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Research Article

Exploring Potential Mechanisms of Action of Mesenchymal Stem Cells in Parkinson's Disease: *In Vivo* Study

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ABSTRACT
Parkinson's disease (PD) is a predominant neurodegenerative disease characterized by debilitating movement as a result of dopaminergic (DA) neurons loss. Currently used therapies improve only the disease symptoms without halting or inverting DA neurons deterioration. For this purpose, this study was goaled to explicate the role of adipose tissue derived mesenchymal stem cells (ADMSCs) in promoting dopaminergic neurons repairment in rodent model of PD after 2 months. ADMSCs were engrafted into injured brains and enhanced serum brain derived neurotrophic factor (BDNF) and brain DA levels. Moreover, they amplified the expression level of tyrosine hydroxylase (TH) gene and increased the number of survivin expressing cells. Finally, they were able to recover the neuronal degeneration of striatum. The aforementioned data clearly demonstrated the promising therapeutic action of ADMSCs in PD through their neurotrophic, neuroregenerative and anti-apoptotic properties.

Key words: Parkinson's disease, Adipose tissue derived MSCs, Cerebrolysin, brain derived neurotrophic factor, dopamine, tyrosine hydroxylase, survivin.

INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disease associated with extrapyramidal motor dysfunction[1] because of the progressive and selective loss of dopaminergic neurons and dopamine suppression[2]. This neurodeterioration results in serious motor impairments such as resting tremors, bradykinesia, akinesia and muscular rigidity[3]. Compelling evidences demonstrate that in the early stages of PD, pharmacological interventions have the ability to improve clinical symptoms only, while in the advanced stages of the disease they are less effective. Also, the accessible therapies for PD couldn't reverse or stop dopaminergic neurons degeneration pointing to necessity for efficient therapies[4]. It has been mentioned that, neuronal survival and differentiation needs neurotrophic factors. Among these factors, brain derived neurotrophic factor (BDNF) has potent protective and regenerative effects on dopaminergic neurons[5]. Growing body of evidences indicated that the drop in the supply of BDNF or other neurotrophic agents and the dysfunctional downstream signaling paths leads to degeneration of dopaminergic cells[6]. Mesenchymal stem cells (MSCs) are a heterogeneous subset of stromal stem cells that have the ability of self-renewal and multipotency[7]. Moreover, they produce a wide range of cytokines and growth factors to induce endogenous restorative mechanisms of injured tissues[8]. Recent researches and trials related to MSCs revealed that these cells have great promise in a variety of disorders and tissue regeneration[7]. Several studies have shown that MSCs could exert neuroprotective effect through two mechanisms: (1) releasing of different trophic factor, including brain-derived neurotrophic factors[9], nerve growth factor (NGF)[10], and insulin-like growth factor-1 (IGF-1)[11], that contribute to recovering neurobehavioral function and stimulating endogenous regeneration; (2) accommodation to damaged tissues and immunoregulation[7]. Moreover, the growth factors and cytokines released by MSCs have been shown to enhance neurogenesis[12,13]. Adipose tissue has been latterly identified as an appropriate source of MSCs[14] because this tissue could be obtained easily with little suffering for patient[15]. Adipose tissue-derived MSCs (ADMSCs) have many advantages such as: easy extraction and proliferation in high rate[16]. The main target of the present work was to identify the mechanisms of action of single dose of ADMSCs in retrieving the dopaminergic functions in the experimental model of PD after 2 months.

MATERIALS AND METHODS

Derivation, purification and characterization of ADMSCs

Mesenchymal stem cells were derived from adipose tissue of 6-week-old male *Sprague Dawley* rats and purified according to previously published work[17]. Flow cytometry was performed using CD 34, CD 90 and CD 105 cell surface markers to confirm whether the isolated ADMSCs maintain their phenotype after propagation in culture[18]. The FITC conjugated-CD 34 antibody was procured from Dako Co., Denmark. While, the PE-conjugated CD 90 and

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CD 105 antibodies were purchased from R&D Systems, UK and Miltenyi Biotec, Germany respectively. The cells were incubated with the antibody against each of the surface markers for 30 min at 4 ◦C for CD 34 and CD 90 and 10 min at 4 ◦C for CD 105 followed by flow cytometry analysis using Beckman Coulter Elite XL, USA instrument.

The biological experimentation

Chemicals and drugs

Rotenone was purchased from Sigma, USA.

Cerebrolysin® ampoules, 1 mL contains 215.2 mg cerebrolysin concentrate as active ingredient in aqueous solution was purchased from EBEWE Pharma Ges.m.b.H. Nfg.KG, Austria.

Experimental animals

Thirty-two adult female *Sprague Dawley* rats weighing 130-150 g were obtained from the Animal House Colony of the National Research Centre, Giza, Egypt and acclimatized in a specific area where temperature (25±1ºC) and humidity (55%). Rats were controlled constantly with 12 hours' light/dark cycles at National Research Centre, Animal Facility Breeding Colony. Rats were individually housed with *ad libitum* access to standard laboratory diet consisted of casein 10%, salt mixture 4%, vitamin mixture 1%, corn oil 10%, cellulose 5% and completed to 100 g with corn starch and tap water. Also, they were cared for according to the guidelines for animal experiments which were approved by the Ethical Committee of Medical Research at National Research Centre, Giza, Egypt. The animals were ovariectomized (ovx) surgically in Hormones Department, Medical Research Division at the National Research Centre. Then, after one month from ovariectomy the animals were classified into 4 groups (8 rats/group). The first group was ovx control group. The groups from second to fourth were subcutaneously injected with rotenone in a dose of 12 mg/kg b. wt.[19] daily for 14 days for induction of PD. Then, the second group wasn't received any treatment; the third group was treated intraperitoneally with cerebrolysin in a dose of 1.08 ml/kg b. wt. that is equivalent to the recommended human dose according to Barnes and Paget[20] equation, 5 days/week for one month and thereafter two times per week for another month[21] and the fourth group was infused with a single dose of ADMSCs (3 x 10^6 cells/rat) intravenously[22]. At the end of the experimental period, all animals were fasted for 12 hours and the blood samples were collected from retro-orbital venous plexus under diethyl ether anaesthesia. The blood samples were left to clot and the sera were separated using cooling centrifugation (4ºC) at 1800 x g for 10 min and then stored immediately at -20ºC in clean plastic Eppendorf until analyzed. The whole brain of each rat was rapidly and carefully dissected. Then, each brain was sagitally divided into two portions. The first portion was immediately frozen in liquid nitrogen and stored at −80º C prior to extraction for molecular study and dopamine level determination. While, the second portion was fixed in formalin buffer (10%) for immunohistochemical examination and histological investigation.

Detection of male-derived MSCs in the brain of females

The genomic DNA was isolated from the brain tissues of female rats which were treated with ADMSCs using phenol/chloroform extraction and ethanol precipitation method according to Sambrook et al.[23] with minor modifications. The presence or absence of the sex determination region on the Y chromosome (SRY) gene in recipient female rats was assessed by PCR technique. Primer sequences for SRY gene (F:5′-CATCGAAGGGTTAAAGTGCCA-3′, R:5′-ATAGTGTGTAGGTTGTTGTCC-3′) were obtained from published sequences[24] and amplified to a product of 104 bp. The PCR conditions were as follows: incubation at 94 °C for 4 min; 35 cycles of incubation at 94 °C for 50 s, 60 °C for 30 s, and 72 °C for 1 min; with a final incubation at 72 °C for 10 min. PCR products were separated using 2% agarose gel electrophoresis and stained with ethidium bromide.

Determination of BDNF and dopamine levels

Serum brain derived neurotrophic factor (BDNF) level was determined by enzyme linked immunosorbent assay (ELISA) method using kit purchased from Millipore corporation, USA, according to the method described by Laske et al.[25] Meanwhile, quantitative determination of dopamine in the brain was carried out according to the method described by Ciarlone[26] using a fluorometric method.

Detection of tyrosine hydroxylase gene expression level

Total RNA was isolated from brain tissue of female rats by the standard TRIzol® reagent extraction method (Invitrogen, USA). Then, the complete Poly(A)$^+$ RNA was reverse transcribed into cDNA in a total volume of 20 µL using RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, Germany). An amount of total RNA (5µg) was used with a reaction mixture, termed as master mix (MM). The MM consisted of 50 mM $MgCl_2$, 5x reverse transcription (RT) buffer (50 mM KCl; 10 mM Tris-HCl; pH 8.3; 10 mM of each dNTP, 50 µM oligo-dT primer, 20 U ribonuclease inhibitor (50 kDa recombinant enzyme to inhibit RNase activity) and 50 U M- MuLV reverse transcriptase. The RT reaction was carried out at 25 °C for 10 min, followed by 1 h at 42 °C, and the reaction was stopped by heating for 5 min at 99 °C. Afterwards the reaction tubes containing RT preparations were flash-cooled in an ice chamber until being used for DNA amplification through sqRT-PCR. An iQ5-BIO-RAD Cycler (Cepheid, USA) was used to determine the rat cDNA copy number. PCR reactions were set up in 25 µL reaction mixtures containing 12.5 µL 1× SYBR® Premix Ex TaqTM (TaKaRa, Biotech. Co. Ltd.), 0.5 µL 0.2 µM forward primer, 0.5 µL 0.2 µM reverse primer, 6.5 µL distilled water, and 5 µL of cDNA template. Primer sequences were F:5′-ACTGTGGAATTCGGGCTATG-3′, R:5′-GACCTCAG-GCTCCTCTGACA-3′ for tyrosine hydroxylase (TH)[27] and F:5′-CTGTCTGGCGGCACCACCAT-3′, R:5′-GCAACTAAGTCATAGTCCGC-3′ for β-actin[28]. The reaction program was allocated to 3 steps. First step was at 95.0°C for 3 min. Second step consisted of 40 cycles in which each cycle divided to 3 steps: (a) denaturation at 95.0°C for 15 sec; (b) annealing at 58.0 and 60°C for 30

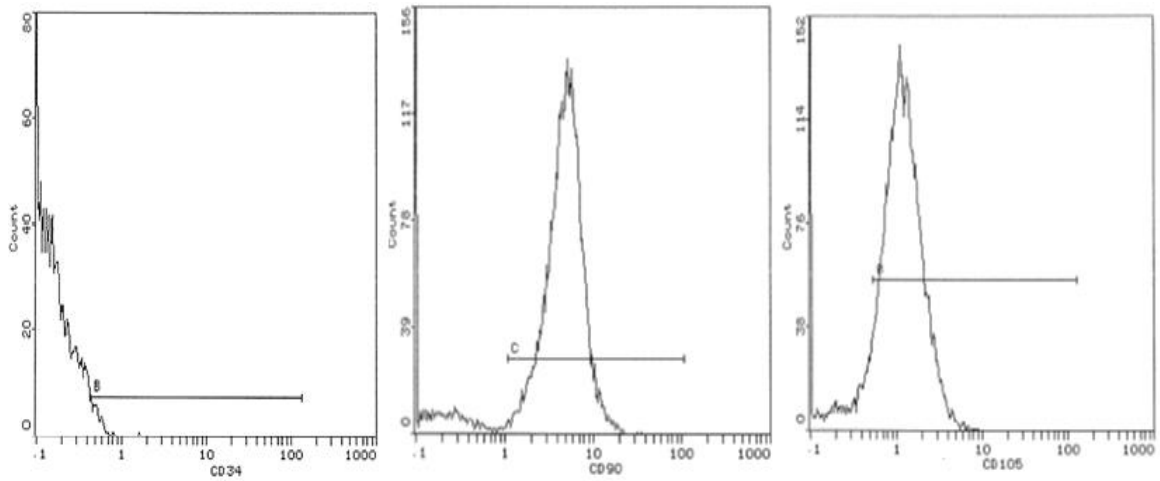


Figure 1: Flow cytometry analysis of ADMSCs after staining with CD34, CD90 and CD105 antibodies: CD90 (82.5%) and CD105 (82.3%).

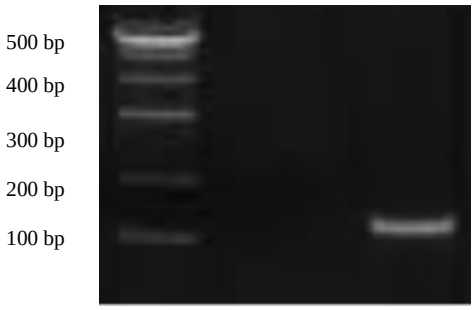


Figure 2: UV transilluminated agarose gel of DNA fragments showed SRY gene in PD rats treated with ADMSCs. (M): DNA ladder; Lane (1): sample from ovx control rats; Lane (2): sample from ADMSCs treated rats.

sec for TH and β-actin genes respectively and (c) extension at 72.0°C for 30 sec.

Immunohistochemical examination of survivin expression

Samples were taken from brain of rats of the different groups and fixed in 10% formalin buffer for 24 hours. Washing was done in tap water then serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56 ºC in hot air oven for 24 hours. Sections were cut into 4µ thick by slidge microtome then fixed on positive slides in a 65 ºC oven for 1 hr. Slides were placed in a coplin jar filled with 200 mL of triology working solution (Cell Marque, CA-USA) which combines the three pretreatment steps: deparaffinization, rehydration and antigen unmasking. Then, the jar is securely positioned in the autoclave which was adjusted so that temperature reached 120 ºC and maintained stable for 15 min after which pressure is released. Thereafter, the coplin jar is removed to allow slides to cool for 30 min. Sections were then washed and immersed in Tris-buffer saline (TBS) to adjust the pH and these were repeated between each step of the IHC procedure. Quenching endogenous peroxidase activity was performed by immersing slides in 3% hydrogen peroxide for 10 min. Broad spectrum LAB-SA detection system (Invitrogen, USA) was used to visualize any antigen-antibody reaction in the tissue. Background staining was blocked by putting 2-3 drops of 10% goat non immune serum blocker on each slide and incubating them in a humidity chamber for 10 min. Without washing, excess serum was drained and the working solution (1:100) of the primary antibody of survivin (mouse monoclonal; Thermo Scientific, USA) was prepared. Two-three drops of the working solution were applied. Then, slides were incubated in the humidity chamber overnight at 4 ºC. Henceforward, biotinylated secondary antibody was applied on each slide for 20 min followed by 20 min incubation with the streptavidin horse reddish peroxidase (HRP) enzyme conjugate. 3,3´-diaminobenzidine (DAB) chromogen was prepared and 2-3 drops were applied on each slide for 2 min. DAB was rinsed, after which counterstaining with Mayer hematoxylin and cover slipping were performed as the final steps before slides were examined under the light microscope (Olympus Cx21 with attached digital camera)[29]. Image analysis was performed using the image J, 1.41a NIH, (USA) analyzer.

Histopathological investigation of brain tissue sections

Samples were taken from brain of rats of the different groups and fixed in 10% formalin buffer for 24 hours. Washing was done in tap water then serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56 ºC in hot air oven for 24 hours. Paraffin wax tissue blocks were prepared for sectioning at 4 µ thick by slidge microtome. The obtained tissue sections were collected on glass slides, deparffinized and stained by hematoxylin and eosin stains[30] for histopathological examination through the electric light microscope.

Statistical analysis

In the present study, all results were expressed as Mean ± S.E of the mean. Data were analyzed by one-way analysis of variance (ANOVA) using the Statistical Package for the Social Sciences (SPSS) program, version 14 followed by least significant difference (LSD) to compare significance between groups.[31] Difference was considered significant when P value was < 0.05.

RESULTS

Characterization of ADMSCs

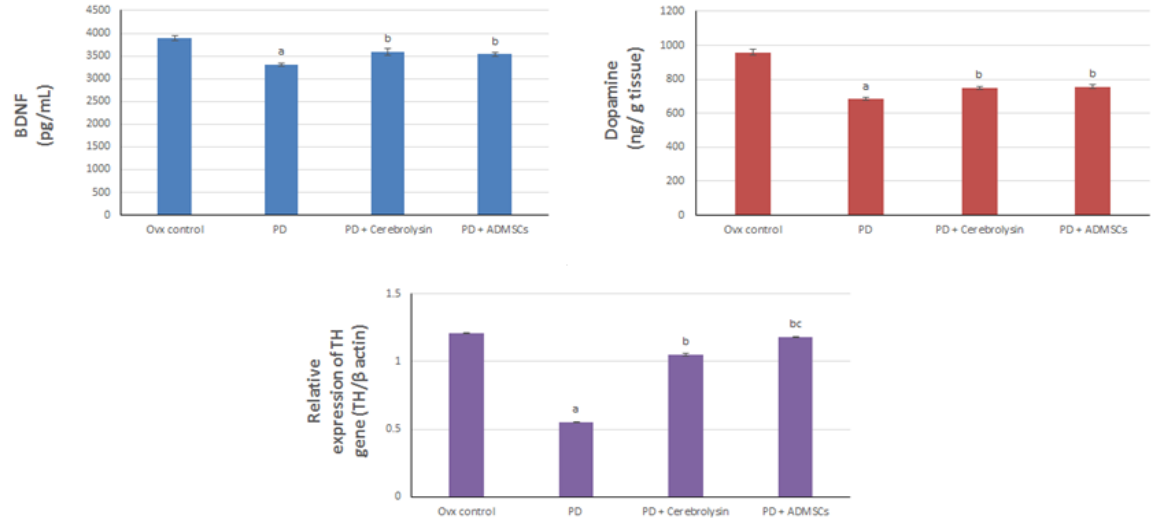


Figure 3: Influence of cerebrolysin and ADMSCs on serum BDNF level, brain dopamine content and tyrosine hydroxylase gene expression level in PD model.
a: Significant change at P< 0.05 versus ovx control group.
b: Significant change at P< 0.05 versus PD group.
c: Significant change at P< 0.05 versus cerebrolysin treated group.

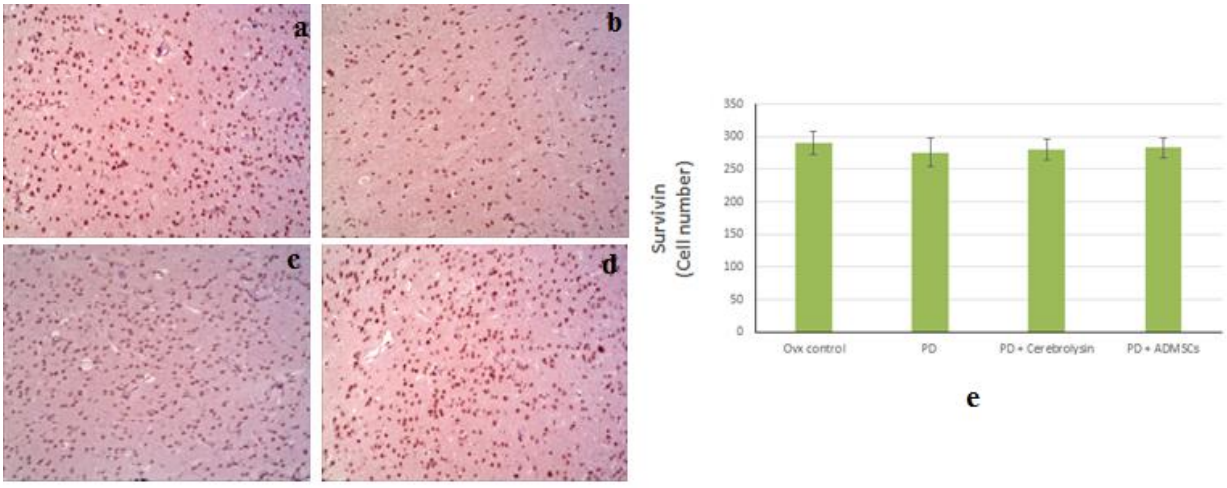


Figure 4: Immunohistochemical examination of survivin expression in PD model groups. (a): ovx control, (b): PD control, (c): PD + cerebrolysin, (d): PD + ADMSCs and (e): number of survivin expressing cells in different experimental groups.

The ADMSCs characterized using flow cytometer were positive for CD90 (82.5%), CD105 (82.3%) and negative for CD34. This result shows that the expanded ADMSCs maintain their phenotype (Fig. 1).

Accommodation of ADMSCs

SRY gene that was used as a marker for accommodation of MSCs into diseased brains is detected in brain tissues of PD bearing rats injected intravenously with ADMSCs (Fig. 2).

Influence of cerebrolysin and ADMSCs on neurotrophic and neurogenic markers

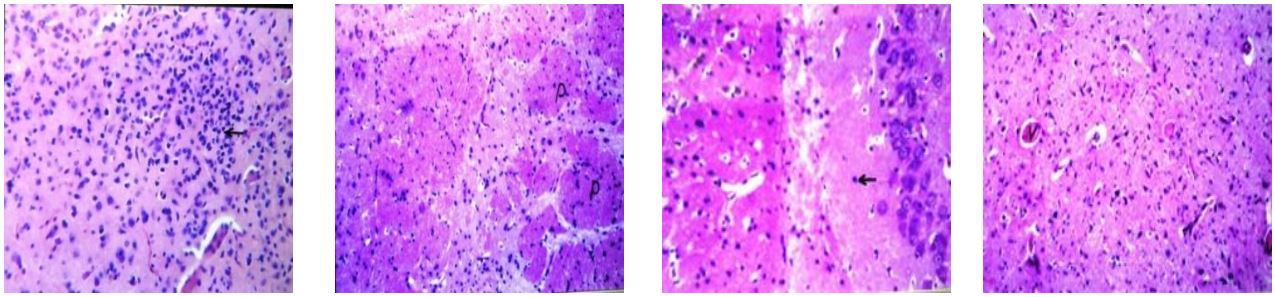
The results of the present study show that rotenone administration evokes significant (P< 0.05) decline in the level of neurotrophic (serum BDNF) and neurogenic (brain DA and TH)[32] markers versus ovx control group. Contrariwise, cerebrolysin and ADMSCs succeeded in recovering the values of these markers significantly (P< 0.05) relative to PD group (Fig. 3).

Influence of cerebrolysin and ADMSCs on anti-apoptotic marker

Rotenone administration causes insignificant (P> 0.05) reduction in the number of survivin expressing cells in comparison with the ovx control group. While, cerebrolysin and ADMSCs enhance the number of survivin expressing cells insignificantly (P> 0.05) relative to the PD group (Fig. 4).

Influence of cerebrolysin and ADMSCs on brain feature

The photomicrograph of brain section of the striatum of ovx control group shows diffuse gliosis (Fig. 5a). While, the photomicrograph of brain section of PD group shows hyalinization with plaque formation in the striatum reflecting neurodegeneration (Fig. 5b). However, the photomicrographs of brain sections of the striatum of treated rats shows diffuse gliosis in case of treatment with



a b c d

Figure 5: Photomicrograph of brain section of (a): ovx control group shows diffuse gliosis (arrow) in the striatum. H&E x 64, (b): PD group shows hyalinization in brain matrix of striatum with plaque formation (p). H&E x 40, (c): PD group treated with cerebrolysin shows diffuse gliosis in the striatum (arrow). H&E x 40 and (d): PD group treated with ADMSCs shows congestion in the blood vessels of striatum (v). H&E x 40

cerebrolysin (Figs. 5c) and congestion in the blood vessels in case of treatment with ADMSCs (Fig. 5d).

DISCUSSION

Latterly, it has been reported that fat is an enormous source of MSCs. These MSCs are specifically interesting because of their rapid proliferation and multidirectional differentiation potential[33]. Therefore, we isolated MSCs from adipose tissues of male rats to be used for treatment of induced PD in female rats. Our investigations revealed that intravenously injected ADMSCs were engrafted into injured brain of rats. This finding is in concert with report from Yousefi et al.[34] that intravenously transplanted ADMSCs could migrate and integrate like neural stem cells in the brain. MSCs could relocate to the inflammatory sites and migrate towards the injured tissues. This process was driven by the connection between growth factors and chemokines released by injured tissue and the extracellular matrix receptors expressed on MSCs surface[35]. Mounting evidences have revealed that MSCs transplantation triggers brain repair through trophic mechanisms[36]. In line with these evidences, the engrafted ADMSCs in the current study produced significant up-regulation of serum BDNF level, brain dopamine content and brain TH gene expression level. Also, they amplified the number of survivin expressing cells and modulated the dopaminergic neurodegeneration in striatum. Han et al.[37] documented that ADMSCs have the ability to secrete BDNF and differentiate into neuron-like cells. Also, McCoy et al.[38] demonstrated that ADMSCs play a pivotal role in the protection against death of dopaminergic neurons through secretion of trophic factors. Moreover, Thejaswi et al.[39] cited that ADMSCs undergo differentiation into neuron like cells and express TH. Since, TH is an enzyme responsible for synthesis of DA, the elevation in TH level would enhance DA production[40]. It has been reported that the BDNF secreted from MSCs elicit neurotrophic and neuroprotective effects on DA neurons.[41] MSCs have the ability to enhance the expression of TH gene and DA content through secretion of growth factors.[42] Bouchez et al.[27] suggested that growth factors are involved in the increase of DA level and TH immunoreactivity after MSCs transplantation. Additionally, Bahat-Stroomza et al.[43] stated that MSCs which were directed *in vitro* toward neurotrophic factor-secreting cells cause a prominent recovery of DA function after transplantation. Furthermore, Trzaska et al.[44] demonstrated that BDNF has potent role in the maturation of DA progenitors. The observed increment in the number of survivin expressing cells in the brain is in parallel to that obtained previously by Okazaki et al.[45] and it might be attributed to the capability of MSCs to secrete BDNF and consequently induce neurogenesis and inhibit apoptosis as documented by Gutiérrez-Fernández et al.[46] Moreover, Kim et al.[47] reported that the anti-apoptotic effect exerted by MSCs could inhibit DA neuronal loss. This effect could be due to the release of vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and BDNF by MSCs and the elevation in the level of pro-survival factor Akt[48]. On the other hand, the amelioration of the neuronal degeneration of striatum by ADMSCs could be imputed to their ability to secrete BDNF[37] which promotes tissue and nerve repair[49] and enhances neurogenesis[46]. Treatment with cerebrolysin evoked significant elevation in serum BDNF level, brain dopamine content and brain TH gene expression level. The elevation in serum BDNF level is in respect with Selianina and Karakulova[50]. This increment could be allied to that cerebrolysin is consisted of neurotrophic factors including BDNF[51]. The neuroprotective and anti-apoptotic effects exerted by BDNF could explain the elevation in DA content and TH gene expression level after treatment with cerebrolysin[52,53]. Also, recently it has been demonstrated that TH gene's transcription is positively regulated by BDNF[54]. Cerebrolysin injection resulted in insignificant increase in the number of survivin expressing cells and neuronal recovery. These effects could be explained by the anti-apoptotic[55] and neurogenic[56] activities of cerebrolysin. Furthermore, Selianina and Karakulova[50] reported that cerebrolysin promotes neurogenesis and brain repair through increasing BDNF. In conclusion, the outcomes of the current work support the hypothesis that ADMSCs would have beneficial effect against PD. It is reasonable to assume that neurotrophic, neuroregenerative and anti-apoptotic effects of ADMSCs are implicated in their neuroprotective activity in this study.

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