

Downregulation of miR-21 suppresses 1-methyl-4-phenylpyridinium-induced neuronal damage in MES23.5 cells

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Abstract. Accumulating evidence suggests that overproduction of oxidative stress, increases neuroinflammation and activates apoptosis. These two processes are associated with the development of Parkinson's disease (PD). The present study aimed to investigate the role of miR-21 in the development of PD. 1-Methyl-4-phenylpyridinium (MPP⁺) was used to induce a PD-like model in MES23.5 cells. The results of the reverse transcription-quantitative PCR assays indicated that miR-21 levels were markedly increased in MES23.5 cells following MPP⁺ treatment. Furthermore, MES23.5 cells were transfected with miR-21 inhibitor, mimics and/or relevant negative control, following MPP⁺ administration. The results of the functional assays revealed that downregulation of miR-21 significantly attenuated the induction of cell apoptosis and reactive oxygen species (ROS) production, while it enhanced the survival of MPP⁺-induced MES23.5 cells. Furthermore, downregulation of miR-21 increased the expression levels of tyrosine hydroxylase, whereas suppression of miR-21 inhibited the production of pro-inflammatory cytokines [interleukin (IL)-6, IL-1 β and tumor necrosis factor- α] in MES23.5 cells. Western blot analysis further indicated that the Bcl-2/Bax protein expression ratio was significantly increased and double luciferase assay analysis confirmed that Bcl-2 was a direct target of miR-21. Taken collectively, the data demonstrated that downregulation of miR-21 protected cells from MPP⁺-mediated cytotoxicity by the inhibition of apoptosis induction, the reduction of the inflammatory response and the suppression of ROS production. The present findings may provide novel approaches for PD clinical treatment.

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by selective loss of dopaminergic neurons in the substantia nigra. This process leads to dopamine (DA) depletion in the striatum affecting several million patients worldwide, notably older adults (1). Although the biochemical and molecular pathogenesis of the loss of dopaminergic neurons in PD have not yet been fully characterized, a number of biochemical processes and molecular mechanisms have been identified as mediators of neuronal cell death in PD, including the overproduction of oxidative stress, the increase in neuroinflammation and the activation of the apoptotic cascade (2). Oxidative stress and apoptosis have been shown to play central roles in the degeneration of dopaminergic neurons in PD (2-4). Therefore, the exploration of novel molecular mechanisms involved in oxidative stress and apoptosis is of great significance for the treatment of PD.

MicroRNAs (miRNAs/miRs), are a class of endogenous highly conserved noncoding RNA molecules, which are ~22 nucleotides in length (5). By binding directly to the 3'-UTR (3'-untranslated region) of target mRNAs, miRNAs are involved in a series of physiological and pathological processes (6). Accumulating evidence suggests that the dysregulation of miRNA plays an important role in the pathogenesis of neurodegenerative disorders, including PD (7,8). Fu *et al* (9) demonstrated that docosahexaenoic acid upregulated the expression of Peroxisome Proliferator Activated Receptor α by inhibiting miR-21 in SH-Y5Y cells. Moreover, a recent study showed that the levels of miR-21 in PD models were significantly higher than those in normal controls in SH-SY5Y cells (10). This evidence suggests that miR-21 may play an important role in PD, while the underlying mechanism remains unclear.

In the present study, the classic neurotoxin 1-methyl-4-phenylpyridinium (MPP⁺) was used to produce a PD cell model *in vitro*. This compound is selectively transported into dopaminergic neurons via the DA transporter and localizes in the mitochondria (11). The aim of the current study was to investigate the role of miR-21 in MPP⁺-induced neurotoxicity of MES23.5 cells and to prove the mechanism of miR-21 in PD to provide novel approaches for clinical treatment.

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Materials and methods

Cell culture and treatment. MES23.5 cells were cultured in DMEM/F12 (Sigma-Aldrich; Merck KGaA) supplemented with 5% fetal bovine serum (Thermo Fisher Scientific, Inc.), 2% of 50X of Sato's solution (Thermo Fisher Scientific, Inc.) at 37°C in a humidified (70–80%) atmosphere containing 5% CO₂. The cells were seeded in 96-well plates at a density of 1×10⁵ cells/well and treated with different concentrations of MPP⁺ (100, 200 or 300 μM) for 3, 6, 12 or 24 h to optimize the experimental conditions.

Experimental groups were as follows: Control group, MES23.5 cells with no treatment and the model group, MES23.5 cells with 200 μM MPP⁺ treatment for 24 h.

Transfection. miR-21 inhibitor (100 nM) and negative control (NC, 100 nM) sequences were obtained from GenePharma and were transfected into MES23.5 cells using Lipofectamine[®] 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The sequences of the inhibitor and the NC samples were as follows: miR-21 inhibitor (5'-UCAACAUCAGUCUGAUAAGCUA-3'), and NC (5'-CAGUACUUUUGU GUAGUACAA-3'). At 24-h following transfection, the cells were treated with 200 μM of MPP⁺ for an additional 24 h at 37°C. Subsequently, the cells were harvested for further experiments. Experimental groups were as follows: Control group, MES23.5 cells with 200 μM MPP⁺ treatment and no transfection; NC group, MES23.5 cells with 200 μM MPP⁺ treatment and inhibitor NC and miR-21 inhibitor group, MES23.5 cells with 200 μM MPP⁺ treatment and miR-21 inhibitor.

Cell Counting Kit-8 (CCK-8) assay. The CCK-8 assay was used to assess cell viability. The cells were seeded in 96-well plates (2×10⁴ cells/well) and incubated at 37°C for 24 h. At 3, 6, 12 and 24-h following MPP⁺ treatment (100, 200 and 300 μM) or MPP⁺ treatment (200 μM) for 24, 48 and 72-h, the cell proliferation indices were measured using a CCK-8 kit (cat. no. C0038, Beyotime Institute of Biotechnology), according to the manufacturer's protocol. The optical density was measured at 450 nm.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies; Thermo Fisher Scientific, Inc.). Single-stranded cDNA was synthesized using the TaqMan MicroRNA Reverse Transcription Kit (Takara) with the special stem-loop primers. The reverse transcription conditions were following 25°C for 10 min, 45°C for 30 min and 5 min at 95°C. RT-qPCR was performed using a Perfect Real Time SYBR Premix Ex Taq Kit (Takara Bio) with an ABI 7500 thermocycler (Thermo Fisher Scientific, Inc.). All procedures were performed according to the manufacturer's protocol. U6 was used as control for the expression levels of miR-21. The reaction conditions for PCR were as follows: Pre-denaturation at 95°C for 3 min and 40 cycles of denaturation at 95°C for 30 sec and annealing at 60°C for 30 sec. The relative expression levels of each gene were calculated by the 2^{-ΔΔC_q} method (12). miRNA-specific reverse transcription primers and quantitative PCR primers were obtained from RiboBio Co. Ltd. The following primer sequences were used: miR-21 stem-loop primer, 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGACTGGATACGACTCAACA-3'

forward, 5'-CACGCACGCATAGCTTATCAGACT-3' and reverse, 5'-CCAGTGCAGGGTCCGAGGTA-3'. U6, stem-loop primer, 5'-GTCGTATCCAGTGCAGGGTCCGAGGTGCAC TGGATACGACAAAATATGG-3', forward, 5'-TGCGGGTGC TCGCTTCGGCAGC-3' and reverse, 5'-CCAGTGCAGGGT CCGAGGTA-3'.

Immunocytochemistry. MES23.5 cells (1×10⁵ cells/well) were fixed with 4% paraformaldehyde for 30 min at 4°C and permeabilized with 0.5% Triton X-100 for 20 min at room temperature. Following blocking with 5% goat serum (Gibco; Thermo Fisher Scientific, Inc.) for 1-h at room temperature and incubation with mouse anti-tyrosine hydroxylase (TH; cat. no. T2928; 1:500; Sigma-Aldrich; Merck KGaA) at 4°C overnight, the cells were further incubated with biotinylated goat anti-mouse secondary antibody (cat. no. A0286; 1,1000; Beyotime Institute of Biotechnology) for 15 min at 37°C. Subsequently, the samples were washed with PBS three times for 15 min at 37°C and visualized by diaminobenzidine (DAB). TH positive cells were counted in three randomly selected images (magnification, x200) using an Olympus Fluoview FV5000 microscope (Thermo Fisher Scientific, Inc.).

Detection of ROS levels. The ROS levels were assessed using the fluorescent dye 2',7'-dichlorofluorescein diacetate (DCFDA; D6883, Sigma-Aldrich; Merck KGaA). MES23.5 cells were seeded at a density of 2×10⁴ cells/well in 96-well plates. The cells were incubated with 2.5 μM of DCFDA for 15 min at 37°C. DCFDA fluorescence was acquired by confocal microscopy at excitation and emission wavelengths of 485 and 535 nm, respectively (Carl Zeiss LSM 700 Meta confocal microscope; Zeiss). The data were analyzed by the Carl Zeiss confocal laser scanning microscopes with the ZEN 2008 software.

ELISA. The culture medium of MES23.5 cells was collected. The levels of IL-6, IL-1β and tumor necrosis factor-α (TNF-α) were measured by the Interleukin-1β ELISA kit (cat. no. ab100562; Abcam) according to the manufacturer's protocol.

Cell apoptosis analysis. An Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (NanJing KeyGen Biotech Co., Ltd) was used to measure cell apoptosis according to the manufacturer's protocol. The samples were analyzed by flow cytometry. A total of 100 nM miR-21 inhibitor and NC sequences were transfected with MES23.5 cells for 24 h at 37°C then the cells were treated with MPP⁺ for an additional 24 h. The cells were subsequently harvested, washed twice with ice-cold PBS and suspended using binding buffer (500 μl; Thermo Fisher Scientific, Inc.). Subsequently, the cells were stained using (Annexin-V-FITC) reagent and propidium iodide (PI). The cells were analyzed by a FACSCalibur flow cytometer with the Cell Quest software (version 3.1; Becton Dickinson).

Western blot analysis. Total protein extraction was performed using RIPA lysis buffer (Beyotime Institute of Biotechnology). The protein concentration was measured using the bicinchoninic acid kit (Bio-Rad Laboratories, Inc.). The protein samples (20 μg/lane) were separated by SDS-PAGE (12% resolving gels, 4% stacking gels) and blotted onto polyvinylidene difluoride membranes (EMD Millipore). The membranes were blocked

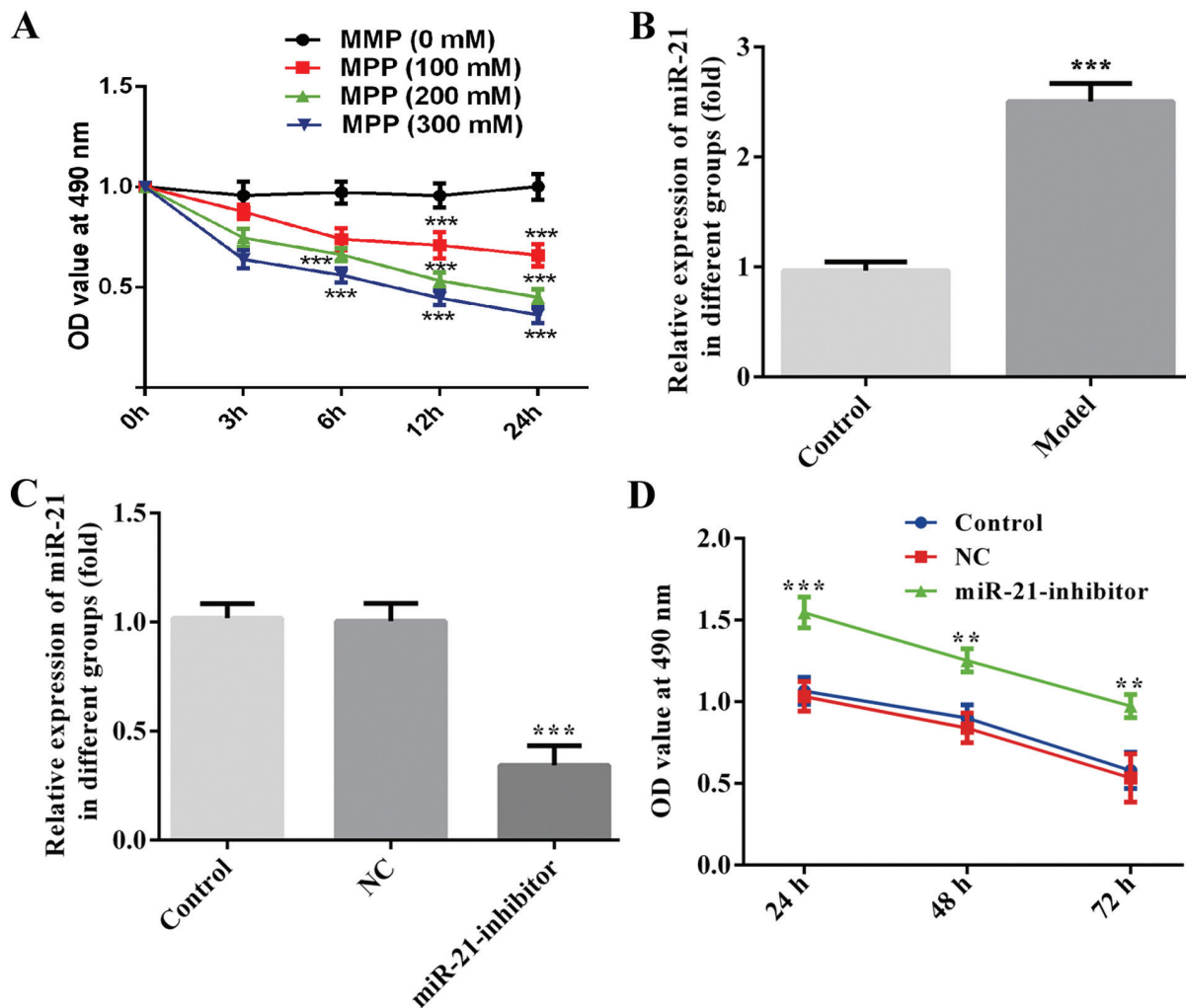


Figure 1. Downregulation of miR-21 enhanced cell survival in MPP⁺-treated MES23.5 cells. MES23.5 cells were treated with MPP⁺ at the concentrations of 100, 200 and 300 μ M. The CCK-8 assay was used to assess cell viability at 0, 3, 6, 12 or 24 h following MPP⁺ treatment. (A) Reverse transcription-quantitative PCR was performed to measure miR-21 levels in MES23.5 cells with MPP⁺ treatment (B) and miR-21inhibitor transfection. (C) CCK-8 assay was used to assess cell viability at 24, 48 and 72 h following transfection of the cells with the miR-21inhibitor (D) **P<0.01, ***P<0.001 vs. control. CCK-8, cell counting kit-8; MPP⁺, 1-methyl-4-phenylpyridinium; miR, microRNA; NC, negative control.

using 5% non-fat milk for 1-h at room temperature, followed by overnight incubation at 4°C with the indicated antibodies against Bax (cat. no. MAB4601, 1:200, EMD Millipore) and Bcl-2 (cat. no. 05-826, 1:500, EMD Millipore). Subsequently, the membranes were incubated with secondary rabbit anti-mouse IgG-horseradish peroxidase antibodies (cat. no. sc-358914, 1:5,000, Santa Cruz Biotechnology, Inc.) at room temperature for a further 2-h. Then, membranes were washed with Tris-buffered saline and Polysorbate 20 seven times (3 min per wash). Chemiluminescent signals were visualized using the enhanced chemiluminescence detection reagent (EMD Millipore). The results were analyzed using ImageJ software 1.4 (National Institutes of Health).

Dual-luciferase reporter assay. TargetScanHuman (www.targetscan.org) was used to predict the putative target site of Bcl-2. The mutant type of the Bcl-2 3'UTR sequence was constructed using a QuickChange Multi Site-Directed Mutagenesis kit (Agilent Technologies, Inc.), according to the manufacturer's protocol. The wild type or mutant types of the Bcl-2 3'UTR sequences were cloned into the firefly

luciferase reporter pGL3-promoter vector (Promega Corp.) to generate the recombinant, wild type (3'UTR-WT) or mutant type (3'UTR-MUT) pGL3-Bcl-2-3'UTR luciferase plasmids. MES23.5 cells (1×10^5 cells/well) were cultured in 24-well plates for 24 h at room temperature, and the cells were co-transfected with 50 ng of 3'UTR-WT (or 3'UTR-MUT) vector and 20 μ M of miR-21 (or NC) mimics (Shanghai GenePharma Co., Ltd.) using LipofectamineTM 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. At 24 h following transfection, the Dual-Luciferase Reporter Assay System (Promega Corp.) was used to determine the luciferase activity, which was normalized to *Renilla* luciferase activity.

Statistical analysis. The data are presented as the mean \pm standard error of the mean. GraphPad Prism version 6 (GraphPad Software, Inc.) was used to perform the statistical analyses. The differences between two groups were carried out by a two-tailed Student's t-test. One-way analysis of variance followed by Bonferroni's multiple comparison tests was used to compare differences between means in more than two groups. P<0.05 was considered to indicate a statistically significant difference.

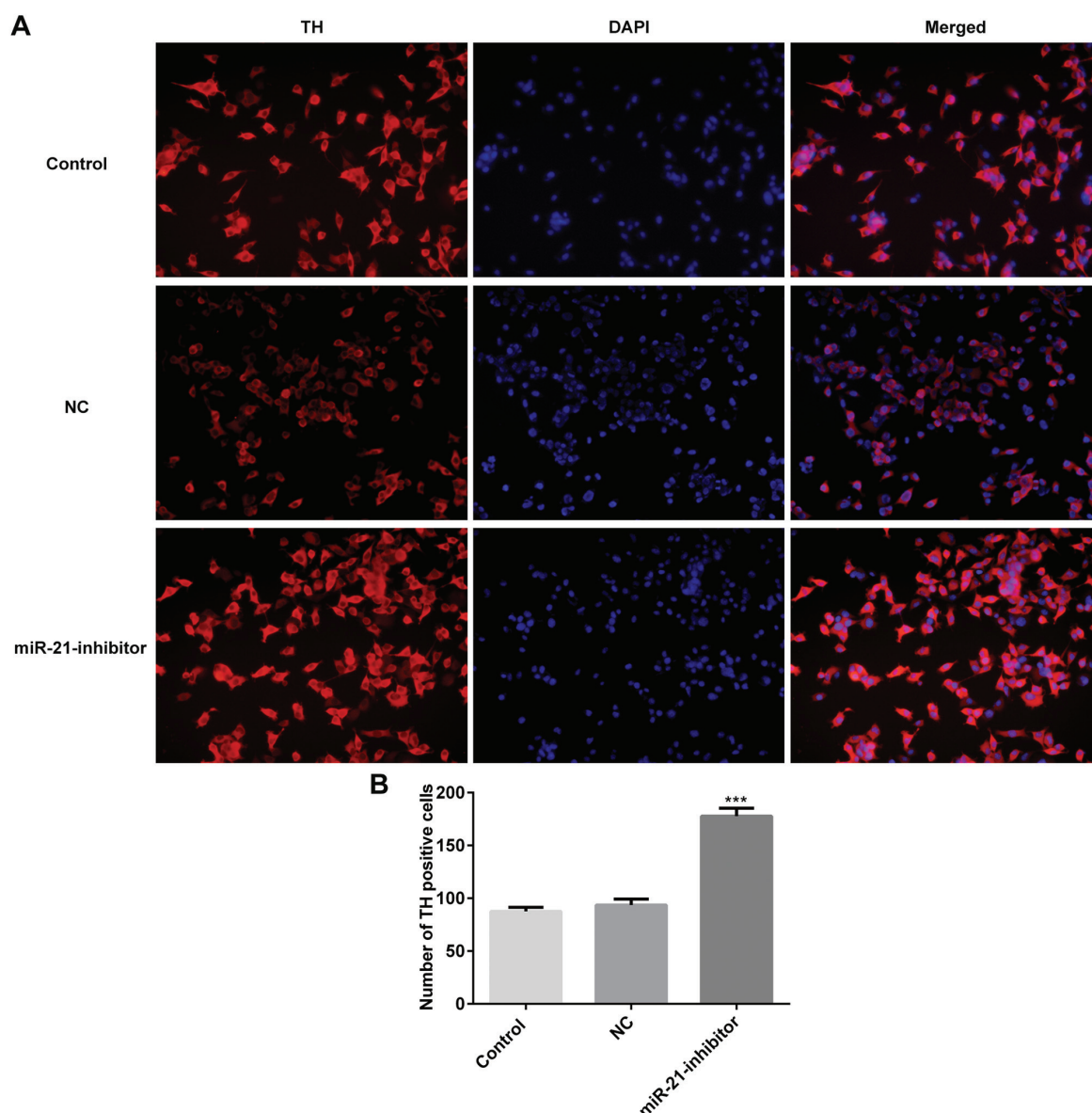


Figure 2. Downregulation of miR-21 increases TH expression in MPP⁺-treated MES23.5 cells. MES23.5 cells were transfected with miR-21 inhibitor and NC sequences for 24 h and the cells. (A) TH expression detected by immunocytochemistry. (B) Quantitative analysis of TH positive cell number. ***P<0.001 vs. control or NC. MPP⁺, 1-methyl-4-phenylpyridinium; miR, microRNA; NC, negative control; TH, tyrosine hydroxylase.

Results

Downregulation of miR-21 enhances cell survival in MPP⁺-treated MES23.5 cells. The optimal concentration of MPP⁺ was determined based on neuronal viability, which was monitored by the CCK-8 assay. MPP⁺ treatment demonstrated dose- and time-dependent cytotoxicity of MES23.5 cells. These effects were noted at the concentrations of 100, 200 and 300 μ M for 0, 3, 6, 12 and/or 24-h time points (Fig. 1A). The optimal concentration (200 μ M) and time point (24 h) of incubation exhibited approximately 50% cell viability following MPP⁺ treatment (Fig. 1A). The expression levels of miR-21 in MPP⁺-treated MES23.5 cells were assessed by RT-qPCR. The results indicated that the expression levels of miR-21 were significantly higher in MES23.5 cells following MPP⁺ treatment compared with those of the control group (Fig. 1B). To further investigate the role of miR-21 in the development of PD,

MES23.5 cells were transfected with miR-21 inhibitor and NC sequences, followed by treatment with 200 μ M of MPP⁺ for 24 h. The expression levels of miR-21 were significantly decreased following transfection with miR-21 inhibitor in MES23.5 cells compared with those of the NC group (Fig. 1C). The CCK-8 assay indicated that the transfection of the miR-21 inhibitor significantly increased the cell survival of the MES23.5 cells following MPP⁺ treatment at the 24, 48 and 72-h time points compared with that noted in the NC group (Fig. 1D). Moreover, the characteristics of active neurons were classified according to the expression of TH (13). The results of the immunohistochemical assay demonstrated that downregulation of miR-21 significantly increased the number of TH positive cells (Fig. 2).

Downregulation of miR-21 causes inhibition of the inflammatory response and ROS production in MPP⁺-treated MES23.5 cells. To investigate the role of miR-21 in MPP⁺-mediated cell

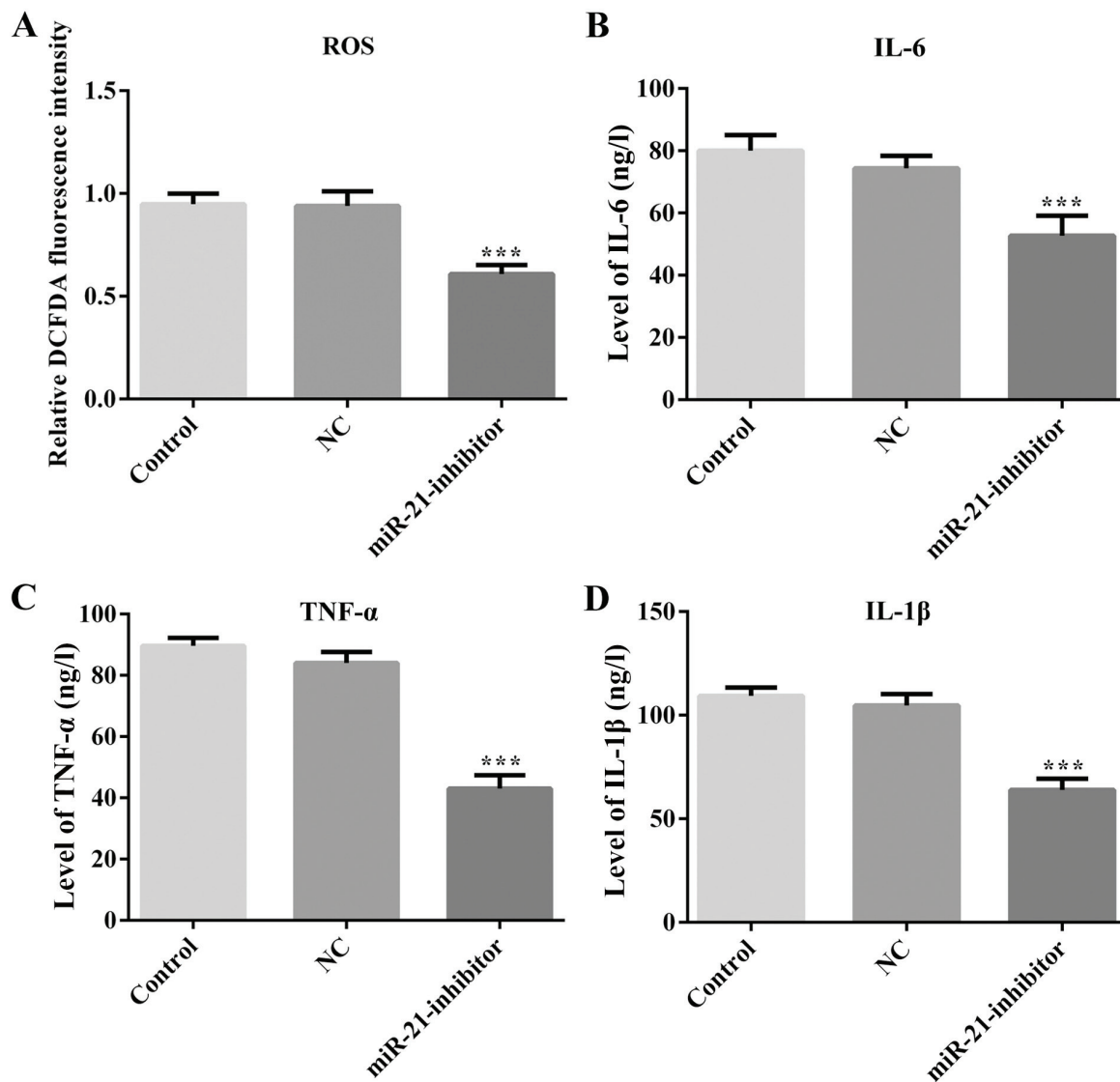


Figure 3. Downregulation of miR-21 inhibits the inflammatory response and ROS production in MPP⁺-treated MES23.5 cells. MPP⁺-treated MES23.5 cells were transfected with miR-21 inhibitor and NC. (A) DCFDA staining was used to assay ROS levels of MES23.5 cells. The ELISA assay was performed to detect IL-6 (B) IL-1β (C) and TNF-α (D) levels. ***P<0.001 vs. control or NC. MPP⁺, 1-methyl-4-phenylpyridinium; miR, microRNA; NC, negative control; IL, interleukin; TNF-α, tumor necrosis factor; ROS, reactive oxygen species.

damage, the production of ROS was determined in MPP⁺-treated MES23.5 cells. The results suggested that downregulation of miR-21 attenuated ROS production compared with that of the NC group (Fig. 3A). Subsequently, the levels of neuroinflammation were assessed by ELISA analysis. The expression levels of the inflammatory markers IL-6, IL-1β and TNF-α were significantly reduced following transfection of the cells with the miR-21 inhibitor (Fig. 3).

Downregulation of miR-21 suppresses cell apoptosis and Bcl-2 is its direct target. The effects of miR-21 in the induction of apoptosis were further evaluated. The data demonstrated that downregulation of miR-21 resulted in a significant inhibition of apoptosis in MPP⁺-treated MES23.5 cells compared with that of the NC group (Fig. 4). In addition, the effects of miR-21 on the expression levels of the apoptotic proteins were also investigated. Inhibition of miR-21 resulted in increased Bcl-2 and decreased Bax levels compared with those of the NC group (Fig. 5).

Moreover, MES23.5 cells were transfected with miR-NC and miR-21 mimic, and the miR-21 mimic significantly increased the levels of miR-21 (Fig. 6A). Furthermore, the results of Dual-luciferase reporter assay showed that cells co-transfected with miR-21 mimics and Bcl-2 3'UTR-WT, exhibited lower luciferase activity compared with those co-transfected with miR-21 mimics and Bcl-2 3'MUT (Fig. 6C). However, no significant difference was noted in the luciferase activity of the NC groups (Fig. 6C). These results confirmed that miR-21 could directly bind to the 3'UTR of the Bcl-2 protein.

Discussion

PD is characterized by progressive and irreversible loss of dopaminergic neurons in the substantia nigra. This disease is the second most common neurodegenerative disorder following Alzheimer disease (1). Current evidence suggests that miRs are involved in diverse biological processes

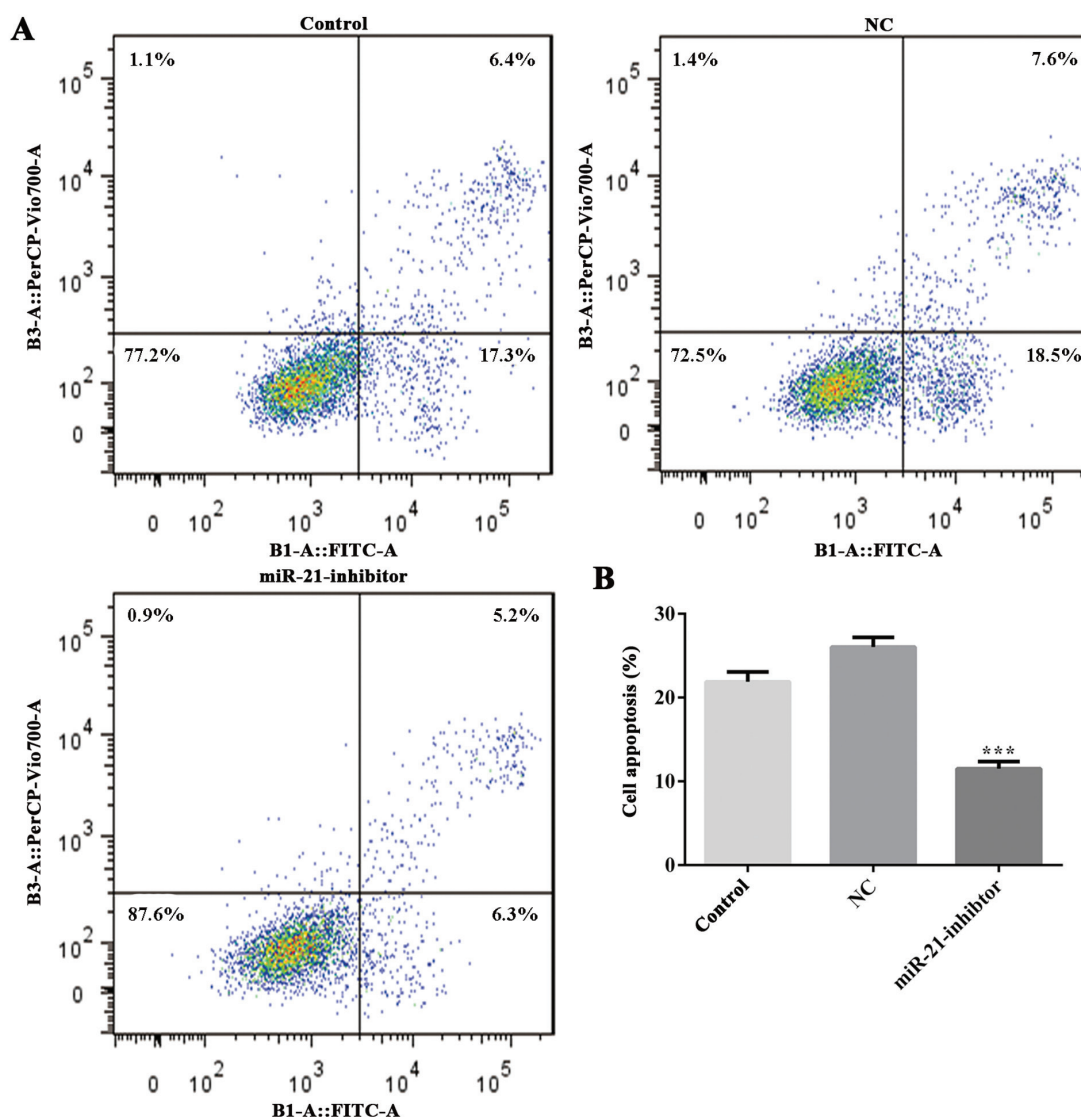


Figure 4. Downregulation of miR-21 inhibits MPP⁺-induced cell apoptosis in MES23.5 cells. MES23.5 cells were transfected with miR-21 inhibitor and NC sequences for 24 h and the cells were treated with MPP⁺ for another 24 h. (A) The percentage of Annexin-V-positive cells was analyzed by flow cytometry. (B) Quantification of cell apoptosis in the different groups investigated. ***P<0.001 vs. NC. MPP⁺, 1-methyl-4-phenylpyridinium; miR, microRNA; NC, negative control.

including pathogenesis of neurodegenerative disorders and abnormal brain function (8,14). With regard to PD, several miRs have been reported to participate and play important roles in PD progression, via the regulation of various processes, such as apoptosis, autophagy, inflammation, mitochondrial dysfunction, and ROS activity (15,16). These molecules can be targeted for the development of future diagnostic tools and treatment strategies. For example, patients with PD and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced animals exhibit a significant decrease in the levels of miR-7 in brain tissue areas associated with dopaminergic neurodegeneration (17). Moreover, miR-7 has recently been shown to target α -synuclein, a protein involved in the pathological process of PD, and can therefore be used for the treatment of this disease (18).

It is important to note that the miR-21 levels of a SH-SY5Y cell PD model were significantly higher than those noted in normal cells as reported by recent studies (9,10). Su *et al* (10) demonstrated that miR-21 upregulated the expression levels

of α -synuclein by directly targeting lysosome-associated membrane protein 2 in SH-SY5Y cells pretreated with MPP⁺. However, the effects and the underlying molecular mechanism of miR-21 in neural cells are still unclear. In the present study, the role and underlying molecular mechanism of miR-21 in PD was investigated using the neurotoxin MPP⁺, a well-established compound in PD cell models (13). It was demonstrated that treatment of MPP⁺ resulted in a dose- and time-dependent cytotoxicity in MES23.5 cells. The present study demonstrated that miR-21 levels were considerably increased in MPP⁺-treated dopaminergic neuronal MES23.5 cells, which is consistent with a previous study (10). To further investigate the role of miR-21, MES23.5 cells were transfected with miR-21 inhibitor sequences. The results suggested that downregulation of miR-21 considerably enhanced cell survival and inhibited cell apoptosis induced by MPP⁺ treatment. Moreover, inhibition of miR-21 significantly increased the TH positive cell number in MPP⁺-treated MES23.5 cells. The levels of the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax

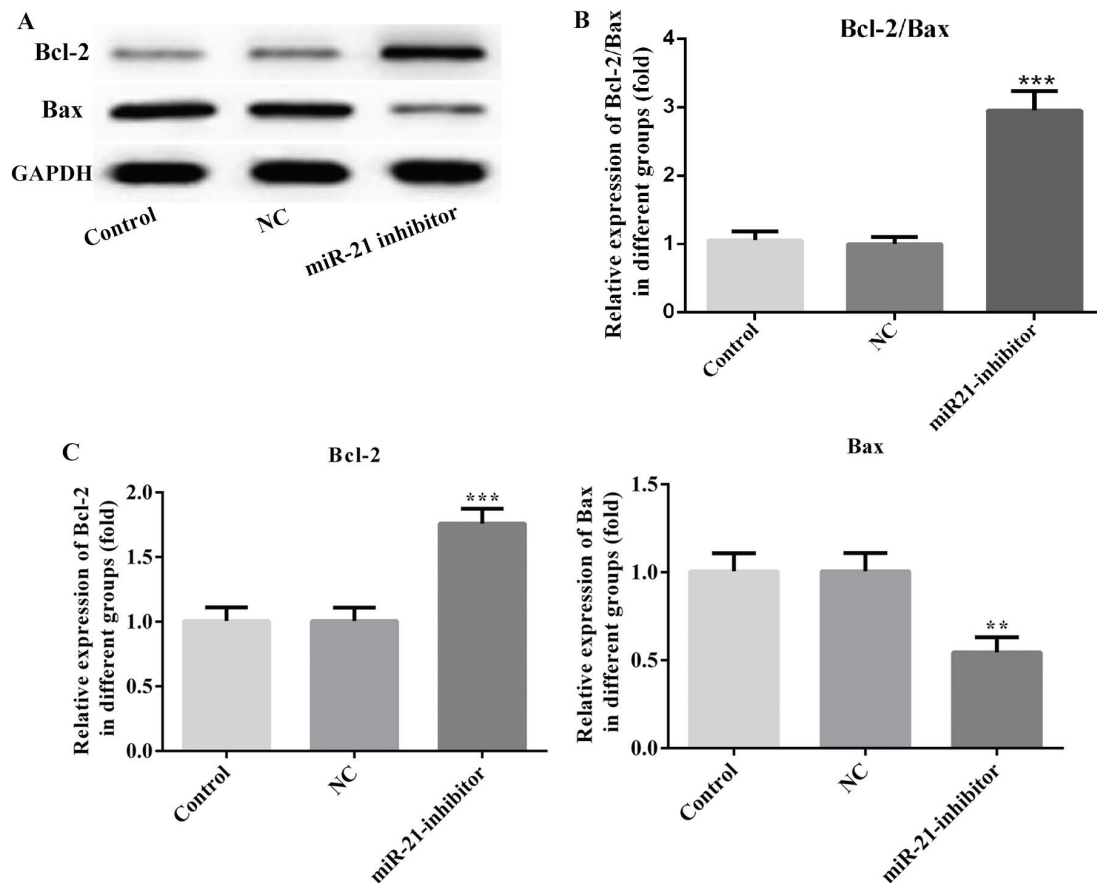


Figure 5. Inhibition of miR-21 expression increased the ratio of Bcl-2/Bax. (A) Western blot analysis was performed to detect the expression levels of Bcl-2 and Bax proteins. (B) Statistical analysis of relative Bcl-2/Bax expression. (C) Statistical analysis of Bcl-2 and Bax levels in the different groups, respectively. **P<0.01, ***P<0.001 vs. NC. miR, microRNA; NC, negative control.

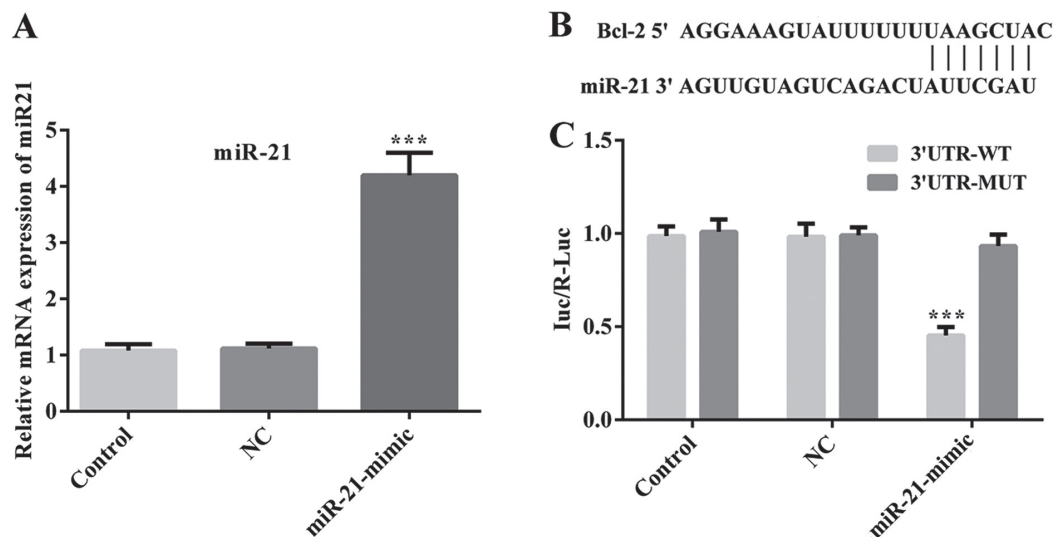


Figure 6. Bcl-2 is a direct target gene of miR-21. (A) MES23.5 cells were transfected with miR-NC and miR-21 mimic, and the expression of miR-21 was detected by reverse transcription-quantitative PCR assay. (B) The putative miR-21 targeted sequences in the 3'-UTR of Bcl-2 mRNA are shown. (C) Dual-luciferase reporter assay was performed to analyze the relative luciferase activity. The results were presented as fold change. ***P<0.001 vs. NC or 3'UTR-WT. miR, microRNA; NC, negative control; WT, wild-type; MUT, mutant type.

were also examined. These two proteins are considered important regulators of apoptosis and the disruption of the balance of the Bcl-2/Bax ratio could lead to the release of pro-apoptotic proteins from the mitochondria to the cytoplasm (19). Western

blot analysis was performed to detect the expression levels of the aforementioned proteins, and the results indicated that downregulation of miR-21 increased the Bcl-2/Bax ratio in the MPP⁺-treated MES23.5 cells. Moreover, dual-luciferase

reporter assay demonstrated that Bcl-2 was a direct target gene of miR-21, which may indicate that miR-21 participated in the induction of cell apoptosis in MES23.5 cells via the regulation of Bcl-2. In addition, MPP⁺ has been shown to cause neurotoxicity by the induction of ROS and the secretion of inflammatory factors (20). Devathasan *et al* (21) demonstrated that patients with PD exhibited a significant increase in the production of ROS in specific brain regions. The overproduction of ROS and the induction of oxidative stress have been shown to be involved in PD and to lead to dopaminergic neuronal cell death and apoptosis (22). The data reported in the present study demonstrated that downregulation of miR-21 resulted in a significant inhibition of ROS production in MPP⁺-induced MES23.5 cells. The results indicated that downregulation of miR-21 may exert a neuroprotective effect in MPP⁺-treated MES23.5 cells by the inhibition of cell apoptosis and the induction of inflammation and ROS production.

In conclusion, the present study demonstrated that miR-21 expression was increased in MPP⁺-treated MES23.5 cells, whereas inhibition of miR-21 alleviated MPP⁺-induced MES23.5 cell damage by suppressing intracellular ROS and inflammatory factor production, and by inhibiting apoptosis. Moreover, the present study reported that Bcl-2 was a direct target gene of miR-21 and that the downregulation of miR-21 could significantly increase TH expression in MPP⁺-treated MES23.5 cells. Although further studies should be conducted, miR-21 may be considered a potential target for PD diagnosis and treatment.

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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

HWM and LDD participated in experiment design, data collection and paper writing.

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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