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# Neuroprotective effects of mesenchymal stem cells through autophagy modulation in a parkinsonian model

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ABSTRACT

Autophagy is a major degradation pathway for abnormal aggregated proteins and organelles that cause various neurodegenerative diseases. Current evidence suggests a central role for autophagy in pathogenesis of Parkinson's disease, and that dysfunction in the autophagic system may lead to α-synuclein accumulation. In the present study, we investigated whether mesenchymal stem cells (MSCs) would enhance autophagy and thus exert a neuroprotective effect through the modulation of α-synuclein in parkinsonian models. In $MPP^+$-treated neuronal cells, coculture with MSCs increased cellular viability, attenuated expression of α-synuclein, and enhanced the number of LC3-II-positive autophagosomes compared with cells treated with $MPP^+$ only. In an MPTP-treated animal model of Parkinson's disease, MSC administration significantly increased final maturation of late autophagic vacuoles, fusion with lysosomes. Moreover, MSC administration significantly reduced the level of α-synuclein in dopaminergic neurons, which was elevated in MPTP-treated mice. These results suggest that MSC treatment significantly enhances autophagolysosome formation and may modulate α-synuclein expression in parkinsonian models, which may lead to increased neuronal survival in the presence of neurotoxins.



## 1. Introduction

Parkinson's disease (PD) is a chronic neurodegenerative disease characterized by selective loss of dopaminergic neurons and the presence of Lewy bodies, proteinaceous inclusions that are composed mainly of α-synuclein in the substantia nigra (SN) (Moore et al., 2005). Although the initial triggering factors remain unknown, recent advances in molecular biology have revealed that the pathogenesis of neuronal degeneration in PD may involve several molecular and cellular events including oxidative stress, accumulation of toxic proteins resulting from the dysfunction of the protein degradation system, proapoptotic mechanisms, and mitochondrial dysfunction.

Like the proteasome system, autophagy is a vital pathway for the refolding or removal of abnormal and aggregated proteins, and thus, acts as a cytoprotective response, particularly under stress or injury conditions (Cuervo, 2010; Levine and Kroemer, 2008; Mizushima et al., 2008; Rubinsztein, 2006). The autophagic pathway delivers intracellular constituents to lysosomes by several processes including chaperone-mediated autophagy, microautophagy, and macroautophagy. Of these, macroautophagy, hereafter referred to as autophagy, is a major intracellular degradation process that acts as the principal mechanism of organelle turnover. Ample evidence has suggested that the autophagic pathway is involved in the pathogenesis of various neurodegenerative diseases, including PD and Alzheimer's disease. Anglade et al. (1997) demonstrated for the first time pathologic evidence that accumulation of autophagic vacuoles (AVs) occurred in the SN of PD patients (Anglade et al., 1997). Thereafter, several pathologic studies reported an increase in the autophagosome marker LC3-II (Crews et al., 2010; Dehay et al., 2010; Higashi et al., 2011; Klucken et al., 2012; Tanji et al., 2011; Yu et al., 2009) and a decrease in the lysosomal marker LAMP1 (Chu et al., 2009; Dehay et al., 2010; Klucken et al., 2012) in patients with PD and dementia with Lewy bodies, suggesting the presence of abundant and dysfunctional autophagosomes and lysosomes in α-synucleinopathies. In cellular and animal studies, overexpression of α-synuclein inhibits macroautophagy in the very early stages of autophagosome formation (Winslow et al., 2010), and α-synuclein is degraded by autophagy in PC12 cell lines with α-synuclein overexpression (Webb et al., 2003). Thus, impairment of these systems may lead to the accumulation and aggregation of proteins resulting in the cellular toxicity and eventual neurodegeneration seen in PD, suggesting a pivotal role for the autophagosome system in α-synucleinopathies.

Mesenchymal stem cells (MSCs) are multipotent stem cells present in adult bone marrow that are capable of differentiating into various cell types under appropriate conditions (Pittenger

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et al., 1999; Sanchez-Romos et al., 2000). Additionally, MSCs secrete various cytotropic factors that, in turn, exert neuroprotective effects (Caplan and Dennis, 2006). Our previous animal studies demonstrated that MSCs had neuroprotective effects via complex mechanisms, such as modulation of neuroinflammation, enhancement of cell survival signals, increased neurogenesis, and modulation of ubiquitinated proteins (Park et al., 2008, 2011, 2012). In the present study, we investigated whether MSCs would enhance autophagy and thus exert a neuroprotective effect through modulation of α-synuclein expression using in vitro and in vivo models of PD.

## 2. Methods

### 2.1. Isolation of MSCs

Bone marrow aspirates (10 mL) were obtained from the iliac crests of human donors. The mononuclear cell layer was isolated by Ficoll–Hypaque, washed in phosphate-buffered saline (PBS), plated in polystyrene plastic 100-mm culture dishes, and cultivated in low-glucose Dulbecco modified Eagles' medium (Gibco-BRL, Grand Island, NY, USA), containing 10% fetal bovine serum (Hyclone, Irvine, CA, USA) and 1% penicillin and/or streptomycin (P and/or S, Sigma, St. Louis, MO, USA) in a humidified incubator at 37 °C under 5% $CO_2$. Nonadherent cells were removed after 24 hours. When these primary cultures reached 80% confluence, the cells were harvested using 0.25% trypsin and subcultured. At passage 6, MSCs were injected into the tail veins of MPTP-treated mice, or cultured into individual wells of 24-transwell plates ($3 \times 10^3$ cells/transwell) or of 6-transwell plates ($3 \times 10^4$ cells/transwell) 1 day before the experiment.

### 2.2. SH-SY5Y cell culture

SH-SY5Y cells (neuroblastoma cell line, Korean Cell Line Bank, Korea) were maintained in Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were cultured in 100-mm dishes and routinely passaged using trypsin–enzyme-linked immunosorbent assay (EDTA) solution. Cells were subcultured into individual wells of 24-well plates ($1 \times 10^5$ cells/well) or 6-well plates ($1 \times 10^6$ cells/well) 1 day before the experiment.

### 2.3. Coculture and treatment

SH-SY5Y cells were treated $MPP^+$ (100 μM; Sigma, St. Louis, MO, USA) for 24 hours (Fan et al., 2006) in a humidified incubator at 37 °C and 5% $CO_2$. Subsequently, the media was refreshed, or else the cells were cocultured with MSCs or SH-SY5Y cells in the transwell. For inhibition of autophagy, either 10 mM 3-methyladenine (3 MA, Sigma) or 200 nM bafilomysin A1 (Sigma) was added to the media. The plates were incubated at 37 °C and 5% $CO_2$ for 24 hours. The cocultured cells were used for immunocytochemistry, immunofluorescence, extraction of α-synuclein protein, and electron microscopy (EM).

### 2.4. Animal studies

To create an MPTP-induced animal model of PD, adult male C57BL/6 mice aged 16 weeks were injected with MPTP (10 mg/kg intraperitoneally, once per day for 2 weeks, 2 groups of $n = 6$). Control mice ($n = 6$) were injected with saline alone. One day after the last MPTP injection, MSCs were injected into the tail vein of one of the MPTP-treated groups ($1 \times 10^6$ cells/200 μL). Histopathological analyses, Western blots, and EM were compared among the 3 groups of mice (control, MPTP-only treated animals, and animals treated with both MSCs and MPTP). The animal work was approved by the Institutional Animal Care and Use Committees of Yonsei University.

### 2.5. Tissue preparation

For immunohistochemistry and immunofluorescence, the mice were perfused with saline solution containing 0.5% sodium nitrate and heparin (10 U/mL) and were fixed with 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (PB, ~ 50 mL/mouse) 2 weeks after MSC injection. The brains were removed from the skulls, post-fixed overnight in buffered 4% paraformaldehyde at 4 °C and stored in a 30% sucrose solution for 1–2 days at 4 °C, until they sank. They were then paraffin-sectioned to obtain 30- or 4-μm coronal sections. The 30-μm coronal sections were stored in tissue stock solution (30% glycerol, 30% ethylene glycol, 30% three volumes of distilled water, 10% 0.2-M PB) at 4 °C until required. For Western blotting, the mice were euthanized at 2 weeks after MSCs injection, and the SN areas were rapidly removed from the brains and stored at −70 °C.

### 2.6. Immunohistochemistry

The 30-μm coronal brain sections was rinsed twice in PBS and incubated in 0.2% Triton X-100 for 30 minutes at room temperature (RT). They were rinsed 3 times with 0.5% bovine serum albumin (BSA) in 1× PBS for blocking. After blocking, they were incubated overnight at 4 °C with primary antibody: mouse anti-tyrosine hydroxylase (TH, 1:2000 dilution for brain tissue, Pel-freez, St. Arkansas, AR, USA). Following this, the brain sections and the cells were rinsed 3 times in 0.5% BSA in 1× PBS (10 min/rinse) and incubated with the appropriate biotinylated secondary antibody and avidin–biotin complex (Elite Kit; Vector Laboratories, Burlingame, CA, USA) for 1 hour at RT. Bound antibodies were visualized by incubating with 0.05% diaminobenzidine–HCl and 0.003% hydrogen peroxide in 0.1-M PB. The brain sections were rinsed with 0.1-M PB for diaminobenzidine inhibition. Immunostained cells were analyzed by bright-field microscopy.

### 2.7. Immunofluorescence

The cocultured cells and the 4-μm sections of embedded paraffin tissues were rinsed twice in PBS and incubated in 0.2% Triton X-100 for 30 minutes at RT. They were rinsed 3 times with 0.5% BSA in 1× PBS for blocking. After blocking, they were incubated overnight at 4 °C with the primary antibodies: mouse α-synuclein (α-syn, 1:200 for immunofluorescence; Invitrogen, Camarillo, CA, USA), rabbit LC3-II (1:500 for immunofluorescence; Sigma), mouse LAMP2 (1:200 for immunofluorescence; Invitrogen), NuMA (1:200 for immunofluorescence; Calbiochem, Germany). The samples were then rinsed 3 times in 0.5% BSA in 1× PBS (10 min/rinse) and incubated with secondary antibody: goat anti-mouse IgG (Alexa Fluor-488, green) and goat anti-rabbit IgG (Alexa Fluor-594, red) for 1 hour at RT. The samples were then washed and mounted using a Prolong Antifade Kit (Molecular Probes, OR, USA). Stained cells and tissues were viewed using an Olympus I×71 confocal laser-scanning microscope (Olympus, Tokyo, Japan).

### 2.8. Labeling of mitochondria

To visualize mitochondria, MitoTrackerRed CMXRos (Invitrogen, Camarillo, CA, USA) was diluted in cell culture media to a final concentration of 50 nM. The cells were incubated under normal culture conditions for 30 minutes, and then visualized by fluorescence microscopy (Axiovert 200 M; Carl Zeiss, Germany).

### 2.9. Hematoxylin and eosin staining

The cocultured SH-SY5Y cells were rinsed twice in distilled water (D.W.), incubated in hematoxylin for 5 minutes at RT, and washed 3 times with D.W. After washing, they were incubated in hematoxylin