Original Article

Transdifferentiation of Bone Marrow Stromal Cells into Tyrosine Hydroxylase Immunoreactive Cells Associated with Angiogenesis in Parkinsonian Rats

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Abstract

Objective: This study is an attempt to examine the transdifferentiation of bone marrow stromal cells (BMSCs) into tyrosine hydroxylase immunoreactive cells in parkinsonian rats associated with angiogenesis.

Materials and Methods: In this study, Sprague-Dawley rats received unilateral stereotaxic injections of 6-hydroxydopamine(6-OHDA) into the left corpus striatum and then were divided into two groups. One group, the negative control, received only medium while the other group was treated with BMSCs. BMSCs were harvested from femur bones, labeled with bromodeoxyuridine (BrdU) and then transplanted into parkinsonian rats, where a behavioral study and immunohistochemistry were used to evaluate the treatment.

Results: The results showed statistically significant improvement in rotational behavior. Anti-BrdU antibody showed engraftment of the transplanted cells at the transplantation site. Additionally, double immunolabeling confirmed that these cells were positive for neurofilament-200 and tyrosine hydroxylase (TH).

Conclusion: It may be concluded that BMSCs transplants could engraft and differentiate into TH immunoreactive cells which may cause recovery from motor deficits. Also, BMSCs may contribute to angiogenesis at the transplantation site.

Keywords: Parkinson's Disease, Bone Marrow Stromal Cells, Stem Cell, Cell Therapy

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Introduction

Parkinson's disease (PD) affects more than 1% of the population over age 60 in the western world. The main clinical features of PD are bradykinesia, tremor, rigidity and postural instability, although significant other deficits are seen in most patients including disturbances of mood, cognition and autonomic dysfunction reflecting the diffuse pathology of advanced PD. PD is a progressive neurodegenerative disease, characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta and a reduction in striatal dopamine. A wide range of pharmacological therapies exists for PD, which are especially effective in the early stages and include levodopa, dopamine agonists and MAO-Binhibitors. Levodopa is the most effective drug therapy that is ultimately used in most patients; however it is associated with problematic side effects such as motor fluctuations with long-term use. Surgical ablative and stimulating therapies, whilst being effective in advanced PD, do not attempt to cure the patient. Neural transplantation however aims to be curative by replacing the missing dopamine neurons and in so doing effectively curing the PD patient, or providing at least a substantial recovery (1-3).

The use of fetal tissues and embryonic stem cells (ESCs) have raised major ethical issues, in addition to the possibility of immunologic rejection, whereas the use of adult stem cells provides the possibility for autologous cell transplantation with a low risk for teratoma formation. Adult stem cells have been isolated from the brain, bone marrow, skin, fat, skeletal muscle and other visceral organs (4). Bone marrow stromal cells (BMSCs) have attracted interest through their possible use for cell therapy in neurological diseases (5, 6). These cells are stem cells derived from the adult marrow that give rise to both mesenchymal and non-mesenchymal lineages. BMSC are multipotent, easily available from aspirates of whole bone marrow and can be isolated by their adherence to the tissue culture surface. Recent reports have demonstrated that BMSCs are able to

migrate extensively throughout the adult animal and have the potential for neuronal differentiation after transplantation into the brain parenchyma. BMSCs are considered to be an option for replacement therapy (7-10). In PD therapy, donor cells should be easily available, capable of rapid expansion in culture, immunologically inert, capable of long-term survival and integration in the host brain. One possible source of such cells is the bone marrow (11, 12).

There are three different approaches responsible for the beneficial effect of stem cell treatment: firstly, cell replacement or direct replacement of degenerated cells with functional cells, (e.g., implantation of differentiated dopaminergic neurons to replace lost cells in the denervated nigrostriatal pathway) (13-16). The second approach is neuroprotection, where transplanted stem cells provide environmental support to the affected brain cells by secreting cytokines and neurotrophic factors. Thirdly, genetically engineered cells capable of producing glial or brain-derived neurotrophic factors to the brain could protect the remaining unaffected neurons and restrain PD (17-21).

The purpose of this study is to evaluate the neuronal phenotype of transplanted BMSCs in parkinsonian rats and to evaluate the vasculogenic activity at the site of BMSCs transplantation.

Materials and Methods

Animals

Adult male Sprague-Dawley rats, weighing 200-250g purchased from Razi Institute, Karaj, Iran, were kept at standard conditions according to the guidelines of the University Animal Care Codes in order to minimize suffering. This study was approved by Damghan University Animal Ethics Committee.

Model induction

Rats were maintained for 15 days at the medical laboratory animal house. Animals were anesthetized (ketamine 60 mg/kg plus xylazine 3 mg/kg, i.p.) and received unilateral stereotaxic injections of 6-OHDA (4µg of 6-OHDA dissolved in 2µl of 0.9% physiological saline containing 0.02% ascorbic acid; Sigma) into the left corpus striatum with 10 µl Hamilton syringes at a rate of 0.5 µl/minute, (the flow rate was controlled by a microsyringe with an EICOM EP-60 pump) (22-26). The injection was carried out using a stereotaxic apparatus according to the atlas of Paxinos and Watson (-3.0 mm lateral, +4.5 mm ventral, +0.2 mm rostral from the bregma with tooth bar at -3.3 mm). The needle was left in place for an additional 5 minutes after the injection, then withdrawn at a rate of 1 mm/min (27-30).

Behavioral test

Two weeks after loading the neurotoxin, a behavioral test was done (31-34). Animals were tested for the rotational behavior test by apomorphine hydrochloride (2.5 mg/kg, i.p.). Those rats that exhibited a net rotational asymmetry (turns ipsilateral to the lesion subtracted from contralateral turns) of at least six full turns per minute, or showing >250-300 turns per hour (contralateral against the lesioned side) were selected as parkinsonian models. Full contralateral rotations were counted in a cylindrical container (33cm diameter by 35cm height) (35).

BMSC culture and injection

Parkinsonian rats were subsequently divided into two groups. Group 1 received only injections of 2 µl of medium, while group 2 received a suspension of 1×10^5 BMSCs (6^{th} passage) into their left dopamine denervated striata (36-38).

For BMSCs isolation, the tibias and femurs from the sacrified rats were removed. Then bone marrow was aspirated according to Rismanchi (39) and cultured in α-minimal essential medium (α-MEM) supplemented with 15% FBS, 1% penicillin and streptomycin, and 25 ng/ml amphotericin B. After 48 hours of incubation, the non-adherent cell population was removed by replacing the medium. Adherent cells formed a confluent layer (80%), which was passaged by 0.25% trypsin/1mM EDTA for 5 minutes, recultured (39, 40), and the sixth passage of BMSCs were identified with sheep anti-fibronectin antibody. The demonstration was performed with donkey anti-sheep antibody conjugated with FITC (Chemicon International, Temecula) (41,42).

Before transplantation, BMSCs were labeled with BrdU at a concentration of 3µg/ml for 3 days (43).

Immunohistochemical and histological study

Six weeks after transplantation, the animals were perfused (4% paraformaldehyde). Their brains were removed, processed for paraffin embedding and sectioned (5µm). The sections were rehydrated, incubated in 50% formamide/2×SSC (standard sodium citrate, SSC: 0.3 M NaCl, 0.03M sodium citrate) for 2 hours at 60°C, washed with 2×SSC for 10 minutes at room temperature, incubated in 2N HCl at 37°C for 30 minutes, rinsed in 0.1 boric acid

(pH 8.5) for 10 minutes and washed in phosphate buffer saline (26). Then, sections were incubated in blocking serum, incubated with mouse anti-BrdU monoclonal antibody (Sigma, St. Louis, MO; B2531) overnight at 4°C and labeled with secondary antibody conjugated with rhodamine for 120 minutes at room temperature. The same sections were labeled either with mouse anti-neurofilament 200 monoclonal antibody (Sigma, St. Louis, MO) for detection of mature neurons, or with anti-tyrosine hydroxylase monoclonal antibody (Chemicon) in order to detect the dopaminergic neurons of transplanted BMSCs. In both instances, FITC conjugated antibodies were used (43). Some sections were stained with either hematoxylin and eosin or with cresyl fast violet.

Statistical analysis

Statistical analysis of the rotation scores for BMSCs-grafted and control animals were done by one-way ANOVA with SPSS (version 13) software. Tukey's HSD was used as a post hoc test. Differences were considered significant at the level of $p<0.05$.

Results

Isolation, proliferation and identification of BMSCs

Pure BMSCs were successfully obtained through several passage cultures. In the primary culture, after 24 hours, cells began to attach to the plastic but proliferated slowly. From the time of the second medium change onwards, cells underwent rapid proliferation and had multiple shapes. One to two weeks later, cells grew to 90% confluence in 25 cm flasks. After several passages, round cells disappeared. Phase contrast micrographs of BMSC at passage 6 revealed spindle-shaped cells (Fig 1A).

In fig 1B, cultured BMSCs were immunostained with anti-fibronectin antibody, a specific marker for BMSCs.

Fig 1C shows the 6th passage of BMSCs immunostained for BrdU marker, of which more than 80% of the cells were BrdU-positive.

Behavioral results

The apomorphine-induced rotational behavior of rats who received culture medium and BMSCs is shown in fig 2. As seen, the rotational behavior was assesed at two weeks after 6-OHDA (6-hydroxydopamine) injection and at intervals of two, four and six weeks after BMSCs transplantation.

One-way ANOVA test showed no significant differences in rotational rate in the control group at two, four and six weeks after medium injection (group 1), however BMSCs-treated rats (group 2) showed significant differences in the rotation scores at two, four and six weeks after transplantation ($p<0.0001$). There was indeed a significant difference in the rotation scores between groups 1 and 2 ($p<0.05$; Fig 2).

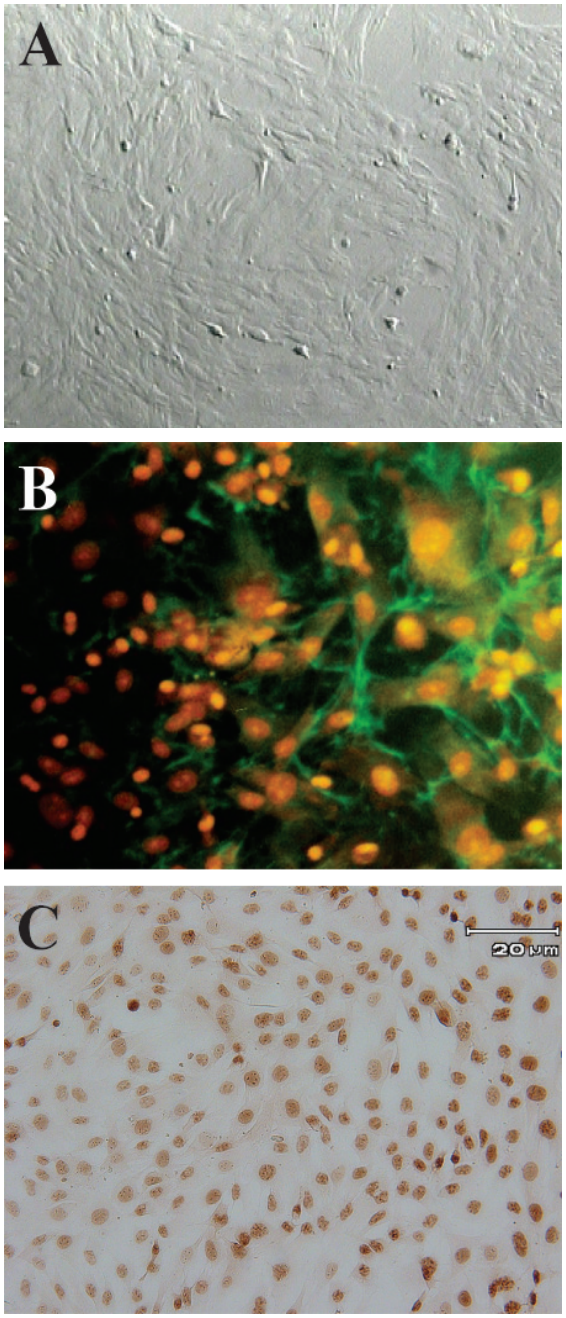

Fig 1: A. Bright-field microscopy of sixth passage cells shows spindle-shaped, large flat cells (×200). B. Immunoreactivity to antibody against fibronectin is a typical marker of marrow stromal cells in culture (×400). C. Sixth passage of cells labeled by BrdU

Immunohistochemistry results

Histological sections stained with hematoxylin and eosin show that the transplanted cells engrafted at the injury site (Fig 3).

Similar sections were stained with cresyl violet, which showed that some of the transplanted cells exhibited neuronal morphology (Figs 3, 4). In both figures angiogenesis was detected. The animals were transplanted with BrdU-labeled BMSCs and double- labeled with anti-BrdU antibody, followed by labeling with secondary antibody conjugated with rhodamine. While the secondary antibody for the anti-neurofilament-200 antibody was conjugated with FITC, the two images were merged (Fig 5A).

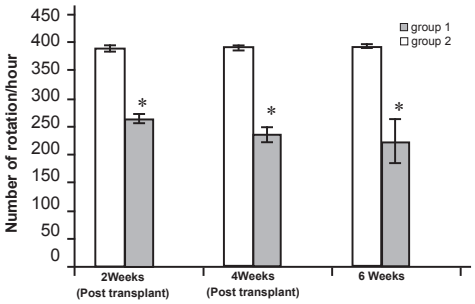

Fig 2: Apomorphine-induced circling behavior evaluated at 2, 4 and 6 weeks after transplantation in two groups (control and BMSCs-treated animals). Animals injected with BMSCs showed significant decrease in rotational rate (rotational improvement) compared with control group. *Significant difference vs. group 1 and group 2 in the same week, p<0.05.

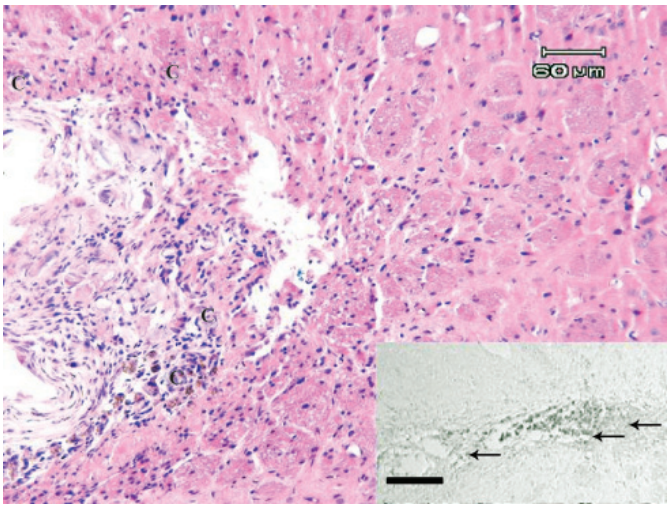

Fig 3: Photomicrograph shows the site of the transplanted cells stained with hematoxylin & eosin, "c" represents neocapillary formation (scale bar: 60 µm). Insert: Photomicrograph shows transplanted cells labeled with BrdU, demonstrated with indirect immunoperoxidase. Arrow indicates engrafted transplanted BMSCs (scale bar: 70 µm).

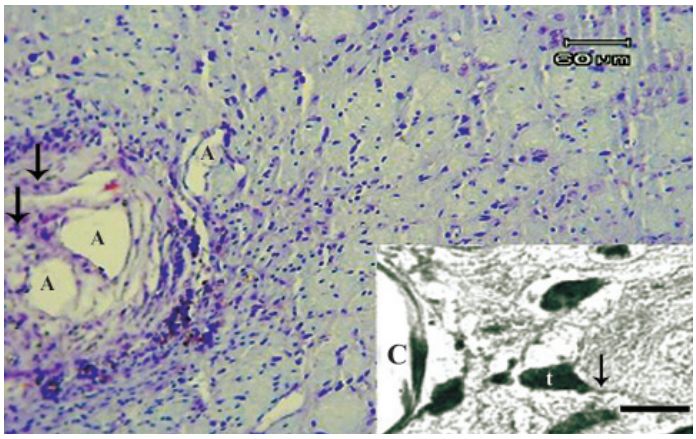

Fig 4: Photomicrograph shows the site of the transplanted cells stained with cresyl violet, "A" represents angiogenesis in the site of transplantation (scale bar: 35 µm). Insert: Photomicrograph shows the site of transplantation stained with cresyl violet, "C" represent the lumen of capillary and "t" represents transdifferentiated transplanted BMSCs into neurons with neuronal extension (arrow) (scale bar: 8 µm).

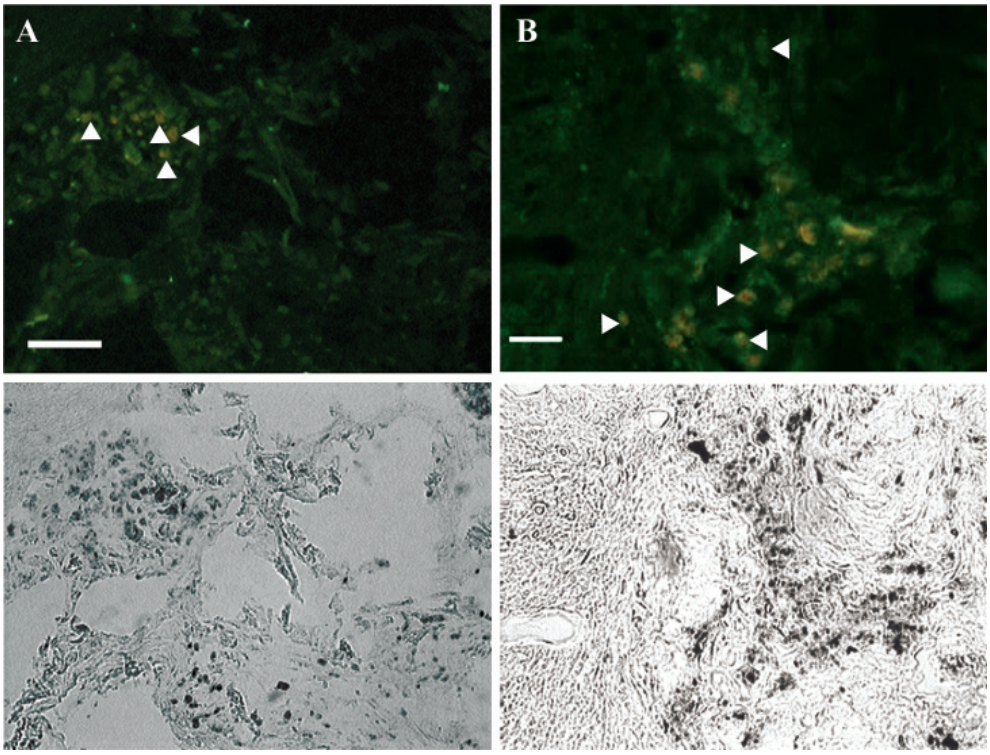

Fig. 5: Immunostaining of the transplanted cells from a parkinsonian rat 6 weeks after transplantation. Upper panel: A fluorescent photomicrograph from the site of transplantation in the striatal region. A: The tissue section was double-labeled with anti-neurofilament 200 (the secondary conjugated with FITC) and anti-BrdU antibody (the secondary conjugated with rhodamine), and the two images were merged. (Scale bar: 25 µm). Lower panel: Phase contrast photomicrograph of the field in the upper panel. B: The tissue section was double-labeled with anti-TH (the secondary conjugated with FITC) and anti-BrdU antibody (the secondary conjugated with rhodamine), and the two images were merged. (Scale bar: 32 µm). Lower panel: Phase contrast photomicrograph of the field in the upper panel.

Accordingly, Fig 5B shows double-labeling with anti-tyrosine hydroxylase antibody and the secondary antibody conjugated with FITC.
Thus some of the cells that were immunopositive for tyrosine hydroxylase (TH) were also positive for BrdU, which demonstrated the presence of dopaminergic neurons as derived from BMSCs.

Discussion

The earliest clinical investigation of BMSCs in the rat stroke model was done by Lu et al. (23), where BMSCs migration and integration in the injured brain resulted in improvement in neurological deficits (14). Hofstetter et al. used BMSCs to treat spinal cord injury which resulted in the recovery of motor activity (4). Replacement therapy of these lost neurons was suggested by transplantation of fetal nigral tissues, ESCs and adult stem cells (3-5). BMSCs have been considered as an option for replacement therapy, and it was reported that these cells have the ability to migrate , differentiate into astrocytes and integrate the brain.
Garcia et al. have suggested the treatment of neurodegenerative diseases such as Parkinson's disease with BMSCs in order to restore lost neurons (16).
In the current study, behavioral data showed recovery in motor disability following BMSCs transplantation. Animals given BMSCs-treated grafts exhibited more rapid recovery from the drug-induced circling behavior than control animals (particularly two weeks after transplantation). Indeed animals treated with BMSCs showed recovery over time from apomorphine-induced turning behavior, whereas control animals did not. Functional recovery required complete neural differentiation and integration with the host's nervous system.
This result was consistent with other studies where fetal tissues were used to treat parkinsonian rats. In this study, the problem of insufficient tissue supply has been considered as a factor in this protocol (19, 20). Recently, cell therapy has been used including ESCs transplantation, which has shown

promising results.
However, the major concern in this strategy is the possibility of rejection due to expression of non-self antigens which may result in immunological rejection (21), as well as the possible development of teratocarcinoma (22). The use of adult stem cells such as BMSCs can be a good alternative for replacement therapy. Lu et al. have reported that intracerebral BMSC transplantation could improve motor deficits in parkinsonian mice, while immunohistochemical studies have shown that the transplanted cells expressed TH (23). Histological and immunohistochemical studies were undertaken to determine the presence of transplanted cells in the cerebral tissues. In order to ensure that the cells which expressed neuronal lineage markers were of BMSCs origin, cells were double-stained for both neuronal markers (NF-200, TH) and BrdU.
With double staining of TH and BrdU, it was possible to identify TH-positive neurons at the implantation site (24).
In addition, the striatum is a target area for DA neurons. It may provide growth factor support that increases the survival of DA neurons compared with other neuronal phenotypes. Hellmann et al. found that transplanted BMSCs survive better in the 6-OHDA-induced damaged hemisphere when compared to the unlesioned side (10).
It was reported the importance of microenvironment in BMSCs transdifferentiation into a neuronal phenotype, however Deng et al. have revealed that the production of neurotrophin proteins from BMSCs may indicate the potential for the autocrine mode of action of these factors to transdifferentiate these cells into neuronal cells, where the neurotrophins and their receptors were reported to be expressed by BMSCs (5, 25, 27). Additionally, it has been reported that BMSCs expressed mRNA encoding brain-derived factor (BDNF), fibroblast growth factor-2 (FGF-2) and glial cell line-derived neurotrophic factor (GDNF). They have been shown to exhibit potent neurotrophic effects on embryonic dopaminergic neurons grafted into the striatum of a rat model of PD (16, 28). As seen in group 2, transplanted cells could differentiate into neural cells and replace lost cells at the implantation site. Possibly these cells, by secreting neurotrophic factors, could protect the remaining unaffected neurons.
BMSCs have been successfully used to treat other neurological disorders such as stroke where the results showed restoration of motor deficits (29).
Moreover, BMSCs have been used for spinal cord injury treatment, where the results showed migration and integration of the cells as well as recovery of motor disabilities (30). One of the advantages of BMSCs is the possible autologous use of these cells in the treatment of neurodegenerative diseases (31). Additionally, BMSCs have been genetically engineered to produce L-DOPA (19, 32) or dopamine, where BMSCs were transfected with TH and recovery was reported (33).
These findings are consistent with the results of this investigation, in which the differentiation of BMSCs into a neuronal phenotype occurred after transplantation in the striatal region with expression of TH. The presence of TH-positive cells with the reduction of apomorphine-induced rotations may show the capacity of dopamine release of grafted cells in vivo and synaptic interaction with the host brain. This indicates that these cells can be a good choice for the treatment of PD.
Moreover, vasculogenic activity was simultaneously noticed with neurogenesis in the transplantation site, consistent with a previous investigation where neuronal transdifferentiation and neocapillary formation could be seen at the transplantation site of BMSCs in a spinal cord injury (33).
Angiogenesis was reported in an animal model for stroke. Chen et al. emphasized the role of vascular formation and the astrocytic microenvironment in the survival and integration of newly formed cells (34). Our results indicated that differentiation of BMSCs into a neuronal phenotype at the implantation site with expression of TH. Moreover, vasculogenic activity was simultaneously noticed with neurogenesis in the transplantation site. The association of angiogenesis with neurogenesis can be explained as due to the enhancement of expression of vascular endothelial growth factor by BMSCs.
The source of the vascular endothelial growth factor can be local cells at the injury site (35). In addition, BMSC transplantation in a hypoxic environment has been reported to increase angiogenesis (36).
For stem-cell therapy to be effective for PD, large numbers of dopaminergic neurons with the characteristics of substantia nigra neurons must be produced. Additionally the survival of transplanted cells was low, thus a variety of different approaches should be investigated to promote graft survival.
However, much research needs to be done with BMSCs before they can be considered as clinical treatment for PD.

Conclusion

Our results confirmed that the reduction of apomorphine-induced rotations may show the differentiation of BMSCs into a neuronal phenotype at

the implantation site with expression of TH. Moreover, vasculogenic activity is noticed simultaneously with neurogenesis at the transplantation site.

Acknowledgments

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