Combination of Human Mesenchymal Stem Cells and Repetitive Transcranial Magnetic Stimulation Enhances Neurological Recovery of 6-Hydroxydopamine Model of Parkinsonian's Disease

Ji Yong Lee¹ · Hyun Soo Kim² · Sung Hoon Kim³ · Han-Soo Kim^{4,5} ·
Byung Pil Cho^{1,6}

Received: 15 October 2019 / Revised: 23 November 2019 / Accepted: 28 November 2019 / Published online: 22 January 2020
© The Korean Tissue Engineering and Regenerative Medicine Society 2020

Abstract

BACKGROUND: Repetitive transcranial magnetic stimulation (rTMS) has been in use for the treatment of various neurological diseases, including depression, anxiety, stroke and Parkinson's disease (PD), while its underlying mechanism is still unclear. This study was undertaken to evaluate the potential synergism of rTMS treatment to the beneficial effect of human mesenchymal stem cells (hMSCs) administration for PD and to clarify the mechanism of action of this therapeutic approach.

METHODS: The neuroprotective effect in nigral dopamine neurons, neurotrophic/growth factors and anti-/pro-inflammatory cytokine regulation, and functional recovery were assessed in the rat 6-hydroxydopamine (6-OHDA) model of PD upon administration of hMSCs and rTMS.

RESULTS: Transplanted hMSCs were identified in the substantia nigra, and striatum. Enhancement of the survival of SN dopamine neurons and the expression of the tyrosine hydroxylase protein were observed in the hMSCs + rTMS compared to that of controls. Combination therapy significantly elevated the expression of several key neurotrophic factors, of which the highest expression was recorded in the rTMS + hMSC group. In addition, the combination therapy significantly upregulated IL-10 expression while decreased IFN- γ and TNF- α production in a synergistic manner. The treadmill locomotion test (TLT) revealed that motor function was improved in the rTMS + hMSC treatment with synergy.

CONCLUSION: Our findings demonstrate that rTMS treatment and hMSC transplantation could synergistically create a favorable microenvironment for cell survival within the PD rat brain, through alteration of soluble factors such as neurotrophic/growth factors and anti-/pro-inflammatory cytokines related to neuronal protection or repair, with preservation of DA neurons and improvement of motor functions.

Keywords Parkinson's disease · Repetitive transcranial magnetic stimulation · Mesenchymal stem cell · Neurotrophic factor · Anti-/pro-inflammatory cytokine

✉ Han-Soo Kim
hankim63@gmail.com

✉ Byung Pil Cho
bpcho@yonsei.ac.kr

¹ Department of Anatomy, Yonsei University Wonju College of Medicine, 20 Ilsan-ro, Wonju, Gangwon-do 26426, Republic of Korea

² FCB-Pharmicell Co. Ltd., 520 Sicox Tower, 484 Dunchon-daero, Jungwon-gu, Seongnam-si, Gyeonggi-do 13229, Republic of Korea

³ Department and Rehabilitation Medicine, Yonsei University Wonju College of Medicine, 20 Ilsan-ro, Wonju, Gangwon-do 26426, Republic of Korea

⁴ Department of Biomedical Sciences, Catholic Kwandong University College of Medical Convergence, 24 Beomil-ro, 579 beon-gil, Gangneung-Si, Gangwon-do 25601, Republic of Korea

⁵ Basic Research Division, Biomedical Institute of Mycological Resource, College of Medicine, Catholic Kwandong University, 24 Beomil-ro, 579 beon-gil, Gangneung-Si, Gangwon-do 25601, Republic of Korea

⁶ Institute of Lifestyle Medicine, Yonsei University Wonju College of Medicine, 20 Ilsan-ro, Wonju, Gangwon-do 26426, Republic of Korea

1 Introduction

Parkinson's disease (PD) is a neurodegenerative disorder that is caused by the progressive loss of dopaminergic (DA) neurons in the substantia nigra. DA neurons in substantia nigra pars compacta (SNc) are part of an interconnected neural circuitry involving other brain areas, such as the striatum (ST), cerebral cortex (CCt), thalamus and subthalamic nuclei, playing an important role in the control of voluntary movement. Therefore, PD is manifested by a combination of motor disturbances such as resting tremor, rigidity, bradykinesia, and gait disturbances [1]. Current therapeutic treatments of PD patients include L-dopa, dopamine receptor agonists, dopamine metabolism inhibitors and stem cell transplantation [2, 3]. Other therapeutic options involve changing the neural excitability in the brain by non-pharmacological methods such as deep brain stimulation (DBS), electroconvulsive therapy (ECT) transcranial direct current stimulation (tDCS), and repetitive transcranial magnetic stimulation (rTMS) [4].

Albeit in their infancy, cell-based therapy holds promise for the treatment of the neurodegenerative disease [5]. Stem cell research underlying 3 different approaches to PD therapy includes the use of therapeutic cells as a cell replacement strategy for damaged or lost cells by transplanted cells, the use of stem cells for protecting the vulnerable neurons, or the utilization of stem cells as a vehicle for neurotrophic factors inducing endogenous neurogenesis for tissue repair [2].

Of the various sources of stem cells, human mesenchymal stem cells (hMSCs) are one of the ideal cell types for clinical applications due to their easy of isolation, culture expansion *in vitro*, stable phenotype maintenance *in vitro* and the ability to home to injury sites upon administration *in vivo*. Moreover, hMSCs possess immunoregulatory properties via their secreted soluble factors [6, 7], and neuroprotective capacity against the degeneration of DA neurons through released neurotrophic factors [8]. Thus, these properties of MSCs make them an ideal option for PD where inflammatory response is deeply associated with the progressive neurodegeneration [9, 10].

For the PD-associated symptom management, the idea of non-invasive brain stimulation by external forces has been present. Of these, repetitive transcranial magnetic stimulation (rTMS) involves magnetic fields to stimulate cerebral cortex or neural networks thereby altering their excitability. This type of non-invasive brain stimulation have been applied to a number of neurological and psychiatric diseases [11–15] to alleviate motor and cognitive symptoms while minimizing risks and adverse effects associated with conventional approaches. Studies have

shown that rTMS as an adjuvant therapy offers beneficial effects in PD animal models as well as PD patients [1, 4, 16–18]. Especially, rTMS increased endogenous dopamine production (in serum and subcortical areas) in both experimental animals and PD patients [18–22]. While clinical studies have demonstrated the beneficial effects of rTMS on PD, its mechanism of action has not been established. Recently, several experiments showed that rTMS elevates synaptogenesis, neuroplasticity and proliferation via complex biochemical events involving induction of immediate early genes, modulation of neurotransmitter release and neurotrophic/growth factor signaling in both PD experimental animals and patients [18, 23–29]. Considering the effect of rTMS *in vivo*, it is anticipated that its therapeutic efficacy can be further enhanced if rTMS is applied to PD animal models or PD patients transplanted with hMSCs.

The present study was designed to provide a rationale for a novel PD therapy combining hMSCs and rTMS (rTMS + hMSC). To that aim, the neuroprotective effect on DA neurons, regulatory effect on neurotrophic/growth factors and anti-inflammatory cytokines, and functional recovery were assessed in a 6-OHDA-induced PD animal model after administration of rTMS and hMSCs.

2 Materials and methods

2.1 Experimental procedures

2.1.1 Animals and housing conditions

All experimental protocols were approved by the institutional Animal Care and Use Committee at Yonsei University Wonju College of Medicine (Identification code: YWC-090828-1) and procedures were performed in accordance with the guidelines of the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. One hundred adult male Sprague–Dawley rats (body weight 250–300 g) were obtained from the Orient Bio Co. (Suwon, Korea) and housed under 12-h light/dark cycle at constant room temperature (20–22 °C) with free access to food and water.

2.1.2 6-Hydroxydopamine (6-OHDA) lesion

After treating with the noradrenalin transporter blocker desipramine [12.5 mg/kg, intraperitoneally (i.p.)]. Sigma Chemical Co., St. Louis, MO, USA) for 30 min, rats were anesthetized with ketamine (40 mg/kg body weight, i.p.) and xylazine (5 mg/kg body weight, i.p.) using a

stereotaxic frame (Stoelting Co., Wood Dale, IL, USA). Animals were kept on a heating pad maintained on 37 °C throughout surgery. Unilateral lesions were produced by stereotaxic injection of 20 µg/4 µl/site dose of 6-OHDA (Sigma Chemical Co., St. Louis, MO, USA) into the two sites (total of 40 µg) of the right striatum [18]. The injection coordinates with reference to bregma were: anterior–posterior (AP) + 0.5 mm, medial–lateral (ML) 2.5 mm, dorsal–ventral (DV) – 5.0 mm; and AP – 0.5 mm, ML 4.2 mm, and DV – 5.0 mm with the rate of 1 µl/min using a 26 G Hamilton syringe [30]. The infusion needle was left in each location for 5 min for 6-OHDA infusion before being slowly retracted, and the skin was sutured immediately. Ten days after the injections, the turning behavior was recorded after i.p. administration of 2.5 mg/kg amphetamine (Sigma Chemical Co., St. Louis, MO, USA) and animals showing more than 100 ipsilateral turns in 50 min were selected [18] and randomly assigned to one of the 4 groups; untreated group (n = 10), rTMS treatment group (n = 10), hMSC transplanted group (n = 10) and rTMS + hMSC treatment/transplanted group (n = 10). Animals were sacrificed 4 weeks after treatment.

2.2 Stem cell transplantation

2.2.1 Isolation and proliferation of hMSCs

Human bone marrow-derived MSCs (hMSCs) were obtained from Pharmicell (Pharmicell Co., Seoul, Korea) and cultured in Dulbecco modified Eagles medium (DMEM, Gibco-BRL, Grand Island, NY, USA) with 10% fetal bovine serum (FBS, Stem Cell Technologies, Vancouver, British Columbia, Canada), supplemented with 2 mM L-glutamine and 100 U/ml of Pen/Strep at 37 °C in 5% humidified CO₂. All experiments were performed with cells at passage 6.

2.2.2 Stem cell transplantation

On treatment day, hMSCs were trypsinized, labeled with PKH26 (PKH26 fluorescent cell linker kit, Sigma Chemical Co., St. Louis, MO, USA) for the cell tracking purpose, washed twice with phosphate-buffered saline (PBS) and resuspended to a final concentration of 2×10^7 cells with Hank's balanced salt solution (HBSS). Two weeks after 6-OHDA administration, the rats were anesthetized with ketamine/xylazine and positioned within a stereotaxic apparatus. The angle between the head and body was 90°, and the punctured surface was positioned horizontally [23]. The skin overlying the cisterna magna at the junction of the head and neck was cut longitudinally along the median line (10–15 mm). The tissues were mechanically displaced from the midline on the neck until dura mater appeared.

The dura mater was perpendicularly punctured using a Hamilton syringe (250 µl, 22 G) at the midpoint of the midline (depth 1 mm). Prior to injecting hMSCs, 50 µl of cerebral spinal fluid (CSF) was withdrawn to prevent intracranial hypertension and regurgitation of the transplanted cells. Two million hMSCs in 50 µl were injected to the subarachnoid space using a Hamilton syringe and a micro-infusion pump at the speed of 10 µl/min. The inserted needle was slowly retracted from the location and the skin was sutured immediately.

2.3 Repetitive transcranial magnetic stimulation (rTMS)

For rTMS stimulation, the rats were placed in a customized acrylic holder for their immobilization during the treatment. The center of the coil was held above the vertex of the rat's skull; the coil was positioned 1 cm away from the head. The rTMS treatment was performed with a BioCon-100 (M-cube Tech, Seoul, Korea) using a circular coil, which produces biphasic pulses lasting 280 µs and a maximum field of 1 Tesla at the center of the coil. rTMS was performed at 10 Hz frequency (on–off interval, 3 s) for a duration of 20 min per day for 4 weeks (Fig. 1).

2.4 Treadmill locomotion test (TLT)

Rats were placed on a motorized treadmill (72 rpm) which was enclosed within a see-through box with rulers drawn from 0 to 40 cm. The treadmill cycled between 20 s on and 20 s off. After adjusting for 1 min, the body position of the rat (tip of the nose) on the ruler was measured. The test was repeated five times [18, 31].

2.5 Immunohistochemistry

At the end of the experiment, rats were anesthetized and transcardially perfused with 100 ml of ice-cold saline followed by 200 ml of 4% paraformaldehyde in PBS. Following fixation with the same fixative 4 for 12 h, brains tissues were incubated with 30% sucrose solution in PBS at 4 °C until they sank. Then, the brains were dissected into a block containing the striatum or the SNc as previously described [23]. The block was then rapidly placed into cold Optimum Cutting Temperature (OCT) compound (Sakura Fine Technical Co., Tokyo, Japan). The samples were serially sectioned with a cryostat microtome (Leica Microsystems Inc., Wetzlar, Germany) at coronal 30 µm sections and the slices were conserved in a cryoprotectant at – 20 °C.

For blocking endogenous peroxidase activity, sections were washed with PBS, treated with 3% hydrogen peroxide for 15 min and incubated with 5% normal serum of the

Fig. 1 Schematic diagram of the experimental schedule. Successful induction of PD was screened by an amphetamine-induced rotation test at ten days after 6-OHDA injection. In rats with PD, rTMS

treatment and hMSC transplantation were performed at 2 weeks after 6-OHDA injection, and behavioral tests were then performed biweekly. *wpt* week post- treatment/transplantation

same host species of primary antibodies for blocking for 1 h. The sections were incubated in rabbit anti-Tyrosine hydroxylase (TH)-polyclonal antibody (1:1000, Chemicon, Temecula, CA, USA) diluted in PBS with 0.2% Tween 20 for 12 h. The slides were then incubated in biotinylated goat anti-rabbit IgG (1:200; Vector, Burlingame, CA, USA) for 1 h and subsequently incubated with avidin–biotin–peroxidase complex (Vector, Burlingame, CA, USA) for 1 h. Sections were then washed, incubated with 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma Chemical Co., St. Louis, MO, USA) and DAB-nickel (Vector) for 3–5 min [18].

2.6 Image analysis

TH-positive neurons on both sides of the SN (/mm²) were counted with the MetaMorph Imaging System (Molecular Device, Sunnyvale, CA, USA) and the effects of rTMS and/or hMSC transplantation on the preservation of TH-neuron was calculated as the percentage of TH-positive neurons in the lesioned sides divided by the percentage of TH-positive neurons of contralateral side. Photomicrographic images were taken on a Nikon Optiphot microscope (Nikon Inc., Tokyo, Japan) equipped with Nikon

digital camera (DXM1200), using Nikon ACT-1 image capture software (ver. 2.2) and subsequently processed with Adobe Photoshop (ver. 7.0, Adobe Systems Inc., San Jose, CA, USA).

2.7 Molecular analysis

2.7.1 Western blot analysis

In preparation for Western blot analysis, the lesioned SNc of brain sections were dissected, homogenized in 500 µl of cold radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris–HCl, pH 7.5, 1% Triton X-100, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), and 1% sodium deoxycholate) with a protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO, USA). Total protein concentration was determined by Quant-iT protein assay kit (Molecular Probes, Eugene, OR, USA) and equal amount of protein (50 µg) was separated in SDS-PAGE. Upon transfer to polyvinylidene difluoride (PVDF) membranes (Invitrogen, Carlsbad, CA, USA) using the trans-blot system, blots were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) for 1 h at room temperature, washed with TBS and incubated overnight at 4 °C with the

following primary antibodies in TBS with 0.02% Tween 20 (TBST) containing 5% nonfat dry milk: rabbit anti-BDNF (1:1000, Abcam, Cambridge, MA, USA), rabbit anti-glial cell-derived neurotrophic factor (GDNF, 1:1000, Abcam, Cambridge, MA, USA), rabbit anti-TH (1:1000; Abcam, Cambridge, MA, USA) and anti-GAPDH (1:3000, Cell signaling, Boston, MA, USA). Next, the blots were rinsed with TBST and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:3000, Santa Cruz Biotech, Santa Cruz, CA, USA) for 1 h in TBST containing 3% nonfat dry milk at room temperature. Antigen detection was performed using enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

2.7.2 Multiplex ELISA assay

To identify growth factors and cytokines regulated by rTMS, hMSCs or rTMS + hMSCs, an array based multiplex ELISA assay (Quantibody[®] array, RayBio-tech, Norcross, GA, USA) was used. The following growth factors and cytokines were detectable in the lesioned SNc: β -nerve growth factor (β -NGF), ciliary neurotrophic factor (CNTF), platelet-derived growth factor AA (PDGF-AA), interferon gamma (IFN- γ), interleukin 1 alpha (IL-1 α), interleukin-2 (IL-2), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF- α). Expression of growth factors and cytokines was detected using an array scanner (Gene PIX[™] 4000B, Axon instruments, Union City, CA, USA).

2.8 Statistical analysis

The data were expressed as a mean $\pm$ standard error of the mean (SEM). Statistical analysis was performed using the *t* test (Prism Graph Pad Software, San Diego, CA, USA) and two-way repeated ANOVA, followed by a Bonferroni post hoc comparison (SAS version 9.2, SAS Institute Inc., Cary, NC, USA). The significance level was assumed at $p < 0.05$, unless otherwise indicated.

3 Results

3.1 Recruitment of transplanted hMSCs to the lesions

To determine the migration of transplanted hMSCs into the brain parenchyma, the presence of PKH26-labeled hMSCs was directly assessed under a fluorescence microscope. A few PKH26-labeled hMSCs were observed in the ipsilateral striatum (ST) (Fig. 2A, B) and SNc (Fig. 2C–G) area at 4 weeks post-treatment/transplantation (wpt). It was

confirmed that a few transplanted hMSCs were co-localized with DAPI at 4 wpt (Fig. 2G).

3.2 Improvement of motor functions following rTMS treatment and/or hMSC transplantation in PD animal models

In order to evaluate the recovery of locomotor activity after rTMS and/or hMSC transplantation in PD rats, treadmill locomotion test was performed. Naïve control rats showed active and smooth motor function during the 60-s treadmill session. On the contrary, 6-OHDA-lesioned rats exhibited robust motor deficits that are typically characterized by slow and passive stepping motion on the treadmill due to limb-sue asymmetry. As a consequence of 6-OHDA lesion, rats was pushed back to the rear wall during the treadmill session. The rTMS treatment ($t = 3.678$, $p < 0.01$; $t = 8.229$, $p < 0.0001$), hMSC transplantation ($t = 3.033$, $p < 0.05$; $t = 6.144$, $p < 0.0001$) and rTMS + hMSC treatment groups ($t = 8.492$, $p < 0.001$; $t = 13.30$, $p < 0.0001$) all showed significant improvement in locomotor function 2 and 4 wpt, compared to the 6-OHDA (untreated) group (Fig. 3), respectively. The locomotor function of the rTMS + hMSC treatment group significantly was the highest among the experimental groups throughout the entire experimental period ($F = 24.71$, $p < 0.0001$) (Table 1). However, the sham control had no change in the locomotor function. The increase in score implies that rTMS treatment and/or hMSC transplantation induced the improvement of the motor function in the PD rats, and suggests that the combined rTMS + hMSC therapy was able to increase the motor function in a synergistic manner.

3.3 Neuroprotective effect of rTMS treatment and/or hMSC transplantation on DA neurons of the lesioned SNc

The neuroprotective effects of rTMS treatment, hMSC transplantation and rTMS + hMSC treatment on DA neurons in the SNc were assessed by TH immunohistochemistry, as well as TH protein quantification with Western blot analysis. Unilateral injection of 6-OHDA to the ST led to the marked decrease in the number of TH-positive neurons in the ipsilateral SNc, compared to that of contralateral SNc. TH immunohistochemical analysis of the SNc showed that the survival of TH-positive neurons in the ipsilateral SNc was significantly higher in the rTMS (19.41 ± 1.35 , $t = 5.155$, $p < 0.0001$), hMSC ($23.49 \pm 1.26\%$, $t = 7.934$, $p < 0.0001$) and rTMS + hMSC ($31.42 \pm 1.56\%$, $t = 13.34$, $p < 0.0001$) groups than that of 6-OHDA group ($11.85 \pm 0.65\%$) at 4 wpt. Notably, the highest survival rate significantly was

Fig. 2 Recruitment of hMSCs to the ipsilateral hemisphere.

A PKH26-labeled hMSCs, which were transplanted into the cisterna magna, are recruited to the ST at 4 wpt. Right the figures **B** is a higher magnification of the ST area demarcated with a rectangle in (**A**) is shown. **C** Recruitment of hMSCs to the SN area. PKH26-labeled hMSCs are recruited to the ipsilateral SN of PD rats at 4 wpt. **D** Is higher magnification of the SN area demarcated with a rectangle in (**C**). **E–G** PKH26 and DAPI double labeling in the ipsilateral SN. PKH26-labeled hMSCs are recruited to the SN at 4 week after hMSC transplantation. **C–E** DAPI labeled cells in the SN (**F**). A few PKH26-labeled hMSCs co-localized with DAPI (arrows) in the ipsilateral SN (**G**) of PD rats at 4 week after transplantation. *ST* striatum, *SN* substantia nigra

recorded in the rTMS + hMSC treatment group, indicating that this combinatorial treatment has synergistic effects on neuroprotection (Fig. 4A, B). The TH protein expression in the rTMS ($t = 17.47$, $p < 0.0001$), hMSC ($t = 24.11$, $p < 0.0001$), and rTMS + hMSC ($t = 37.08$, $p < 0.0001$) groups were higher than that of the 6-OHDA group, and the difference was statistically significant at 4 wpt. In sham control, the survival rate of dopamine neuron in the ipsilateral SN was comparable to that of contralateral SN. Notably, the rTMS + hMSC group significantly exhibited the highest TH expression, confirming the synergistic effects on neuroprotection of the combination of rTMS treatment and hMSC transplantation (Fig. 4C, D).

3.4 Modulation of neurotrophic/growth factors upon rTMS treatment and/or hMSC transplantation

To identify the neurotrophic/growth factors regulated by rTMS treatment and/or hMSC transplantation, the expression of BDNF, GDNF, β -NGF, CNTF, PDGF-AA, and VEGF in ipsilateral SNc was measured by Western blot analysis or ELISA assay at 4 wpt. Among the tested, the levels of BDNF, GDNF, NGF and PDGF were significantly elevated 4 wpt in the rTMS, hMSC, and rTMS + hMSC groups compared to the 6-OHDA group (Fig. 5).

BDNF expression was significantly increased in the rTMS ($t = 4.429$, $p < 0.01$) and rTMS + hMSC groups

Fig. 3 Treadmill locomotion test (TLT) in PD rats. Distance between the tip of the rat's nose and the rear wall of the treadmill chamber during 20 s treadmill walking periods in the untreated, rTMS, hMSC, and rTMS + hMSC groups. The distance to the nose tip is markedly increased in the rTMS, hMSC, and rTMS + hMSC groups. Note the greatest distance in the rTMS + hMSC group at all experimental time points. There was no changes in sham control group. *6-OHDA*: PD animals without any treatment; *rTMS*: PD animals treated with 10 Hz rTMS; *hMSC*: PD animals transplanted with hMSCs via cisterna magna; *rTMS + hMSC*: PD animals transplanted with hMSCs via cisterna magna and treated with 10 Hz rTMS. * $p < 0.05$ compared to the 6-OHDA group; ** $p < 0.01$ compared to the 6-OHDA group, *** $p < 0.001$, **** $p < 0.001$ comparison with the rTMS and rTMS + hMSC group, ***** $p < 0.001$ comparison with the hMSC and rTMS + hMSC group

($t = 6.498$, $p < 0.0001$) compared to the 6-OHDA group at 4 wpt. However, hMSC group showed not significance than 6-OHDA. The highest BDNF expression was significantly observed in the rTMS + hMSC group ($p = 0.0002$) (Fig. 5A, B).

Similarly, GDNF expression was significantly increased in the rTMS ($t = 3.30$, $p < 0.05$), hMSC ($t = 4.364$, $p < 0.001$), and rTMS + hMSC groups ($t = 6.213$, $p < 0.0001$) compared to the 6-OHDA group at 4 wpt. In particular, the highest expression of GDNF was significantly recorded in the rTMS + hMSC group ($p < 0.05$), indicating that the combination treatment has synergistic effect on the increased expression of these neurotrophic factors (Fig. 5A, C).

To quantify the level of CNTF, β -NGF, PDGF and VEGF, multiplex ELISA assays were performed on ipsilateral SN homogenates of the experimental groups (Fig. 5D). In general, β -NGF and PDGF protein levels were significantly increased in the rTMS ($t = 3.979$, $p < 0.001$; $t = 3.378$, $p < 0.05$), and rTMS + hMSC groups ($t = 5.163$, $p < 0.0001$; $t = 3.883$, $p < 0.001$) compared to the 6-OHDA group at 4 wpt. The highest NGF and PDGF levels were significantly recorded in the rTMS + hMSC group at 4wpt ($p = 0.0046$; $p = 0.0011$) (Fig. 5E, F). However, CNTF and VEGF expression levels showed no significant changes following rTMS treatment and/or hMSC transplantation (data not shown).

3.5 Changes in pro-/anti-inflammatory cytokine expression upon rTMS treatment and/or hMSC transplantation

To quantify several anti-/pro-inflammatory cytokines modulated by rTMS treatment and/or hMSC transplantation, five candidate cytokines (IL-10, IL-6, IFN- γ , TNF- α , and IL-2) were analyzed by an array-based multiplex ELISA assay using the ipsilateral SNc (Fig. 6). In general, rTMS treatment and/or hMSC transplantation tended to upregulate and suppress anti-inflammatory and pro-inflammatory cytokines, respectively (Fig. 6B–D). Four weeks after only combined rTMS + hMSC therapy, IL-10 level was significantly increased compared to the 6-OHDA group ($t = 3.839$, $p = 0.029$) (Fig. 6A, B).

In contrast to the upregulation of anti-inflammatory cytokines, the level of IFN- γ was significantly decreased in hMSC ($t = 3.142$, $p < 0.05$) and rTMS + hMSC groups ($t = 4.070$, $p = 0.0087$) compared to that of 6-OHDA group at 4 wpt. The lowest level of IFN- γ was noted in rTMS + hMSC group (Fig. 6A, C). In addition, TNF- α expression was significantly decreased in the hMSC groups ($t = 2.340$, $p = 0.034$) at 4 wpt (Fig. 6A, D). IL-6 and IL-2 showed little change in expression following rTMS treatment and/or hMSC transplantation (data not shown).

Table 1 Treadmill locomotion test (TLT) scores (mean $\pm$ SD; mm)

Period	6-OHDA	rTMS	hMSC	rTMS + hMSC	Sham control
Baseline	145.0 $\pm$ 19.8	143.6 $\pm$ 21.7	147.1 $\pm$ 20.79	142.9 $\pm$ 17.9	396.4 $\pm$ 9.9
2 weeks	170.7 $\pm$ 16.9	211.4 $\pm$ 13.8**	204.3 $\pm$ 11.7*	264.7 $\pm$ 33.2**	395.4 $\pm$ 4.8
4 weeks	209.7 $\pm$ 11.5	295.0 $\pm$ 27.5*****	272.9 $\pm$ 10.7*****	346.4 $\pm$ 21.7***	392.1 $\pm$ 6.9

6-OHDA: PD animals without any treatment; *rTMS*: PD animals treated with 10 Hz rTMS; *hMSC*: PD animals transplanted with hMSCs via cisterna magna; *rTMS + hMSC*: PD animals transplanted with hMSCs via cisterna magna and treated with 10 Hz rTMS

* $p < 0.05$ compared to the 6-OHDA group; ** $p < 0.01$ compared to the 6-OHDA group, *** $p < 0.0001$, **** $p < 0.001$ comparison with the rTMS and rTMS + hMSC group, ***** $p < 0.0001$ comparison with the hMSC and rTMS + hMSC group

Fig. 4 Quantitative analysis of tyrosine hydroxylase (TH) in the SNc. **A** TH immunohistochemistry in the SNc. Distribution of TH-positive neurons in the contralateral (CONT) and ipsilateral SN of the untreated, rTMS, hMSC, and rTMS + hMSC groups. Relatively large numbers of TH-positive neurons are found in the ipsilateral SN of the rTMS + hMSC group. In sham control, the survival rate of dopamine neuron did not difference in the ipsilateral SN compare with contralateral SN. **B** Survival rate of TH-positive neurons of the ipsilateral SN compared with the CONT side in the 6-OHDA, rTMS, hMSC, and rTMS + hMSC groups. The survival rate of TH-positive neurons was significantly increased in the rTMS, hMSC, and rTMS + hMSC groups compared to the 6-OHDA group. In particular, the highest survival rate was significantly recorded in the rTMS + hMSC group than rTMS and hMSC group at 4 weeks after

treatment. **C** TH protein expression in the SNc. Representative images of TH Western blot analysis of the SNc from 4 rats/group are shown. **D** Ratios of TH to GAPDH density values in C. TH expression was significantly increased in the rTMS, hMSC, and rTMS + hMSC groups compared to the 6-OHDA group at 4 wpt. The highest TH protein expression was significantly recorded in the rTMS + hMSC group than rTMS and hMSC group after treatment. *6-OHDA*: PD animals without any treatment; *rTMS*: PD animals treated with 10 Hz rTMS; *hMSC*: PD animals transplanted with hMSCs via cisterna magna; *rTMS + hMSC*: PD animals transplanted with hMSCs via cisterna magna and treated with 10 Hz rTMS. *** $p < 0.001$ comparison with the 6-OHDA group. **** $p < 0.001$ comparison with the rTMS and rTMS + hMSC group, **** $p < 0.001$ comparison with the hMSC and rTMS + hMSC group

4 Discussion

The present study demonstrated the therapeutic potential of combining rTMS and hMSC transplantation via cisterna magna to improve motor function and preserve DA neuronal survival through the modulation of neurotrophic factors and pro-/anti-inflammatory cytokines in a 6-OHDA-lesioned PD model.

The striatal infusion of 6-OHDA caused early damage of dopaminergic terminals during first 2 weeks, followed by a slowly evolving loss of dopaminergic neurons in the SN until 8 weeks [32, 71]. For protection of degenerating dopaminergic neurons in 6-OHDA-injected animal model, the therapeutic time windows was set to 2 weeks after 6-OHDA injection [18]. For the administration of hMSC, we selected intrathecal injection that has been have been used to deliver therapeutic substances and cells to the central nervous system (CNS) [33, 34] for enhanced

transplantation efficiency of therapeutic cells for neurodegenerative diseases with avoiding potential brain damage produced by a needle or cannula [35, 36]. In addition, the human intrathecal transplantation of therapeutic stem cells showed promising outcome with no marked adverse effects in clinical trials [37]. We did find some PKH26 labeled hMSCs and fragments of hMSCs in the ipsilateral SNc, ST and around of the lateral ventricle (LV)/subventricular zone (SVZ) after hMSC transplantation via the cisterna magna. This result suggests that the hMSCs injected through the cisterna magna spread to each part of the brain following the circulation of CSF, and some of them migrated to the SNc, ST, LV and SVZ. This finding aligns with previous studies showing the migration of administered cells into the parenchyma in the neurodegenerative diseases, such as PD, multiple systemic atrophy, amyotrophic lateral sclerosis, traumatic brain, and ischemic spinal cord injury [34–37]. However, after 4 weeks of stem

Fig. 5 Modulation of neurotrophic/growth factors upon rTMS treatment and/or hMSC transplantation. **A** Western blot analysis of BDNF and GDNF in the ipsilateral SN. **B** Representative Western blot analysis from 4 rats/group are shown BDNF Western blots (**A**) were quantified using densitometry analysis, normalized to GAPDH and graphed as mean $\pm$ SEM ($n = 4$). BDNF was significantly increased in the rTMS, hMSC, and rTMS + hMSC groups compared to the 6-OHDA group at 4 wpt. **C** GDNF expression in (**A**) were quantified using densitometry analysis, normalized to GAPDH and graphed as mean $\pm$ SEM ($n = 4$). GDNF was significantly increased in the rTMS, hMSC, and rTMS + hMSC groups compared to the 6-OHDA group at 4 wpt. **D, E** Multiplex ELISA assays for β -NGF and PDGF-AA in the ipsilateral SN NGF was significantly increased in the

rTMS, hMSC, and rTMS + hMSC groups compared to the 6-OHDA group, and significantly increased in the rTMS + hMSC groups than rTMS and hMSC group at 4 weeks after treatment. **F** PDGF-AA expression levels were significantly increased in the rTMS, hMSC, and rTMS + hMSC groups at 4 wpt. **6-OHDA**: PD animals without any treatment; **rTMS**: PD animals treated with 10 Hz rTMS; **hMSC**: PD animals transplanted with hMSCs via cisterna magna; **rTMS + hMSC**: PD animals transplanted with hMSCs via cisterna magna and treated with 10 Hz rTMS. * $p < 0.05$ compared to the 6-OHDA group; ** $p < 0.01$ compared to the 6-OHDA group. # $p < 0.05$ comparison with the rTMS and rTMS + hMSC group, § $p < 0.05$ comparison with the hMSC and rTMS + hMSC group

cell transplantation, most of the PKH26 labeled stem cells were deemed dead. Nevertheless, it can be presumed that the therapeutic effect in PD animals is due to the paracrine effect of the stem cells, rather than to their replacement of neuronal cells.

We verified that rTMS and hMSCs elicited neurorestorative effects on functional outcomes in the treadmill locomotion test. In particular, the treatment showing the highest efficacy was observed in the combination of rTMS and hMSC transplantation. The impairment of treadmill locomotion test reflects the dysfunction of the basal ganglia, including dopamine depletion in the striatum, indirectly leading to cortical dysfunction [38]. In addition, previous studies have shown that rTMS significantly improved motor function in unilateral DA-lesioned rats by treadmill locomotion test [31, 39, 40]. We speculated that while rTMS may control the excitability of the basal ganglia motor loops accompanied by neurorestorative effects, leading to improved locomotor function, the observed improvement of locomotor function upon hMSC

transplantation appears to be simply caused by the neurorestorative effects. It can also be postulated that if rTMS and hMSC transplantation are combined, the respective therapeutic effects are added, and thus appear to be more effective in restoring motor functions.

The survival rate of DA neurons and TH protein expression of were higher in the rTMS, hMSC and rTMS + hMSC groups than in the untreated group. It is noteworthy that the survival rate and the dopamine protein expression were the highest in the rTMS + hMSC. Our data suggest that rTMS and hMSC treatment might prevent or delay neurodegeneration induced by 6-OHDA, rather than increase the number of DA neurons in the lesion. It is also plausible that the neurorestorative effect was partially amplified by the combination of rTMS and hMSC. We evaluated endogenous neurorestorative proteins and inflammatory cytokines released from the 6-OHDA-lesioned brains after rTMS, hMSC transplantation, and rTMS + hMSC combination treatment. We demonstrated that the level of neurotrophic growth factors, such as NGF,

Fig. 6 Quantification of anti-/pro-inflammatory cytokines in the ipsilateral SNc. **A** Cytokine levels were quantified by multiplex ELISA. **B** The level of IL-10 was significantly increased in the hMSC and rTMS + hMSC groups compared to the 6-OHDA group at 4 wpt. The level of IL-10 was not significantly different in any treatment group. **C** The level of INF- γ was significantly decreased in the rTMS, hMSC, and rTMS + hMSC groups compared to the 6-OHDA group at 4 wpt. **D** The level of TNF- α was significantly decreased in the

rTMS, hMSC, and rTMS + hMSC groups compared to the 6-OHDA group at 4 wpt, but rTMS + hMSC group was no statistically significant difference in the among groups. *6-OHDA*: PD animals without any treatment; *rTMS*: PD animals treated with 10 Hz rTMS; *hMSC*: PD animals transplanted with hMSCs via cisterna magna; *rTMS + hMSC*: PD animals transplanted with hMSCs via cisterna magna and treated with 10 Hz rTMS. * $p < 0.05$ compared to the 6-OHDA group; ** $p < 0.01$ compared to the 6-OHDA group

BDNF, GDNF, PDGF and VEGF was significantly increase by these treatments. The increases of NGF, BDNF, GDNF and PDGF levels were the highest among the groups at 4 wpt. From these results, it can be speculated that rTMS treatment, hMSC transplantation and the combined rTMS + hMSC therapy exhibit neuroprotective effects on degenerated DA neurons, and the effects of the combined rTMS + hMSC therapy are greater than those of each therapy alone.

Neurotrophic or growth factors are key regulators in development, survival, function and regeneration of nervous systems. These soluble factors working in harmony with other neurotrophic factors significantly affect fates of given neurons. As the pathophysiologic PD progression is strongly associated with the alterations in the striatal neurotrophic factor levels leading to regional loss of striatal dopamine, a number of therapeutic strategies utilizing these factors for PD have been proposed [30, 41, 42]. For example, BDNF produced by DA neurons in the SN and the ventral tegmental area (VTA) is known to play an essential role for the survival, proper function and synaptic

plasticity of DA neurons in the SN [43, 44]. Expression of BDNF, at mRNA and protein levels, in postmortem brain tissues of PD was lower in the SN of PD patients than in controls implying its association with the DA neuron loss in PD [45]. In line with this, BDNF significantly exhibited a protective role on DA neuron survival in *in vitro* model of PD [46, 47]. GDNF has also been demonstrated to enhance the survival of DA neurons in animal model of PD and in clinical trials [42, 48–53]. The level of NGF, an essential cytokine for the proliferation, survival and developmental plasticity of neurons in the central nervous system as well as in the peripheral nervous system, was significantly reduced in PD experimental models and patients [43, 54] indicating that the loss of DA neurons and NGF level is strongly associated. The protective role of NGF for nigrostriatal DA neurons was further demonstrated by NGF administration to the striatum of 6-OHDA-lesioned rat model of PD [55]. PDGF, a pleiotrophic cytokine acting on fibroblasts, smooth muscle cells, and other cells [56], has been shown to have a neuroprotective function in both *in vitro* cultured rat DA neurons as well as *in vivo* DA

neurons in the rat SN against 6-OHDA-induced lesions [57–60].

In addition to their multipotency, MSCs have become therapeutic interest due to their ability to secrete various neurotrophic factors, including BDNF, NGF and GDNF, known to be involved in neuronal cell growth, axonal regeneration, neural stem/progenitor mobilization and neuronal protection [61, 62]. Transplanted MSCs may also directly or indirectly stimulate the synthesis or release of these neurotrophic/growth factors from host tissues. Up-regulated neurotrophic/growth factors might partially contribute to motor improvement and neuronal cell survival [Jin, 2008 #213, 63]. In addition, a number of studies reported the neurotrophic effects of high-frequency rTMS, including changes of BDNF concentration *in vitro*, *in vivo* animal models and in patients with neurologic disorders [18, 24–27, 29].

Studies have shown that inflammatory cytokines play an important role in the pathogenesis of PD. Indeed, the levels of pro-inflammatory cytokines, such as TNF- α , IL-1 and IL-6, were significantly upregulated in the SN region of postmortem brains and cerebrospinal fluid from PD patients, and in animal PD models [54, 64, 65] suggesting their deleterious effects on the DA neurons and/or glial cells. The anti-inflammatory and immunomodulatory functions probably one of the most studied properties of MSCs in translational research. Indeed, studies indicate that the observed neuroprotective effects of MSCs are mostly mediated by their paracrine expression of neurotrophic and anti-inflammatory cytokines in the absence of engraftment [66–69]. In the context of PD, administration of MSCs protected DA neuronal death by suppressing microglial activation and thereby modulating subsequent neuroinflammation [63, 70–73]. While anti-inflammatory activity via paracrine factors appears to be a key to the therapeutic benefits of MSCs [74, 75], the molecular mechanisms underlying the therapeutic effects of rTMS remain unclear. The safety and efficacy of long-term rTMS was demonstrated in a number of experimental models and clinical studies for PD [76–81]. The present study revealed that rTMS and transplanted hMSCs modulated various neurotrophic growth factors, and that BDNF, GDNF, NGF, NGF and PDGF-AA expression levels were increased by the rTMS and hMSC treatments in PD rats. In addition, the combined rTMS and hMSC treatment was found to be more effective in neurotrophic/growth factor expression in PD rats. An increase in the neurotrophic/growth factors thus seems to protect and restore the damaged DA neurons in the SNc.

In this study, the effects of rTMS, hMSC, and rTMS + hMSC treatment on the levels of inflammatory mediators in PD rats were investigated by multiplex ELISA. In general, rTMS treatment and/or hMSC

transplantation tended to enhance the expression of anti-inflammatory cytokines (IL-10), but to suppress that of pro-inflammatory cytokines (IFN- γ , TNF- α). Overall, the rTMS + hMSC treatment was more effective in immune regulation than either treatment alone. These results suggest that rTMS and hMSC transplantation may modulate inflammatory cytokines with different mechanisms, and each treatment may inhibit inflammation and encourage repair in the rat SN, preventing dopaminergic cells from being impaired by 6-OHDA. In addition, the combined rTMS treatment and hMSC transplantation were able to amplify the immune modulation effects in a synergistic manner. Our results suggests that rTMS treatment and/or hMSC transplantation increase neurotrophic/growth factor and anti-inflammatory cytokines, and suppress pro-inflammatory cytokines, to prevent or delay the neuronal degeneration arising from 6-OHDA, rather than repopulate the DA neurons. We speculate that rTMS treatment and hMSC transplantation may exert their prominent role by altering secreting factors in the PD brain microenvironment to prevent DA neurons from 6-OHDA-induced damage. Similarly, the combination of rTMS with hMSC transplantation has a synergistic effect on the neuroprotection of degenerated DA neurons. The mechanisms underlying this effect require further investigation.

One of the limitation in the present study is the long-term evaluation of the combination therapy. While the sustained behavioral recovery was also seen at 8 weeks of rTMS and cell transplantation, this was not accompanied with enhanced level of neurotrophic factors, except BDNF, and anti-inflammatory cytokines (data not shown). Since the transplanted hMSCs could not be detected in the ipsilateral SN at 8th week, the improved outcome may reflect the extension of the therapeutic effects of combined rTMS and hMSCs treatment during the first 4 weeks. Further study is essential for the elucidation of underlying mechanism. In addition to this, the reduced inflammation upon combination therapy during 4 weeks needs be at immunohistochemically examined. Although we performed the immunohistochemical staining of microglia in the SN tissues at 4 weeks, the activated microglia with intense OX6-immunoreactivity were not observed in the ipsilateral SN. This was, in part, due to conversion of activated microglia of SN into resting microglia or migration 6 weeks after 6-OHDA injection [32, 71].

In summary, the major findings of our study were: rTMS treatment, hMSC transplantation and their combination could induce a positive environment beneficial to cell survival within the PD rat brain, through the modulation of neurotrophic/growth factors and anti-/pro-inflammatory cytokines associated with neuronal protection or repair. These changes lead to DA neurons preservation and improvement of motor functions in PD rats. Furthermore,

combining rTMS treatment and hMSC transplantation produced partially synergistic effects. While the results of our study provide a theoretical framework for the development of novel therapeutic strategies utilizing combination of cell therapy with non-invasive brain stimulation for PD and other neurodegenerative diseases, further studies are warranted to elucidate the mechanisms involved in their synergistic effects.

Acknowledgements This study was supported by grants from the National Research Foundation (2017M3A9B4042583, 2010-0024334) and the Ministry of Science and Technology, Republic of Korea.

Compliance with ethical standards

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethical statement All experimental procedures performed in this work involving animals were approved by the institutional Animal Care and Use Committee at Yonsei University Wonju College of Medicine (Identification code: YWC-090828-1) and procedures were performed in accordance with the guidelines of the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

References

- Wu AD, Fregni F, Simon DK, Deblieck C, Pascual-Leone A. Noninvasive brain stimulation for Parkinson's disease and dystonia. *Neurotherapeutics*. 2008;5:345–61.
- Mendes Filho D, Ribeiro PDC, Oliveira LF, de Paula DRM, Capuano V, de Assunção TSF, et al. Therapy with mesenchymal stem cells in Parkinson disease: history and perspectives. *Neurologist*. 2018;23:141–7.
- Wijeyekoon R, Barker RA. Cell replacement therapy for Parkinson's disease. *Biochim Biophys Acta*. 2009;1792:688–702.
- Siebner HR, Mentschel C, Auer C, Conrad B. Repetitive transcranial magnetic stimulation has a beneficial effect on bradykinesia in Parkinson's disease. *Neuroreport*. 1999;10:589–94.
- Kramer BC, Woodbury D, Black IB. Adult rat bone marrow stromal cells express genes associated with dopamine neurons. *Biochem Biophys Res Commun*. 2006;343:1045–52.
- Wu J, Sun Z, Sun HS, Wu J, Weisel RD, Keating A, et al. Intravenously administered bone marrow cells migrate to damaged brain tissue and improve neural function in ischemic rats. *Cell Transplant*. 2008;16:993–1005.
- Krampera M, Pasini A, Pizzolo G, Cosmi L, Romagnani S, Annunziato F. Regenerative and immunomodulatory potential of mesenchymal stem cells. *Curr Opin Pharmacol*. 2006;6:435–41.
- Hellmann MA, Panet H, Barhum Y, Melamed E, Offen D. Increased survival and migration of engrafted mesenchymal bone marrow stem cells in 6-hydroxydopamine-lesioned rodents. *Neurosci Lett*. 2006;395:124–8.
- Monahan AJ, Warren M, Carvey PM. Neuroinflammation and peripheral immune infiltration in Parkinson's disease: an autoimmune hypothesis. *Cell Transplant*. 2008;17:363–72.
- Liau MT, Amini F, Ramasamy TS. The therapeutic potential of stem cells and progenitor cells for the treatment of Parkinson's disease. *Tissue Eng Regen Med*. 2016;13:455–64.
- Zwanzger P, Ella R, Keck ME, Rupprecht R, Padberg F. Occurrence of delusions during repetitive transcranial magnetic stimulation (rTMS) in major depression. *Biol Psychiatry*. 2002;51:602–3.
- Fitzgerald PB, Daskalakis ZJ. The effects of repetitive transcranial magnetic stimulation in the treatment of depression. *Expert Rev Med Devices*. 2011;8:85–95.
- Bentwich J, Dobronevsky E, Aichenbaum S, Shorer R, Peretz R, Khaigrekht M et al. Beneficial effect of repetitive transcranial magnetic stimulation combined with cognitive training for the treatment of Alzheimer's disease: a proof of concept study. *J Neural Transm (Vienna)*. 2011;118:463–71.
- Theilig S, Podubecka J, Bösl K, Wiederer R, Nowak DA. Functional neuromuscular stimulation to improve severe hand dysfunction after stroke: does inhibitory rTMS enhance therapeutic efficiency? *Exp Neurol*. 2011;230:149–55.
- Corti M, Patten C, Triggs W. Repetitive transcranial magnetic stimulation of motor cortex after stroke: a focused review. *Am J Phys Med Rehabil*. 2012;91:254–70.
- Arias-Carrión O. Basic mechanisms of rTMS: implications in Parkinson's disease. *Int Arch Med*. 2008;1:2.
- Yang YR, Tseng CY, Chiou SY, Liao KK, Cheng SJ, Lai KL, et al. Combination of rTMS and treadmill training modulates corticomotor inhibition and improves walking in Parkinson disease: a randomized trial. *Neurorehabil Neural Repair*. 2013;27:79–86.
- Lee JY, Kim SH, Ko AR, Lee JS, Yu JH, Seo JH, et al. Therapeutic effects of repetitive transcranial magnetic stimulation in an animal model of Parkinson's disease. *Brain Res*. 2013;1537:290–302.
- Strafella AP, Paus T, Barrett J, Dagher A. Repetitive transcranial magnetic stimulation of the human prefrontal cortex induces dopamine release in the caudate nucleus. *J Neurosci*. 2001;21:RC157.
- Strafella AP, Paus T, Fraraccio M, Dagher A. Striatal dopamine release induced by repetitive transcranial magnetic stimulation of the human motor cortex. *Brain*. 2003;126:2609–15.
- Khedr EM, Rothwell JC, Shawky OA, Ahmed MA, Foly N, Hamdy A. Dopamine levels after repetitive transcranial magnetic stimulation of motor cortex in patients with Parkinson's disease: preliminary results. *Mov Disord*. 2007;22:1046–60.
- Cho SS, Strafella AP. rTMS of the left dorsolateral prefrontal cortex modulates dopamine release in the ipsilateral anterior cingulate cortex and orbitofrontal cortex. *PLoS One*. 2009;4:e6725.
- Lee JY, Park HJ, Kim JH, Cho BP, Cho SR, Kim SH. Effects of low- and high-frequency repetitive magnetic stimulation on neuronal cell proliferation and growth factor expression: a preliminary report. *Neurosci Lett*. 2015;604:167–72.
- Baek A, Kim JH, Pyo S, Jung JH, Park EJ, Kim SH, et al. The differential effects of repetitive magnetic stimulation in an in vitro neuronal model of ischemia/reperfusion injury. *Front Neurol*. 2018;9:50.
- Müller MB, Toschi N, Kresse AE, Post A, Keck ME. Long-term repetitive transcranial magnetic stimulation increases the expression of brain-derived neurotrophic factor and cholecystokinin mRNA, but not neuropeptide tyrosine mRNA in specific areas of rat brain. *Neuropsychopharmacology*. 2000;23:205–15.
- Angelucci F, Oliviero A, Pilato F, Saturno E, Dileone M, Versace V, et al. Transcranial magnetic stimulation and BDNF plasma levels in amyotrophic lateral sclerosis. *Neuroreport*. 2004;15:717–20.
- Gedge L, Beaudoin A, Lazowski L, du Toit R, Jokic R, Milev R. Effects of electroconvulsive therapy and repetitive transcranial magnetic stimulation on serum brain-derived neurotrophic factor levels in patients with depression. *Front Psychiatry*. 2012;3:12.

28. Doi W, Sato D, Fukuzako H, Takigawa M. c-Fos expression in rat brain after repetitive transcranial magnetic stimulation. *Neuroreport*. 2001;12:1307–10.
29. Wang HY, Crupi D, Liu J, Stucky A, Cruciani G, Di Rocco A, et al. Repetitive transcranial magnetic stimulation enhances BDNF-TrkB signaling in both brain and lymphocyte. *J Neurosci*. 2011;31:11044–54.
30. Peterson AL, Nutt JG. Treatment of Parkinson's disease with trophic factors. *Neurotherapeutics*. 2008;5:270–80.
31. Chang JY, Shi LH, Luo F, Woodward DJ. High frequency stimulation of the subthalamic nucleus improves treadmill locomotion in unilateral 6-hydroxydopamine lesioned rats. *Brain Res*. 2003;983:174–84.
32. Lee JS, Lee JY, Cho WG, Yang YC, Cho BP. Relationship between microglial activation and dopaminergic neuronal loss in 6-OHDA-induced Parkinsonian animal model. *Korean J Phys Anthropol*. 2013;26:13–23.
33. Hylden JL, Wilcox GL. Intrathecal morphine in mice: a new technique. *Eur J Pharmacol*. 1980;67:313–6.
34. Habisch HJ, Janowski M, Binder D, Kuzma-Kozakiewicz M, Widmann A, Habich A, et al. Intrathecal application of neuroectodermally converted stem cells into a mouse model of ALS: limited intraparenchymal migration and survival narrows therapeutic effects. *J Neural Transm (Vienna)*. 2007;114:1395–406.
35. Morita E, Watanabe Y, Ishimoto M, Nakano T, Kitayama M, Yasui K, et al. A novel cell transplantation protocol and its application to an ALS mouse model. *Exp Neurol*. 2008;213:431–8.
36. Kim H, Kim HY, Choi MR, Hwang S, Nam KH, Kim HC, et al. Dose-dependent efficacy of ALS-human mesenchymal stem cells transplantation into cisterna magna in SOD1-G93A ALS mice. *Neurosci Lett*. 2010;468:190–4.
37. Saito F, Nakatani T, Iwase M, Maeda Y, Hirakawa A, Murao Y, et al. Spinal cord injury treatment with intrathecal autologous bone marrow stromal cell transplantation: the first clinical trial case report. *J Trauma*. 2008;64:53–9.
38. Pellis SM, Pellis VC, Chesire RM, Rowland N, Teitelbaum P. Abnormal gait sequence in locomotion after atropine treatment of catecholamine-deficient akinetic rats. *Proc Natl Acad Sci U S A*. 1987;84:8750–3.
39. Johnson RE, Schallert T, Becker JB. Akinesia and postural abnormality after unilateral dopamine depletion. *Behav Brain Res*. 1999;104:189–96.
40. Lee HY, Hsieh TH, Liang JI, Yeh ML, Chen JJ. Quantitative video-based gait pattern analysis for hemiparkinsonian rats. *Med Biol Eng Comput*. 2012;50:937–46.
41. Lorigados Pedre L, Pavón Fuentes N, Alvarez González L, McRae A, Serrano Sánchez T, Blanco Lescano L, et al. Nerve growth factor levels in Parkinson disease and experimental parkinsonian rats. *Brain Res*. 2002;952:122–7.
42. Herrán E, Requejo C, Ruiz-Ortega JA, Aristieta A, Igartua M, Bengoetxea H, et al. Increased antiparkinson efficacy of the combined administration of VEGF- and GDNF-loaded nanospheres in a partial lesion model of Parkinson's disease. *Int J Nanomedicine*. 2014;9:2677–87.
43. Baquet ZC, Bickford PC, Jones KR. Brain-derived neurotrophic factor is required for the establishment of the proper number of dopaminergic neurons in the substantia nigra pars compacta. *J Neurosci*. 2005;25:6251–9.
44. Mogi M, Togari A, Kondo T, Mizuno Y, Komure O, Kuno S, et al. Brain-derived growth factor and nerve growth factor concentrations are decreased in the substantia nigra in Parkinson's disease. *Neurosci Lett*. 1999;270:45–8.
45. Howells DW, Porritt MJ, Wong JY, Batchelor PE, Kalnins R, Hughes AJ, et al. Reduced BDNF mRNA expression in the Parkinson's disease substantia nigra. *Exp Neurol*. 2000;166:127–35.
46. Spina MB, Hyman C, Squinto S, Lindsay RM. Brain-derived neurotrophic factor protects dopaminergic cells from 6-hydroxydopamine toxicity. *Ann N Y Acad Sci*. 1992;648:348–50.
47. Shults CW, Kimber T, Altar CA. BDNF attenuates the effects of intrastriatal injection of 6-hydroxydopamine. *Neuroreport*. 1995;6:1109–12.
48. Hoffer BJ, Hoffman A, Bowenkamp K, Huettl P, Hudson J, Martin D, et al. Glial cell line-derived neurotrophic factor reverses toxin-induced injury to midbrain dopaminergic neurons in vivo. *Neurosci Lett*. 1994;182:107–11.
49. Tomac A, Widenfalk J, Lin LF, Kohno T, Ebendal T, Hoffer BJ, et al. Retrograde axonal transport of glial cell line-derived neurotrophic factor in the adult nigrostriatal system suggests a trophic role in the adult. *Proc Natl Acad Sci U S A*. 1995;92:8274–8.
50. Cohen AD, Tillerson JL, Smith AD, Schallert T, Zigmond MJ. Neuroprotective effects of prior limb use in 6-hydroxydopamine-treated rats: possible role of GDNF. *J Neurochem*. 2003;85:299–305.
51. Gill SS, Patel NK, Hotton GR, O'Sullivan K, McCarter R, Bunnage M, et al. Direct brain infusion of glial cell line-derived neurotrophic factor in Parkinson disease. *Nat Med*. 2003;9:589–95.
52. Slevin JT, Gerhardt GA, Smith CD, Gash DM, Kryscio R, Young B. Improvement of bilateral motor functions in patients with Parkinson disease through the unilateral intraputamenal infusion of glial cell line-derived neurotrophic factor. *J Neurosurg*. 2005;102:216–22.
53. Lang AE, Gill S, Patel NK, Lozano A, Nutt JG, Penn R, et al. Randomized controlled trial of intraputamenal glial cell line-derived neurotrophic factor infusion in Parkinson disease. *Ann Neurol*. 2006;59:459–66.
54. Nagatsu T, Sawada M. Inflammatory process in Parkinson's disease: role for cytokines. *Curr Pharm Des*. 2005;11:999–1016.
55. Chaturvedi RK, Shukla S, Seth K, Agrawal AK. Nerve growth factor increases survival of dopaminergic graft, rescue nigral dopaminergic neurons and restores functional deficits in rat model of Parkinson's disease. *Neurosci Lett*. 2006;398:44–9.
56. Heldin CH, Westermark B. Mechanism of action and in vivo role of platelet-derived growth factor. *Physiol Rev*. 1999;79:1283–316.
57. Nikkha G, Odin P, Smits A, Tingström A, Othberg A, Brundin P, et al. Platelet-derived growth factor promotes survival of rat and human mesencephalic dopaminergic neurons in culture. *Exp Brain Res*. 1993;92:516–23.
58. Othberg A, Odin P, Ballagi A, Ahgren A, Funa K, Lindvall O. Specific effects of platelet derived growth factor (PDGF) on fetal rat and human dopaminergic neurons in vitro. *Exp Brain Res*. 1995;105:111–22.
59. Pietz K, Odin P, Funa K, Lindvall O. Protective effect of platelet-derived growth factor against 6-hydroxydopamine-induced lesion of rat dopaminergic neurons in culture. *Neurosci Lett*. 1996;204:101–4.
60. Funa K, Yamada N, Brodin G, Pietz K, Ahgren A, Victorin K, et al. Enhanced synthesis of platelet-derived growth factor following injury induced by 6-hydroxydopamine in rat brain. *Neuroscience*. 1996;74:825–33.
61. Mahmood A, Lu D, Chopp M. Marrow stromal cell transplantation after traumatic brain injury promotes cellular proliferation within the brain. *Neurosurgery*. 2004;55:1185–93.
62. Arnold S, Klein H, Klinz FJ, Absenger Y, Schmidt A, Schinköthe T, et al. Human bone marrow stroma cells display certain neural characteristics and integrate in the subventricular

- compartment after injection into the liquor system. *Eur J Cell Biol.* 2006;85:551–65.
63. Kim YJ, Park HJ, Lee G, Bang OY, Ahn YH, Joe E, et al. Neuroprotective effects of human mesenchymal stem cells on dopaminergic neurons through anti-inflammatory action. *Glia.* 2009;57:13–23.
 64. Liberatore GT, Jackson-Lewis V, Vukosavic S, Mandir AS, Vila M, McAuliffe WG, et al. Inducible nitric oxide synthase stimulates dopaminergic neurodegeneration in the MPTP model of Parkinson disease. *Nat Med.* 1999;5:1403–9.
 65. Cicchetti F, Brownell AL, Williams K, Chen YI, Livni E, Isacson O. Neuroinflammation of the nigrostriatal pathway during progressive 6-OHDA dopamine degeneration in rats monitored by immunohistochemistry and PET imaging. *Eur J Neurosci.* 2002;15:991–8.
 66. Bai L, Lennon DP, Eaton V, Maier K, Caplan AI, Miller SD, et al. Human bone marrow-derived mesenchymal stem cells induce Th2-polarized immune response and promote endogenous repair in animal models of multiple sclerosis. *Glia.* 2009;57:1192–203.
 67. Uccelli A, Prockop DJ. Why should mesenchymal stem cells (MSCs) cure autoimmune diseases? *Curr Opin Immunol.* 2010;22:768–74.
 68. Zhang R, Liu Y, Yan K, Chen L, Chen XR, Li P, et al. Anti-inflammatory and immunomodulatory mechanisms of mesenchymal stem cell transplantation in experimental traumatic brain injury. *J Neuroinflammation.* 2013;10:106.
 69. Lo Furno D, Mannino G, Giuffrida R. Functional role of mesenchymal stem cells in the treatment of chronic neurodegenerative diseases. *J Cell Physiol.* 2018;233:3982–99.
 70. Chao YX, He BP, Tay SS. Mesenchymal stem cell transplantation attenuates blood brain barrier damage and neuroinflammation and protects dopaminergic neurons against MPTP toxicity in the substantia nigra in a model of Parkinson's disease. *J Neuroimmunol.* 2009;216:39–50.
 71. Lee JS, Song DY, Cho WG, Lee JY, Park YS, Yang YC, et al. Transplantation of human mesenchymal stem cells into the cisterna magna and its neuroprotective effects in a parkinsonian animal model. *Mol Cell Toxicol.* 2015;11:373–85.
 72. Schwerk A, Altschüler J, Roch M, Gossen M, Winter C, Berg J, et al. Adipose-derived human mesenchymal stem cells induce long-term neurogenic and anti-inflammatory effects and improve cognitive but not motor performance in a rat model of Parkinson's disease. *Regen Med.* 2015;10:431–46.
 73. Gugliandolo A, Bramanti P, Mazzon E. Mesenchymal stem cell therapy in Parkinson's disease animal models. *Curr Res Transl Med.* 2017;65:51–60.
 74. Khalilpourfarshbafi M, Hajiaghaalipour F, Selvarajan KK, Adam A. Mesenchymal stem cell-based therapies against podocyte damage in diabetic nephropathy. *Tissue Eng Regen Med.* 2017;14:201–10.
 75. Kim D, Cho GS, Han C, Park DH, Park HK, Woo DH, et al. Current understanding of stem cell and secretome therapies in liver diseases. *Tissue Eng Regen Med.* 2017;14:653–65.
 76. Okada K, Matsunaga K, Yuhi T, Kuroda E, Yamashita U, Tsuji S. The long-term high-frequency repetitive transcranial magnetic stimulation does not induce mRNA expression of inflammatory mediators in the rat central nervous system. *Brain Res.* 2002;957:37–41.
 77. Yang X, Song L, Liu Z. The effect of repetitive transcranial magnetic stimulation on a model rat of Parkinson's disease. *Neuroreport.* 2010;21:268–72.
 78. Dong Q, Wang Y, Gu P, Shao R, Zhao L, Liu X, et al. The neuroprotective mechanism of low-frequency rTMS on nigral dopaminergic neurons of Parkinson's disease model mice. *Parkinsons Dis.* 2015;2015:564095.
 79. Ba M, Ma G, Ren C, Sun X, Kong M. Repetitive transcranial magnetic stimulation for treatment of lactacystin-induced Parkinsonian rat model. *Oncotarget.* 2017;8:50921–9.
 80. Wang HJ, Tan G, Zhu LN, Chen D, Xu D, Chu SS, et al. The efficacy of repetitive transcranial magnetic stimulation for Parkinson disease patients with depression. *Int J Neurosci.* <https://doi.org/10.1080/00207454.2018.1495632>.
 81. Aftanas LI, Gevorgyan MM, Zhanaeva SY, Dzmidovich SS, Kulikova KI, Al'perina EL, et al. Therapeutic Effects of repetitive transcranial magnetic stimulation (rTMS) on neuroinflammation and neuroplasticity in patients with Parkinson's disease: a placebo-controlled study. *Bull Exp Biol Med.* 2018;165:195–9.

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.