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Cell viability and dopamine secretion of 6-hydroxydopamine-treated PC12 cells co-cultured with bone marrow-derived mesenchymal stem cells*☆

Yue Tang[1], Yongchun Cui[1], Fuliang Luo[1], Xiaopeng Liu[1], Xiaojuan Wang[2], Aili Wu[1], Junwei Zhao[1], Zhong Tian[1], Like Wu[2]

1*State Key Laboratory of Translational Cardiovascular Medicine, Fuwai Hospital & Cardiovascular Institute, Chinese Academy of Medical Sciences, Peking Union Medical College, Beijing 100037, China*
2*Beijing Wu Stem Cells Medical Center, Beijing 100070, China*

Abstract
In the present study, PC12 cells induced by 6-hydroxydopamine as a model of Parkinson's Disease, were used to investigate the protective effects of bone marrow-derived mesenchymal stem cells bone marrow-derived mesenchymal stem cells against 6-hydroxydopamine-induced neurotoxicity and to verify whether the mechanism of action relates to abnormal α-synuclein accumulation in cells. Results showed that co-culture with bone marrow-derived mesenchymal stem cells enhanced PC12 cell viability and dopamine secretion in a cell dose-dependent manner. MitoLight staining was used to confirm that PC12 cells co-cultured with bone marrow-derived mesenchymal stem cells demonstrate reduced levels of cell apoptosis. Immunocytochemistry and western blot analysis found the quantity of α-synuclein accumulation was significantly reduced in PC12 cell and bone marrow-derived mesenchymal stem cell co-cultures. These results indicate that bone marrow-derived mesenchymal stem cells can attenuate 6-hydroxydopamine-induced cytotoxicity by reducing abnormal α-synuclein accumulation in PC12 cells.

Key Words
bone marrow-derived mesenchymal stem cells; α-synuclein; 6-hydroxydopamine; PC12 cells; dopamine; cell apoptosis; neurotoxicity; neural regeneration

Abbreviations
BMSC, bone marrow-derived mesenchymal stem cell; PD, Parkinson's disease; α-SYN, α-synuclein; 6-OHDA, 6-hydroxydopamine

INTRODUCTION

Studies of bone marrow-derived mesenchymal stem cell (BMSCs) transplantation for treatment of Parkinson's disease (PD), have focused mainly on cell replacement and nutritional support[1-4]. However, the influence of BMSCs on the specific pathogenic mechanism of PD, for example α-synuclein (α-SYN) accumulation, misfolding-induced dopaminergic neuron degeneration or necrosis in corpus striatum and reduced dopamine production[5], remain poorly understood. The present study utilized the PC12 cell PD model[6], to investigate the protective mechanism of BMSCs on 6-hydroxydopamine (6-OHDA)-induced cytotoxicity. PC12 apoptosis and α-SYN accumulation were assessed to verify our hypothesis that BMSCs may treat PD through reducing α-SYN accumulation and attenuating PC12 cell injury.

RESULTS

BMSCs attenuated 6-OHDA-induced PC12 injury

To determine the appropriate experimental

Yue Tang☆, M.D., State Key Laboratory of Translational Cardiovascular Medicine, Fuwai Hospital & Cardiovascular Institute, Chinese Academy of Medical Sciences, Peking Union Medical College, Beijing 100037, China

Yongchun Cui and Yue Tang equally contributed to this work.

Corresponding authors: Like Wu, M.D., Beijing Wu Stem Cells Medical Center, Beijing 100070, China; Yue Tang, State Key Laboratory of Translational Cardiovascular Medicine, Fuwai Hospital & Cardiovascular Institute, Chinese Academy of Medical Sciences, Peking Union Medical College, Beijing 100037, China
likewu66@vip.sina.com
tangyue1226@vip.sina.com

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dose of 6-OHDA, concentrations of 100 nM, 1 μM, 10 μM, 100 μM, 1 mM, 10 mM and 100 mM were exposed to cultured PC12 cells for 48 hours. The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to detect PC12 cell viability. A 50% inhibiting concentration for 6-OHDA was 150 μM according to the MTT results analyzed using Graphpad software (Figure 1A). The survival rate of PC12 cells in BMSCs+PC12 co-culture at a ratio of 1: 10 group ($M_1/P_{10}/O$), BMSCs+PC12 at a ratio of 1: 1 group ($M_1/P_1/O$) and BMSCs+PC12 at a ratio of 10: 1 group ($M_{10}/P_1/O$) was significantly increased compared with the PC12+6-OHDA (P/O) group ($P < 0.01$; Figure 1B).

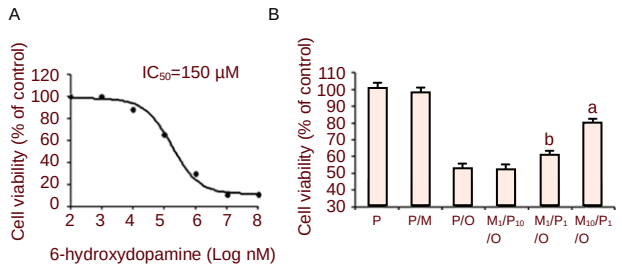

Figure 1 PC12 cell viability (MTT assay).

(A) Determination of the 50% inhibiting concentration of 6-hydroxydopamine for PC12 cells.

(B) Determination of 6-hydroxydopamine-treated PC12 cell viability after co-culture with bone marrow-derived mesenchymal stem cells (BMSCs) for 48 hours. [a]$P < 0.01$, [b]$P < 0.05$, *vs.* P/O group.

P: PC12 cells; P/M: PC12 + BMSCs; P/O: PC12 + 6-hydroxydopamine; $M_1/P_{10}/O$, $M_1/P_1/O$, $M_{10}/P_1/O$: percentage of BMSC and PC12 cells was 1:10, 1:1 and 10:1, respectively. MTT: Methylthiazolyldiphenyl-tetrazolium bromide

BMSCs attenuated 6-OHDA-induced PC12 functional injury to secrete dopamine

After PC12 cells were exposed to 6-OHDA (150 μM) for 24 hours, the supernatant was discarded and BMSCs were added for further 24, 48 and 72 hours culture. After co-culture for 24 hours, dopamine content in the supernatant was similar among groups; while after co-culture for 48 and 72 hours, dopamine concentration was significantly increased in $M_1/P_1/O$ and $M_{10}/P_1/O$ groups compared with the P/O group ($P < 0.05$; Table 1).

BMSCs relieved 6-OHDA-induced neurotoxicity by reducing PC12 cell apoptosis

PC12 cells were incubated with 6-OHDA for 48 hours in the presence or absence of BMSCs, followed by MitoLight staining. PC12 cell apoptosis was determined using flow cytometry. Results showed that 51%, 49%, 35% and 16% of PC12 cells in the P/O, $M_1/P_{10}/O$, $M_1/P_1/O$ and $M_{10}/P_1/O$ groups respectively had undergone apoptosis (Figure 2).

Table 1 Comparison of PC12 cell function to produce dopamine (ng/mL) after co-culture with BMSCs at different ratios for different time durations

Group	Time of co-culture (hour)		
	24	48	72
P	9.3±0.2	10.8±0.2	11.3±1.1
P/M	9.8±0.1	11.2±0.5	11.8±0.5
P/O	8.3±0.2	4.3±0.2	2.3±0.5
$M_1/P_{10}/O$	7.5±0.3	4.8±0.5	2.5±0.3
$M_1/P_1/O$	9.3±0.5	6.3±0.1[a]	5.6±0.2[a]
$M_{10}/P_1/O$	8.3±0.2	8.6±0.2[a]	7.6±1.2[a]

Supernatants were collected after 6-hydroxydopamine (O)-treated PC12 cells (P) were co-cultured with bone marrow-derived mesenchymal stem cells (BMSCs) (M) for 24, 48 and 72 hours to determine dopamine concentration. Data are expressed as mean ± SD of triplicate experiments.

[a]$P < 0.05$, *vs.* P/O group (student's *t*-test). $M_1/P_{10}/O$, $M_1/P_1/O$, $M_{10}/P_1/O$: percentage of BMSCs and PC12 cells was 1: 10, 1:1 and 10:1, respectively.

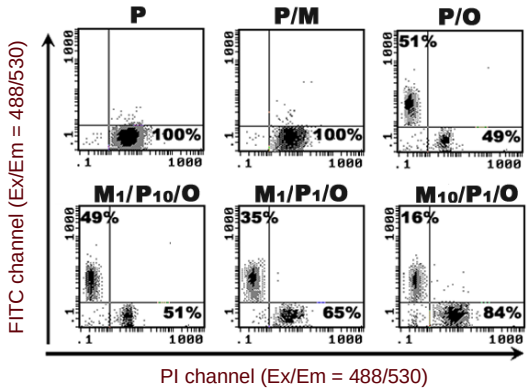

Figure 2 PC12 cell apoptosis (flow cytometry, MitoLight staining).

6-hydroxydopamine-treated PC12 apoptosis was determined after co-culture with bone marrow-derived mesenchymal stem cell (BMSCs). The propidium iodide (PI) channel displays surviving cells with red fluorescence and the fluorescein isothiocyanate (FITC) channel displays apoptotic cells with green fluorescence.

The percentage of apoptosis was 50%, 49%, 35% and 16% in the P/O, $M_1/P_{10}/O$, $M_1/P_1/O$ and $M_{10}/P_1/O$ groups respectively. Experiments were conducted in triplicate. P: PC12 cells; P/M: PC12 + BMSCs; P/O: PC12 + 6-hydroxydopamine; $M_1/P_{10}/O$, $M_1/P_1/O$, $M_{10}/P_1/O$: percentage of BMSCs and PC12 cells was 1: 10, 1:1 and 10:1, respectively.

BMSCs relieved 6-OHDA-induced neurotoxicity by reducing abnormal α-SYN accumulation in PC12 cells

To investigate whether the neuroprotective effects of BMSCs on 6-OHDA-induced cytotoxicity were associated with abnormal α-SYN accumulation in cells, PC12 cells were collected from each group and α-SYN expression was determined using immunocytochemistry and western blot analysis. Immunocytochemistry showed that the number of α-SYN-positive PC12 cells was less in

the $M_1/P_1/O$ and $M_{10}/P_1/O$ groups compared with the P/O group (Figure 3A). Similarly, western blot analysis found normalized α-SYN protein expression was significantly lower compared to the P/O group in the $M_1/P_1/O$ group (110 ± 8% *vs.* 60 ± 5%, $P < 0.05$) and $M_{10}/P_1/O$ group (110 ± 8% *vs.* 20 ± 6%, $P < 0.01$; Figure 3B).

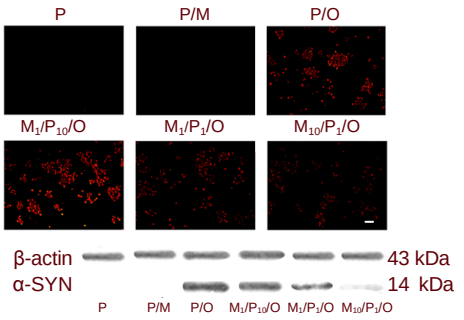

Figure 3 α-synuclein (α-SYN) expression in PC12 cells.

(A) After co-culture of PC12 cells (P; 2×10^5/well) with bone marrow-derived mesenchymal stem cells (BMSCs) (M) at different ratios for 48 hours, the number of α-SYN-positive PC12 cells was less in $M_1/P_1/O$ and $M_{10}/P_1/O$ groups compared with the P/O group (PE fluorescent staining, fluorescence microscope). Scale bar: 20 μm.

(B) Western blot analysis was used to determine α-SYN expression in PC12 cells, represented by the gray value ratio of α-SYN to β-actin. Experiments were carried out in triplicate.

P/O: PC12 cells + 6-hydroxydopamine; $M_1/P_{10}/O$, $M_1/P_1/O$, $M_{10}/P_1/O$: percentage of BMSCs and PC12 cells was 1: 10, 1:1 and 10:1, respectively.

DISCUSSION

6-OHDA is an endogenous neurotoxic factor, up-regulated in patients with PD and has been frequently used to establish *in vitro* PD models[7]. In the present study, 6-OHDA-induced cytotoxicity in PC12 cells was exhibited in a dose-dependent manner. A 50% PC12 cell inhibiting concentration for 6-OHDA of 150 μM was used, as this concentration damaged the cells but did not induce a large amount of cell death.

Cell apoptosis analysis showed that the percentage of PC12 cell apoptosis, when co-cultured with BMSCs, was lower in comparison to PC12 cells alone. This indicates BMSCs exhibit protective effects on 6-OHDA-induced cytotoxicity in PC12 cells. BMSCs inhibit PC12 cell apoptosis to attenuate 6-OHDA-induced cytotoxicity in PC12 cells in a quantity-dependent manner. Possible protective mechanisms of BMSCs include: (1) cytokine secretion, such as vascular endothelial cell growth factor, stem cell growth factor and insulin like growth factor I[8-10]; (2) inhibition of mitochondria membrane potential changes. The present study utilized MitoLight dye, a lipophilic cation and indicator of mitochondrial activity. Depending on the membrane potential, MitoLight can accumulate in the mitochondria of living cells and emit a red fluorescence. However, MitoLight dye emits green fluorescence in response to changes in mitochondrial membrane potential. The percentage of PC12 cells with green fluorescence was significantly reduced in the $M_1/P_1/O$ and $M_{10}/P_1/O$ groups compared with the P/O group. This indicates the neuroprotective effects of BMSCs involve the mitochondrial pathway, associated with the effect of 6-OHDA at appropriate concentration to completely inhibit mitochondrial respiratory chain NADH dehydrogenase and cytochrome c oxidase activity[11]. Immunocytochemistry and western blot analysis showed that BMSCs exert protective effects against neurotoxicity by reducing abnormal α-SYN accumulation in PC12 cells. α-SYN accumulation can induce cell apoptosis by transporting and inhibiting mitochondrial complex I function, inducing mitochondrial membrane potential changes and reducing ATP and active oxygen production. Reduction in mitochondrial transmembrane potential results in opening of membrane transition pores and release of pro-apoptotic proteins from the mitochondria[5]. These proteins can induce apoptosis upon entering the cell cytoplasm[12]. In addition, α-SYN protofibril-like bacteriotoxin can form holes in the mitochondrial membrane, inducing transmembrane potential extension and release of proapoptotic factors, thereby promoting cell apoptosis[13-14]. Recent clinical studies demonstrate that overexpression and abnormal accumulation of α-SYN can aggravate PD through cell-cell interactions[15-17].

BMSCs attenuate 6-OHDA-induced injury in PC12 cell viability and function and the effect was enhanced with increasing percentage ratio of BMSCs to PC12 cells in co-cultures. BMSCs exert neuroprotection on PC12 cells by reducing abnormal accumulation of α-SYN, thus inhibiting cell apoptosis.

The present study provides important insight on the biological effects of BMSCs as a cell therapy for PD.

MATERIALS AND METHODS

Design

An *in vitro* comparative observation.

Time and setting

The experiment was performed in Fuwai Hospital for Cardiovascular Diseases, Chinese Academy of Medical Sciences, China, from December 2010 to October 2011.

Materials

Healthy adult, male Sprague-Dawley rats, weighing

200–300 g, purchased from Vital River Laboratories, Beijing, China (License No. SCXK (Jing) 2006-0009), were used to extract BMSCs. Experimental procedures were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China[18].

Methods

Cell culture

BMSCs were isolated and cultured as previously described[3-6]. The quality was determined (supplementary Figure 1 online).

The BMSCs and PC12 co-culture system was purchased from Millipore, Billerica, MA, USA. The co-culture method was as follows: PC12 cells in log phase were collected, treated in culture medium containing 3% fetal bovine serum overnight, followed by cell quantification on the next day. Cell density was adjusted to 2 × 10^5/well (96 well plate) and incubated with 6-OHDA for 24 hours. BMSCs at a ratio of 1:10, 1:1 or 10:1 with PC12 cells were added to the upper layer of the culture system, with 3–5 parallel wells in each group. After incubation with 5% CO_2 at 37°C for 24, 48 and 72 hours, the BMSCs in the upper layer were collected and the PC12 cells in the lower layer were detected.

MTT assay for PC12 cell viability

Four hours before the end of experimentation, 20 μL MTT solution (5 mg/mL, *i.e.* 0.5% MTT) was added to each well containing PC12 cultured cells (500 μL culture medium). Cells were incubated for 4 hours, then supernatants were discarded and 400 μL dimethyl sulfoxide was added to each well. Plates were shaken for 10 minutes to completely dissolve the crystal. Absorbance for each well was determined at 490 nm using an enzyme-linked immunosorbent assay reader (ELISA, Infinite™ 200 series Microplate Readers, Tecan, Ltd, Männedorf, Switzerland). Blank control wells were included. Cell viability (%) = (absorbance of experimental group – absorbance of blank group)/(absorbance of control group – absorbance of blank group) × 100%.

ELISA for dopamine secretion from PC12 cells

Dopamine secretion function of PC12 cells was detected according to the manufacturer's instructions (40-371-25013, GenWay Biotech, San Diego, USA). Briefly, antibody was diluted using 500× coating buffer overnight at 4°C. The well plate was rinsed the next day and standards and samples were added for incubation at 37°C for 1 hour. Negative control wells were included. Horseradish peroxidase-labeled antibody was added to each well, incubated at 37°C for 0.5–1 hour, rinsed, treated with 0.1 mL freshly prepared tetramethylbenzidine substrate coloration solution at 37°C for 10–30 minutes, followed by 2 M sulfuric acid to terminate the reaction. Absorbance of each well was determined at 405 nm using the Infinite™ 200 series Microplate Reader after the blank control well was zero adjusted. Dopamine content in the samples was determined according to the standard curve.

MitoLight staining for PC12 cell apoptosis

MitoLight staining was used to detect PC12 cell apoptosis in each group using flow cytometry. After co-culture for 48 hours, PC12 cells in the lower layer were collected by centrifugalization at 1 000 r/min for 5 minutes. Cell density was adjusted to 1 × 10^6/mL, treated with 100 μL MitoLight working solution (Cat No. APT142, Chemicon, Tamecula, CA, USA) in 5%CO_2 at 37°C for 20 minutes. The supernatant was discarded and 100 μL PBS was added to the cell precipitation, which was shaken to suspend the cells. Cells were transferred to special tubes for flow cytometry using FACSCalibur™ (BD Biosciences, San Jose, CA, USA).

Immunocytochemistry for α-SYN accumulation in PC12 cells

Immunocytochemistry was used to detect α-SYN expression and distribution in PC12 cells. Briefly, PC12 cells were cultured to 50% confluence on collagen (5 μg/cm^2)-coated coverslip (Solarbio, Beijing, China) in the lower layer of the culture plate. After cells had adhered, they were fixed with 3.7% paraformaldehyde (Sigma, St. Louis, MO, USA), permeabilized with 0.02% Triton X-100 (Amresco, Solon, Ohio, USA), blocked with 10% goat serum, hybridized with rabbit anti-rat α-SYN primary antibody (1:500; Zymed, San Francisco, CA, USA) at room temperature for 2 hours, rinsed, incubated with phycoerythrin-labeled goat anti-rabbit secondary antibody (Invitrogen, Carlsbad, CA, USA) at room temperature for 1 hour and observed and photographed using a Leica fluorescence microscope (DM2500-3HF-FL1, Germany).

Western blot assay for quantification of α-SYN protein expression

PC12 cells were collected and resuspended with cell lysate (containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 100 μg/mL phenylmethyl sulfonylfluoride, 33 μg/mL aprotinin and 1 mM sodium orthovanadate, pH 7.4) at 4°C for 30 minutes and centrifuged at 1 200 × *g* at 4°C for 10 minutes. The supernatant was collected and protein concentration was determined using the bicinchoninic acid protein assay kit (Solarbio). Protein of equal amounts from parallel wells of each group was mixed with 2 × sodium dodecyl sulfate sample buffer and boiled for 5 minutes. Samples of 10 μL were electrophoresed in sodium dodecyl sulfate

polyacrylamide gel, with a separation gel concentration of 10%. Proteins were transferred to a membrane at 50 mA for 1 hour, blocked with 5% defatted milk powder for 2 hours, incubated with primary antibody rabbit anti-rat α-SYN (1:800, Zymed, USA) and β-actin (1:1 000, Zymed), agitated at room temperature for 2 hours, rinsed 3 times with Tris-buffered saline Tween-20 for 5 minutes each, followed by exposure to horseradish peroxidase-labeled goat anti-rabbit secondary antibody (1:1 000; Beijing Zhongshan Biotechnology, Beijing, China) at room temperature for 1 hour. Immunoreactive bands were detected using Super signal femto chemiluminescent detection reagent (Pierce, Rockford, IL, USA) and α-SYN protein expression was quantitatively analyzed using an image analysis system (Sigma Scan Pro. SPSS, Inc. Chicago, IL, USA).

Statistical analysis

Data were collected from at least three independent experiments and analyzed using SPSS 11.0 package (SPSS, Chicago, IL, USA). Data were expressed as mean ± SD and compared using a Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

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Author contributions: Yue Tang was in charge of funds, conceived and designed the study, and revised the manuscript. Yongchun Cui conceived and designed the study, analyzed experimental data and drafted the manuscript. Fuliang Luo conducted statistical analysis. Xiaopeng Liu and Zhong Tian analyzed the data. Xiaojuan Wang guided the study. Aili Wu revised the manuscript. Junwei Zhao provided and integrated experimental data. Like Wu conceived and designed the study, provided technical support and revised the manuscript.

Conflicts of interest: None declared.

Ethical approval: This study received permission from the Animal Ethics Committee of Fuwai Hospital for Cardiovascular Diseases, Chinese Academy of Medical Sciences, China.

Supplementary information: Supplementary data associated with this article can be found in the online version, by visiting www.nrronline.org and entering Vol. 7, No. 14, 2012 after selecting the "NRR Current Issue" button on the page.

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