# Effect of intranasal stem cell administration on the nigrostriatal system in a mouse model of Parkinson's disease

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**Abstract.** Parkinson's disease (PD) is the second most common neurodegenerative disease worldwide. It affects the locomotor system, leading to a final severe disability through degeneration of dopaminergic neurons. Despite several therapeutic approaches used, no treatment has been proven to be effective; however, cell therapy may be a promising therapeutic method. In addition, the use of the intranasal (IN) route has been advocated for delivering various therapies to the brain. In the present study, the IN route was used for administration of mesenchymal stem cells (MSCs) in a mouse model of PD, with the aim to evaluate IN delivery as an alternative route for cell based therapy administration in PD. The PD model was developed in C57BL/6 mice using intraperitoneal rotenone administration for 60 consecutive days. MSCs were isolated from the mononuclear cell fraction of pooled bone marrow from C57BL/6 mice and incubated with micrometer-sized iron oxide (MPIO) particles. For IN administration, we used a 20 $\mu$l of $5x10^5$ cell suspension. Neurobehavioral assessment of the mice was performed, and after sacrifice, brain sections were stained with Prussian blue to detect the MPIO-labeled MSCs. In addition, immunohistochemical evaluation was conducted to detect tyrosine hydroxylase (TH) antibodies in the corpus striatum and dopaminergic neurons in the substantia nigra pars compacta (SNpc). The neurobehavioral assessment revealed progressive deterioration in the locomotor functions of the rotenone group, which was improved following MSC administration. Histopathological evaluation of brain sections in the rotenone+MSC group revealed successful delivery of MSCs, evidenced by positive Prussian blue staining. Furthermore, rotenone treatment led to significant decrease in dopaminergic neuron number in SNpc, as well as similar decrease in the corpus striatum fiber density. By contrast, in animals receiving IN administration of MSCs, the degeneration caused by rotenone treatment was significantly counteracted. In conclusion, the present study validated that IN delivery of MSCs may be a potential safe, easy and cheap alternative route for stem cell treatment in neurodegenerative disorders.

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## Introduction

Parkinson's disease (PD) is a degenerative disorder affecting the central nervous system (CNS), which results from the death of dopamine-generating cells in the substantia nigra of the midbrain. Thus, PD is characterized by depletion of dopaminergic cell bodies in substantia nigra that are subsequently lost in the nigrostriatal system. The nigrostriatal system is composed of the Substantia Nigra and Corpus striatum and is the affected dopaminergic system in cases of PD. The reported incidence rates of PD vary largely. The lowest PD incidence was reported to be 4.5/1,000000 in Libya, while the highest incidence was reported in the USA at 20/1,000000 (1). The early symptoms of PD include slowness of movement, rigidity, shaking and walking difficulties. Symptoms presented at later stages of the disease may include thinking and behavioral problems, with dementia and depression arising in the advanced stages (1). Other symptoms, including sensory, sleep and emotional problems, may also occur. The majority of PD cases occur after the age of 50 years, and the disorder is more prevalent among men rather than women. The pathological hallmark of the disease is accumulation of the protein α-synuclein into inclusions known as Lewy bodies in neurons (2). The intensity of the Lewy bodies is directly associated with the clinical symptoms of each individual. Mechanisms underlying PD may include mitochondrial dysfunction, oxidative stress, inflammation and defective

protein handling. PD is the second most common neurodegenerative disorder after Alzheimer's disease (3). Upon clinical diagnosis of PD, the loss in dopaminergic neurons has already reached 80%, and thus neuroprotective therapies for PD are of little significance as the majority of dopaminergic neurons are lost. By contrast, the use of regenerative agents in PD patients appears to be more promising (4).

Cell-based therapy has been advocated for PD due to the high failure rate of other therapeutic strategies (5). Despite the improvement imparted by developing novel dopamine receptors agonists, these agents are considered weak in comparison with L-DOPA, thus resulting in their use as a complementary treatment rather than a substitute to the classical L-DOPA therapy (6). Cellular therapy, which was initially investigated using fetal tissues with limited long-term success, is currently the central component of regenerative treatment for PD. However, the identification of stem cells increased the possibility of more successful cellular therapy for neuroregeneration (7). However, to date, advances in stem cell research have failed to offer a successful regenerative therapy for PD patients due to various limiting factors. The most important problem for stem cell therapy in neurodegenerative diseases such as PD is the method of administration. The first mode of stem cell transplantation is through their direct introduction into the corpus striatum using stereotaxis, with the corpus being the preferred site for transplantation over the substantia nigra and subthalamic nucleus (8). However, this route is considered inapplicable in humans due to inconvenience, risk of several possible complications and high costs (9). By contrast, systemic administration of stem cells has not demonstrated sufficient encouraging results, which may be due to the difficulty of cells crossing the blood-brain barrier (BBB) (10).

The search for efficient delivery route for neurological diseases appears to be crucial. The majority of candidate drugs for CNS diseases that showed promising results on *in vitro* and *in vivo* studies failed to show similar efficacy in humans, leading to high attrition rates of novel CNS active drugs in clinical trials (11). The main reason for such failure is the presence of the BBB, which prevents the passage of the right concentration of the drug to the target tissue (12).

One approach for resolving this issue is targeted intranasal (IN) delivery, which is an applicable method used to circumvent BBB rather than attempting to cross it (13). The nasal passage is the only direct connection between the brain and the external environment. This connection occurs through the extension of axons from the olfactory bulb to the nose, allowing direct contact with the external environment. Another potential route is the nose to brain pathway, which is a controversial pathway suggesting the passage of medication through the deep structures in the nose that are innervated by cranial nerves (14). Based on this pathway, the IN route has been used for the delivery of a variety of agents for the treatment of different CNS conditions. For instance, drugs delivered using IN delivery system include growth factors, neuropeptides, genes and small molecules (9). Notably, previous animal studies showed the successful IN delivery of mesenchymal stem cells (MSCs) to the brain. In addition, animal models of Parkinson's disease have been successfully treated through IN administration of L-DOPA (15).

Based on these previous findings, the present study aimed to investigate the use of the IN route for administration of MSCs in a mouse model of PD.

## Materials and methods

*Rotenone mouse model and stem cells administration*. A total of 30 B57BL/6 mice (age, 8 weeks; weight, 16-20 g) were provided by the Medical Experimental Research Center of Mansoura University (Mansoura, Egypt) and maintained in conditions of 21-23˚C, with a humidity of 40-55% and a 12 h dark/light cycle. All animal experiments were performed according to the Guidelines for the Care and Use of Mammals in Neuroscience (2003), and were approved by the Ethical Committee for research at Mansoura University. All efforts were made to minimize animal suffering and to reduce the number of animals used.

A PD model was developed in mice through the intraperitoneal administration of 3 mg/kg/day body weight rotenone (Sigma-Aldrich, St. Louis, MO, USA), for 60 consecutive days. The mice were divided into three groups (10 mice in each) as follows: Control group, which received daily intraperitoneal injection of 0.5% carboxymethyl cellulose (El-Nasr Chemicals Co., Cairo, Egypt); PD model group (rotenone group), receiving rotenone (3 mg/kg body weight) dissolved in 0.5% carboxymethyl cellulose intraperitoneally; and rotenone+MSC group, which received rotenone administration similarly to the PD model group, followed by IN bone marrow MSCs derived on day 60. All animals were sacrificed by perfusion through the aorta with 50 ml of 10 mM phosphate-buffered saline (PBS), followed by 150 ml of a cold fixative consisting of 4% paraformaldehyde, 0.35% glutaraldehyde and 0.2% picric acid in 100 mM phosphate buffer, under deep anesthesia with pentobarbital (100 mg/kg, intraperitoneally). Animals were sacrificed on day 70, thus after 10 days of MSCs treatment for the rotenone+ MSC group.

*Stem cells isolation*. MSCs were isolated from the mononuclear cell fraction of pooled bone marrow from healthy C57BL/6 mice. Mice were sacrificed by cervical dislocation and their femurs and tibiae were carefully cleaned from the skin and cut at the ankle bone. The muscle and connective tissue were scraped, and the bones were placed in 10% ethyl alcohol for sterilization and left for a few seconds. Next, the ends of the tibia and femur were cut by sharp scissors and a 27-gauge needle was inserted, after which the sample was flushed with Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and collected in a 15-ml tube. The cell suspension was then filtered through a 70-$\mu$m filter mesh. Bone marrow cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution (Thermo Fisher Scientific, Inc.) in a 25-$cm^2$ tissue culture flask and incubated at 37˚C and 5% $CO_2$. Subsequent to adhesion to the plastic wall of the dish and culture in DMEM, the stem cells were tested for plasticity through *in vitro* differentiation, including investigation of adipogenesis, chondrogenesis and osteogenesis (16). Isolated MSCs were then incubated overnight with micrometer-sized iron oxide (MPIO) particles (Bangs Laboratories, Inc., Fishers, IN, USA). For IN application, a 20 $\mu$l cell suspension

($5x10^5$ cells) was carefully placed on one nostril of each animal, allowing the mice to insufflate the MSC suspension, as previously described (17).

*Behavioral evaluation*

*Behavioral assessment*. To evaluate the therapeutic effects of IN delivery of MSCs, neurobehavioral investigation of all animals was performed by monitoring their general activity, presence of tremors, akinesia and cataplexy, as well as using the open field and parallel rod tests.

*Tremors*. Tremors were monitored immediately after the administration of rotenone. Three trained examiners monitored the animals in a blind study. Tremors were quantified on a modified intensity-score basis in a scale of 0-5, as described previously (18).

*Akinesia*. Akinesia was measured by recording the latency (in seconds) of the animals to move all four limbs, and the test was terminated when the latency exceeded 180 sec (19).

*Catalepsy*. The term catalepsy indicates the inability of rodents to correct an externally imposed posture (19). It was measured by placing the animals on a flat horizontal surface with the two hind limbs on a square wooden block (3-cm high), and the latency to move the hind limbs from the block to the ground was estimated in seconds.

*Open field analysis*. Mice were adapted to the open field enclosure prior to the test, and monitored. All tests were performed for 1 h, using an ANY-maze video tracking system (Stoelting Co., Wood Dale, IL, USA). This software, with the complementary Open Field Cage, was used to analyze the behavioral changes in the mice objectively, including the following parameters: Average speed, total time immobile, total number of immobility episodes, rotations of body and efficient paths.

*Parallel rod test*. Mice were adapted to the parallel rod enclosure prior to the assessment, and monitored. All tests were performed for 15 min, using an ANY-maze video tracking system. This software, with the complementary Open Field Cage, was used to analyze locomotor changes in mice in the form of number of slips.

*Tissue analysis*. After the mice were sacrificed on day 70, all animals were evaluated for stem cells tracking and tyrosine hydroxylase (TH) antibody binding. TH is the key enzyme in the dopamine synthesis process, staining against TH is the preferred antibody-based method to detect dopaminergic neurons. For stem cell tracking, brain sections of IN MSC-treated mice were stained with Prussian blue (Sigma-Aldrich) in order to detect the MPIO-labeled MSCs (18). After perfusion, the brain was quickly removed and post-fixed for 2 days with paraformaldehyde in 100 mM PBS, and then transferred to 15% sucrose solution in 100 mM PBS containing 0.1% sodium azide at 4˚C. The brain specimens were processed into paraffin blocks, and then cut by a microtome at 4-5 micron on glass slides. Next, the specimens were deparaffinized, and then endogenous peroxidase was blocked using 30% hydrogen peroxide in methanol for 10 min, followed by serum blocking solution (10% non-immune serum) for 10 min. Antigen retrieval was performed with EDTA solution for 20 min at 90˚C in a water bath. Subsequently, the slides were incubated with primary mouse monoclonal anti-TH antibody (dilution, 1:1,000; cat no. AMAB91112; Sigma-Aldrich) overnight at 4˚C. Following several washes with PBS, the samples were incubated with the required biotinylated secondary antibody (1:10,000; cat no. A4416; Sigma-Aldrich) for 10 min, followed by the avidin-biotin-peroxidase complex for 10 min at room temperature. All the sections were washed several times with PBS between each incubation, and labeling was then revealed by addition of diaminobenzidine, which was used as a chromogen. Slides were counterstained with Meyer's hematoxylin (Sigma-Aldrich), dehydrated and covered with the cover slip.

Immunohistochemical analysis was also performed to investigate TH antibody binding in substantia nigra pars compacta (SNpc) and corpus striatum. Subsequently, TH immunostained brain sections were evaluated as follows: i) Striatal TH-fiber density measurement was performed using Image J software (http://imagej.nih.gov/ij/) as previously described (19); and ii) number of dopaminergic neurons in the SNpc were counted as previously described (20), and were reported as percentages of the control neurons (with the control set to 100%).

*Image analysis*. All analyses were performed by an investigator blinded to the experimental design. Various areas were subjected to image analysis. In order to bilaterally evaluate the TH-positive fiber innervation in the striatum, mean optical density measurements were performed using the Image J software (version 1.33-1.34; National Institutes of Health, Bethesda, MD, USA). In addition, images of coronal sections were captured at seven rostral-caudal levels, in order to cover the entire striatal complex. The striatum was included from the lateral ventricle to the external capsule and a horizontal line connecting the ventral end of the ventricle via the anterior commissure to the external capsule. The data are expressed as percentage of the controls and represent the average of the seven levels (20). Furthermore, assessment of dopaminergic neurons in the substantia nigra pars compacta (SNpc) was determined by counting the number of TH-positive cells in the SNpc of both hemispheres, in every fourth section throughout the entire nucleus. The anatomical levels considered in the anteroposterior (AP) extension were within -5.20 and -5.80 mm with respect to bregma. Results are expressed as the percentage of TH-positive cells in the lesioned SNpc with respect to the control (21).

*Statistical analysis*. All data are presented as the mean ± standard deviation. Two groups of data were analyzed by Student's t-test. Three groups of data were analyzed by analysis of variance with a Tukey's post-hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*Behavioral evaluation*. Behavioral assessment revealed progressive deterioration in the locomotor functions of the rotenone group (PD model group) compared with the control group. However, IN administration of MSCs (rotenone+MSC group) was found to improve the locomotor performance of the animals when compared with the rotenone-only treated animals. In general, increased salivation and hyperpnea were observed following injections, but with no accompanied convulsions or mortality in any group.

Table I. Open field test parameters.

| Parameter | Group 1 | Group 2 | Group 3 |
|---|---|---|---|
| Average speed | 0.11±0.03 | 0.07±0.01[a] | 0.10±0.02 |
| Total time immobile | 4.21±2.14 | 13.03±5.24[a] | 6.02±4.03 |
| Total immobile episode | 2.01±1.21 | 7.21±3.22[a] | 3.91±2.12 |
| Clockwise rotations | 1.03±0.63 | 5.89±1.90[a] | 2.10±0.74 |
| Efficient path | 0.04±0.02 | 0.01±0.00[a] | 0.02±0.02[a] |

[a]P<0.05 vs. control group.

Table II. SNpc neuronal counting and stratial OD.

| Parameter | Rotenone group | Rotenone+MSC group |
|---|---|---|
| SNpc neurons, % | 37±8 | 80±6[a] |
| Stratial OD, % | 53±7 | 91±5[a] |

[a]P<0.05, vs. rotenone group. The values reported are the percentage compared with the control (where control was 100%). MSC, mesenchymal stem cell; SNpc, substantia nigra pars compacta; OD, optical density.

The maximum tremor intensity was only up to a score of 4, with a maximum tremor duration of 20 min and a peak in intensity at 10 min. Tremors were rarely observed in the MSC-treated group, and the intensity did not exceed a score of 1.

Rotenone administration resulted in akinesia in the rotenone treated mice at 34+7 sec, while the MSC-treated group displayed an improved performance (12+5 sec). In addition, catalepsy was evident in the animals treated with rotenone, with a latency period was 41+5 sec. By contrast, the MSC-treated group displayed a better performance (13+4 sec).

The results of the open field test indicated a significant deterioration between the control and the rotenone treated groups with regard to various parameters, including the average speed, total time immobile, total immobility episodes, rotations of body and efficient paths (Table I). However, mice in the IN MSC-treated group showed results that were comparable to the control mice, revealing behavioral improvement. These parameters were determined by the original software ANY-maze, compatible with the open field test.

Furthermore, a parallel rod test was performed, and the results indicated a significant increase in the number of slips in the rotenone group (6 slips) compared with the control group, which had an average of 1 slip. IN MSC treatment improved the performance of the rotenone-treated mice to an average of 2 slips.

*Stem cell tracking*. Histopathological evaluation of treated animal brain sections revealed successful IN delivery of stem cells to the brain tissues of the rotenone+MSC mice. This successful delivery was evidenced by the positive staining with Prussian blue in different areas of the brain tissues (Fig. 1).

*Immunohistochemical analysis*. Rotenone treatment resulted in a significant decrease in dopaminergic neuron number in the SNpc (37±8% of neurons compared with the control; Table II). Similarly, rotenone treatment reduced the corpus striatum fiber density to 53±7% of the control value (Table I; Fig. 2A and B). However, in the animals receiving IN MSCs, the degeneration caused by rotenone treatment was significantly counteracted by stem cells administration, with a SNpc neuron number at 80±6% and corpus striatum fiber density at 91±5% of the values reported in the control group (Table I; Fig. 2A and C).

## Discussion

Cellular therapy for PD has been previously suggested as a promising treatment option (22) However, the limitations of cellular transplantation methods render this approach unsuitable for clinical applications (23). Experimentally, the most successful approach for cell transplantation is the intralesional route, performed by stereotaxis-aided injection of cells into the corpus striatum (24). However, despite the efficiency of this route, it requires complex surgical procedures that makes it clinically difficult to advocate for PD cases. Furthermore, longitudinal follow-up of transplanted cells revealed the need of booster doses, due to the loss of the majority of transplanted cells with time (25). The requirement for repetitive doses results in difficult repeat surgical procedures in human cases. Systemic administration of cells has not been proven to be effective in PD, and certain positive results have been attributed to systemic effects due to growth factor upregulation rather than local regenerative effects imparted by the transplanted cells (26).

A new and attractive alternative route is the intranasal administration of drugs for CNS diseases (27). The IN route efficiently introduces drugs intracranially through different suggested pathways. Initially, direct delivery of IN therapeutics was attributed to the olfactory nerve, but more recently, the trigeminal nerve was suggested as another contributing route. In addition, the quick time required for therapeutics to reach the brain through the IN route suggests extracellular delivery rather than axonal transport. Recently, diffusion within perineural and lymphatic channels or perivascular space has been suggested as a more reliable hypothesis (28).

The ease and efficacy of IN administration, besides the rapid delivery of therapeutic agents, resulted in several trials investigating the use of this route in various CNS disorders, particularly in neurodegenerative diseases. The

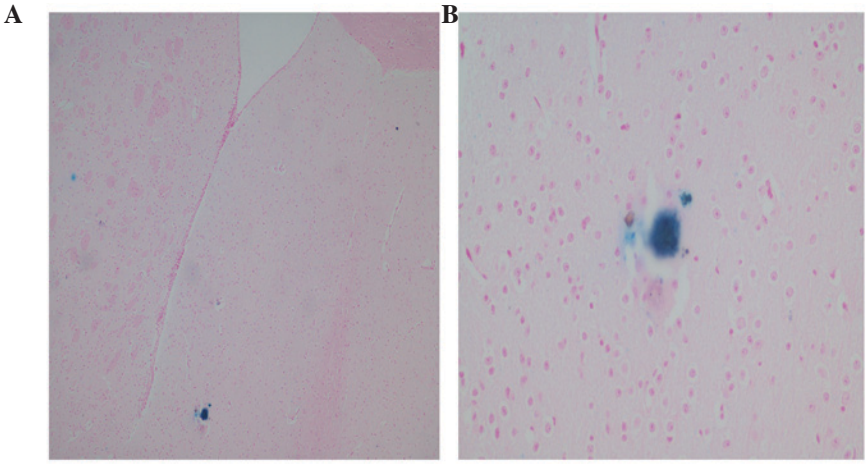


Figure 1. Tracking micrometer-sized iron oxide-labeled mesenchymal stem cells in the brain of the mice after intranasal administration. Prussian blue staining of brain tissue sections was performed, and images show the sections at a (A) low magnification of x4, and (B) high magnification of x20.

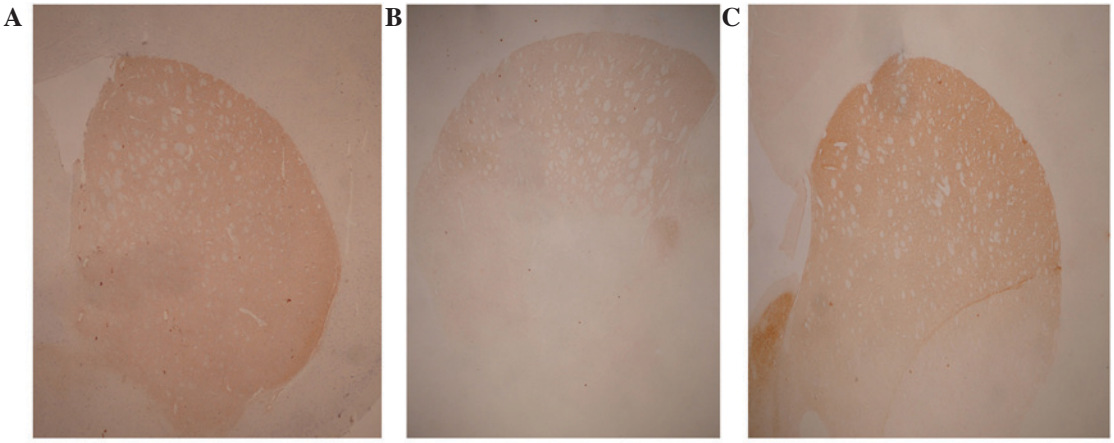


Figure 2. Immunohistochemical staining for tyrosine hydroxylase (TH) antibody in the striatum tissue samples of the (A) control group, (B) rotenone-only group and (C) rotenone+mesenchymal stem cell (MSC) group after 70 days. The intranasal MSC treatment was found to protect dopaminergic neurons, as evidenced by the preservation of TH fiber density in the striatum compared with the rotenone group.

IN administration of therapeutic agents was attempted with numerous vehicles and tracking methodologies (29,30). In the majority of studies, IN administration (alone or assisted with drug delivery agents) successfully reached the intracranial region and led to significant improvement in the disease (31). In addition, investigation of the role of IN stem cell delivery in PD has been attempted in several animal models, with promising results. Although long-term follow-up of transplanted stem cells revealed disappearance of cells after a certain period of time, this is predicted in neurodegenerative diseases, which mandates repetitive doses, thus further supporting the use of the easy IN route.

In spite of the positive results obtained from previous studies addressing the use of IN stem cells in PD, translation into clinical practice has yet to be achieved. This is due to the lack of an ideal animal model of PD that can recapitulate the pathology that occurs in human cases (32). Therefore, it is important to study new therapeutic agents for PD on different animal models. Previously, the IN route was assessed in 6-OHDA rat model (21) and in transgenic mice (22). The main issue with animal models of PD is the absence of an ideal model that can recapitulate all PD pathological findings (23). Therefore, it appears that investigating new therapeutic approaches on different animal models may be more effective (24).

Although the 6-OHDA and transgenic models are important tools to study PD in animals, they present major limitations that may reduce the credibility of their use for therapeutic agent testing (33). The 6-OHDA model involves the local injection of the agent into the nigrostriatal system, leading to immediate and severe degeneration in this area. With the exception of damage caused in the dopaminergic system, this model is not associated with other characteristics of PD. It lacks the cascade of events or pathogenic pathways that lead to PD in human cases, including lack of the neuro-inflammatory nature of PD which is important when studying cell transplantation. Furthermore, it does not represent the progressive nature of the disease, and since PD is a unilateral disease, the model does not accurately represent the loco-motor disturbances occurring in PD. By contrast, transgenic models of PD can serve as successful models for the rare familial type of the disease; however, they lack the sequence

of events that lead to the development of typical PD. In idiopathic PD, the role of environmental exposure is major, and thus this is a limiting point of transgenic models. An alternative model that carries numerous of the PD characteristics is the rotenone-induced model. Rotenone can induce a PD model in animals that has a chronic and progressive nature. The disease process is accompanied by various features of PD pathogenesis, particularly the neuroinflammatory effects and BBB influence, which are critical points in evaluating cell therapy.

Based on the aforementioned observations and limitations, the present study used the IN route for delivering MSCs in a rotenone-induced PD model in mice. To evaluate the therapeutic efficiency of this route, animals were evaluated behaviorally using a variety of neurobehavioral tests (such as the open field and parallel rod tests) and histopathological evaluation of brain sections through immunostaining against TH, which is the main marker of dopaminergic cells. Behavioral assessment assists in the study of the symptom-relieving effects of therapy, as behavioral tests can be translated into clinical performance in human cases. In addition, histopathological evaluation helps to study the improvement of disease pathology following treatment. In the present study, the transplanted stem cells were tracked to ensure their successful delivery intracranially and that they reached the site of the lesion. This step is of paramount importance to verify that any therapeutic effects of MSCs are caused by their direct regenerative effects and not due to a systemic body reaction.

In the current study, IN delivery of MSCs administered to a rotenone animal model were found to result in improvements in all affected neurobehavioral tests, which indicates the efficient therapeutic effect of this treatment. The successful passage of IN stem cells, as observed by stem cell tracking in the mouse brain tissue, shows that the therapeutic effects observed on the behavioral level can be attributed to the physical presence of MSCs inside the target brain tissues. The therapeutic efficiency of IN delivery of MSCs was then verified by immunostaining with TH antibodies, showing reduced degenerative effects compared with the rotenone-only treated group.

The results reported in the present study complement previous research findings that denote the success of IN delivery of stem cells in animal PD models. The use of a rotenone PD model appears to be of great importance, as this model carries certain important features of PD, such as environmental contribution and the chronic progressive pattern of the disease (25).

In conclusion, the present study identified the positive effects of IN delivery of MSCs in a progressive mouse model of PD. Thus, this treatment may have a possible similar effect in clinical practice, suggesting potential application in human cases of PD. According to the present results along with those of previous studies, IN delivery of stem cells appears to be a potential safe, easy and cheap route for stem cell treatment in neurodegenerative disorders. Although this study offered proof of the potential therapeutic benefit of IN route for delivering MSCs as a treatment for PD, further investigation is required prior to clinical application, such as comparison between different drug delivery vehicles, evaluation of nanosubstance addition, and investigation of the ideal type of stem cells and timing of transplantation.

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