in adipogenic medium, staining with oil red showed lipid-containing adipocytic vacuoles within the cells (Figure 1C.c and d). These results indicated the potential of UCMSCs to differentiate to both adipocytic and osteoblastic fates.

CPECs express transthyretin 1 (TTR1) and a range of growth factors

Choroid plexus tissues were extracted from rat brain and digested by trypsin. CPECs initially appeared as round cells in culture dishes but gradually gained polygonal morphologies covering the entire surface. To prevent fibroblastic contamination, we used cytosine arabinoside (Ara C). Immunocytochemical analysis indicated that CPECs are positive for TTR1 (Figure 2A) a reliable marker for detection of CPECs *in vitro* and *in vivo*. This data was also confirmed by RT-PCR analysis that detected TTR1 mRNA (Figure 2B). The technique also revealed that CPECs expressed mRNA of GDNF and a few other factors including brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), FGF2 and vascular endothelial growth factor (VEGF) (Figure 2B). However, they did not express those of conserved DAergic neurotrophic factor (CDNF), sonic hedgehog (SHH) and fibroblast growth factor 8 (FGF8) (Data not shown).

Synergy between CPECs-CM and retinoic acid/FGF2 effectively induces neuronal and glial differentiation of UCMSCs

The UCMSC treated with CPECs-CM+RA+FGF2 underwent morphological changes within 4 hours. A number of cells lost

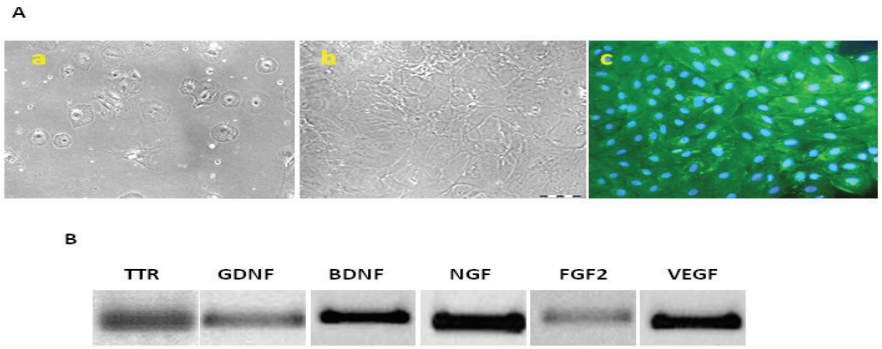

Figure 2. Isolation, culture and expression analysis of choroid plexus epithelial cells (CPECs). **(A)** Isolated CPECs shown on (a) day 3, (b) day 6, and (d) stained for transthyretin 1 (TTR1). Magnification: 100 µM. **(B)** RT-PCR images of amplified rat TTR and glial cell line-derived neurotrophic factor (GDNF) sequences using samples of three independent CPEC isolates. The samples were run on separate gels for different molecules and so DNA marker lanes were deleted and the images combined as presented. Fragment sizes are 126 bp (rGDNF), 269 bp (rTTR1), 50 bp (rBDNF), 100 bp (rNGF), 47 bp (rFGF2), 158 bp (rVEGF). See table (2).

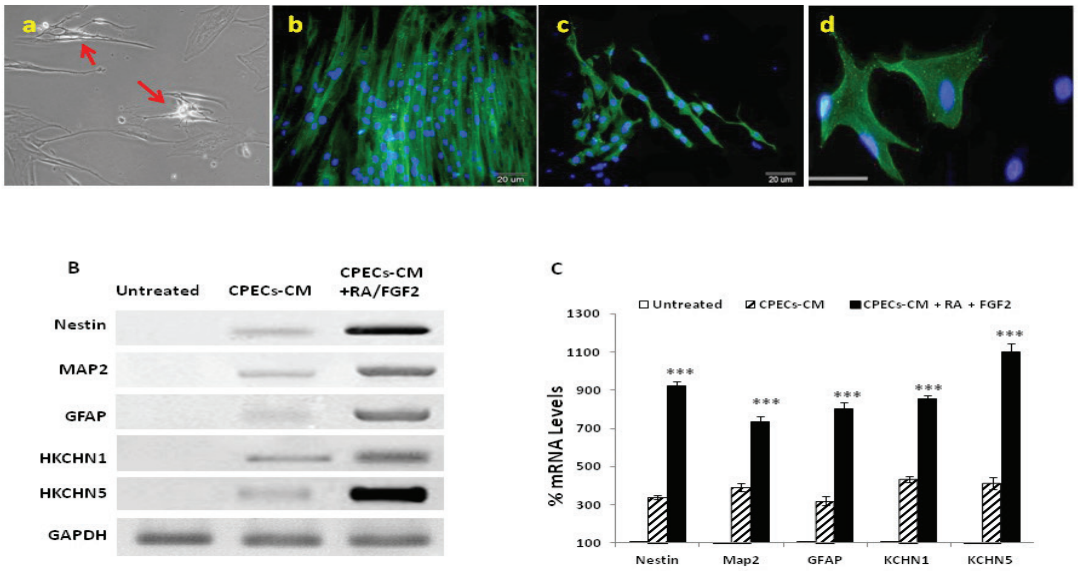

Figure 3. Neuronal differentiation of UCMSCs. **(A)** Cells after treatment with CPECs- conditioned medium (CPECs-CM), retinoic acid (RA) and fibroblast growth factor 2 (FGF2): **a.** treated unstained cells with neuritogenic changes shown by arrows, **b-d** stained with: **b.** DAPI (fluorescent color of DAPI was inverted to black and white), **c.** anti-β–tubulin-3 antibody, **d**. anti-GFAP antibody. DAPI was used for counterstaining. Magnification: 100 µM (**a-c**) and 200 µM (**d**). **(B)** RT-PCR analysis of UCMSCs samples treated with either CPECs-CM alone or CPECs-CM+RA+FGF2. The gel image was prepared as outlined in Fig.2. Fragment sizes are 240 bp (hNestin), 152 bp (hMap2), 107 bp (hGFAP), 100 bp (KCNH1 and KCNH5) and 126 bp (hGAPDH). **(C)** Densitometric analysis of RT-PCR gel images shown in **(B)**. Pixel densities of each band were calculated as percent of mRNA levels expressed in untreated control cells. Each column represents an average of three independent experiments. Statistical differences in percent increase of mRNA levels from untreated to CPECs-CM-treated cell groups are shown by ### and from CPECs-CM-treated to CPECs-CM+RA+FGF2-treated groups by ***.

their spindle-like morphologies and formed small projections (Figure 3A.a) that were clearly visualized when the cells were subjected to DAPI staining (Figure 3A.b). These observations suggested that such a treatment can rapidly induce neuronal differentiation. To demonstrate this phenomenon, we analyzed the cells using immunocytochemistry: they expressed neuronal marker β-tubulin-3 (Figure 3A.c) and also tested positive for expression of astrocytic marker GFAP (Figure 3A.d).

A 3-day exposure of UCMSCs to RA+FGF2 was followed by treatment with CPECs-CM for a period of 14 days. Primarily, we found that incubation with CPECs-CM alone was sufficient to induce neuronal differentiation of UCMSCs. RT-PCR analyses indicated that two routinely-tested neuro-progenitor (Nestin) and neuronal (MAP2) markers began their expression in the presence of CPECs-CM (Figure 3B). These analyses also showed that the differentiated cells express ionic channels HKCHN1 and HKCHN5. Interestingly, however, the UCMSCs treated with CPECs-CM and RA+FGF2 expressed several-fold higher levels of the markers than did their counterparts that only received CPECs-CM: 2.7-fold for Nestin, 1.9-fold for MAP2, 2.5-fold for GFAP, 2-fold for HKCHN1 and 2.7-fold for HKCHN5 (Figure 3C; $P < 0.001$, Duncan's Multiple-comparison Test).

CPECs-CM induces dopaminergic differentiation of UCMSCs

In order to determine the neuronal identity of the UCMSCs treated with RA+FGF2 and CPECs-CM, the differentiated cells

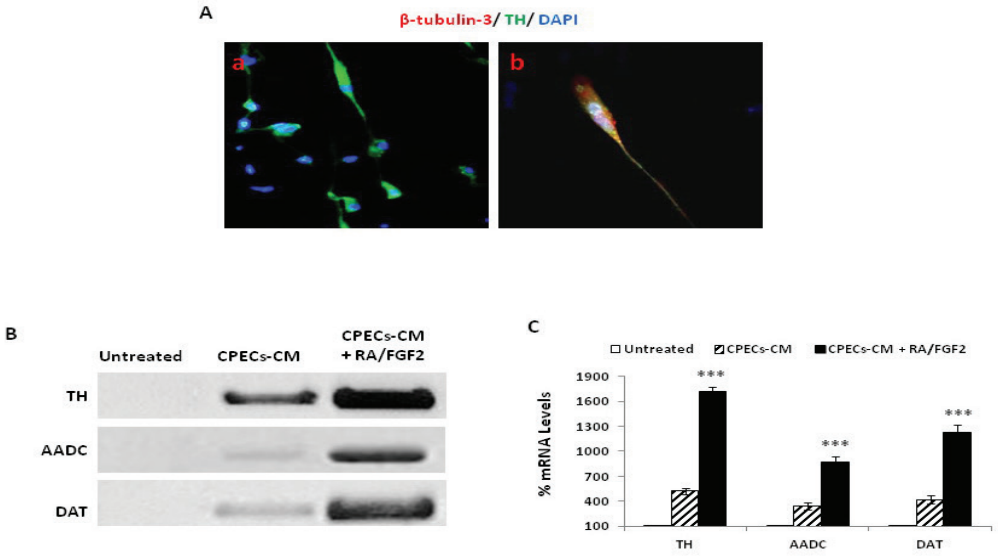

Figure 4. Dopaminergic differentiation of UCMSCs. **(A)** Immunocytochemical analysis of UCMSCs after incubation with CPECs-CM+RA+FGF2, **a.** stained against TH (green), **b.** double-stained against TH (green) and β-tubulin-3 (red). In both images, nuclei stained with DAPI are shown in blue. Magnification: 100 µM. **(B)** RT-PCR analysis of UCMSCs samples treated with either CPECs-CM or CPECs-CM+RA+FGF2. The gel image was prepared as outlined in Fig.2. Fragment sizes are 194 bp (human tyrosine hydroxylase, hTH), 241 bp (human aromatic acid decarboxylase, hAADC) and 471 bp (human dopamine transporter, hDAT). **(C)** Densitometric analysis of RT-PCR gel images shown in **(B)** carried out as in Figure 3.

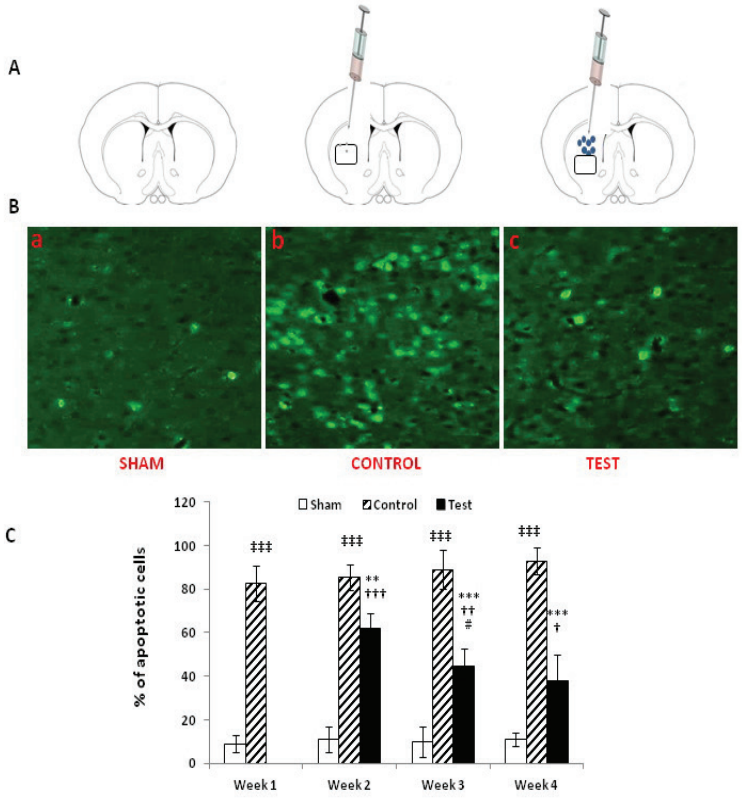

Figure 5. Striatal transplantation of UCMSCs and subsequent analyses. **(A)** A schematic diagram showing brain injections for three groups of animals under study: *Sham* (no injection), *Control* (Saline injection) and *Test* (Cell injection). **(B)** TUNEL staining of the coronal sections prepared from the rat striatum with transplanted dopaminergic neurons, derived from human UCMSCs, in injection week 3, and **(C)** analysis of images in **(B)** for the rate of apoptosis. Statistical differences between *Control* and *Test* groups in graph **(C)** are shown by *, ** or ***. Symbol # shows differences of data between week 2 and week 3. Differences between *Control* and *Sham* and between *Test* and *Sham* are shown, respectively, by ‡‡‡ and †, †† or †††. Magnification: 100 µM.

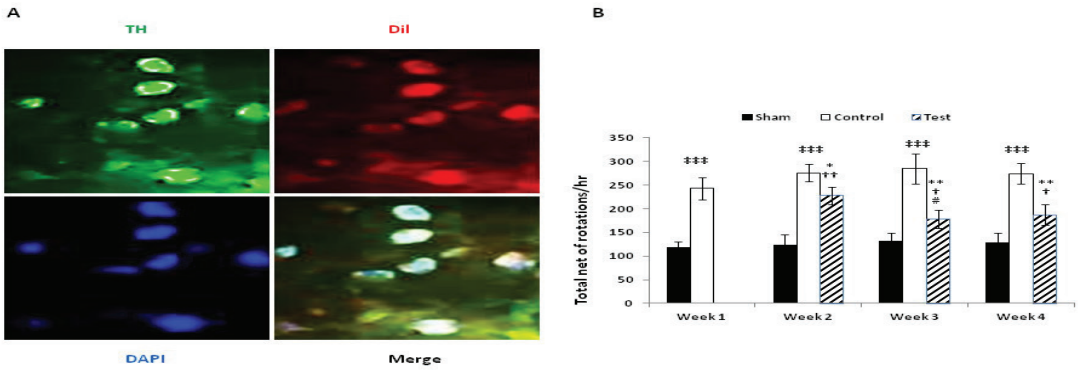

Figure 6. Detection of transplanted UCMSCs in the brain. **(A)** Dopaminergic staining of tissue sections incubated with anti-tyrosine hydroxylase antibody. DiI labeling of cell membrane done before transplantation is shown in red. **(B)** A graph showing data collected from Apomorphine-induced rotation test. Symbols show statistical differences as outlined in **(5C).** Magnification: 200 μM.

were subjected to immunocytochemical and RT-PCR analyses that confirmed the expression of mRNAs for tyrosine hydroxylase (TH), aromatic acid decarboxylase (AADC) and dopamine transporter (DAT) – three key molecules involved in dopamine metabolism. The differentiated cells were primarily co-stained for TH and β-tubulin-3 (Figure 4A). Random cell count on microscopic fields determined an average of 45 ± 4% of the cells to be co-stained for the two proteins. The differentiation process was further confirmed by RT-PCR analysis (Figure 4B). In the presence of CPECs-CM only, the mRNA levels of DAergic markers increased significantly: 5-fold for TH, 3.5-fold for AADC, and 4-fold for DAT (Figure 4C; $P < 0.001$, Duncan's Multiple-comparison's Test). Compared to these treated cell groups, co-treatment of UCMSCs with CPECs-CM and RA+FGF2 further induced expression levels of the DAergic markers to several fold higher: 3.3-fold for TH, 2.5-fold for AADC, and 2.9-fold for DAT (Figure 4C; $P < 0.001$). These data indicate that CPECs-CM and RA+FGF2 can act in synergy to induce DAergic fate of UCMSCs.

Differentiated UCMSCs survive post-transplantation in rat brain

Table 1 shows the time frame of all animal work including toxin injection, cell transplantation and rotational analyses. The *in vivo* portion of the study was designed with the aim of simulating the scenario that usually occurs in parkinsonian brains (Figure 5A). To this end, we injected 6-OHDA into the left medial forebrain bundle (MFB) where the majority of DAergic fibers reside[22] so they can be specifically targeted. Next, we grafted the differentiated UCMSCs into the striatal region as previously reported.[25] Striatal sections were prepared from the injected site to examine the survival of transplanted cells. TUNEL assay showed highly significant increase of apoptosis rate in *Control* animals bearing 6-OHDA lesions compared to the *Sham* group (Figure 5B, and C; $P < 0,001$, Duncan Multiple Comparison's Test). The rate of apoptosis stood high within the 4-week period of our study. However, the *Test* animals that had received cell transplants showed significantly diminished rates of apoptosis compared to the un-transplanted (*Control*) group (Figure 5C; $P < 0.01$ in week 2 and $P < 0.001$ in weeks 3 and 4). In the *Test* group, the apoptosis rate also reduced significantly from week 2 to week 3 (Figure 5C; $P < 0.05$) but remained constant in week 4. These data demonstrate the protecting activity of transplants against 6-OHDA toxicity in the brain tissue and their parallel potential to survive and possibly integrate into the striatal neural network for their biological function.

Differentiated UCMSCs maintain DAergic phenotype in rat brain

Two weeks post-transplantation, immunohistochemical analyses showed ongoing TH expression by transplanted cells (Figure 6A). However, the pattern of TH staining was different from what we observed in culture dish (see Figure 4C). This difference occurred due to the striatal microenvironment new to the injected cells where they needed extra time to abandon their round morphology and spread to settle for local integration. Since differentiated UCMSCs were labeled with DiI for their cell membrane before transplantation, the red stain in Figure 6A demonstrates the fact that the cells expressing TH are our genuine transplants.

Transplanted UCMSCs promote behavioral recovery in parkinsonian rats

Behavioral function of the rats was evaluated based on apomorphine-induced rotation just before cell transplantation plus 1, 3 and 4 weeks post-transplantation. We found that throughout the 4-week time window, apomorphine caused a significant contra-lateral turning in the un-transplanted (*Control*) group that were lesioned by 6-OHDA injection compared to the *Sham* group (Figure 6B; $P < 0.001$; Duncan's Multiple Comparison's Test). However, the compound was unable to induce parallel number of rotations in the transplanted (*Test*) group, so that the total net number of rotations in this group was significantly reduced in comparison with the *Control* group ($P < 0.05$ in week 1 and $P < 0.01$ in weeks 3 and 4). This reduction was not significant enough to reach the levels in the *Sham* group; however, it was an indication that UCMSCs bear the capacity to correct behavioral deficits. There was no major difference in the number of rotations between weeks 3 and 4 – an observation that was compatible with the apoptosis data outlined above.

Discussion

We demonstrated that conditioned medium taken from epithelial cells of choroid plexus (CPECs-CM) is capable of inducing neuronal and DAergic differentiation of UCMSCs. The medium co-applied with RA+FGF2 significantly intensified both neuronal and DAergic differentiation shown by upregulated expression of relevant markers. Upon transplantation of these differentiated cells into the striatum of 6-OHDA-lesioned rats, they survived, reduced apoptosis rate and further induced behavioral recovery from 6-OHDA toxicity.

UCMSCs have potential of differentiation similar to bone

marrow MSCs and are further advantageous in that they are derived from redundant postnatal tissue and so their collection does not require invasive surgical procedures. Studies have shown that UCMSCc can acquire DAergic phenotype upon induction by dopamine-signaling molecules such as SHH, FGF8 and Lmx1a, or growth factors including NGF, VEGF, neurturin and hepatocyte growth factor.[26–29]

In order to induce neuronal differentiation of UCMSCs, we applied conditioned medium of growing CPECs. This medium is indeed comprised of a cocktail of potent inducing factors, as shown by our RT-PCR analysis. Our results showed that 71 ± 3% of UCMSCs incubated only with CPECs-CM expressed neuronal marker MAP2. This figure further increased to 81 ± 5% when the cells were pre-differentiated with RA+FGF2 before receiving CPECs-CM. Similar increases were detected for DAergic marker TH and astrocytic marker GFAP. These observations indicated that co-presence of CPECs-CM and RA+FGF2 could cooperatively induce neuronal, DAergic and glial differentiation of UCMSCs.

The CSF provides signaling molecules for proliferation of neural progenitor cells required for neurogenesis.[30] The CPECs secrete 70% of proteins present in CSF and are suspected to express a range of neurotrophic factors that drive cells towards various neuronal or non-neuronal fates.[31] Our CPECs tested positive for BDNF, NGF, VEGF and FGF2 beside DAergic-inducing GDNF. Embryonic stem cells co-cultured with Leydig cells that endogenously secrete GDNF can differentiate to DAergic neurons.[32] However, the inducing capacity of Leydig cells is not significant. In comparison and based on our current findings, CPECs-CM has powerful effects on DAergic and glial differentiation of UCMSCs.

GDNF is a protectant and inducer of DAergic neurons. We have previously applied conditioned medium from engineered astrocytes for both protection[33–35] and differentiation of stem cells to DAergic neurons (unpublished data). Similarly and in continuation of our current study, CPECs can be engineered to oversecrete GDNF and/or other inducers. Such cells could supply conditioned medium with more inducing power to boost DAergic differentiation and generate an enriched collection of TH^+ neurons.

The use of exogenous mitogenic and growth factors to induce neuronal differentiation of MSCs has not been effective in ameliorating PD symptoms upon cell transplantation.[20] However, our UCMSCs fed with CPEC-CM did better in alleviating PD symptoms in rat brain. Shetty *et al.* have reported that DAergic neurons derived from UCMSCs have the capacity to reduce parkinsonian symptoms in model animal.[37] Similarly, our current study demonstrated that differentiated cells derived from Wharton's jelly reduce the rate of apoptosis in the injected location in 6-OHDA-lesioned rats. Moreover, 6-OHDA-lesioned animals that received differentiated UCMSCs showed significant behavioral recovery when tested by apomorphine-induced rotations.

The rehabilitating function of transplanted UCMSCs can be attributed to their DAergic as well as neuroprotective activities. Further analyses could determine at what levels the injected cells can regionally supply dopamine and whether this dopamine source is directly involved in functional restoration in the brain. Also, the grafted USMSCs might induce behavioral recovery by protecting endogenous neurons. This paracrine effect occurs due to USMSCs capacity to secrete vital growth factors and cytokines[38] that foster DAergic neuroprotection in the striatal site of cell injection and/ or upon retrograde transport to the SNpc sub-region.[25]

Cell replacement therapy offers advantages for cure of neurodegenerative disorders, but also suffers from its specific pitfalls that may include chromosomal/genetic instability of cell sources, poor integration into or rejection from the neural network and unfavorable functionality and performance in the new microenvironment in the long-term[40] Dopaminergic differentiation for CRT purposes is induced either by manual application of conditioned/defined media[40–42] and exogenous factors or by ectopic expression of inducing factors in target cells.[43,44]

Our method of cell differentiation is advantageous to many other methods in that it relies on natural conditioned medium for induction and utilizes cell sources that carry no foreign genetic material, are freshly made from postnatal tissue without need for invasive surgery and have minimum risk of genetic instability compared to adult cells. Chromosomal stability and the post-mitotic status of these cells upon neuronal differentiation would certainly minimize their risk of tumorigenicity upon transplantation. In the case of gene transfer, our strategy demands no *in vivo* gene delivery and if needed *in vitro*, genetic material can be delivered to helper cells (i.e., CPECs) but not to target cells (UCMSCs). Therefore, the method we developed in this study does not carry many risks involved with ordinary procedures applied for gene therapy of the nervous system.[45]

In conclusion, we demonstrated that conditioned medium of CPECs induced dopaminergic differentiation of growing UCMSCs, and, RA+FGF2 accelerated this induction. Within the lesioned rat brain, the differentiated cells survived well, resisted 6-OHDA toxicity and ultimately improved parkinsonian symptoms. For clinical uses, these differentiated cells would have to be enriched *in vitro* to pure dopaminergic neurons, a step that requires dissecting the interplay between neurotrophic and dopaminergic-specific factors absent from the scene. Enlightenment of these interactive mechanisms will determine fine-tuned concentrations of specific dopamine-signaling molecules critical for dopaminergic differentiation. *In vivo*, molecular events leading to neural integration of transplants and biochemical changes they make to alleviate disease symptoms require detailed investigations to improve their preclinical and clinical effectiveness.

Declaration of interest

The authors report no declarations of interest.

Acknowledgment

We appreciate kind assistance made by Dr. F. Khodagholi of Neuroscience Research Center and her team members to this study. Guidelines set by the Ethical Committee in Shahid Beheshti University of Medical Sciences were followed in handling, injecting and sacrificing animals. A to-be-discarded fresh umbilical cord was obtained from Taleghani hospital in Tehran with the consent of the parents and was used in the study. This study was financially supported in part by a grant from NRC (Project-134). The authors declare that they have no conflict of interests.

References

1. Hirsch EC, Faucheux B, Damier P, Mouatt-Prigent A, Agid Y. Neuronal vulnerability in Parkinson's disease. *J Neural Transm.* 1997; 50: 79 – 88.
2. Lindvall O, Björklund A. Cell therapy in Parkinson's disease. *NeuroRx.* 2004; 1(4): 382 – 393.
3. Kitada A, Dezawa M. Parkinson's disease and mesenchymal stem cells: potential for cell-based therapy. *Parkinson's Dis.* 2012; 2012: 873706.
4. Kassis I, Grigoriadis N, Gowda-Kurkalli B, Mizrachi-Kol R, Ben-Hur T, Slavin S, et al. Neuroprotection and immunomodulation with mesenchymal stem cells in chronic experimental autoimmune encephalomyelitis. *Arch Neurol.* 2008; 65(6): 753 – 761.
5. Munoz JR, Stoutenger BR, Robinson AP, Spees JL, Prockop DJ. Human stem/progenitor cells from bone marrow promote neurogenesis of endogenous neural stem cells in the hippocampus of mice. *Proc Natl Acd Sci U.S.A*. 2005; 102(50): 18171 – 18176.
6. Lin YT, Chern Y, Shen CK, Wen HL, Chang YC, Li H, et al. Human mesenchymal stem cells prolong survival and ameliorate motor deficit through trophic support in Huntington's disease mouse models. *PLoS One*. 2011; 6(8): 22924.
7. Olson SD, Pollock K, Kambal A. Genetically Engineered Mesenchymal Stem Cells as a Proposed Therapeutic for Huntington's Disease. *Mol Neurobiol*. 2012; 45(1): 87 – 98.
8. O. Sadan O, Bahat-Stromza M, Barhum Y. Protective effects of neurotrophic factor-secreting cells in a 6-OHDA rat model of Parkinson disease. *Stem Cells Dev*. 2009; 18(8): 1179 – 1190.
9. Bouchez G, Sensebé L, Vourc'h P, Garreau L, Bodard S, Rico A, et al. Partial recovery of dopaminergic pathway after graft of adult mesenchymal stem cells in a rat model of Parkinson's disease. *Neurochem IntNatl.* 2008; 52(7): 1332 – 1342.
10. Wang HS, Hung SC, Peng ST, Huang CC, Wei HM, Guo YJ, et al. Mesenchymal Stem Cells in the Wharton's Jelly of the Human Umbilical Cord. *Stem Cells.* 2004; 22(7): 1330 – 1337.
11. Frausin S, Viventi S, Verga Falzacappa L, Quattromani MJ, Leanza G, Tommasini A, et al. Wharton's jelly derived mesenchymal stromal cells: Biological properties, induction of neuronal phenotype and current applications in neurodegeneration research. *Acta Histochem*. 2015; 117(4-5): 329 – 338.
12. Fu YS, Cheng YC, Lin MY, Cheng H, Chu PM, Chou SC, et al. Conversion of human umbilical cord mesenchymal stem cells in Wharton's jelly to dopaminergic neurons *in vitro*: potential therapeutic application for parkinsonism. *Stem Cells*. 2006; 24(1): 115 – 124.
13. Ou Y, Yu S, Kaneko Y, Tajiri N, Bae EC, Chheda SH, et al. Intravenous infusion of GDNF gene-modified human umbilical cord blood CD34+ cells protects against cerebral ischemic injury in spontaneously hypertensive rats. *Brain Res*. 2010; 1366: 217 – 225.
14. Shang AJ, Hong SQ, Xu Q, Wang HY, Yang Y, Wang ZF, et al. NT-3-secreting human umbilical cord mesenchymal stromal cell transplantation for the treatment of acute spinal cord injury in rats. *Brain Res*. 2011; 1391: 102 – 113.
15. Ryu CH, Park SH, Park SA, Kim SM, Lim JY, Jeong CH, et al. Gene therapy of intracranial glioma using interleukin 12-secreting human umbilical cord blood-derived mesenchymal stem cells. *Hum Gene Ther.* 2011; 22(6): 733 –743.
16. Dohrmann GJ. The choroid plexus: a historical review. *Brain Res.* 1970; 18(2): 197 – 218.
17. Timmusk T, Mudo G, Metsis M, Belluardo N. Expression of mRNAs for neurotrophins and their receptors in the rat choroid plexus and duramater. *Neuroreport*. 1995; 6(15): 1997 – 2000.
18. Huang X, Ketova T, Fleming JT, Wang H, Dey SK, Litingtung Y, et al. Sonic hedgehog signaling regulates a novel epithelial progenitor domain of the hindbrain choroid plexus. *Development.* 2009; 136(15): 2535 – 2543.
19. Parada C, Martin C, Alonso MI, Moro JA, Bueno D, Gato A. Embryonic cerebrospinal fluid collaborates with the isthmic organizer to regulate mesencephalic gene expression. *J Neurosci Res*. 2005; 82(3): 333 – 345.
20. Baehr C, Reichel V, Fricker G. Choroid plexus epithelial monolayers – a cell culture model from porcine brain. *Cerebrosp Fluid Res.* 2006; 3: 13.
21. Esmaeilzadeh E, Gardaneh M, Gharib E, Sabouni F. Shikonin protects dopaminergic cell line PC12 against 6-hydroxydopamine-mediated neurotoxicity via both glutathione-dependent and independent pathways and by inhibiting apoptosis. *Neurochem Res*. 2013; 38(8): 1590 – 1604.
22. Torres EM , Lane EL, Heuer A, Smith GA, Murphy E, Dunnett SB. Increased efficacy of the 6-hydroxydopamine lesion of the median forebrain bundle in small rats, by modification of the stereotaxic coordinates. *J Neurosci Methods*. 2011; 200(1): 29 – 35.
23. da Conceição FS, Ngo-Abdalla S, Houzel J, Rehen SK. Murine Model for Parkinson's Disease: from 6-OH Dopamine Lesion to Behavioral Test. *J Vision Expt.* 2010; 35: 1376.
24. Roghani M, Niknam A, Jalali-Nadoushan MR, Kiasalari Z, Khalili M, Baluchnejadmojarad T. Oral pelargonidin exerts dose-dependent neuroprotection in 6-hydroxydopamine rat model of hemi-parkinsonism. *Brain Res Bullet*. 2010; 82(5-6): 279 – 283.
25. Connor B, Kozlowski DA, Schallert T, Tillerson KL, Davidson BL, Bohn MC. Differential effects of glial cell line-derived neurotrophic factor (GDNF) in the striatum and substantia nigra of the aged Parkinsonian rat. *Gene Ther*. 1999; 6(12): 1936 – 1951
26. Xiong N, Zhang Z, Huang J, Chen C, Zhang Z, Jia M. VEGF-expressing human umbilical cord mesenchymal stem cells, an improved therapy strategy for Parkinson's disease. *Gene Ther*. 2010; 18(4): 394 – 402.
27. Li M, Zhang SZ, Guo YW, Cai YQ, Yan ZJ, Zou Z, et al. Human umbilical vein derived dopaminergic-like cell transplantation with nerve growth factor ameliorates motor dysfunction in a rat model of Parkinson's disease. *Neurochem Res*. 2010; 35(10): 1522 – 1529.
28. Yan M, Sun M, Zhou Y, Wang W, He Z, Tang D, et al. Conversion of human umbilical cord mesenchymal stem cells in Wharton's jelly to dopamine neurons mediated by the lmx1a and neurturin *in vitro*: potential therapeutic application for Parkinson's disease in a rhesus monkey model. *PLoS One* 2013; 8(5): 64000.
29. Li JF, Yin HL, Shuboy A, Duan HF, Lou JY, Li J, Wang HW, Wang YL. Differentiation of hUC-MSC into dopaminergic-like cells after transduction with hepatocyte growth factor. *Mol Cell Biochem*. 2013; 38(1-2): 83 – 90.
30. Lehtinen MK, Zappaterra MW, Chen X, Yang YJ, Hill AD, Lun M. The cerebrospinal fluid provides a proliferative niche for neural progenitor cells. *Neuron.* 2011; 69(5): 893 – 905.
31. Matsumoto N, Taguchi A, Kitayama H, Watanabe Y, Ohta M, Yoshihara T, et al. Transplantation of cultured choroid plexus epithelial cells via cerebrospinal fluid shows prominent neuroprotective effects against acute ischemic brain injury in the rat. *Neurosci Lett.* 2010; 469(3): 283 – 288.
32. Yue F, Cui L, Johkura K, Ogiwara N, Sasaki K. Induction of midbrain dopaminergic neurons from primate embryonic stem cells by coculture with sertoli cells. *Stem Cells.* 2006; 24(7): 1695 – 1706.
33. Sandhu JK, Gardaneh M, Iwasiow R, Lanthier P, Gangaraju S, Ribecco-Lutkiewicz M, et al. Astrocyte-secreted GDNF and glutathione antioxidant system protect neurons against 6OHDA cytotoxicity. *Neurobiol Dis*. 2009; 33(3): 405 – 414.
34. Safi R, Gardaneh M, Maghsoudi N, Zaefizadeh M, Panahi Y, Gharib E. Optimized quantities of GDNF overexpressed by engineered astrocytes are critical for protection of neuroblastoma cells against 6-OHDA toxicity. *J Mol Neurosci*. 2011; 46(3): 654 – 665.
35. Gharib E, Gardaneh M, Shojaei S. Upregulation of glutathione peroxidase-1 expression and activity by glial cell line-derived neurotrophic factor promotes high-level protection of PC12 cells against 6-hydroxydopamine and hydrogen peroxide toxicities. *Rejuven Res.* 2013; 16(3): 185 – 199.
36. Khoo ML, Tao H, Meedeniya ACB, Mackay-Sim A, and Ma DDF. Transplantation of neuronal-primed human bone marrow mesenchymal stem cells in Hemiparkinsonian rodents. *PLoS One* 2006; 6(5): 19025.
37. Shetty P, Thakur AM, Viswanathan C. Dopaminergic cells, derived from a high efficiency differentiation protocol from umbilical cord derived mesenchymal stem cells alleviate symptoms in a Parkinson's disease rodent model. *Cell Biol IntNatl.* 2013; 37(2): 167 – 180.
38. Joyce N, Annett G, Wirthlin L, Olson S, Bauer G, Nolta JA. Mesenchymal stem cells for the treatment of neurodegenerative disease. *Regen Med*. 2011; 5(6): 933 – 946.
39. Herberts CA, Kwa MS, Hermsen HP. Risk factors in the development of stem cell therapy. *J Transl Med*. 2011; 9: 29 – 42.
40. Borkowska P, Fila-Danilow A, Paul-Samojedny M, Kowalczyk M, Hart J, Ryszawy J, et al. Differentiation of adult rat mesenchymal stem cells to GABAergic, dopaminergic and cholinergic neurons. *Pharmacol Rep*. 2015; 67(2):179 – 186.

41. Yang S, Sun HM, Yan JH, Xue H, Wu B, Dong F, et al. Conditioned medium from human amniotic epithelial cells may induce the differentiation of human umbilical cord blood mesenchymal stem cells into dopaminergic neuron-like cells. *J Neurosci Res*. 2013; 91(7): 978 – 986.
42. Paldino E, Cenciarelli C, Giampaolo A, Milazzo L, Pescatori M, Hassan HJ, et al. Induction of dopaminergic neurons from human Wharton's jelly mesenchymal stem cell by forskolin. *J Cell Physiol*. 2014; 229(2): 232 – 244.
43. Friling S, Andersson E, Thompson LH, Jönsson ME, Hebsgaard JB, Nanou E, et al. Efficient production of mesenchymal dopamine neurons by Lmx1a expression in embryonic stem cells. *Proc Natl Acad Sci U S A*. 2009; 106(18): 7613 – 7618.
44. Sánchez-Danés A, Consiglio A, Richaud Y, Rodríguez-Pizà I, Dehay B, Edel M, et al. Efficient generation of A9 midbrain dopaminergic neurons by lentiviral delivery of LMX1A in human embryonic stem cells and induced pluripotent stem cells. *Hum Gene Ther.* 2012; 23(1): 56 – 69.
45. Bowers EJ, Breakefield XO, Sena-Esteves M. Genetic therapy for the nervous system. *Hum Mol Genet.* 2011;20 : R28 – R41.