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Protective effects of Mito-TEMPO against rotenone-induced neurotoxicity in SH-SY5Y neuroblastoma cells

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The effects of mitochondria-targeted treatments have great promise in prevention of Parkinson Disease (PD). This study aimed to explore the possible protective effects of Mito-TEMPO, a mitochondria-targeted chemical against neurotoxic damage induced by rotenone in SH-SY5Y cells. SH-SY5Y cells were exposed to varying concentrations of rotenone (10 nM, 50 nM, 125 nM, 250 nM, 500 nM, 1000 nM) for 24 and 48 h. Mito-TEMPO (10, 100, and 1000 µm) was administered to the cultures at concentrations of 10, 100, and 1000 µM 2 h prior to rotenone exposure. Cell viability across groups was measured using the MTT assay. Apoptosis was analyzed through Hoechst 33258 staining and Western blot techniques, and reactive oxygen species (ROS) levels were quantified via the DCFH-DA method. Mitochondrial activation was examined with MitoTracker Green staining.All concentrations of Mito-TEMPO significantly protected cells against rotenone toxicity. There were significant apoptotic marks such as nuclear fragmentation and bax/bcl-2 & cleaved caspase-3 increase in rotenone group. Mito-TEMPO exhibited protective effects by reducing apoptotic alterations and decreasing ROS levels significantly. The alterations of mitochondria density and localization in rotenone-treated cells were prominent while there was no difference observed in Mito-TEMPO group. Overall, Mito-TEMPO exhibited protective effects against rotenone-induced toxicity.

Keywords: Mitochondria-targeted treatment, Apoptosis, Parkinson's disease, ROS, MitoTracker

Parkinson's disease (PD) is a common neurodegenerative disorder characterized by the degeneration of dopaminergic neurons in the basal ganglia and the formation of Lewy bodies due to the buildup of α-synuclein[1].As the primary energy producers in cells, mitochondria play a vital role in neurodegeneration. The production of reactive oxygen species (ROS) disrupts the electron transport chain in complex I, impacts mitochondrial dynamics, and activates pro-apoptotic signaling within the substantia nigra[2,3]. Specifically, defects in complex I are key contributors to PD pathogenesis, leading to apoptosis and loss of dopaminergic neurons. Additionally, increased ROS levels damage mitochondrial DNA and result in mutations in genes such as Parkinson's disease protein 7 (PARK7), PARK2 (which encodes Parkin), and phosphatase and tensin homolog (PTEN)-induced kinase 1 (PINK-1), all of which are linked to PD[4].

Rotenone, an organic lipophilic pesticide decreases mitochondria respiration by inhibiting complex I[5,6]. Several studies have indicated that chronic exposure to rotenone causes oxidative stress through α-synuclein accumulation and caspase-induced cell death[7–9]. Despite extensive research, the mechanisms underlying PD pathogenesis remain largely unclear. SH-SY5Y cell line derived from a human neuroblastoma, possesses many properties of dopaminergic neurons, and have been used to investigate the effects of PD-related toxins and genetic mutations on cellular processes such as mitochondrial function, protein aggregation, and oxidative stress[10–12]. Therefore, the SH-SY5Y cell line represents a valuable tool for studying the cellular mechanisms underlying rotenone neurotoxicity related to PD and for the development of novel therapeutics for this debilitating disease.

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Recently, the significance of mitochondria dysfunction in PD pathogenesis has drawn attention to mitochondria-targeted therapies in the treatment. These therapies can occasionally have more than one effect such as changing redox homeostasis, electron transport chain activity, and adenosine triphosphate (ATP) synthesis[13,14]. In the mechanism of one of these therapies, lipophilic cation compounds such as triphenylphosphonium (TPP) bind to bioactive components. TPP cations are composed of phosphorus atoms with large hydrophobic surfaces, thus, they easily pass through the lipid membrane of the cell using the cell membrane potential (30-60 mV). TPP cations begin to accumulate 100-150 folds in the negatively charged mitochondrial matrix using high mitochondrial membrane potential (150- 180 mV) created by the proton gradient. As a result, that gradient difference allows TPP to enter the mitochondria from the cytoplasm in a much higher concentration[7,8].

Mito-TEMPO is a TPP-conjugated agent formed with TEMPO (2,2,6,6-tetramethylpiperidin-1-oxyl-4-ylamino), an intracellular antioxidant and a mitochondrial redox cycle nitroxide[13,15]. Several studies have indicated that Mito-TEMPO facilitates ROS detoxification, restores ATP, and protects against necrosis and apoptosis[16–18]. Given the role of mitochondrial dysfunction in neurodegeneration, this study sought to examine the potential therapeutic effects of Mito-TEMPO on neurotoxicity induced by rotenone in SH-SY5Y cells.

Materials and Methods

Chemicals

SH-SY5Y cells were sourced from the American Type Culture Collection in Germany. Fetal Bovine Serum (FBS), Dulbecco's Modified Eagle's Medium Mixture F-12 Ham (DMEM/F-12, 1:1), Phosphate Buffered Saline (PBS), Mito-TEMPO, and additional cell culture reagents were obtained from Sigma Aldrich GmbH, Germany. MitoTrackerTM Green FM and the DCFH-DA kit were supplied by Thermo Fisher.

Cell culture

Cells were maintained in 75 cm² flasks using DMEM/F12 medium supplemented with 10% FBS, 1% L-glutamine, 1% penicillin-streptomycin, and 1% amphotericin. Incubation took place in a controlled environment of 5% CO_2 and 95% air at 37°C. The culture media was changed 3 times weekly. Cell density was monitored under an inverted light microscope (Olympus CKX53), and a confluence level of 85-90% was deemed adequate. Following trypsinization and centrifugation, cell viability was determined via Trypan blue staining, and cell counting was performed.

Rotenone treatment

Rotenone was initially dissolved in dimethylsulfoxide (DMSO) and then diluted in DMEM/F-12 medium to prepare varying concentrations for testing its effective dose. Cells were seeded into 96-well plates at a density of 5×10^3 cells per well (n=8) and treated with rotenone at concentrations of 10 nM, 50 nM, 125 nM, 250 nM, 500 nM, and 1000 nM, followed by incubation for 24 and 48 h The half-maximal inhibitory concentration (IC50) for rotenone was calculated to be 250 nM at the 24 h mark using the (MTT) assay[19,20].

Mito-TEMPO treatment

Mito-TEMPO was initially dissolved in PBS and then diluted in DMEM/F-12 medium to achieve concentrations ranging from 10 to 1000 µM. It was applied to cells seeded in 96-well plates at a density of 5×10^3 cells per well. One hour after Mito-TEMPO application, rotenone (250 nM) was introduced, and the cells were incubated for 24 h to evaluate the protective effects of Mito-TEMPO[14,21]

Cell viability assessment

Cell viability was assessed using the MTT assay, where the formation of coloured formazan, generated by mitochondrial dehydrogenase activity, directly correlates with the number of viable cells[22]. Cells were seeded at a density of 5×10^3 cells per well in 96-well plates and incubated for 24 h. Following the removal of the supernatant, 250 nM rotenone was added to the cells after pre-treatment with Mito-TEMPO, while untreated cells served as controls. Subsequently, cells were incubated with 1 mg/mL MTT for 4 h. Dimethyl sulfoxide (100 µL) was then added, and plates were shaken for 10 min. Optical density (OD) readings were taken at 490 nm using a microplate reader (Multiscan Go, Thermo Fisher Scientific Inc., USA), and cell viability (%) was calculated using the formula: (ODtreatment / ODcontrol) × 100. DMSO was used as positive control group while control group did not receive any treatment.

Morphological analysis

Cells were plated in 12-well plates at a density of 1.5×10^3 cells per well with culture medium and incubated for 24 h. After the medium was removed, cells were treated with effective concentrations of Mito-TEMPO prior to exposure to the toxic concentration of rotenone. Morphological changes were observed using an inverted light microscope after a 24 hr incubation period, and the findings were compared with DMSO and control groups.

Hoechst 33258 staining

Hoechst 33258 staining (Sigma Aldrich Co.), which binds to DNA, was employed to identify apoptotic cells. Cells were plated at 5×10^3 cells per well, then fixed with 10% formalin for 30 min following a PBS wash and stained with 1 mg/mL Hoechst 33258 for 10 min in the dark. Nuclear condensation and fragmentation were observed using an inverted fluorescence microscope, and images were captured with Olympus software (CKX53). Image analysis was conducted using ImageJ (https://imagej.nih.gov/ij). The percentage of Hoechst-positive nuclei was determined by counting stained nuclei in 20 randomly selected optical fields, and the average count was recorded

Western blotting

Experimental groups (rotenone, Mito-TEMPO at 10, 100, and 1000 μM) were seeded into 6-well plates at a density of 1.5×10^3 cells per well. Following 24 h of incubation, cells were trypsinized, centrifuged, and rinsed with PBS. Cell lysis buffer was then added, and the samples were centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was collected, and the DC Protein Assay Kit (Bio-Rad, Hercules CA) was used to quantify total protein content. Protein samples (40 μg) were loaded onto a 4-15% SDS-PAGE gel and subsequently transferred onto polyvinylidene difluoride (PVDF) membranes. Blocking of the membranes was carried out with 0.1% Triton X-100 and 5% nonfat dry milk in Tris-buffered saline with Tween 20 for 1 h. For immunodetection, membranes were incubated overnight at 4°C with primary antibodies (anti-Bax, anti-Bcl-2, anti-caspase 3, rabbit monoclonal antibody) from Cell Signaling Technology, diluted 1:1000 in Tris-buffered saline. The following day, membranes were treated with horseradish peroxidase-conjugated anti-rabbit antibody, and anti-β-actin mouse monoclonal antibody was applied as a loading control. Imaging was conducted using the ChemiGlow West Chemiluminescence Substrate Kit (1:1 mixture), and images were captured with the FluorChem 8900 Imager. Densitometric analysis was performed using ImageJ software[23].

Assessment of ROS levels

The levels of ROS production were measured using the 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) method. DCFH-DA (Thermo Fisher Scientific) penetrates the cell and is deacetylated by cellular esterases into a non-fluorescent form, which is then oxidized by ROS to form the highly fluorescent 2',7'-dichlorofluorescein (DCF)[24] . Thus, fluorescence intensity serves as an indicator of ROS generation. After removing the supernatant and washing the cells with PBS, cells (5×10^3 per well) were incubated with a 5 μM DCFH-DA solution for 30 min. Extracellular DCFH-DA was then removed, and the cells were visualized under an inverted fluorescence microscope. Quantitative analysis was carried out using ImageJ software (https://imagej.nih.gov/ij).

MitoTracker Green FM staining

MitoTracker Green FM (Thermo Fisher Scientific), a green-fluorescent dye, diffuses through the plasma membrane with the thiol-reactive chloromethyl moiety and selectively accumulates in the mitochondria matrix. Viable cells (5×10^3 per well) were rinsed twice with PBS and then incubated in the dark for 30 min with a 100 nM solution of MitoTracker Green FM in PBS. Mitochondrial density and morphology were examined under live cell imaging fluorescence microscope using a ×40 objective.

Statistical analysis

The analysis of the data was conducted utilizing SPSS version 22.0 (IBM SPSS, Chicago, USA). Comparisons between groups for datasets adhering to a normal distribution were conducted using one-way ANOVA accompanied by Tukey's post hoc analysis. In instances where data failed to satisfy normality assumptions, the Kruskal-Wallis and Mann-Whitney tests were utilized. A P value below 0.05 was deemed to be statistically significant. Results are expressed as mean ± standard error of the mean (SEM).

Results

Cytotoxicity of rotenone

MTT assay demonstrated the effects of rotenone at different concentrations for 24 and 48 hs.As seen in

Fig. 1, rotenone significantly decreased cell viability both at 24 and 48 h ($P< 0.05$) (Fig. 1). The toxic concentration of rotenone was determined as 250 nM (46.06 ± 5.52 %) at 24 h and used in experimental groups. MTT assay results showed that rotenone produced excessive damage on SH-SY5Y cells dependent on time and concentration. Additionally, observation of the cells through an inverted light microscope revealed that rotenone exposure led to a marked reduction in cell number and loss of cellular connections, which was statistically significant ($P< 0.05$) (Fig. 2).

Neuroprotective effects of Mito-TEMPO

SH-SY5Y cells were treated with varying concentrations of Mito-TEMPO for 24 h, and three effective doses–10 μM, 100 μM, and 1000 μM–were selected for further analysis based on results from the MTT assay. As seen in Fig. 3, Mito-TEMPO application did not cause any cytotoxicity on cells at all concentrations. Rotenone (250 nM) was added to cells 1 h after Mito-TEMPO pre-treatment. Cell viability was significantly decreased in rotenone treated cells (40.67 ± 1.74 %, $P<0.005$) compared to DMSO (90.83 ± 4.44 %), while 10 μM (54.09 ± 3.91 %, $P< 0.05$) 100 μM (60.5 ± 4.91 %, $P< 0.005$) and 1000 μM (86.63 ± 5.07 %, $P<0.0005$) of Mito-TEMPO protected cells against rotenone toxicity (Fig. 4).

Assessment of apoptosis-related cell death

Hoechst 33258 (bisbenzimide) staining was used to reveal DNA fragmentation and condensation in cells undergoing apoptosis. Compared to the control group, rotenone exposure significantly elevated nuclear fragmentation ($P< 0.0005$), whereas 100 μM and 1000 μM concentrations of Mito-TEMPO enhanced cell survival ($P< 0.005$). Although 10 μM Mito-TEMPO reduced apoptotic cell death, this reduction was not statistically significant ($P> 0.05$). These findings indicate that pre-treatment with Mito-TEMPO offers protective effects against rotenone-induced damage (Fig. 5).

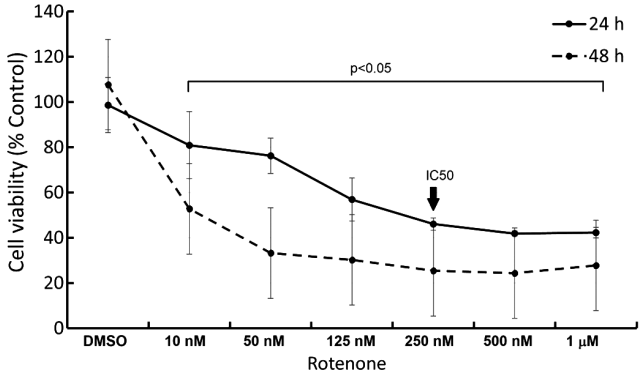

Fig. 1 — Rotenone exhibited concentration and time-dependent toxicity compared to control and DMSO groups. (n = 8 wells for each group, *$P< 0.05$).

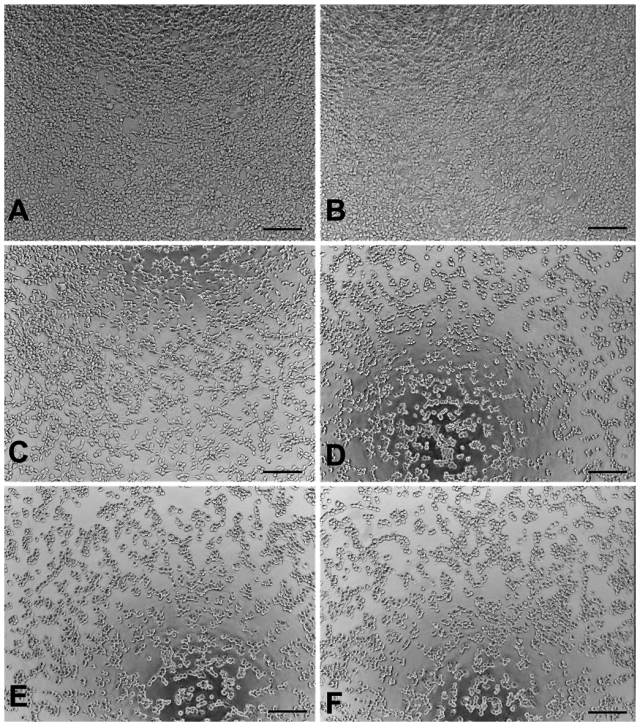

Fig. 2 — Rotenone treatment caused cytotoxicity after 24 h under inverted phase-contrast microscope. (A) Control (B) DMSO (C) 10 nM (D) 125 nM (E) 250 nM (F) 500 nM (n = 8 wells for each group, scale bars = 200 μm)

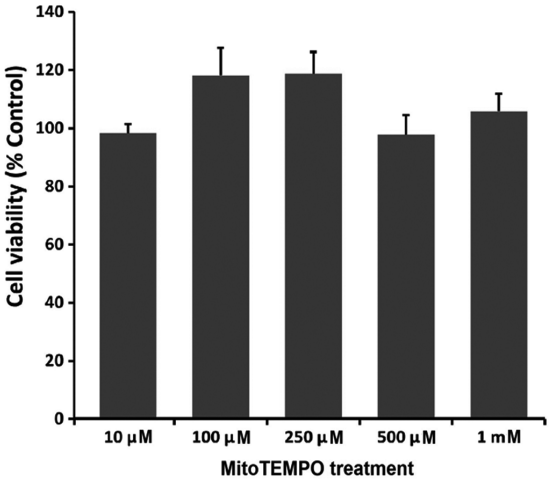

Fig. 3 — The MTT assay results indicated no statistically significant difference between the Mito-TEMPO-treated groups and the control group (n = 8 wells per group, $P> 0.05$).

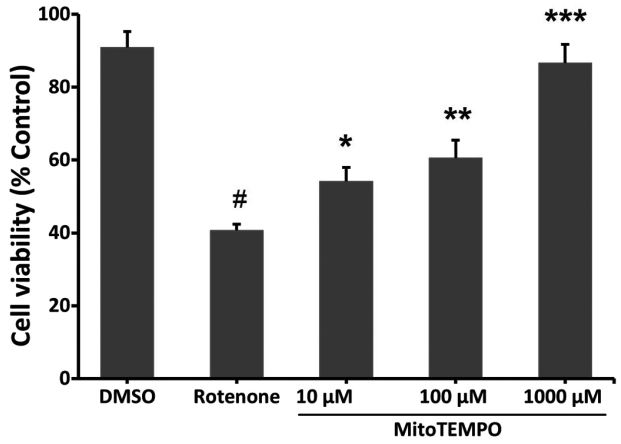

Fig. 4 — Mito-TEMPO protected cells against rotenone toxicity compared to DMSO group. (n = 8 wells for each group, # $P<$ 0.0005 *vs*. DMSO, * $P<$ 0.05 *vs*. rotenone, ** $P<$ 0.005 vs. rotenone, *** $P<$ 0.0005 *vs*. rotenone).

Assessment of bcl-2, bax, and caspase-3 expression levels.

Western blot analysis was performed to assess levels of bcl-2, bax, and cleaved caspase-3 as markers of apoptosis. In alignment with Hoechst staining findings, bax and cleaved caspase-3 were significantly elevated in the rotenone-treated group compared to controls ($P<$ 0.05). Mito-TEMPO treatment notably decreased bax expression and restored anti-apoptotic bcl-2 levels, which were reduced by rotenone exposure ($P<$ 0.05). In summary, the bax/bcl-2 ratio indicated that rotenone induced apoptotic cell death, whereas pre-treatment with Mito-TEMPO exerted protective effects (Fig. 6).

Effects of Mito-TEMPO on ROS production

The DCFH-DA oxidation technique was employed to assess ROS production in SH-SY5Y cells, with fluorescence intensity analyzed using

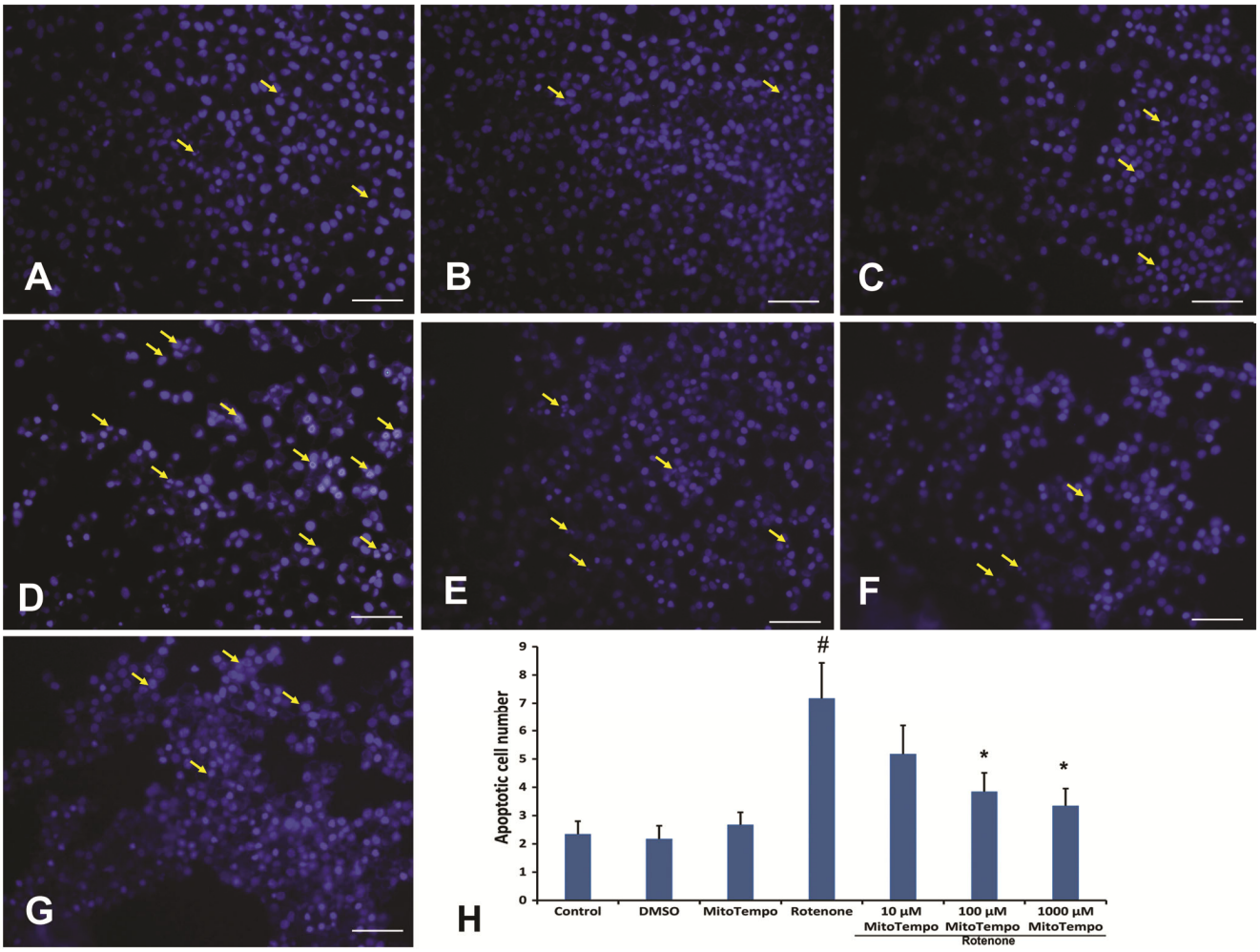

Fig. 5 — Hoechst 33258 staining results showed that Mito-TEMPO reduced apoptotic cell death. (A) Control (B) DMSO (C) Mito-TEMPO (D) Rotenone (E) 10 µM Mito-TEMPO + rotenone (F) 100 µM Mito-TEMPO + rotenone (G) 1000 µM Mito-TEMPO + rotenone (H) Evaluation of the apoptotic cell number in groups. ($^{\#}$ < 0.0005 *vs*. control, * $P<$ 0.005 *vs*. rotenone) (n = 4 for each group, scale bars = 40 µm)

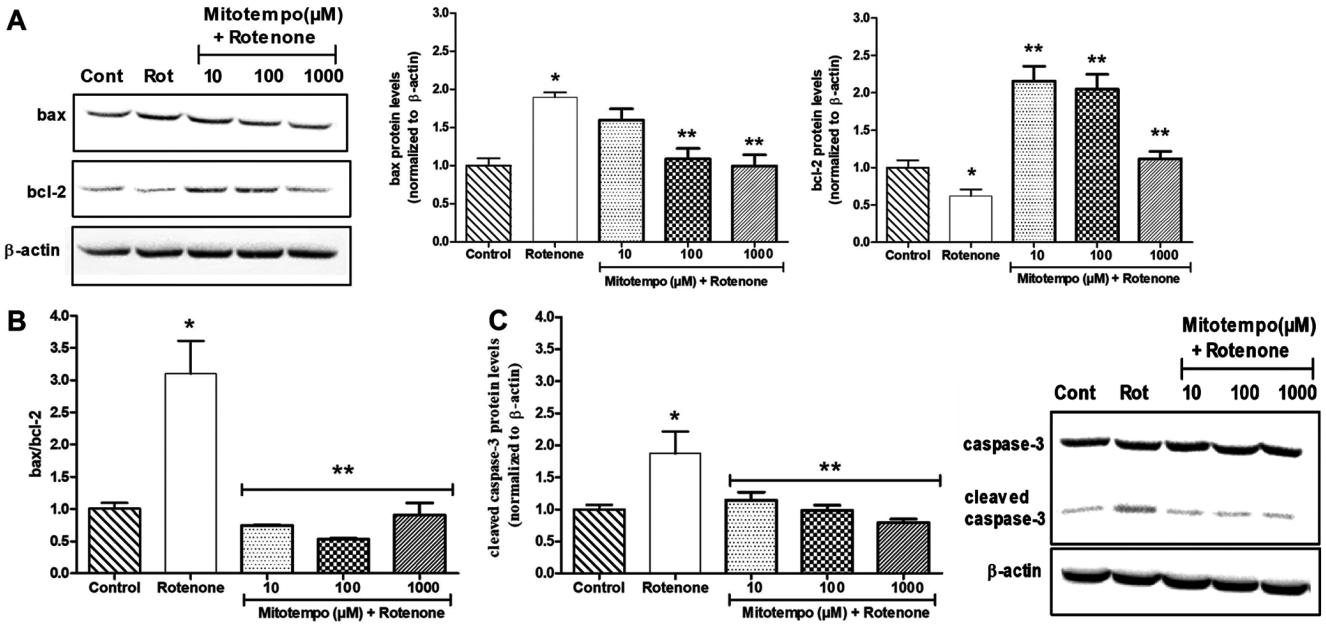

Fig. 6 — The effects of rotenone and Mito-TEMPO. (A) Bax and Bcl-2 protein levels (*P< 0.0005 *vs*. control, ** P< 0.05 *vs*. rotenone) (B) Bax / Bcl-2 ratio shows apoptotic alterations in rotenone treated cells (*P< 0.0005 *vs*. control, ** P< 0.05 *vs*. rotenone) (C) Cleaved caspase- 3 protein levels exhibited protective effects of Mito-TEMPO against apoptosis triggered by rotenone treatment. (*P< 0.0005 *vs*. control, ** P< 0.05 *vs*. rotenone) (n = 3 for each group)

Image J. There was no significant difference in DMSO (105.39 ± 5.26 %) and Mito-TEMPO groups (107.58 ± 5.27 %) compared to control while rotenone (221.68 ± 11.86 %) significantly increased ROS levels (P< 0.00005). When compared to the rotenone group, 10 μM (179.04 ± 13.72 %, P< 0.05), 100 μM (144.73 ± 3.51 %, P< 0.0005), and 1000 μM (109.04 ± 7.96 %, P< 0.00005) Mito-TEMPO significantly reduced ROS levels (Fig. 7). These results revealed that rotenone caused ROS formation in SH-SY5Y cells, while Mito-TEMPO effectively suppressed ROS production in a concentration-dependent manner.

Effects of rotenone and Mito-TEMPO on mitochondrial mass and distribution

Effects of Mito-TEMPO on mitochondrial mass and distribution against rotenone toxicity were evaluated qualitatively by using MitoTracker Green staining. Observation under a fluorescence microscope revealed that mitochondrial distribution appeared uniform in the control group, while it was irregular and reduced in cells treated with rotenone (Fig. 8). On the other hand, an improvement in mitochondrial density and distribution was observed in all Mito-TEMPO treated groups, especially in the 1000 μM group. These results suggest that rotenone decreased mitochondrial density and distribution in SH-SY5Y cells while Mito-TEMPO protected cells by preventing mitochondrial damage. Mito-TEMPO may exert neuronal protective effects against rotenone via suppressing excessive ROS generation and apoptosis cascade in mitochondria. These results suggest that Mito-TEMPO reduced oxidative stress and promoted cell survival as presented in Fig. 9.

Discussion

Mitochondria is the main organelle for cellular energy production and are particularly vulnerable to oxidative damage. Mitochondrial dysfunction is a primary contributing factor in neurodegenerative diseases[25]. Decrease of complex I activity and ATP formation produce excessive ROS that defects mitobiogenesis and activates mitophagy. Mutation and accumulation of alfa-synuclein as well as mutations in PINK1, DJ1 and Parkin lead to mitochondrial dysfunction in PD. ROS release in dopaminergic neurons also impairs proteins, lipids and DNA, therefore inadequate ATP formation causes necrosis or apoptosis at the onset of the disease[25]. In addition, defects in mtDNA, elevated oxidative stress, and the formation of Lewy bodies due to alpha-synuclein accumulation in the substantia nigra lead to motor symptoms in Parkinson's disease (PD)[26].

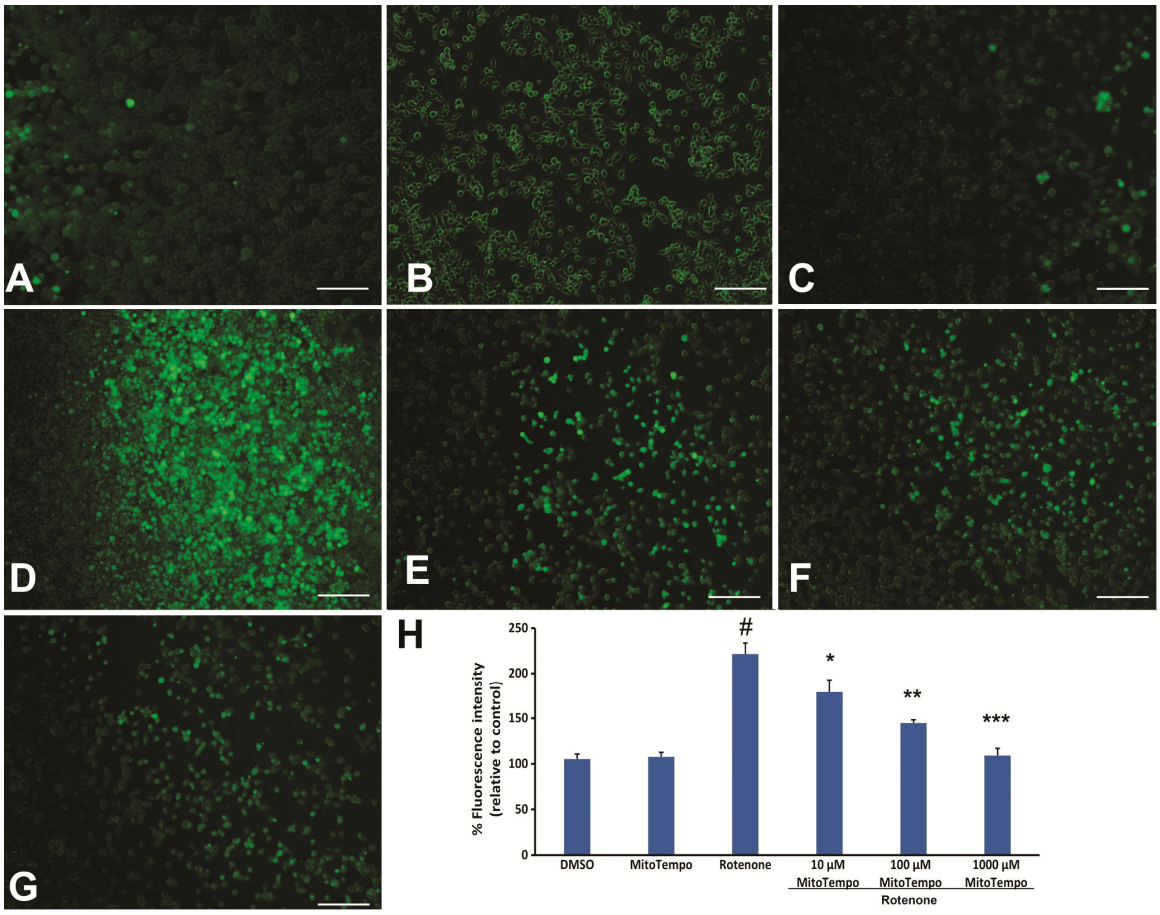

Fig. 7 — Mito-TEMPO reduced ROS production significantly. (A) Control (B) DMSO (C) Mito-TEMPO (D) Rotenone (E) 10 µM Mito-TEMPO + rotenone (F) 100 µM Mito-TEMPO + rotenone (G) 1000 µM Mito-TEMPO + rotenone, (H) Evaluation of the % fluorescence intensity (relative to control) in groups treated with Mito-TEMPO and rotenone. (#P< 0.00005 *vs*. DMSO, * P< 0.05 *vs*. rotenone, ** P< 0.0005 *vs*. rotenone, *** P< 0.00005 *vs*. rotenone) (n = 4 for each group, scale bars = 20 µm)

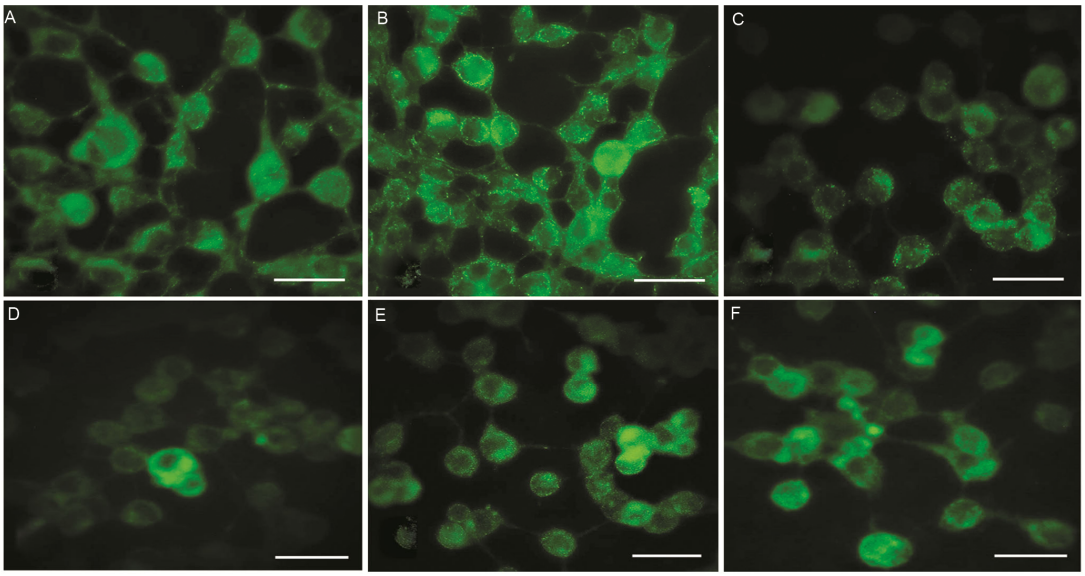

Fig. 8 — Evaluation of mitochondrial mass and distrubution via MitoTracker Green FM staining. (A) Control (B) Mito-TEMPO (C) Rotenone (D) 10 µM Mito-TEMPO + rotenone (E) 100 µM Mito-TEMPO + rotenone (F) 1000 µM Mito-TEMPO + rotenone (scale bars = 10 µm)

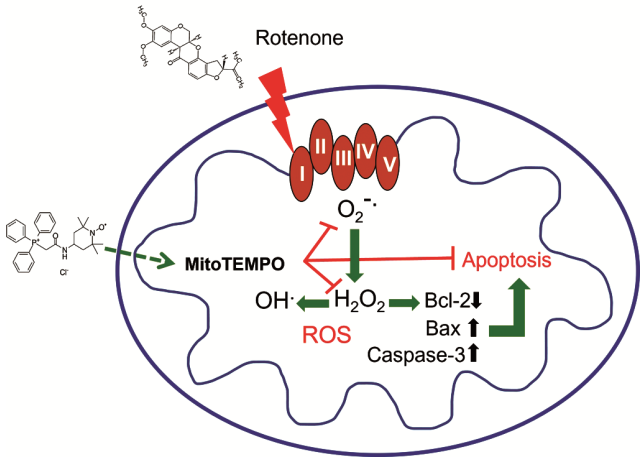

Fig. 9 — The possible mechanism of Mito-TEMPO against rotenone toxicity in SH-SY5Y cells.

Given the critical role of mitochondrial dysfunction in the pathogenesis of Parkinson's disease (PD), numerous chemicals and probes have been developed to specifically target mitochondria. Mito-TEMPO, a mitochondria-targeted chemical has been studied in many diseases and found protective against hepatotoxicity[27], cardiomyopathy[28,29] and oxalate induced kidney damage[30]. On the other hand, in a mouse sepsis model, Mito-TEMPO was shown to alleviate kidney damage and sepsis in the short term[31], while in another study its long-term effects on sepsis were not found to be sufficient[32]. However, the impact of Mito-TEMPO on neurodegenerative diseases, particularly PD, remains uncertain. This study, therefore, aimed to examine the effects of Mito-TEMPO on rotenone-induced neurotoxicity in SH-SY5Y cells.

In the preliminary study, it is determined the toxic concentration of rotenone as 250 nM and effective concentrations of Mito-TEMPO as 10 µM, 100 µM and 1000 µM at 24 h. Cell viability was significantly decreased in rotenone-induced cells compared to DMSO group while Mito-TEMPO pre-treatment protected cells against rotenone in all concentrations, especially at 1000 µM. Similarly, Mito-TEMPO had protective effects against MPTP-induced neurotoxic model in dopaminergic neurons and it reduced mitochondrial dysfunction in DJ-1 mutant dopaminergic neurons[33].

Apoptosis is important for eliminating damaged cells in the body by maintaining tissue homeostasis. One of the key proteins involved in apoptosis is caspase-3, a final executioner of the cell death process by cleaving various cellular proteins. Bax and Bcl-2 also play important role to regulate apoptosis. Bax forms pores in mitochondrial membrane to promote apoptosis while Bcl-2 inhibits apoptosis by preventing Bax activation. Bax and Bcl-2 balance is essential for regulating apoptosis and maintaining cellular homeostasis[34]. This study explored the impact of Mito-TEMPO on apoptotic cell death, revealing that 100 µM and 1000 µM concentrations of Mito-TEMPO significantly reduced nuclear fragmentation and condensation induced by rotenone toxicity. Moreover, the increase in anti-apoptotic Bcl-2 indicated that Mito-TEMPO effectively suppressed apoptotic cell death in SH-SY5Y cells exposed to rotenone. In contrary, pro-apoptotic bax was increased in rotenone group while 10 and 100 µM Mito-TEMPO reduced it. Furthermore, Mito-TEMPO suppressed cleaved caspase-3 levels in rotenone group, thus exhibiting inhibitory effects on the apoptotic process. Similarly, Mito-TEMPO inhibited apoptosis by suppressing cleaved caspase-3 activation and reducing ROS release in SH-SY5Y cells[21].

ROS, generated by mitochondrial respiration play a critical role in regulating various cellular processes. Excessive ROS production damage mitochondrial proteins, lipids, and DNA, leading to oxidative stress, mitochondrial dysfunction and cell death. A defect in Complex I within the electron transport chain leads to an increase in ROS, which damages mitochondrial DNA and subsequently nuclear[35]. DNA Therefore, ROS generation was examined in SH-SY5Y cells after Mito-TEMPO and rotenone treatments by DCFH-DA oxidation method and monitored the cells under fluorescence microscope. These results showed that ROS release was significantly increased in rotenone treated cells, while Mito-TEMPO suppressed ROS production in all concentrations. Previously, consistent with current results, it has been demonstrated that Mito-TEMPO works by scavenging ROS and inhibiting oxidative damage, particularly in mitochondria. Mito-TEMPO can also improve mitochondrial function by stabilizing mitochondrial membrane potential and reducing mitochondrial fragmentation. For instance, it has been reported that Mito-TEMPO protected dopaminergic neurons by reducing superoxide formation in PD and decreased ROS release by altering mitochondria dynamics against amyloid beta toxicity[36,37]. Similarly, the MTT results showed that Mito-TEMPO increased the cell viability via suppressing ROS production. According to Zhang *et al*., mitochondrial H_2O_2 combined with

Mito-TEMPO can enhance the function of abnormal NADPH-oxidase-2 (NOX2) and positively modulate the AMPK/Akt-mTOR signaling pathway[38] . Another study showed that pre-treatment of PC12, SH-SY5Y cells, and primary neurons with Mito-TEMPO (10 μM) significantly attenuated the PD toxins-induced generation of H_2O_2 in the cells[39]. Overall, these studies support that Mito-TEMPO is successful in preventing the formation of mitochondrial ROS.

Mitochondria dynamics such as fusion, fission, and mobility are crucial in determining the density and distribution of mitochondria. Mito-TEMPO can prevent or reduce mitochondrial damage induced by oxidative stress and preserve mitochondrial mass and function. Disruption of this activation has been shown to cause axonal degeneration and synaptic dysfunction in PD and can be reversed with antioxidant treatment[40]. Studies have shown that treatment with Mito-TEMPO can prevent or reduce mitochondrial damage induced by oxidative stress and preserve mitochondrial mass and function[41]. Cid-Castro and Moran reported that Mito-TEMPO reduced ROS release associated with the fission protein Drp1 although it did not affect mitochondrial morphology in cerebellar granular cells[41] . In the current study, the distribution and density of mitochondria were qualitatively assessed in live cells via Mitotracker Green staining. The findings demonstrated that pre-treating SH-SY5Y cells with Mito-TEMPO preserved mitochondrial mass and distribution in the face of rotenone toxicity, particularly at the 1000 μM concentration, and decreased ROS generation. *In vivo* studies demonstrating the effects of Mito-TEMPO in PD are limited. Ahmed *etal*. showed that Mito-TEMPO treatment inhibited NLRP3 inflammasome requires mitochondria activation and supports dopaminergic neuron survival[43].Further quantitative studies comparing the effects of different mitochondria targeted treatments *in vitro* and *in vivo* are needed to yield deeper insights into mitochondrial functionality in PD.

Conclusion

The present data suggests that Mito-TEMPO pre-treatment for 24 h increased cell viability against rotenone toxicity, suppressed apoptotic cell death by changing Bax, Bcl-2 and cleaved caspase 3 levels and increased mitochondria activation by reducing oxidative stress. Overall, Mito-TEMPO pre-treatment has promising therapeutic potential for PD. The effects of Mito-TEMPO on different properties of mitochondria such as ATP production, mitochondrial DNA and membrane potential are the limitations of this study. Additional *in vivo* studies are required to clarify the potential mechanisms of Mito-TEMPO in neurodegeneration process. The potential benefits of Mito-TEMPO pre/post-treatment in reducing oxidative stress and protecting mitochondrial function make it an exciting avenue for future research and therapeutic development.

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