

MFN2 ameliorates cell apoptosis in a cellular model of Parkinson's disease induced by rotenone

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Abstract. A number of studies indicated that apoptosis, a specific type of programmed cell death, contributed to the loss of dopaminergic neurons during progression of Parkinson's disease (PD). Previously, the authors of the present study demonstrated that apoptosis of dopaminergic neurons was mainly achieved via the mitochondria-mediated apoptosis pathway, however, the precise molecular mechanisms remain to be elucidated. The present study aimed to determine whether mitofusin-2 (MFN2), a mitochondrial protein, participated in the apoptosis of dopaminergic neurons in a cellular model of PD induced by rotenone. The present study demonstrated that the expression of MFN2 was relatively stable following treatment with rotenone. Lentiviral knockdown and overexpression experiments for the first time, to the best of the authors knowledge, revealed that MFN2 prevented rotenone-induced cell death by amelioration of apoptosis. These results revealed a protective role of MFN2 against apoptosis in an *in vitro* model of PD and may be used to establish MFN2 as a potential therapeutic target for the treatment of this disease.

Introduction

As the second most common type of neurodegenerative disorder, Parkinson's disease (PD) is characterized by evident motor symptoms including rigidity, bradykinesia, resting tremor and postural instability (1). These symptoms are mainly due to the selective dopaminergic neuron loss within basal ganglia structures (2).

Previous studies indicated that apoptosis, a specific type of programmed cell death, contributed to the loss of dopaminergic neurons during PD progression (3,4). In mammalian cells, apoptosis is closely modulated by two different signaling pathways including the receptor-dependent apoptotic pathway and the mitochondria-mediated apoptotic pathway (5). Previously, the authors of the present study demonstrated that apoptosis of dopaminergic neurons was primarily achieved via the mitochondria-mediated apoptotic pathway (6), however, the precise molecular mechanisms remain to be elucidated.

A member of the family of GTPases, mitofusin-2 (MFN2), is widely distributed in numerous tissues including heart, kidney, liver and brain (7). At the subcellular level, MFN2 is located predominantly in the outer membrane of the mitochondria, and is required for mitochondrial fusion, integrity and metabolism (8,9). Previous reports suggested that MFN2 was involved in the process of mitochondria-mediated apoptosis and, therefore, contributed to the pathogenesis of several neurological disorders including Charcot-Marie-Tooth disease (CMT), ischemic stroke and Alzheimer's disease (10-12). However, the role of MFN2 in the apoptosis of dopaminergic neurons during the progression of PD remains unclear.

The present study therefore aimed to assess whether MFN2 participates in dopaminergic neuronal apoptosis using a cellular model of PD induced by rotenone (13).

Materials and methods

Cell culture. A human neuroblastoma cell line SH-SY5Y was purchased from American Type Culture Collection (Manassas, VA, USA; stock no. CRL-2266). Cells were cultured in RPMI 1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal calf serum (Thermo Fisher Scientific, Inc.) containing 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere of 5% CO₂ at 37°C, as previously described (13).

Lentiviral particles and cell transduction. For MFN2 knockdown and overexpression, lentiviral particles containing MFN2 short hairpin (sh) RNA (cat. no. sc-43928-V), lentiviral particles encoding human wild-type MFN2 cDNA (sc-400536-LAC) and their control lentiviral particles (cat. nos. sc-108080 and sc-437282, respectively) were purchased from Santa Cruz

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Biotechnology, Inc. (Dallas, TX, USA). The cell transduction was performed as previously described (14). The efficiency of transduction was determined by western blotting 72 h later as described below.

Rotenone treatment. SH-SY5Y cells were treated with vehicle (0.01% DMSO) and rotenone (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany; 20, 100 or 500 nM dissolved in 0.01% DMSO) for 12 h, as previously described (15). Subsequently, the cells were collected and used for the experiments described below.

Cell viability assay. The viability of SH-SY5Y cells was assessed by the MTT method, as previously described (4). The SH-SY5Y cells were seeded at a density of 1.5×10^5 cells/cm² and received the indicated treatment. Subsequently, MTT was added to the culture medium to reach a final concentration of 0.5 mg/ml. After incubation at 37° for 4 h, the culture medium containing MTT was removed. DMSO was added into each well and the absorbance was measured at a wavelength of 490 nm using a microplate reader.

Colorimetric assay for caspase-3 activity. A colorimetric assay was carried out as previously described (3). The SH-SY5Y cells were seeded at a density of 1.5×10^5 cells/cm². After receiving the indicated treatment, the cells were harvested and lysed in an extraction buffer (included in the caspase-3 colorimetric assay kit mentioned below). The activity of caspase-3 was detected by a colorimetric assay kit (cat. no. ab39401; Abcam, Cambridge, UK) according to the manufacturer's protocol.

Western blotting. Western blotting was carried out as previously described (16). The SH-SY5Y cells were seeded at a density of 1.5×10^5 cells/cm² and received the indicated treatment. Subsequently, the cells were harvested and lysed in an extraction buffer containing complete protease inhibitor cocktail. Samples with equal amounts of protein were separated via SDS-PAGE on a 10% gel, transferred to nitrocellulose membranes and blocked with 5% bovine serum albumin (Thermo Fisher Scientific, Inc.). Following washing, membranes were incubated with a primary antibody against MFN2 (cat. no. ab101055; 1:500; Abcam) at 4°C overnight, washed again and incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (cat. no. A0208; 1:1,500; Beyotime Institute of Biotechnology, Haimen, China) for 2 h. Following washing, protein bands were detected with a chemiluminescent HRP substrate and exposed to an X-ray film. The signal intensity of primary antibody binding was analyzed using Quantity One software 4.6.2 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and normalized to a loading control GAPDH (cat. no. sc-47724; 1:1,000; Santa Cruz Biotechnology, Inc.).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The SH-SY5Y cells were seeded at a density of 1.5×10^5 cells/cm² and received the indicated treatment. Subsequently, total RNA was extracted from SH-SY5Y cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Equal amounts of total RNA were reverse transcribed in a final volume of 10 µl with random primers under standard

conditions (described in the manufacturer's protocol) using the PrimeScript® RT Master Mix (Takara Biotechnology Co., Ltd., Dalian, China). Reverse transcription reaction was carried out as previously described (17). Subsequently, qPCR reactions were performed with SYBR® Premix Ex Taq™ (Takara Biotechnology Co., Ltd.) and a specific primer (forward: 5'-TGGCTCAAGACTATAAGCTGCG-3', reverse: 5'-GAG GACTACTGGAGAAGGGTGG-3') to detect MFN2 mRNA expression levels. The thermo cycling conditions were the same as previously described (17). GAPDH (forward: 5'-AAG GTGAAGGTCGGAGTCAAC-3', reverse: 5'-GGGGTCATT GATGGCAACAATA-3') was used as an internal control. Relative levels were determined using the $2^{-\Delta\Delta C_q}$ method (18).

Statistical analysis. Data are presented as the mean ± standard deviation of at least four independent experiments. Statistical significance was detected by one-way analysis of variance followed by Turkey's post hoc test using SPSS software 18.0 (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Apoptosis contributes to the decrease in viability of SH-SY5Y cells induced by rotenone. The effects of rotenone on the viability of SH-SY5Y cells were assessed in the present study. Cells were incubated with different doses of rotenone (20, 100 and 500 nM), and MTT assay was used to evaluate cell viability after 12 h. Rotenone (100 and 500 nM) significantly reduced the viability of SH-SY5Y cells by 38.3% ($P < 0.05$) and 45.6% ($P < 0.05$), respectively (Fig. 1A). This result implied that rotenone induced cell death in a dose-dependent manner. To investigate whether apoptosis contributed to the rotenone-induced cell death, activity of caspase-3, a crucial executioner of apoptosis, was detected using a colorimetric assay kit. Rotenone (100 and 500 nM) significantly increased caspase-3 activity 2.91-fold ($P < 0.05$) and 3.47-fold ($P < 0.05$), respectively (Fig. 1B). These observations indicated that apoptosis contributed to the death of SH-SY5Y cells induced by rotenone.

Expression of MFN2 in SH-SY5Y cells is stable following treatment with rotenone. The effect of rotenone on the expression of MFN2 was detected in SH-SY5Y cells. Treatment with rotenone (20, 100 and 500 nM) did not significantly affect the mRNA and protein expression levels of MFN2 compared with the vehicle control (Fig. 2A-C).

Knockdown of MFN2 exacerbates apoptosis induced by rotenone in SH-SY5Y cells. To further investigate the role of MFN2 in rotenone-induced apoptosis, a lentiviral transduction strategy was used to knock down MFN2 expression in SH-SY5Y cells. The alterations in MFN2 protein expression levels were confirmed by western blotting (Fig. 3A and B). Subsequently, cells were incubated with 100 nM rotenone for 12 h. Knockdown of MFN2 further increased the activity of caspase-3 1.72-fold ($P < 0.05$) in SH-SY5Y cells following treatment with rotenone, compared with the control shRNA group (Fig. 3C). Furthermore, MFN2 knockdown further reduced the viability of SH-SY5Y cells by 52% ($P < 0.05$) following

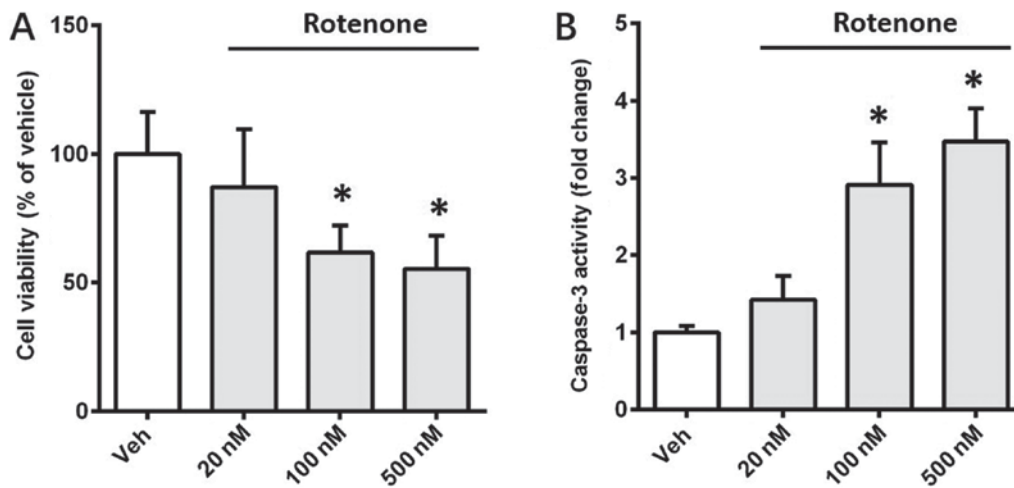


Figure 1. Apoptosis contributes to the death of SH-SY5Y cells induced by rotenone. SH-SY5Y cells were incubated with different doses of rotenone (20, 100 and 500 nM) for 12 h. (A) MTT assay was used to evaluate the viability of SH-SY5Y cells. Data are presented as a percentage of the vehicle control group. (B) A colorimetric assay was used to detect the activity of caspase-3 in SH-SY5Y cells. Data are presented as a fold change relative to the vehicle control group. All figures are representative of at least four independent experiments. All data were analyzed by one-way analysis of variance followed by Tukey's post hoc test. Values are presented as the mean \pm standard deviation. * P <0.05 vs. the vehicle control group. Veh, vehicle control.

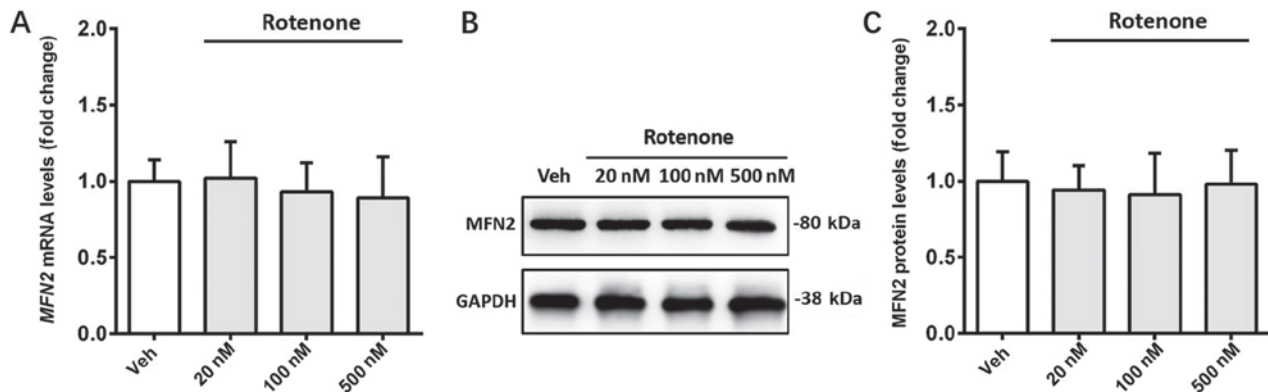


Figure 2. Expression of MFN2 in SH-SY5Y cells is stable following treatment with rotenone. SH-SY5Y cells were incubated with different doses of rotenone (20, 100 and 500 nM) for 12 h. (A) mRNA expression levels of MFN2 were detected by reverse transcription-quantitative polymerase chain reaction. Data were expressed as a fold change relative to vehicle control group. GAPDH was used as an internal control. Protein expression levels of MFN2 were (B) detected by western blotting and (C) quantified. Data are presented as a fold change relative to the vehicle control group. GAPDH was used as a loading control. All data were analyzed by one-way analysis of variance followed by Tukey's post hoc test. Values are presented as the mean \pm standard deviation. MFN2, mitofusin-2; veh, vehicle control.

treatment with rotenone, compared with the control shRNA group (Fig. 3D). Knockdown of MFN2 did not significantly affect the viability of SH-SY5Y cells untreated with rotenone (data not shown). Taken together, the above results indicated that MFN2 knockdown exacerbated cell apoptosis caused by rotenone, suggesting that MFN2 may serve a protective role against rotenone-induced cell apoptosis.

Overexpression of MFN2 ameliorates apoptosis induced by rotenone in SH-SY5Y cells. To validate the protective role of MFN2 against rotenone-induced cell apoptosis, MFN2 was overexpressed in SH-SY5Y cells using a lentiviral strategy. The alterations in MFN2 protein levels were confirmed by western blotting (Fig. 4A and B). Subsequently, cells were incubated with 100 nM rotenone for 12 h. The increase in caspase-3 activity induced by rotenone was attenuated by MFN2 overexpression (P <0.05; Fig. 4C). Furthermore, overexpression of MFN2 ameliorated the rotenone-induced reduction

in cell viability (P <0.05; Fig. 4D). MFN2 overexpression did not significantly affect the viability of SH-SY5Y cells without rotenone treatment (data not shown).

Discussion

Rotenone, a mitochondrial complex I inhibitor, was reported to reproduce a number of neuropathological features of PD, including loss of dopaminergic neurons (19). In the present study, rotenone resulted in decreased viability of human neuroblastoma SH-SY5Y cells, in a dose-dependent manner. Furthermore, expression levels of caspase-3, a crucial executioner of apoptosis, were markedly increased following treatment with rotenone, suggesting that apoptosis contributed to the loss of SH-SY5Y cells. These results are in agreement with previous reports by the authors of the present study, where rotenone induced apoptosis in human and mouse dopaminergic cell lines (13,15). It has been previously demonstrated that apoptosis

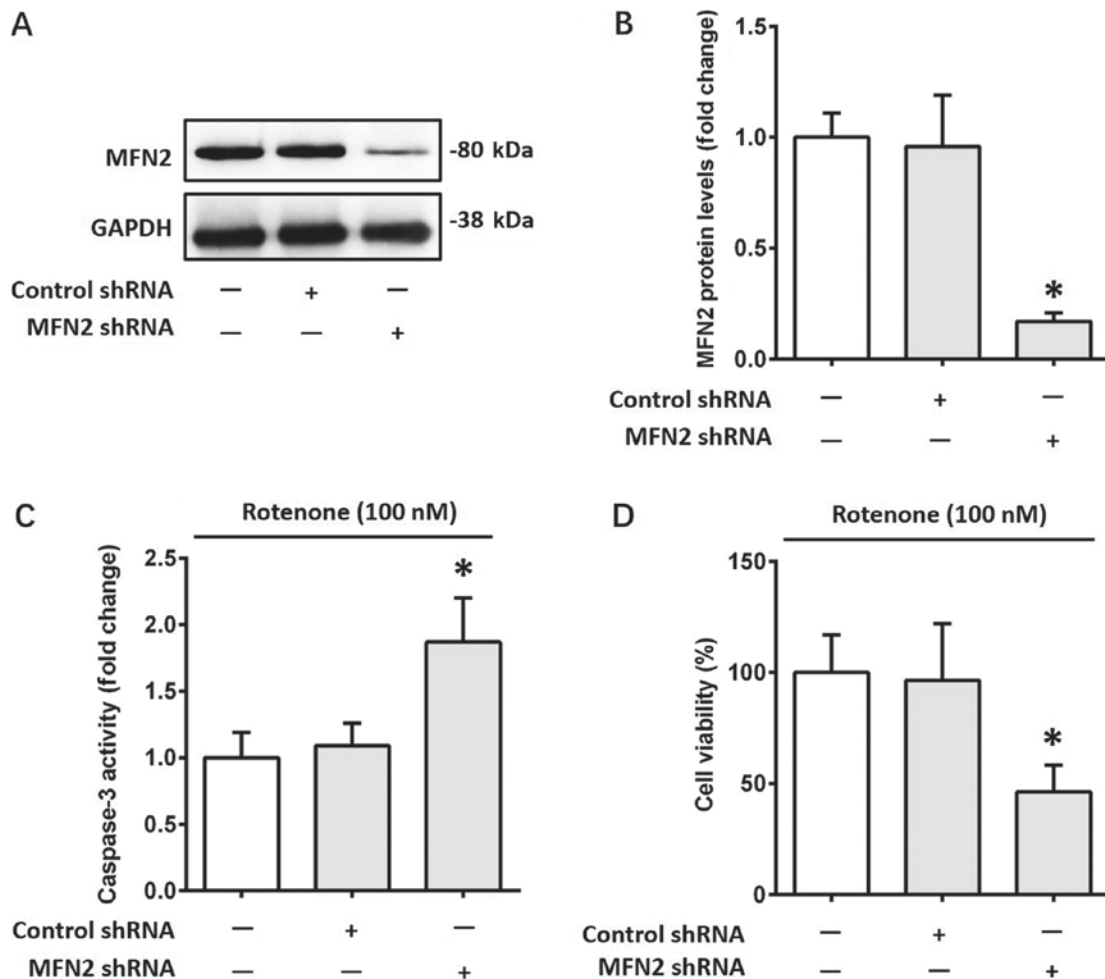


Figure 3. Knockdown of MFN2 exacerbates apoptosis induced by rotenone in SH-SY5Y cells. A lentiviral strategy was used to knock down the expression of MFN2 in SH-SY5Y cells. (A) The alterations in MFN2 protein levels were confirmed by western blotting. (B) The protein expression levels were quantified. Subsequently, the SH-SY5Y cells were incubated with 100 nM rotenone for 12 h. (C) A colorimetric assay was used to detect the activity of caspase-3 in SH-SY5Y cells. Data are presented as a fold change relative to untransfected cells. (D) MTT assay was used to evaluate the viability of SH-SY5Y cells. Data are presented as a percentage of the untransfected group. All data were analyzed by one-way analysis of variance followed by Tukey's post hoc test. Values are presented as the mean \pm standard deviation. * $P < 0.05$ vs. the control shRNA group. shRNA, short hairpin RNA; MFN2, mitofusin-2.

is closely modulated by two pathways: The receptor-dependent apoptotic pathway and the mitochondria-mediated apoptotic pathway (5). Recently, the authors of the present study suggested that apoptosis of dopaminergic neurons was primarily achieved via the mitochondria-mediated apoptotic pathway, since cytochrome c, a mitochondrial pro-apoptotic factor, was observed in the cytoplasm of dopaminergic neurons (6). However, the underlying molecular mechanisms remain unclear.

MFN2, a mitochondrial protein that belongs to the family of GTPases, is widely distributed in numerous tissues and organs including heart, kidney, liver, and brain (7). At the subcellular level, MFN2 is located predominantly in the outer membrane of the mitochondria (8). A previous study has indicated that MFN2 was required for several physiological processes of mitochondria including fusion and metabolism (9). Furthermore, several lines of evidence indicated that MFN2 may be involved in the process of mitochondria-mediated apoptosis and participated in the pathogenesis of several neurological disorders (10-12). Loss-of-function mutations of MFN2 gene led to the onset of CMT type 2A, a neurological disease characterized by the degeneration of axons in the

peripheral nervous system (20). In an *in vitro* model of ischemic stroke, decreased MFN2 expression was closely associated with increased neuronal apoptosis (21). In addition, reduced MFN2 level was associated with increased oxidative stress and apoptosis of neurons during neurodegeneration (22,23). In the present study, knockdown of MFN2 in a cellular model of PD induced by rotenone aggravated cell apoptosis. The above results implied a protective role of MFN2 against apoptosis. To further validate the antiapoptotic effect of MFN2 in the *in vitro* model of PD, MFN2 was overexpressed in SH-SY5Y cells prior to treatment with rotenone. For the first time to the best of the authors' knowledge, it was demonstrated that MFN2 ameliorated apoptosis induced by rotenone. This observation was supported by previous reports that MFN2 overexpression attenuated neuronal apoptosis under the conditions of ischemic stroke or neurodegeneration (21-23), indicating the antiapoptotic effect of MFN2.

The present study has certain limitations. The antiapoptotic effect of MFN2 was evaluated in a cellular model of PD. Therefore, the results require further confirmation *in vivo* using animal models of PD. Additionally, the precise signaling

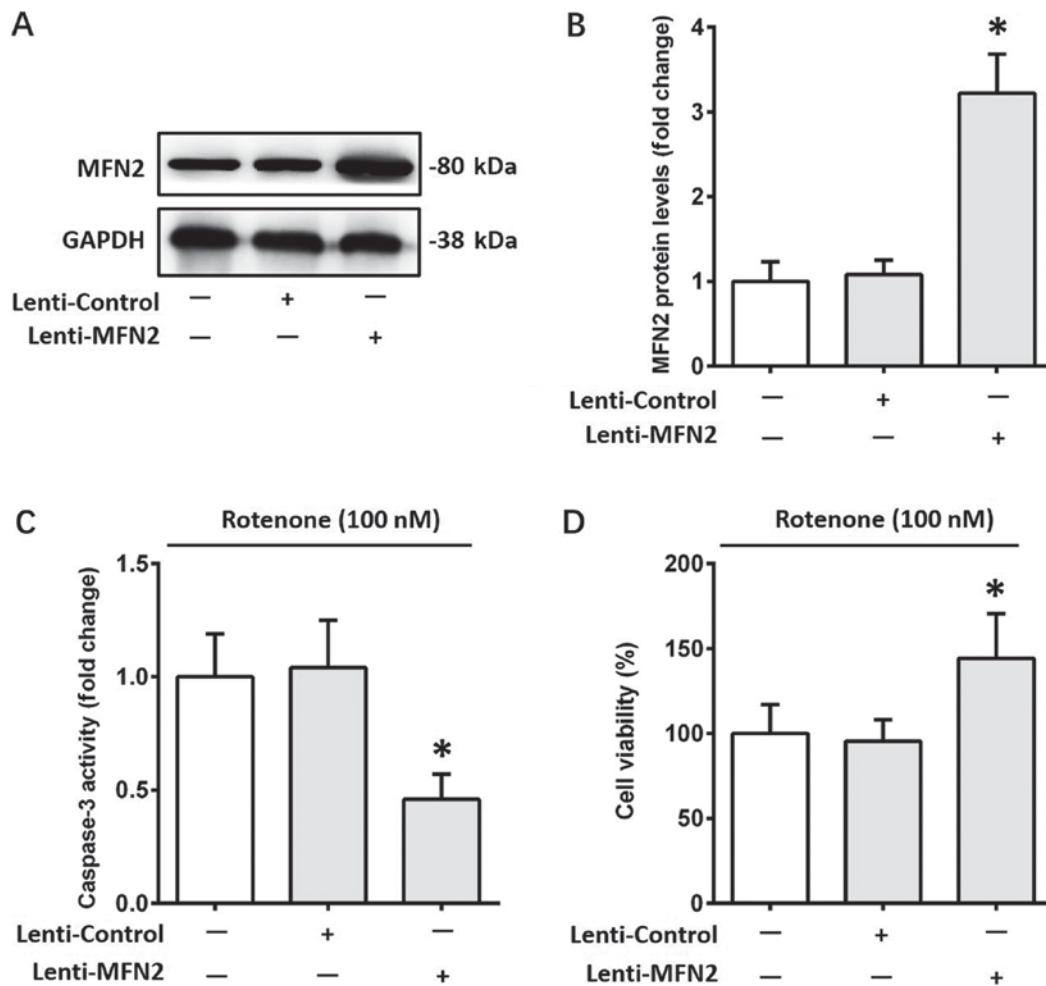


Figure 4. Overexpression of MFN2 ameliorates apoptosis induced by rotenone in SH-SY5Y cells. A lentiviral strategy was used to overexpress MFN2 in SH-SY5Y cells. The alterations in MFN2 protein levels were (A) confirmed by western blotting and (B) quantified. Subsequently, the SH-SY5Y cells were incubated with 100 nM rotenone for 12 h. (C) A colorimetric assay was used to detect the activity of caspase-3 in SH-SY5Y cells. Data are presented as a fold change relative to untransfected cells. (D) MTT assay was used to evaluate the viability of SH-SY5Y cells. Data are presented as a percentage of the untransfected group. All data were analyzed by one-way analysis of variance followed by Tukey's post hoc test. Values are presented as the mean \pm standard deviation. * $P < 0.05$ vs. the control lentivirus group. Lenti, lentivirus; MFN2, mitofusin-2.

underlying the antiapoptotic effect of MFN2 was not identified in the present study and this should be investigated in the future.

In conclusion, the present study indicated that the expression of MFN2 was stable following treatment with rotenone in a cellular model of PD. Using a lentiviral knockdown and overexpression strategy, it was demonstrated that MFN2 prevented rotenone-induced cell death by amelioration of apoptosis. These results revealed a protective role of MFN2 against apoptosis in an *in vitro* model of PD and may be used to establish MFN2 as a potential therapeutic target for the treatment of this disease.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

YZ and TJ designed the present study. YY and LX performed the experiments. ZO and XX analyzed the data and prepared all figures. TJ wrote the manuscript. All authors have read and approved this manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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