Effects of intravenous human umbilical cord blood CD34+ stem cell therapy versus levodopa in experimentally induced Parkinsonism in mice

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Abstract

Introduction: Parkinsonism is a neurodegenerative disease with impaired motor function. The current research was directed to investigate the effect of CD34+ stem cells versus levodopa in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced Parkinsonism.

Material and methods: Mice were divided into 4 groups; saline-injected, MPTP: received four MPTP injections (20 mg/kg, *i.p.*) at 2 h intervals, MPTP groups treated with levodopa/carbidopa (100/10 mg/kg/twice/day for 28 days) or single intravenous injection of 10^6 CD34+ stem cells/mouse at day 7 and allowed to survive until the end of week 5.

Results: Levodopa and stem cells improved MPTP-induced motor deficits; they abolished the difference in stride length, decreased percentage of foot slip errors and increased ambulation, activity factor and mobility duration in parkinsonian mice ($p < 0.05$). Further, they significantly ($p < 0.05$) increased striatal dopamine (85.3 ±4.3 and 110.6 ±5.3) and ATP levels (10.6 ±1.1 and 15.5 ±1.14) compared to MPTP (60.1 ±3.9 pmol/g and 3.6 ±0.09 mmol/g, respectively) ($p < 0.05$). Moreover, mitochondrial DNA from mice treated with levodopa or stem cells was in intact form; average concentration was (52.8 ±3.01 and 107.8 ±8.6) and no appreciable fragmentation of nuclear DNA was found compared to MPTP group. Regarding tyrosine hydroxylase (TH) immunostaining, stem cell group showed a marked increase of percentage of TH-immunopositive neurons (63.55 ±5.2) compared to both MPTP (37.6 ±3.1) and levodopa groups (41.6 ±3.5).

Conclusions: CD34+ cells ameliorated motor, biochemical and histological deficits in MPTP-parkinsonian mice, these effects were superior to those produced by levodopa that would be promising for the treatment of PD.

Key words: CD34+ stem cells, levodopa, mice, 1-methyl-4-phenyl-1,2,3,6-tetra-hydropyridine, Parkinsonism.

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disease with impaired motor function including bradykinesia, rigidity, postural instability and resting tremors [1]. The development of clinically identifiable PD symptoms results from loss of more than 50% of the dopaminergic neurons in the SNpc and a marked reduction in the release of dopamine by those neurons that project into the neostriatum [2].

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The causes of the majority of PD cases remain undefined, but multiple factors such as age-related neurodegeneration, genetic constitution and toxin exposure may play a role [3]. In the absence of a genetic cause, environmental influences appear to play an important role in PD [4]. Whether caused by toxin exposure or genetic susceptibility, evidence suggests that subsequent oxidative stress, inflammation, and apoptosis play a major role in promoting neurodegeneration [5]. In addition, mitochondrial dysfunction has been implicated as decreased activity of complex I of the electron transport chain (ETC) and the more recent description of mtDNA deletions was reported in PD patients [6].

The MPTP mouse model replicates many neuropathologic and neurochemical features of PD. 1-Methyl-4-phenylpyridinium ion (MPP+), the metabolite of MPTP, inhibits complex I of the mitochondrial ETC [6]. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine causes selective loss of dopaminergic neurons in the SNpc leading to dopamine depletion in the caudate putamen and selective motor impairment in rodents [7].

Levodopa has had an established position in the treatment of Parkinson's disease for the last 40 years. The treatment is relatively easy in the first uncomplicated stage of PD, but at the late stages the levodopa treatment is a challenge due to the changes in reactivity to single doses as a result of changes in its pharmacokinetics, reactivity of dopamine receptors and progressive loss of dopaminergic neurons [8]. Surgical treatment is not better than drugs [9]. Cell transplantation has emerged as a therapeutic alternative. In order to make cell therapy in PD successful, the cells must produce dopamine in appropriate amounts, survive and make connections with the host tissue and they should be effective in reducing PD symptoms [10].

Human umbilical cord blood (HUCB) is a major source for hematopoietic stem cells used in regenerative medicine to repair loss of organ function. Possible targets for regenerative medicine include CNS applications [11]. Human umbilical cord blood cells present multiple benefits including their simple and economical retrieval and their immaturity, which may play a significant role when transplanted into a mismatched host [12]. Further, they are considered to be an accessible and less immunogenic source for mesenchymal, unrestricted somatic and for other stem cells with pluri/multipotent properties [13]. Using rodent models, intravenous HUCB stem cells have been reported to migrate and accumulate in injured areas of spinal cord, amyotrophic lateral sclerosis, heat and cerebral strokes, and to differentiate into neural cells, and thus improve functional recovery of these animals [14–17].

To the best of our knowledge studies of potential use of stem cells in the setting of PD mostly use embryonic stem cells [18]. Furthermore, relatively few studies have used HUCB stem cells and those studies examined the effect of mesenchymal stem cells but not of hematopoietic stem cells. Therefore, the present study aimed to evaluate the ameliorating effect of intravenous HUCB CD34+ stem cell therapy on experimentally induced Parkinsonism in mice.

Material and methods

Animals

Forty-eight female Swiss mice weighing 20–25 g were used. Mice were purchased from the National Centre of Research (Cairo, Egypt), housed in clean cages under hygienic conditions and allowed to acclimatize for 7 days before starting the experiment. Mice were kept on standard chow and water ad libitum with reversed dark-light cycle. The behavioral tests were done daily at 4 p.m. to minimize the circadian influence on animal behavior. All experimental protocols were approved by the Institutional Animal Care and Use Committee at Suez Canal University. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Drugs and chemicals

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (Sigma-Aldrich, MO, USA) was dissolved in sterile saline. Levodopa/carbidopa was kindly provided by Global Napi Pharmaceuticals (Cairo, Egypt) and dissolved in distilled water. mtDNA isolation kit (Bio Vision, USA) and Wizard® nDNA purification kit (Promega Corporation, Madison, WI, USA) were used according to the manufacturer's instructions. CD34 separation kit (Dynabeads M-450 CD34, DETACHaBEAD CD34, Dynal Biotech ASA, Oslo, Norway). The biotinylated rabbit anti-mouse tyrosine hydroxylase antibodies were purchased from R&D Systems® (Minneapolis, USA), whereas horseradish peroxidase (HRP) was purchased from DakoCytomation (Carpinteria, USA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Induction of experimental Parkinsonism

Experimental Parkinsonism was induced in groups II, III and IV by injections of four doses of MPTP hydrochloride (20 mg/kg/2 h, *i.p.*) in a volume of 2 ml/kg [19].

Experimental protocol

Mice were randomly allocated into four groups, twelve mice each: group I (saline group): mice received 4 *i.p.* injections of sterile saline (2 ml/kg) parallel to MPTP. Group II (MPTP group): 1 week after MPTP injection, mice received distilled water

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(1 ml/kg/twice a day, *p.o.*) parallel to levodopa treatment. Group III (Levodopa group): 1 week after MPTP injection, mice received levodopa/carbidopa (100/10 mg/kg/twice a day, *p.o.*) in a volume of 1 ml/kg [20]. Distilled water or levodopa was administered by oral gavage starting at day 7 – after the baseline evaluation of motor function – and continued until the end of week 5. Group IV (Stem cell group): 1 week after MPTP injection, mice received a single intravenous injection of 10^6 CD34+ stem cells/mouse at day 7 and allowed to survive until the end of week 5 [21].

Technique of collection, separation and transplantation of CD34+ stem cells

CD34+ cell collection

The HUCB was used as the source of CD34+ cells. After obtaining informed consent, HUCB was collected ex-utero from healthy full-term pregnant women with a male fetus during normal vaginal delivery. The HUCB was collected from four volunteers in four 50 ml graded sterile plastic Falcon tubes (Greiner Bio-One, Germany) containing 7 ml of citrate phosphate dextrose adenine-1 (CPDA-1) anticoagulant. Immediately after the cord cutting, and before the placental separation, the maternal end of the umbilical cord was sterilized with ethyl alcohol (70%) and the blood was allowed to flow into the tube. Blood samples were kept at a temperature of 4°C during the transport and storage procedures until being processed within 24 h after collection [22].

CD34+ cell separation and purification

Isolation of low density mononuclear cells (MNC)
UCB was collected in presence of anticoagulant and was diluted 1 : 1 in isolation buffer (100 ml phosphate buffered saline (PBS) + 10 ml richen placental medium (RPMI) + 1 ml 10% fetal calf serum (FCS) (Lonza Bioproducts, Belgium). Seven millilitres of diluted UCB sample was layered drop by drop using sterile Pasteur pipettes into a sterile centrifuge tube (50 ml, Greiner Bio-One, Germany) containing 15 ml of lymphocyte separation medium, Ficoll-Hypaque solution (Sigma Aldrich, Cat# H8889), and submitted to density gradient centrifugation at 2500 rpm for 20 min at room temperature in order to isolate the buffy coat containing the low-density MNC. The MNC were transferred carefully to a new sterile tube using sterile Pasteur pipettes and washed twice with PBS through centrifugation at 2000 rpm for 10 min. Then the pellet was resuspended in cold isolation buffer. The total volume was 1 ml and was maintained at 2–8°C [23].

Dynabeads washing procedure
The Dynabeads M-450 CD34, supplied as a suspension of 4×10^8 beads/ml in phosphate buffered saline (PBS), pH 7.4, containing 0.1% human serum albumin (HSA) and 0.02% (NaN$_3$), were resuspended thoroughly in the vial. The desired amount of Dynabeads was transferred into a washing tube that was placed in a Dynal MPC for 1 min and the fluid was pipetted off. The tube was removed from the Dynal MPC and 2 ml of washing buffer (PBS/0.1% BSA) was added and resuspended, the previous step was repeated, and the washed Dynabeads were resuspended in an equal volume of washing buffer that was originally pipetted from the vial.

Positive selection and characterization of CD34+ cells
The optimal concentration of Dynabeads M-450 CD34 was 4×10^7 beads/ml. The cells were resuspended thoroughly with a pipette with a narrow tip to prevent cells from aggregating before adding them to the beads; the cells were added to the beads then the mixture was vortexed for 2–3 s and the cell-bead suspension was incubated for 30 min at 2–8°C with gentle tilt rotation. The cell-bead complexes were resuspended then vortexed for 2–3 s, the tube was placed in a DYNAL MPC for 2 min to separate Dynabeads M-450 CD34-rosetted cells from non-target cells, the isolation buffer was added to the height of the magnet, and the supernatant containing non-rosetted cells was aspirated while the tube was still exposed to the magnet. This step was repeated. After the final wash, the rosettes were resuspended in 100 µl of isolation buffer per 4×10^7 beads used.

Detachment of beads from purified cells
One hundred microlitres DETACHaBEAD CD34 was added per 4×10^7 Dynabeads M-450 CD34 in a volume of 100 µl and the mixture was vortexed for 2–3 s then incubated for 15 min at 37°C with gentle tilt rotation. After incubation, 2 ml of isolation buffer was added and vortexed for 2–3 s to enhance detachment of Dynabeads M-450 CD34 from the cells; the tube was placed on the DYNAL MPC and the Dynabeads M-450 CD34 were allowed to accumulate on the tube wall for 2 min. The released cells were transferred to a new tube and the previous step was repeated three times then pooling released cells into a single tube. The tube with the pooled isolated cells was placed on the DYNAL MPC for 2 min to remove any residual beads and the supernatant containing released cells was transferred to a fresh conical tube. Finally 50 µl of NH$_3$Cl was added to the supernatant to hemolyse any red blood cells [24].

Assessment of the quantity and quality of the separated CD34+ cells

The quantity of the isolated CD34+ cells was assessed by putting the sample on automated cell counters (Hitachi auto analyzer, Japan) [25]. The quality of the isolated CD34+ cells was determined

by using the trypan blue dye exclusion test: the viable cells were not stained [26].

Transplantation of CD34+ cells

A single dose of 10^6 CD34+ stem cells/mouse were injected intravenously in the tail vein after inducing venodilatation by rubbing the tail with alcohol [21].

Functional assessment

At the end of week 1, mice were screened for the motor function in the following tests and revaluated again at the end of the therapeutic period (at the end of week 5).

Stride length quantitative gait analysis test

Mice were habituated to the apparatus for 3 days prior to starting the experiments. The apparatus was composed of an open field (60 cm × 60 cm × 40 cm) illuminated by a light (80 lx), in which a runway (4.5 cm wide, 42 cm long, borders 12 cm high) was arranged to lead out into a dark wooden box (20 cm × 17 cm × 10 cm). Stride lengths were measured by wetting animals' fore- and then hind paws with pencil black ink and letting them trot on a strip of paper (4.5 cm wide, 40 cm long) down the brightly lit runway towards the dark goal box. The forelimb stride lengths were first measured for all animals, then the hind-limbs on a new strip of paper, once the forelimb inked paws had dried. Stride lengths were measured manually as the distance between two paw-prints. The mean of the three longest stride lengths (corresponding to maximal velocity) were measured from each run. Paw-prints made at the beginning (7 cm) and the end (7 cm) of the run were excluded because of velocity changes. Runs in which the mice made stops or obvious decelerations observed by the experimenter were excluded from the analysis [27].

Grid walking test

It assesses the ability to accurately place the forepaws during spontaneous exploration of an elevated grid by analyzing the frequency the mice failed to accurately grasp the rungs. Mice were placed on a wire circular grid (330 mm in diameter with 15 mm × 15 mm grid squares) and allowed to freely explore for 3 min. The mice were videotaped and at a later date an experimenter blinded to the treatment group scored the percentage of foot slips out of the first 50 steps taken with the left and right fore and hind paws. A foot slip was scored either when the paw completely missed a rung and thus the limb fell between the rungs and the animal lost balance, or when the paw was correctly placed on the rung but slipped off during weight bearing. The animals required no pretraining but were placed on the grid twice prior to MPTP injection for habituation and to obtain baseline scores [28].

Open field test

The open-field arena (113 cm × 113 cm × 44 cm) was made of dark glass. The floor was painted with white lines that formed a 5 cm × 5 cm pattern. Mice were introduced individually in the open field arena and behavioral parameters were observed for 5-min sessions; the test was performed under high-light conditions (80 lx). Mice were observed for ambulation (the number of squares crossed), number of stops and mobility duration. An experienced observer, who was blinded to the treatment groups, quantified the open-field locomotive behavior. In addition, an activity factor (number of squares divided by the total number of stops) was calculated [29].

Processing of the brain

After assessment of the motor performance, mice were anesthetized by injection of thiopental sodium (50 mg/kg) [30] and killed by decapitation. Brains were quickly dissected and washed with ice-cold saline and prepared as 300 μm slices using Vibratome 1000 Plus (United States). The compact part of the SN was dissected from these sections [31]. The SN from the right hemisphere of each mouse was immediately frozen at –80°C and used to detect the male-specific SRY gene (in the stem cell group), dopamine, ATP and extraction of mtDNA and nDNA (in all groups) as described later. The SN from the left hemisphere was prepared for histopathological staining with hematoxylin + eosin (H + E) and cresyl violet for Nissl staining as well as for immunohistochemical staining for TH.

Real-time polymerase chain reaction analysis of the male-specific SRY gene

At the end of 5 weeks, the SRY gene (on the Y chromosome) was used to identify male donor-derived cells in female recipients. Genomic DNA was extracted from the SNpc of female recipients by Jena Bioscience (Germany) according to the manufacturer's guidelines. Real-time polymerase chain reaction (RT-PCR) was done using (Biocompare, Light Cycler, Germany). The primer sequences used were 5'-CAGCTAACACTGATCTTTTC-3' and 5'-TTAC-TGAGCCAGAATCATAG-3' and concentrations were optimized according to the manufacturer's guidelines (Light Cycler-DNA Amplification Kit SYBR Green I). DNA from normal human females and males was used as negative and positive controls, respectively [32].

Determination of dopamine level

Sample preparation

Frozen tissue was homogenized for 20 s using a tapered motorized pestle in 500 µl of ice-cold mobile phase (50 mM sodium acetate buffer, pH 4.3, containing 35 mM citric acid, 0.13 mM disodium EDTA, 0.45 mM 1-octanesulfonate and 10% methanol) containing a known amount of 3,4-dihydroxybenzylamine as an internal standard. The homogenized tissue suspension was centrifuged at 10 000 × g for 1 min, and the supernatant was removed and filtered through a 0.2 µm Teflon syringe filter for HPLC analysis.

HPLC analysis

Levels of dopamine were measured in the reverse-phase HPLC coupled to ECD. First, the mobile phase was oxidized by a guard cell (V = +/450 mV). The guard cell serves to perform a partial clean up of the mobile phase prior to sample injection. The samples were introduced into the autosampler carrousel (CMA 200, CMA Microdialysis, Solna, Sweden) with the flow rate of mobile phase being 0.8 ml/min (ESA 2200 Analytic Pump, Bischoff, Leonberg, Germany). Separation of the neurotransmitter was carried out by means of a reverse-phase column (Prontosil 33 × 4.0 mm, pore diameter: 3.0 mm; Bischoff). The substrates were detected by an ESA detector (ESA Coulochem 5100A, Analytical cell ESA 5010). Typical values of applied potential used in the present experiment were ±20 mV at electrode 1 and ±320 mV at electrode 2.

Determination of ATP

ATP was extracted from the tissues with 10 ml of 0.6 M perchloric acid in the ice bath for 1 min [33]. The extraction mixture was centrifuged for 10 min at 6000 × g and 4°C, and 6 ml of the supernatant was taken and quickly neutralized to pH = 6.5 with 1 M KOH solution. The neutralized supernatant was then allowed to stand for 30 min in an ice bath to precipitate most of the potassium perchlorate, which was removed by paper filtration. The filtrate solution was filtered again through a 0.45 µm filter. The final filtrate solution was made up to 8 ml and then stored at –30°C prior to the analysis. The concentration of ATP was determined as described previously [34].

Assessment of mtDNA integrity

Isolation of mitochondria

Mitochondria were extracted by differential centrifugations [35]. One part (100 mg) of tissue was homogenized in 0.25 M sucrose in 0.7 M Tris-HCl buffer (pH = 7.4) at 1 g tissue : 9 ml of Tris-sucrose. EDTA was added to aid disruption of cells. Tissue homogenate was spun at 2500 × g for 10 min to remove nuclei and unbroken cells. Supernatant fluid was decanted into centrifuge tubes and spun at 10000 × g for 10 min to form a primary mitochondrial pellet. Supernatant fluid was decanted and the pellet was gently resuspended in 10 ml Tris-sucrose for washing. The pellet was recentrifuged and supernatant fluid was decanted. This washing cycle was repeated several times to improve the degree of mitochondrial purity. The final mitochondrial pellet was resuspended (1 ml Tris-sucrose/1 g of original sample).

Isolation of mtDNA

The mtDNA samples were subjected to 1% agarose gel electrophoresis at 4 V/cm using TAE solution (40 mM Tris-acetate, pH 8.0, 1 mM EDTA) as a running buffer. The gel was stained with 0.5 µg/ml ethidium bromide and photographed by the G:Box Gel Documentation system (Syngene, USA) [36]. Furthermore, mtDNA quantity and purity were determined using NanoDrop™ 1000 spectrophotometer V3.7 (Thermo Fisher Scientific Inc., Wilmington, DE, USA).

DNA laddering assay

Endogenous endonucleases, a group of enzymes that cleave double-stranded DNA in the linker region between nucleosomes, generating mono- and oligonucleosomes of 180 bp or multiples, are one of the characteristics of apoptosis. To assess endonuclease-dependent ladder-like DNA fragmentation by gel electrophoresis, genomic DNA was extracted from the tissue by Jena Bioscience (Germany) according to the manufacturer's guidelines then loaded onto agarose gel (15 µg/lane). DNA laddering was determined by constant voltage mode electrophoresis (in a large submarine at 4 V/cm, for 4 h) on 1.5% agarose gel containing 0.5 µg/ml ethidium bromide. A 1 kbp ladder served as a DNA base pair marker [37]. Gels were visualized by the G:Box Gel Documentation system (Syngene, USA).

Histopathology and immunohistochemistry

The SN of the left hemispheres from each brain was fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH = 7.4) by immersion for 48 h at 4°C. Tissue samples were processed to prepare 5-µm thick paraffin sections. Sections were stained with H + E and cresyl violet stain. Immunostaining was performed using antibodies against neuronal TH. Briefly, sections were incubated in 0.3% H_2O_2/0.3% goat serum/PBS for 30 min to block endogenous peroxidase. After washing in PBS, sections were incubated in 10% goat serum/0.3% Triton X 100/PBS for 1 h and then incubated in the primary antibodies (rabbit anti-tyrosine hydroxylase, 1 : 1000) at 4°C overnight. Sections were washed in PBS and then
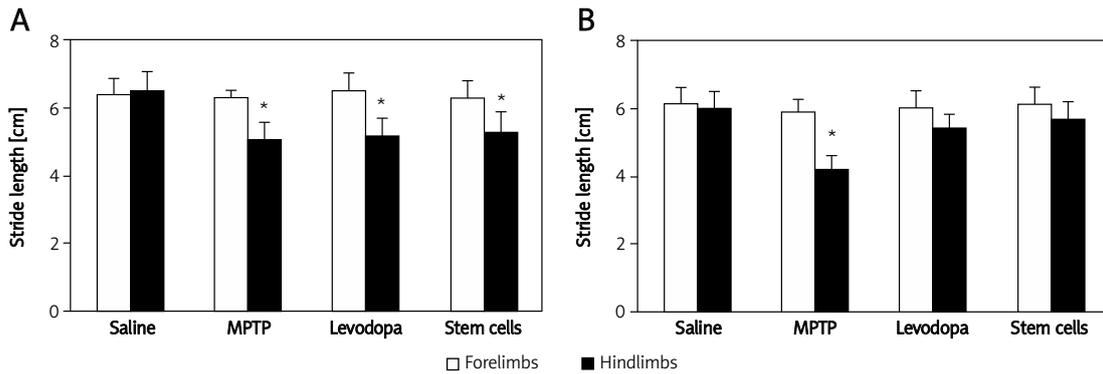
Figure 1. Forelimbs and hind limbs stride length (cm) in the experimental groups. **A** – Stride length measured at the end of week 1. **B** – Stride length measured at the end of week 5. MPTP induced a difference between the forelimbs and hind limbs mean stride length at the end of week 1 and 5. Levodopa or stem cell therapy ameliorated the difference between the forelimbs and hind limbs stride length compared to the MPTP group at week 5. Results are mean ± SEM and were analyzed using one-way ANOVA and Bonferroni post-hoc test. Six multiple comparisons were made among groups

*$p < 0.05$ compared to forelimbs stride length in the MPTP group, MPTP – 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

incubated in the secondary antibody (biotinylated goat antimouse IgG, 1 : 500) for 1 h. The avidin-biotin complex method was used to detect the secondary antibody (ABC elite kit, Vector laboratories, Burlingame, CA) and the reaction product was visualized by 3,3'-diaminobenzidine tetrachloride. Finally sections were counterstained in Mayer's hematoxylin, dehydrated, cleared and mounted. Control sections for TH were incubated with mouse IgG (0.2 µg/ml, Sigma). Using the light microscope, histological sections of SNpc were examined for quantitative assessment of neuronal population and percentage in TH immunostained sections. The number of TH positive neurons with distinct nuclei was estimated by manual counting and then divided by the total number of neurons in the examined fields to obtain the percentage of positive cells. Five high power fields were assessed per section and 3 sections were examined per mouse.

Statistical analysis

Data were expressed as mean ± SEM and analyzed using the Statistical Package of Social Sciences (SPSS program, version 17, SPSS Inc., Chicago, IL, USA). The difference of mean values among groups was assessed by using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test. All p values reported are two-tailed and $p < 0.05$ was considered significant. All possible comparisons were made among groups, amounting to six multiple comparisons for each variable.

Results

Functional assessment

Stride length quantitative gait analysis test

The results of the current study showed that there was a difference between the stride length

of forelimbs and hind limbs of mice at week 1 in group II, III and IV (Figure 1 A). However, at week 5, a difference in mean stride length was observed only in group II (MPTP group) ($p < 0.05$, Figure 1 B). Treatment with levodopa/carbidopa or stem cells abolished the difference in the stride length compared to the MPTP group (Figure 1 B).

Grid walking test

At the end of week 1, group II, III and IV showed more foot slip errors in the grid walking test as compared with the saline group. At the end of week 5, mice treated with levodopa/carbidopa or stem cells decreased the percentage of foot slip errors compared to the MPTP group at the end of week 5. Importantly, mice treated with stem cells showed
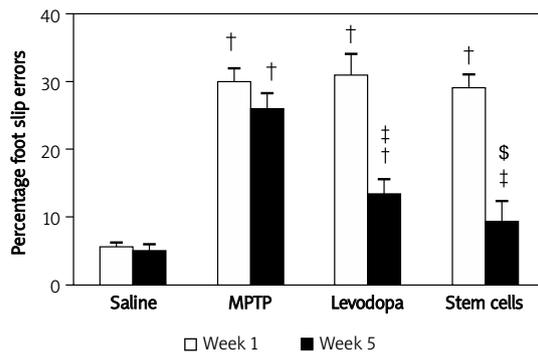

Figure 2. The percentage of foot slips in the experimental groups in the grid walking test. The MPTP induced higher foot slip errors compared to the saline group at week 1 and 5. Treatment with levodopa or stem cells decreased the percentage of foot slip errors at the end of week 5 compared to the MPTP group. Results are mean ± SEM and were analyzed using one-way ANOVA and Bonferroni post-hoc test. Six multiple comparisons were made among groups

†$p < 0.05$ compared to saline group, ‡$p < 0.05$ compared to MPTP group, $p < 0.05$ compared to levodopa group, MPTP – 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

fewer errors as compared to the levodopa group ($p < 0.05$, Figure 2).

Open field test

Mice treated with MPTP showed poor mobility in the open field test. At the end of week 1, the ambulation (Figure 3 A), activity index (Figure 3 C) and mobility duration (Figure 3 D) were decreased, whereas the number of stops did not differ significantly compared to the saline group (Figure 3 B). At the end of week 5, the ambulation, the activity factor and the mobility duration were greater, whereas the number of stops was lower in levodopa and stem cell groups compared to the MPTP group. Stem cell therapy was superior compared to levodopa/carbidopa in improving all the above-mentioned parameters, with the exception of the number of stops ($p < 0.05$, Figures 3 A–D).

Real-time PCR analysis for identification of human Sry gene in female mice

Genomic DNA was isolated from the SNpc of female mice recipients, of the stem cell group, at the end of the study and HUCB CD34+ stem cell transplantation. DNA from normal human females and males was used as negative and positive controls, respectively.

Within the stem cell injected group (12 mice), the PCR product could be amplified from the regenerating SNpc of nine female mice, so 75% of SNpc samples were positive for presence of the Sry gene while 25% of SNpc samples were negative for presence of the Sry gene.

The PCR product was also detected in DNA from normal human males but was absent in DNA from normal human untransplanted females, demonstrating the specificity of the assay (Figure 4).

Dopamine and ATP levels

MPTP-treated mice showed a decrease in dopamine and ATP levels compared to the saline group. Treatment with levodopa/carbidopa or stem cells increased these parameters compared to the MPTP group. Dopamine and ATP levels are less reduced by MPTP after CD34+ stem cell injection ($p < 0.05$, Figures 5 A, B).

Mitochondrial DNA integrity and fragmentation of nuclear DNA

In saline-treated mice, the intact form of mtDNA was electrophoresed as a major band of approximately 16.5 kb (lanes 1, 2). On the other hand, administration of MPTP markedly decreased the amounts of intact mtDNA in the brain tissue (lanes
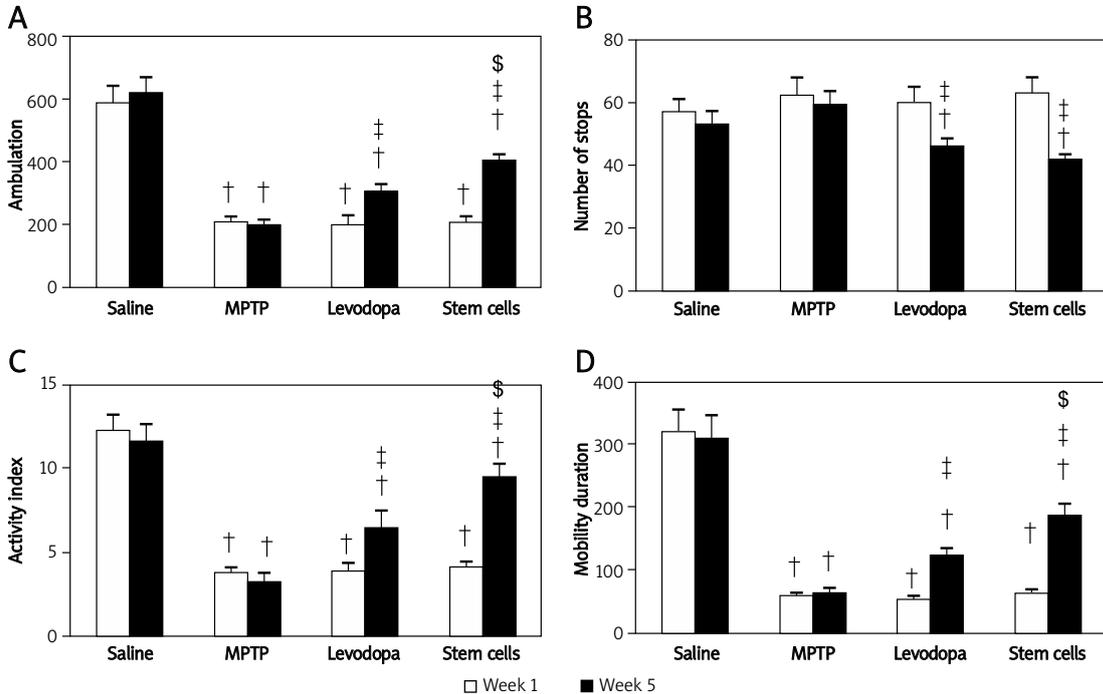

Figure 3. The motor performance of mice in the open field test. Mice were observed for the ambulation (**A**), number of stops (**B**), activity factor (**C**) and mobility duration (**D**) in a 5-minute session. The MPTP group showed a decrease in ambulation, activity factor and mobility duration compared to the saline group at week 1 and 5. Treatment with levodopa or stem cells improved these parameters as compared to the MPTP group at the end of week 5. Results are mean ± SEM and were analyzed using one-way ANOVA and Bonferroni post-hoc test. Six multiple comparisons were made among groups for each variable

†$p < 0.05$ compared to saline group, ‡$p < 0.05$ compared to MPTP group, $$p < 0.05$ compared to levodopa group, MPTP – 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

5, 6). However, mtDNA from mice treated with levodopa/carbidopa (lanes 7, 8) and stem cells (lanes 3, 4) was electrophoresed in its intact form (single band) (Figure 6 A). The MPTP administration resulted in a marked decrease in the amount of intact nDNA in brain tissues (Figure 6 B). Moreover, a dramatic oligonucleosome-length degradation of DNA was observed, characterized by mixed smearing and laddering (lane 5). No appreciable fragmentation of nDNA was found to occur in animals treated with levodopa/carbidopa (lanes 6, 7, 8) and stem cells (lanes 2, 3, 4). However, only 5 mice treated with levodopa/carbidopa showed mild smearing (data not shown), while the other 7 mice of this group showed total ladder and smear negativity. Such mild smearing may be attributed to the residual damage in the heavily injured cells associated with the ongoing recovery, repair and renovation processes. In the MPTP-treated group, a decline was observed in the brain tissue content of mtDNA, as compared to that of the saline group ($p < 0.05$, Figure 6 C). However, improvement of the mtDNA content was observed upon treatment with stem cells. The integrity of mtDNA from the levodopa/carbidopa group (lanes 7, 8) was apparently damaged.

Histopathological examination and immunostaining

H + E stained sections in the SNpc of the saline group showed a cell-rich region with abundant large and medium sized neurons with vesicular nuclei. Sections from MPTP and levodopa groups showed similar neuronal degeneration. In the CD34+ stem cell treated animals, the reduction of the dopamine cells produced by the MPTP is counteracted (Figure 7 A).

With cresyl violet stain, abundant purple Nissl granules were detected in the perikarya and dendrites of nigral neurons in the saline group. The MPTP group revealed decreased purple Nissl granules. Treatment with levodopa/carbidopa resulted in increased purple Nissl granules; however, stem cell therapy revealed
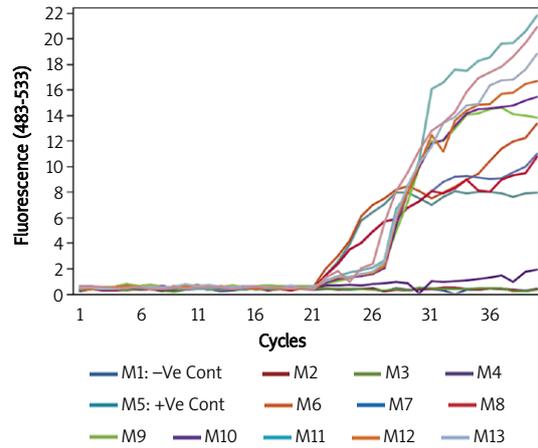

Figure 4. Real-time PCR analysis for identification of human Sry gene in the female mice brain of the stem cell group. The PCR product could be amplified from the regenerating SNpc of nine female mice, so 75% of SNpc samples were positive for presence of the Sry gene while 25% of SNpc samples were negative for presence of the Sry gene

purple Nissl granules of nigral neurons more or less similar to those of the saline group (Figure 7 B).

Regarding TH immunostaining, the saline group revealed abundant TH-immunopositive neurons in the SNpc. The MPTP group showed a marked loss in TH-immunopositive neurons (Figure 8 A) with a decrease in the mean percentage of TH-positive neurons compared to the saline group (Figure 8 B). TH-immunopositive neurons in the levodopa group showed a microscopic picture similar to the MPTP group with a mean percentage of 38.5 ±3.14% (Figures 8 A, B), while in the stem cell group there was a marked increase of TH-immunopositive neurons with a mean percentage of 56.6 ±4.18% that was greater compared to the levodopa group ($p < 0.05$, Figure 8 B).

Discussion

The current study aimed to explore the curative effect of intravenous HUCB CD34+ stem cell
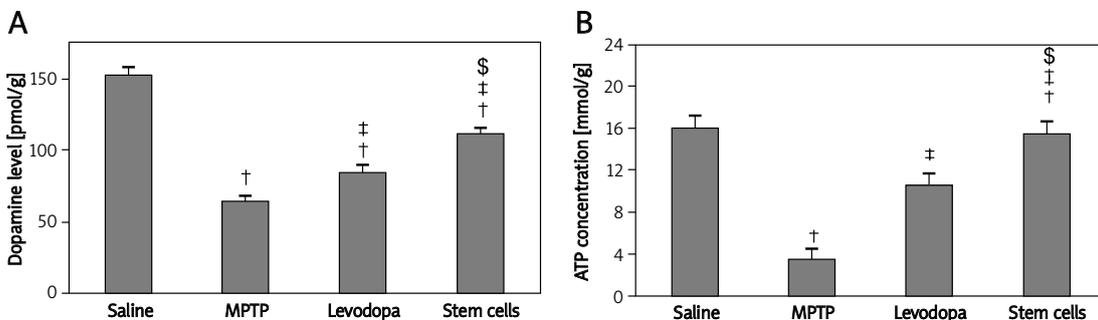

Figure 5. Dopamine (**A**) and ATP (**B**) levels in the experimental groups at the end of week 5 dopamine and ATP levels were markedly decreased in the MPTP group compared to the saline group. Treatment with levodopa or stem cells increased the dopamine and ATP levels compared to the MPTP group. Results are expressed as mean ± SEM and were analyzed using one-way ANOVA and Bonferroni post-hoc test. Six multiple comparisons were made among groups for each variable

†$p < 0.05$ compared to saline group, ‡$p < 0.05$ compared to MPTP group, $$p < 0.05$ compared to levodopa group, MPTP – 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

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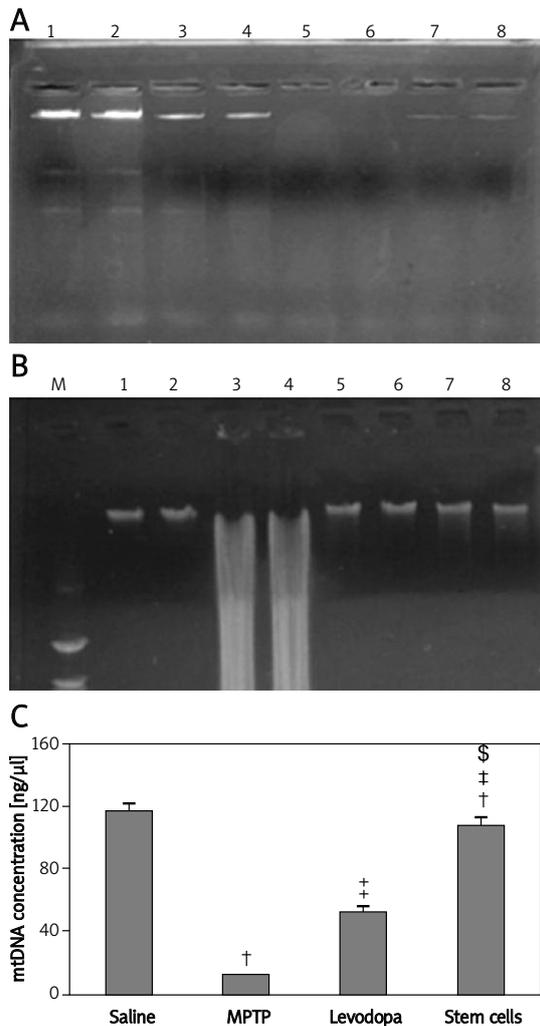

Figure 6. Mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) in the experimental groups. **A** – mtDNA of the experimental groups, lanes 1, 2: intact mtDNA isolated from saline group; lanes 3, 4: mtDNA samples isolated from stem cell group; lanes 5, 6: mtDNA samples isolated from MPTP group; lanes 7, 8: mtDNA samples isolated from levodopa group. **B** – The integrity of nDNA of the experimental groups. Lane M, 1 kb DNA ladder; lane 1, 2 intact nDNA isolated from of saline group; lane 3 and lane 4, nDNA; isolated from MPTP group, lane 5, lane 6, nDNA isolated from sample from stem cell group; lane 7, lane 8, nDNA isolated from levodopa group. **C** – mtDNA concentration in the brain tissues of experimental groups. MPTP induced significant mtDNA damage compared to the saline group. The MPTP-induced mtDNA damage was significantly decreased upon administration of stem cells. Results are expressed as mean ± SEM and were analyzed using one-way ANOVA and Bonferroni post-hoc test. Six multiple comparisons were made among groups
†$p < 0.05$ compared to saline group, ‡$p < 0.05$ compared to MPTP group, $$p < 0.05$ compared to levodopa group, MPTP – 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

therapy to treat parkinsonian mice experimentally. In agreement with previous studies, MPTP-treated mice exhibited impaired motor activity in the gait analysis, grid walking and open-field tests

along with a decrease in striatal dopamine level [38, 39].

Studies demonstrated that soon after the systemic administration of MPTP, its active metabolite, MPP+, concentrates in the mitochondrial matrix, where it binds to complex I of the ETC. This binding interrupts the movement of electrons along the ETC, leading to increased production of reactive oxygen species, mitochondrial injury and decrease in respiratory function associated with a drop in ATP production [40]. This phenomenon is found only in susceptible areas of the brain such as the ventral midbrain and striatum [41]. Similarly, the current study revealed that administration of MPTP disrupted the integrity and amount of intact mtDNA and striatal ATP level. Moreover, a decrease in genomic nDNA and a dramatic oligonucleosome-length degradation of nDNA were observed. In accordance, Hoang *et al.* [42] found that not only mtDNA but also nDNA is a target for the MPTP insult and DNA damage that may contribute to the neurodegenerative process in PD.

Tyrosine hydroxylase immunoreactivity in SNpc dopaminergic neurons was affected by administration of MPTP. Consistently, MPTP through mitochondrial complex 1 inhibition induced a loss in TH immunoreactivity and a consequent decline in dopamine [43, 44]. In neurotoxic models of Parkinsonism, parkinsonism-like symptoms are detectable only when markers of dopaminergic neurons in the SN fall below 20–40% of normal values [45].

Levodopa produces behavioral enhancement/reversal of Parkinsonian symptoms in animal models of PD, as well as in PD patients [46, 47]. In contrast, Hwang *et al.* [48] reported a negative effect of levodopa in an open-field test and explained that as the potential nigrostriatal pathway-dependent motor deficits could be detected by measuring special tasks as stride length and grid walking tests that are considered among the sensitive measures of behavioral changes in an MPTP mouse model of PD [49]. In the current study, treatment with levodopa increased striatal dopamine, ATP and the amount of intact mtDNA, while it abolished the ladder pattern of nDNA cleavage in brain tissues of MPTP-treated mice. Therefore, it apparently provided treatment against MPTP-induced apoptosis. The beneficial effects of levodopa can be explained according to the fact that within SN, MPTP leads to the formation of free radicals that react with membrane lipids and cause cell death [50], and levodopa treatment correlates negatively with peripheral endogenous and/or exogenous lipid peroxidation in a dose-dependent way [50]. These results may indicate that levodopa can act as an antioxidant in plasma.

Levodopa showed a microscopic picture that is similar to the MPTP group in spite of improving the motor function and the biochemical parameters.
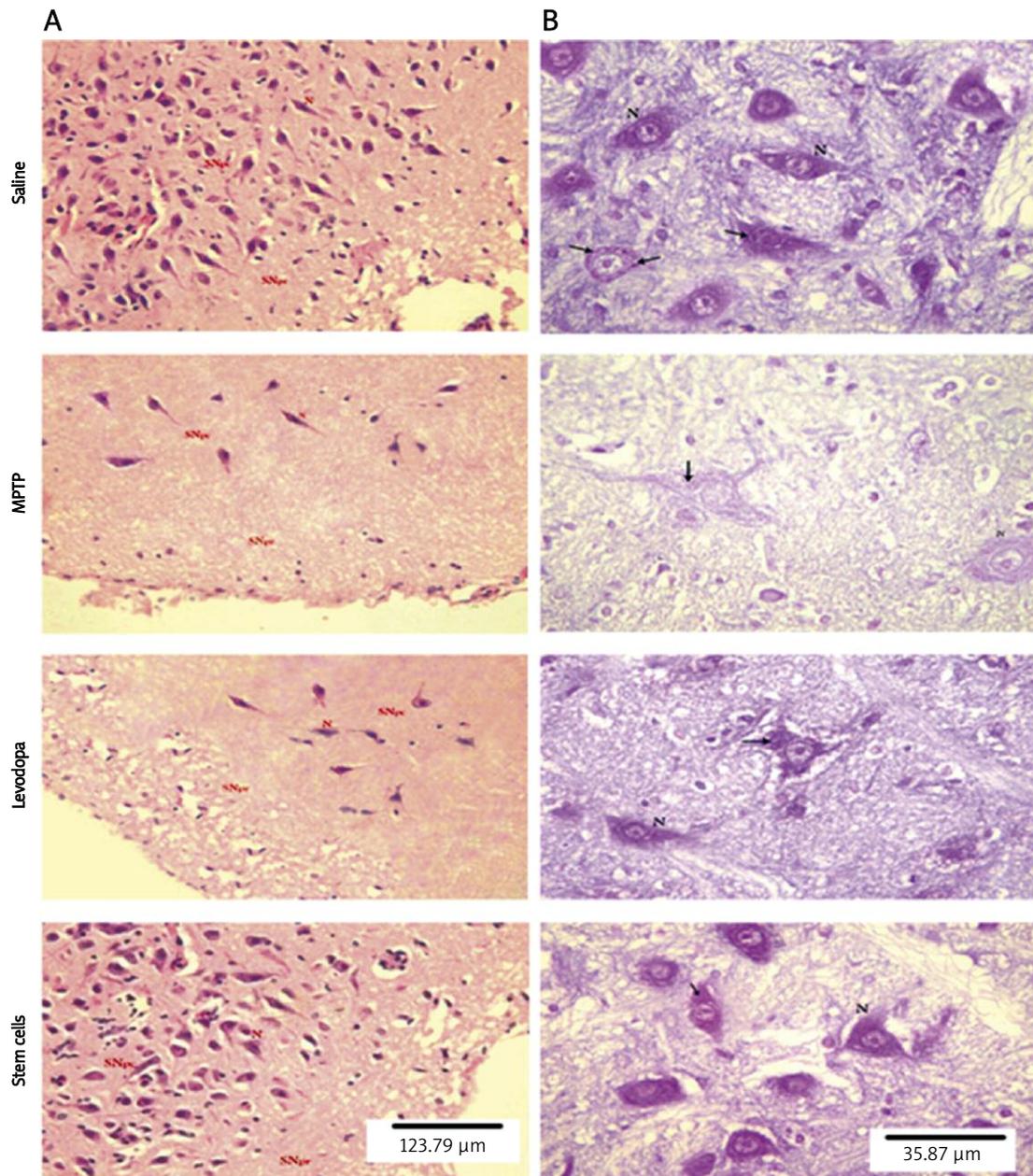
Figure 7. Sections from the substantia nigra in experimental groups stained with hematoxylin and eosin (H + E; 400×) (**A**) or cresyl fast violet (600×) (**B**). **A** – H + E staining of substantia nigra from saline-treated mice showed a cell-rich region full of abundant large and medium sized neurons with vesicular nuclei, substantia nigra pars compacta (SNpc) and a cell-poor substantia nigra pars reticularis (SNpr). The MPTP group showed neuronal degeneration in SNpc. The levodopa group showed neuronal degeneration in the SNpc similar to the MPTP group. The stem cell group showed a marked increase of the neuronal population in SNpc. **B** – Cresyl fast violet staining indicated that SNpc taken from the saline group showed large abundant purple Nissl granules (arrows) in the perikarya and dendrites of nigral neurons. The MPTP group showed decreased purple Nissl granules (arrows) in the perikarya and dendrites of nigral neurons. The levodopa group showed purple Nissl granules (arrows) in the perikarya and dendrites of a few nigral neurons. The stem cell group showed purple Nissl granules (arrows) in the perikarya and dendrites of nigral neurons similar to that of the saline group

MPTP – 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

Levodopa led to an increase in striatal dopamine level and this was reflected by motor improvement without a beneficial effect on the damaged dopaminergic neurons. This can be explained as levodopa is a catechol-containing compound that may undergo autoxidation *in vivo* in the striatal extra-cellular compartment, with the consequent generation of quinone derivatives and superoxide anion that cause oxidative stress and lead to neuronal destruction by necrosis [51]. Moreover, patients with early PD often respond well to dopaminergic medications with few clinical fluctuations. This is likely
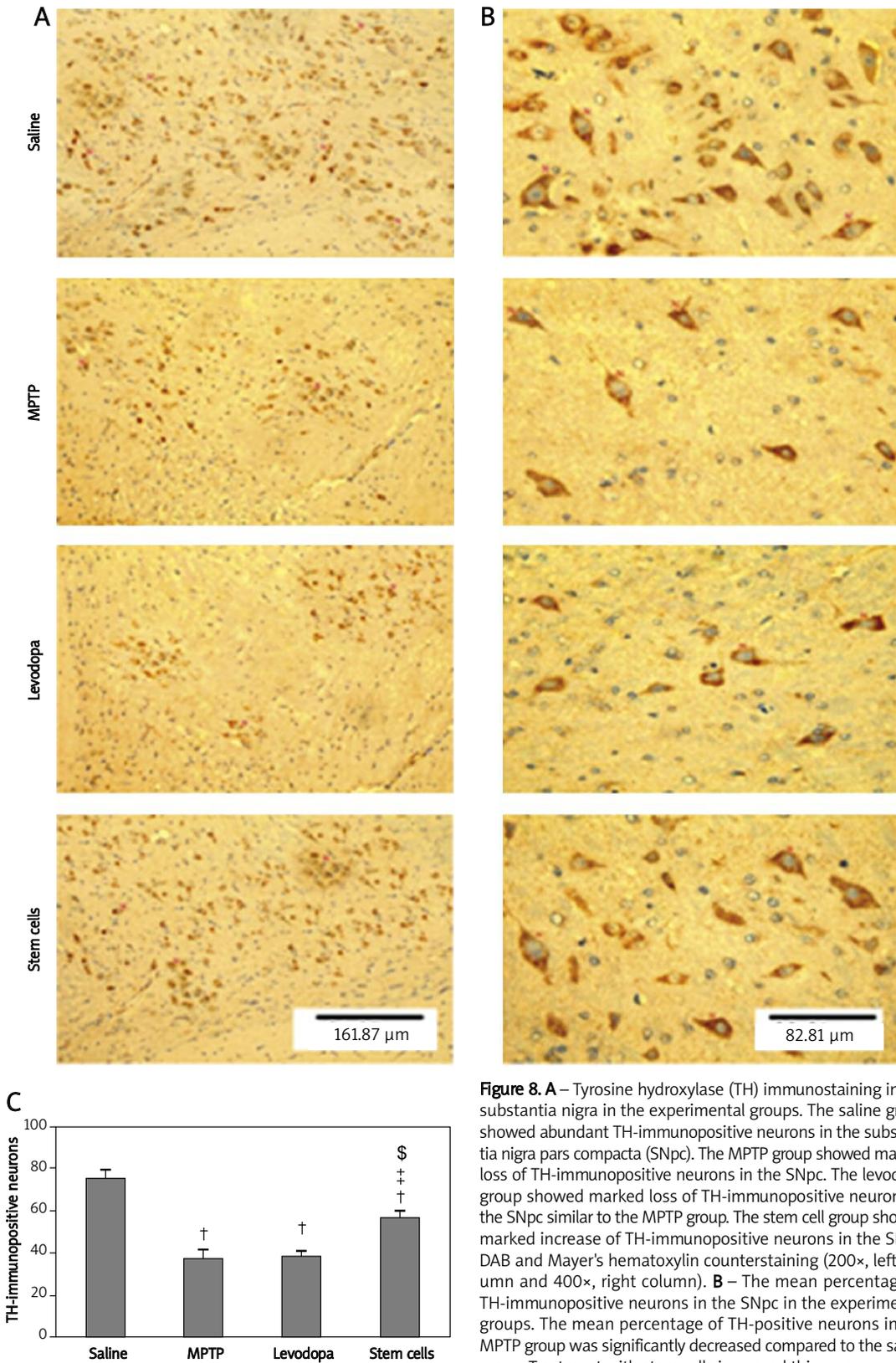
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Figure 8. A – Tyrosine hydroxylase (TH) immunostaining in the substantia nigra in the experimental groups. The saline group showed abundant TH-immunopositive neurons in the substantia nigra pars compacta (SNpc). The MPTP group showed marked loss of TH-immunopositive neurons in the SNpc. The levodopa group showed marked loss of TH-immunopositive neurons in the SNpc similar to the MPTP group. The stem cell group showed marked increase of TH-immunopositive neurons in the SNpc. DAB and Mayer's hematoxylin counterstaining (200×, left column and 400×, right column). **B** – The mean percentage of TH-immunopositive neurons in the SNpc in the experimental groups. The mean percentage of TH-positive neurons in the MPTP group was significantly decreased compared to the saline group. Treatment with stem cells increased this mean percentage compared to the MPTP group. Results are mean ± SEM and were analyzed using one-way ANOVA and Bonferroni post-hoc test. Six multiple comparisons were made among groups

†$p < 0.05$ compared to saline group, ‡$p < 0.05$ compared to MPTP group, $$p < 0.05$ compared to levodopa group, MPTP – 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

due to the up-regulation of nigropallidal dopaminergic projection to the globus pallidus internus (GPi), reducing inhibitory output from GPi to the thalamus. In more advanced Parkinson's, loss of nigropallidal upregulation may result in alteration of the firing pattern of GPi from tonic to burst firing, heralding the onset of motor complications [52].

In the present study, treatment with CD34+ stem cells improved locomotor activity, dopamine and ATP levels, as well as mtDNA and nDNA integrity. In accordance, I.V. HUCB stem cell therapy was reported to restore dopaminergic function in humans [53] and delayed the onset of symptoms and death of Parkinsonian mice [54].

DNA damage and cell death after injection of MPTP lead to a dramatic increase in the levels of secreted stress signals – chemokines, cytokines, and proteolytic enzymes – in many organs as part of the regeneration and repair process, which have profound impacts on stem cell migration and repopulation [55]. Stem cells were reported to grow in the presence of different tissue types such as heart, lung and nerve tissue. The stem cells differentiate into the same type of cells that were surrounding them [56].

The beneficial effects of stem cells in neuronal damage can be explained also as they produce neurotrophic factors, enhance angiogenesis, secrete numerous angiogenic factors, and reduce inflammation, apoptotic protection and nerve fiber reorganization [57, 58]. However, neuroregenerative therapies should be tested for their potential clinical use [59, 60].

In the current study, stem cell therapy improved MPTP-induced dopaminergic degeneration and induced a marked increase of TH-immunopositive neurons. Similarly, stem cells can differentiate into dopaminergic neurons that reinnervate the denervated striatum and become functionally integrated, restoring striatal dopamine release [61]. Stem cell transplantation and activation of endogenous neurogenesis represents currently one of the most promising therapeutic approaches to CNS repair in neuroregenerative medicine [62]. In the present study, functional assessment agreed with the biochemical and histopathologic assessment for CD34+ stem cell therapy. The beneficial effect of stem cells over levodopa was also in accordance with Lindvall and Björklund [61], who stated that transplantation of human embryonic stem cells has provided proof of the principle that neuronal replacement can work in PD patients.

In conclusion, CD34+ stem cells improved the motor function, ameliorated striatal dopamine and ATP levels, and restored mtDNA and nDNA integrity, in addition to increasing TH immunostaining in the SNpc of MPTP-parkinsonian mice. These effects were superior to those produced by levodopa. Stem cells

as an alternative neuronal source can be employed to generate mature dopaminergic neurons to innervate the affected striatum in PD. Further studies are needed to explore other possible mechanisms involved in the neuroprotective effect of CD34+ stem cells in experimental models of PD and to determine the clinical benefit of using such therapy.

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