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Original Article

# Cerium Oxide Nanoparticles Rescue Dopaminergic Neurons in Parkinson's Disease Model of SH-SY5Y Cells via Modulating Nrf2 Signaling and Ameliorating Apoptotic Cell Death

Betül Çiçek[1], Betül Danışman[2]

[1]Department of Physiology, Erzincan Binali Yıldırım University, Faculty of Medicine, Erzincan, Türkiye
[2]Department of Biophysics, Atatürk University, Faculty of Medicine, Erzurum, Türkiye

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**ORCID IDs of authors:** B.C. 0000-0003-1395-1326, B.D. 0000-0002-3812-9884.

## ABSTRACT

**Objective:** This study was designed to explore the potential neuroprotective role of cerium oxide nanoparticles, which are known to have antioxidant properties, in 6-hydroxydopamine-induced Parkinson's disease in SH-SY5Y cells.

**Methods:** SH-SY5Y cells were exposed to 200 μM 6-hydroxydopamine for 24 hours to mimic Parkinson's disease model in vitro. Cells were treated with cerium oxide nanoparticles (25, 50, and 100 μg/mL) for 30 minutes before 6-hydroxydopamine administration. Cell viability was determined by (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) and lactate dehydrogenase assays. The neuroprotective effect of cerium oxide nanoparticles on 6-hydroxydopamine-related oxidative damage was assessed with malondialdehyde, superoxide dismutase, and catalase analyses. The effects of cerium oxide nanoparticles on *NRF2* expression in 6-hydroxydopamine-associated neurotoxicity were evaluated by the reverse transcriptase polymerase chain reaction. Moreover, *BAX* and *BCL-2* expression levels were analyzed to determine the apoptosis.

**Results:** Cerium oxide nanoparticles at concentrations of 50 and 100 μg/mL showed protective effects against 6-hydroxydopamine-induced cell damage. Moreover, cerium oxide nanoparticles (at 50 and 100 μg/mL) attenuated oxidative injury evoked by 6-hydroxydopamine as determined by decreased malondialdehyde level probably through the improvement of antioxidant capacity by elevating superoxide dismutase and catalase. Cerium oxide nanoparticles promoted the *NRF2* transcriptional activity. Finally, we found that this nanoparticle (at 50 and 100 μg/mL) reversed the 6-hydroxydopamine-related elevation of *BAX* levels and reduction of *BCL-2* level.

**Conclusion:** Collectively, cerium oxide nanoparticles prevented 6-hydroxydopamine-induced toxicity in SH-SY5Y cells in a dose-dependent manner by activating Nrf2 signaling-related antioxidant pathways and alleviating apoptotic neuronal cell death.

**Keywords:** Parkinson's disease, SH-SY5Y cell line, CeO2NP, *NRF2*, antioxidant, apoptosis

## INTRODUCTION

Parkinson's disease (PD) is a type of disorder that impacts the nervous system and the regions of the body innervated by the nerves.[1] The fundamental biochemical deterioration in PD is the depletion of striatal dopamine associated with the progressive degeneration of dopaminergic neurons (DANs) in substantia nigra.[2] The mechanisms underlying the pathogenesis of PD are not fully understood.[1] However, free radical-related damage to neurons has been proposed as the main hypothesis for PD-related pathogenesis.[3] Multiple risk factors lead to mitochondrial dysfunction and ultimately increase free radical formation by releasing apoptosis-inducing factors.[4]

6-Hydroxydopamine (6-OHDA), a supremely oxidizable dopamine analog, is a neurotoxin and is commonly employed to research the pathogenesis of PD in cellular and animal models.[5] It induces elevated generation of free radicals and activates mitochondrial-associated apoptotic

**Corresponding Author:** Betül Çiçek, **E-mail:** betulll.cicekk@gmail.com



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signaling in DANs that initiate pathological alterations resembling PD.[6,7] The drugs employed for therapy for PD demonstrate suboptimal results, and those representing some variety of neuroprotection in vitro and in vivo research failed to indicate any considerable efficacy in clinical trials.[8] Nowadays, nanotechnology is gaining notability in research related to the treatment of neurodegenerative disorders (NDs). Especially, cerium oxide nanoparticles ($CeO_2$NPs) have characteristic features that can be efficacious in nanotherapeutics.[6] Because $CeO_2$NPs can bind oxygen reversibly and switch between oxidation conditions (Ce3+/Ce4+), they can scavenge free radicals.[9] $CeO_2$NPs are superior to other antioxidants compounds because of their SOD and glutathione (GSH) enzyme mimetic properties in a redox-state contingent manner.[10] Unlike conventional antioxidants, the radical scavenging feature of $CeO_2$NPs is regenerative and allows prolonged activity over long periods of time.[11] Reports have declared that $CeO_2$NPs preserve neurons and other kinds of cells from free radicals-related damage through their antioxidant properties.[6,11] Regarding NDs including Alzheimer's disease (AD),[12] traumatic brain injury,[13] etc. in which oxidative stress has a main role, $CeO_2$NPs have shown promising therapeutic potential via regulating diverse signaling pathways including neuronal survival, differentiation, and death. However, although $CeO_2$NPs have been suggested as a disease-modifying therapy for the in vivo model of PD induced by 6-OHDA, the pathological mechanism needs further investigation.

Therefore, the aim of the current study was to investigate the impact of $CeO_2$NPs on 6-OHDA related in vitro model of PD with the main focus on oxidative damage-regulated apoptotic pathways.

## METHODS

### *Cell Culture and Exposure of* 6-Hydroxydopamine

All experiments were performed using human neuroblastoma SH-SY5Y cells originally from ATCC, Virginia, USA. About 10 μM retinoic acid was used for their differentiation in Dulbecco's modified Eagle's medium (DMEM) -F12 medium for 5 days. Ascorbic acid (0.02%) was employed to dissolve the 6-OHDA (Sigma-Aldrich, St. Louis, USA).

**MAIN POINTS**

- 6-Hydroxydopamine (6-OHDA) manifested its toxicity in SH-SY5Y cells.
- Cerium oxide nanoparticles ($CeO_2$NPs) could ameliorate the oxidative stress induced by 6-OHDA in SH-SY5Y cells.
- The neuroprotective features of $CeO_2$NPs against 6-OHDA might be mediated by the activation of Nrf2 signaling.
- Cerium oxide nanoparticles inhibited apoptosis through the modulation of *BAX* and *BCL-2* genes.

### Cell Death/Viability Assessment

SH-SY5Y cells were seeded at a density of $5 \times 10^4$ cells/well into 96-well plates and allowed to attach overnight. Cerium oxide nanoparticles, which had a hydrodynamic diameter of 14 nm, were kindly donated by Assoc. Prof. Dr. Kemal Volkan Özdokur (Erzincan Binali Yildirim University, Erzincan, Turkey). SH-SY5Y cells were pretreated with $CeO_2$NPs (25, 50, and 100 μg/mL) half an hour before the 200 μM 6-OHDA application for 24 hours. After incubation, MTT solution was applied to cells for 4 hours (Sigma-Aldrich). The absorbance was determined at 570 nm with a microplate reader (Multiskan GO, Thermo Scientific, Elabscience, Texas, USA Multiskan GO, Canada, USA). On the other hand, cell death was determined by using LDH released from cells with damaged membranes in agreement with the manufacturer's instructions (Elabscience, USA). Optic density was determined at 450 nm with a spectrophotometer plate reader (Multiskan GO, Thermo Scientific, USA).

### Measurement of Oxidative Parameters

Malondialdehyde (MDA), SOD, and CAT levels were determined through commercial assay kits (Elabscience, USA). The cells were seeded at a density of $5 \times 10^5$ cells/well in 6-well plates. Different concentrations of $CeO_2$NPs (25, 50, and 100 μg/mL) were applied to the cells and incubated together for 24 hours by adding 200 μM 6-OHDA half an hour later. The assays were performed in line with the manufacturer's guidelines. Optic density was determined by a spectrophotometer plate reader (Multiskan GO, Thermo Scientific, USA).

### Gene Expression by Quantitative Reverse Transcription Polymerase Chain Reaction

SH-SY5Y cells were seeded and treated as mentioned above. Afterward, total RNA was extracted using an RNeasy kit (Thermo Scientific, USA) relative to the manufacturer's directions, and mRNA was employed for cDNA synthesis kit (Thermo Scientific, USA) as specified previously.[14] Levels of *NRF2, BAX,* and *BCL-2* mRNA expression of the cells were assigned by Rotor-Gene 6000 (Corbett Life Science, Mortlake, Australia). β-actin was employed as the standard gene. *NRF2, BAX,* and *BCL-2* expressions were normalized to β-actin using the $2-\Delta\Delta Ct$ method. Primer sequences are presented in Table 1.

### Statistical Analysis

Data were evaluated with one-way analysis of variance with post hoc Tukey's test Statistical Package for Social Sciences 22.0, IBM SPSS Corp., Armonk, NY, USA), and $P < .05$ was accepted to be significant.

**Table 1.** Sequence List of the Primers Used for RT-PCR

| Gene Name | Primer Sequence (5′–3′) |
|---|---|
| ***NRF2*** | Forward:5′-ACACGGTCCACAGCTCATA-3′<br>Reverse: 5′-TGTCAATCAAATCCATGTCCTG-3′ |
| ***BAX*** | Forward:5′-GGACGAACTGGACAGTAACATGG-3′<br>Reverse: 5′-GCAAAGTAGAAAAGGGCGACAAC-3′ |
| ***BCL-2*** | Forward: 5′-ATCGCCCTGTGGATGACTGAG-3′<br>Reverse: 5′-CAGCCAGGAGAAATCAAACAGAGG-3′ |
| ***β-ACTIN*** | Forward: 5′-TTGTTACAGGAAGTCCCTTGCC-3′<br>Reverse: 5′-ATGCTATCACCTCCCCTGTGTG-3′ |

RT-PCR, reverse transcriptase polymerase chain reaction.

## RESULTS

### Cerium Oxide Nanoparticles *Ameliorate* 6-Hydroxydopamine-*Related Neurotoxicity in SH-SY5Y Cells*

After the addition of 200 μM 6-OHDA, the cell viability markedly dropped to 55.09% ($P < .0001$), while cell viabilities were 86.79% and 94.03% when 50 and 100 μg/mL concentrations of $CeO_2$NPs nanoparticles were added, respectively ($P < .01$ and $P < .001$; Figure 1A). On the other hand, 25 μg/mL of $CeO_2$NPs nanoparticles demonstrated partial protection from the deleterious effects of 6-OHDA on SH-SY5Y cells; however; this was not statistically significant.

Furthermore, 6-OHDA promoted marked LDH release from the cells in comparison to the control group ($P < .0001$). When 50 and 100 μg/mL concentrations of $CeO_2$NPs were added, LDH release was markedly lower than the LDH release prompted by exposure to 6-OHDA alone ($P < .01$ and $P < .001$, respectively; Figure 1B), showing prevention of the injury to membrane integrity induced by 6-OHDA. In line with the MTT findings, when a concentration of 25 μg/mL of $CeO_2$NPs was added, LDH levels were not remarkably different from those induced by treatment with 6-OHDA.

### Cerium Oxide Nanoparticles *Attenuate Oxidative Stress Induced by* 6-Hydroxydopamine

In order to determine whether $CeO_2$NPs could suppress 6-OHDA-induced oxidative damage, we measured the MDA levels and CAT and SOD activities. As shown in Figure 2, after being treated with 6-OHDA, MDA levels were markedly elevated, while CAT and SOD activities were importantly reduced as compared with the control group ($P < .0001$), suggesting that 6-OHDA could promote oxidative damage. When SH-SY5Y cells were pretreated with $CeO_2$NPs (50 and 100 μg/mL) followed by exposure to 6-OHDA, MDA levels were importantly reduced in comparison to the only 6-OHDA group ($P < .01$ and $P < .001$, respectively; Figure 2A). Furthermore, 50 and 100 μg/mL $CeO_2$NPs elevated CAT and SOD activities in 6-OHDA-exposure SH-SY5Y cells notably (Figure 2B and 2C). However, when a concentration of 25 μg/mL of CeO2NPs was applied, SOD and CAT activities were not different from only 6-OHDA-treated cells.

### Cerium Oxide Nanoparticles *Induce the Expression of NRF2 in 6-OHDA-Treated SH-SY5Y Cells*

As shown in Figure 3, after 24 h exposure to SH-SY5Y cells with 200 μM 6-OHDA, we detected a marked reduction in comparison to the control value of the *NRF2* expression level ($P < .0001$). SH-SY5Y cells treated with 50 and 100 μg/mL $CeO_2$NPs nanoparticles for 30 minutes prior to exposure to 6-OHDA led to a marked elevation in *NRF2* expression levels ($P < .001$ and $P < .0001$, respectively) compared with 6-OHDA-treated cells. On the other hand, the treatment of SH-SY5Y cells with 25 μg/mL $CeO_2$NPs

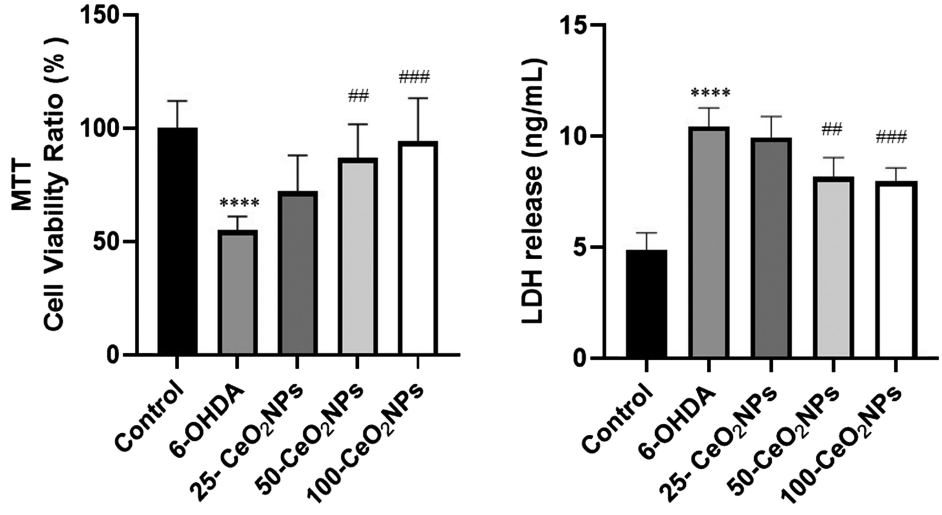


**Figure 1.** Effects of $CeO_2$NPs on cell viability test results. Data are expressed as the means (SD). $^{****}P < .0001$ vs. control group, $^{##}P < .01$ vs. 6-OHDA group, $^{###}P < .001$ vs. 6-OHDA group. $CeO_2$NPs, cerium oxide nanoparticles; 6-OHDA, 6-hydroxydopamine.

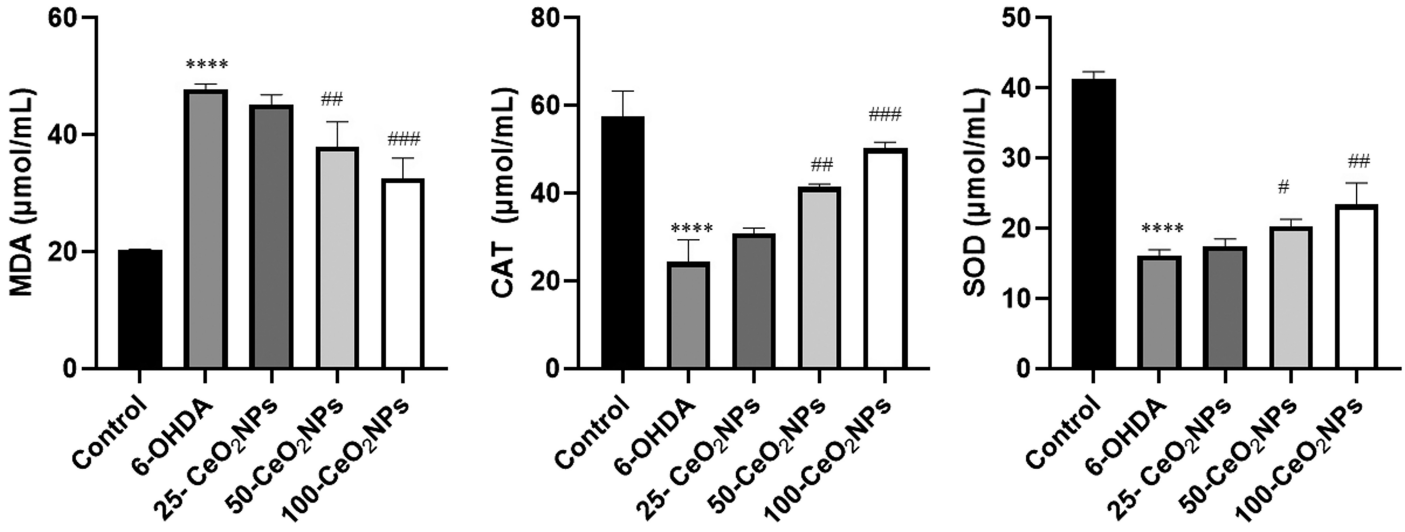


**Figure 2.** Effects of $CeO_2$NPs on oxidative stress marker MDA and the antioxidant enzymes SOD and CAT. Data are expressed as the means (SD). $^{****}P < .0001$ vs. control group, $^{\#}P < .05$ vs. 6-OHDA group, $^{\#\#}P < .01$ vs. 6-OHDA group, $^{\#\#\#}P < .001$ vs. 6-OHDA group. $CeO_2$NPs, cerium oxide nanoparticles; 6-OHDA, 6-hydroxydopamine; MAD, malondialdehyde; SD, standard deviation.

30 min before 6-OHDA exposure did not change significantly *NRF2* levels with respect to cells treated only with 6-OHDA.

### *$CeO_2$NPs Demonstrate Antiapoptotic Properties in* 6-Hydroxydopamine-*Treated SH-SY5Y Cells*

As demonstrated in Figure 4, after 24 hours of incubation with 200 µM 6-OHDA, the findings demonstrated that in 6-OHDA treatment alone, level of *BAX* expression was elevated, and level of *BCL-2* expression was reduced in comparison with the control ($P < .001$ and $P < .0001$ respectively). Pretreatment with 50 and 100 µg/mL $CeO_2$NPs induced a dramatic decrease in *BAX* levels ($P < .05$ and $P < .01$, respectively) and a marked increase in *BCL-2* levels ($P < .01$ and $P < .001$, respectively) in treated SH-SY5Y cells with 6-OHDA. However, pretreatment with 25 µg/mL $CeO_2$NPs showed partial alterations in levels of *BAX* and *BCL-2*, which was not statistically significant.

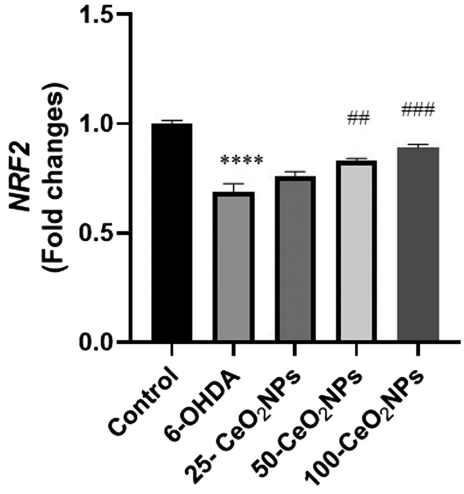


**Figure 3.** Effects of $CeO_2$NPs on *NRF2* expression. Data are expressed as the means (SD). $^{***}P < .001$ vs. control group, $^{****}P < .0001$ vs. control group, $^{\#}P < .05$ vs. 6-OHDA group, $^{\#\#}P < .01$ vs. 6-OHDA group, $^{\#\#\#}P < .001$ vs. 6-OHDA group. $CeO_2$NPs, cerium oxide nanoparticles; 6-OHDA, 6-hydroxydopamine; SD, standard deviation.

## DISCUSSION

The present study represents that $CeO_2$NPs protect SH-SY5Y cells against apoptosis induced by 6-OHDA through the Nrf2 signaling by modulating antioxidant enzymes such as CAT and SOD and inhibiting the generation of MDA. Numerous pharmacological properties have been attributed to the $CeO_2$NPs, with the inclusion of antioxidant and anti-inflammatory effects.[2,6,15] A limited number of studies have reported that $CeO_2$NPs have neuroprotective effects in models of PD;[2,6] however, further studies are needed to demonstrate the detailed mechanism of action of $CeO_2$NPs.

6-Hydroxydopamine prompted neurotoxicity in SH-SY5Y cells is an extensively employed in vitro PD model.[5,8] It is widely known that this neurotoxin caused neuronal injury by inducing oxidative damage which ultimately leads to activated apoptosis in DANs.[16] Initially, in order to explore the effects of $CeO_2$NPs on 6-OHDA-induced neurotoxicity, we evaluated cell viability employing the MTT assay in SH-SY5Y cells. The pre-incubation of SH-SY5Y cells with 50 and 100 µg/mL of $CeO_2$NPs 30 minutes before the exposure to 6-OHDA revealed the protective properties of this nanoparticle. Noteworthy, the 25 µg/mL $CeO_2$NPs had no protective effect against the 6-OHDA-related neurotoxicity. Neuronal injury also results in release of

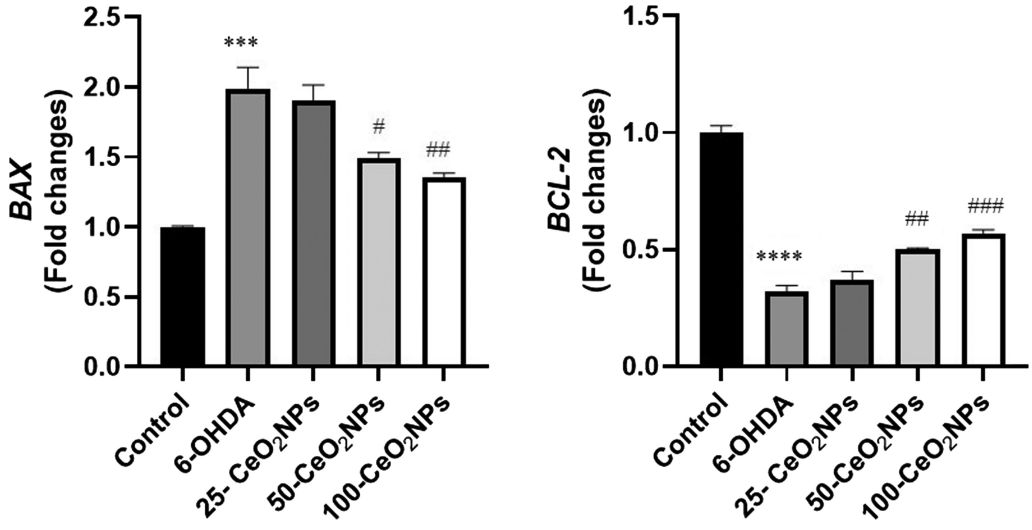


**Figure 4.** Effects of $CeO_2$NPs on apoptosis. Data are expressed as the means (SD). $^{****}P < .0001$ vs control group, $^{\#\#\#}P < .001$ vs. 6-OHDA group, $^{\#\#\#\#}P < .0001$ vs. 6-OHDA group. $CeO_2$NPs, cerium oxide nanoparticles; 6-OHDA, 6-hydroxydopamine; SD, standard deviation.

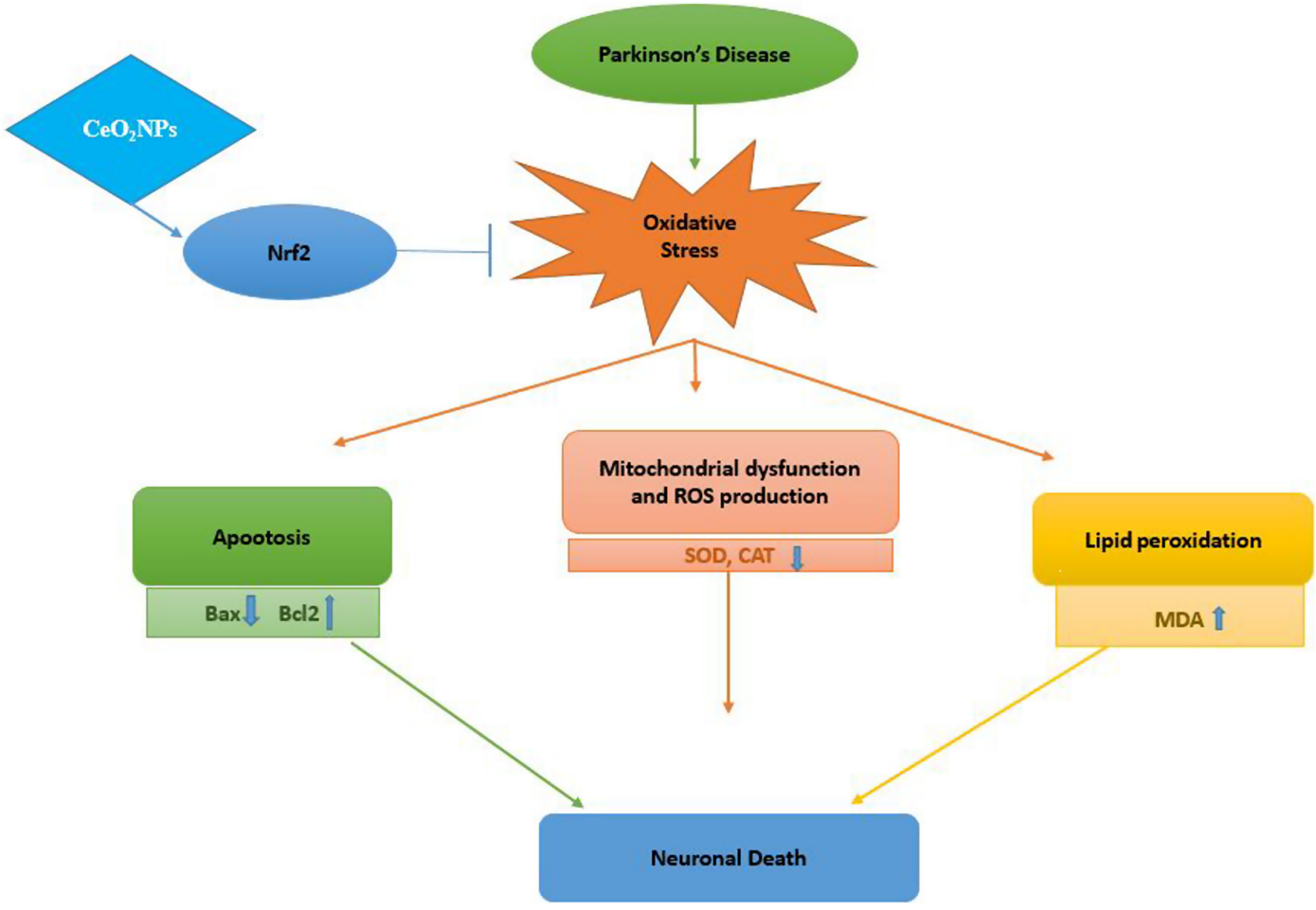


**Figure 5.** $CeO_2$NPs alleviate oxido-inflammatory stress and apoptotic responses by activating Nfr2 signaling in 6-OHDA-induced in vitro model of Parkinson's disease .

LDH into the extracellular space. We measured LDH content in 6-OHDA-treated SH-SY5Y cells and found that pretreatment of 50 and 100 µg/mL of $CeO_2$NPs greatly increased metabolic activity and reduced LDH release. Therefore, these findings demonstrate that $CeO_2$NPs may repress 6-OHDA-associated oxidative damage and apoptosis.

We also examined the effects of $CeO_2$NPs pretreatment on 6-OHDA-induced alterations in antioxidant enzymes and lipid peroxidation induction in SH-SY5Y cells. Many studies have reported that oxidative stress related to 6-OHDA markedly alters the balance between the generation of free radicals and the antioxidant defense system, which ultimately leads to neuronal injury.[16,17] Free radicals stimulated by 6-OHDA react with numerous biological targets and disrupt mitochondrial function. Additionally, excess free radicals diminish the antioxidant enzymes activities, including CAT and SOD.[6,18] In this study, oxidative damage induced by 6-OHDA was manifested with an elevated level of MDA associated with a marked reduction CAT and SOD levels. This oxidative injury associated with 6-OHDA in SH-SY5Y cells is in compliance with previous reports.[19] *NRF2* signaling has the main role in managing oxidative stress during PD via the regulation of antioxidant response.[20] In the current study, disturbed Nfr2 signaling with oxidative stress was demonstrated in 6-OHDA-treated SH-SY5Y cells, in line with previous reports. [21]

In previous reports, high levels of antioxidant enzymes were reported to maintain against damage of mitochondrial membrane damage and cell death.[22] Here, we found that $CeO_2$NPs (at 50 and 100 µg/mL) were able to restore *NRF2* expression which mediates amelioration of CAT and SOD activities, as well as reduction of MDA levels. The useful effects of $CeO_2$NPs on neurodegenerative disease could be attributed to its potential free radicals scavenging characteristics, enhancement of antioxidant capacity.[11,12] Hegazy et al[6] previously demonstrated that $CeO_2$NP reduces MDA together with elevated total antioxidant capacity levels in PD-induced rats.

As mentioned above, the generation of free radicals caused by the 6-OHDA in SH-SY5Y cells results in mitochondrial membrane dysfunction and eventually joins to neuronal cell death associated with the pathogenesis of PD.[23] Bax and Bcl-2 proteins take part in the regulation of membrane permeability, mitochondrial integrity, and eventually apoptosis.[24] Apoptotic process enhances through activation of Bax, which alters membrane permeability. In contrast, Bcl-2 suppresses apoptosis and maintains against neuronal cell death owing to oxidative damage.[25] Consistent with other reports, we found that application of 6-OHDA importantly elevated *BAX* levels and reduced *BCL-2* levels in SH-SY5Y cells.[23] However, pretreatment with $CeO_2$NP (at 50 and 100 µg/mL) dramatically alleviated these alterations in protein levels induced by 6-OHDA. Based on our findings, we speculate that $CeO_2$NP protects from 6-OHDA-related neuronal cell death via the maintenance of mitochondrial activity in SH-SY5Y cells in a dose-dependent manner. Consistent with current results, together with evidence pointing to $CeO_2$NPs attenuating DANs apoptosis, antiapoptotic effects of $CeO_2$NPs were found in 6-OHDA-treated SH-SY5Y cells.

Taken together, our findings demonstrated that $CeO_2$NPs protect against 6-OHDA-induced neuronal cell death through the reduction of oxidative damage and the regulation of apoptotic signaling. In addition, the mechanism of this neuroprotective impact may also involve the activation of *NRF2* signaling (Figure 5). Cerium oxide nanoparticles may be a potential therapeutic or preventive agent for PD; however, further experiments assessing the effects of $CeO_2$NPs on detailed signaling pathways associated with the pathogenesis of PD are required.

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**Informed Consent:** Not applicable.

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