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Selegiline induces neuroprotection via *PGC-1α*/*Nrf2* gene upregulation in H_2O_2 -treated hippocampal-derived neural stem/progenitor cells

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Abstract

Oxidative stress mediated by reactive oxygen species (ROS) is a major contributor to the pathogenesis of neurodegenerative diseases, including Alzheimer's, Parkinson's, Huntington's, multiple sclerosis, and amyotrophic lateral sclerosis. Selegiline, a monoamine oxidase B inhibitor, has been reported to exert neuroprotective effects, although its precise cytoprotective mechanisms remain unclear. In this study, we investigated the effects of selegiline on apoptosis, necrosis, and cell survival in hydrogen peroxide (H_2O_2)-treated hippocampal-derived neural stem/progenitor cells (HD-NSPCs) in vitro. Passage 3 HD-NSPCs were treated with varying concentrations of selegiline (10^{-3} to 10^{-9} M) prior to exposure to 125 µM H_2O_2. Cell viability was assessed using the MTT assay, while apoptosis and necrosis were evaluated using TUNEL and acridine orange/ethidium bromide staining, respectively. Real-time RT-PCR was performed to quantify mRNA levels of PGC-1α, Nrf2, and Bcl-2. Treatment with 10^{-7} M selegiline significantly enhanced HD-NSC viability, reduced apoptotic and necrotic cell fractions, and upregulated PGC-1α, Nrf2, and Bcl-2 expression compared to untreated cells ($P < 0.05$). These findings suggest that selegiline mitigates oxidative stress–induced cytotoxicity by activating Nrf2/PGC-1α signaling and promoting anti-apoptotic gene expression, thereby preserving mitochondrial function and enhancing cell survival. Overall, selegiline may represent a promising therapeutic agent for protecting neural progenitor cells and alleviating neuronal damage in neurodegenerative disorders.

Keywords: Selegiline, HD-NSPCs, oxidative stress, Nrf2, PGC-1α, Bcl-2, neuroprotection.

1. Introduction

Recent investigations have shown that various neurodegenerative conditions, such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and multiple sclerosis, could be due to oxidative damage driven by reactive oxygen species (ROS)[1, 2]. ROS can cause severe harm to essential biological molecules, including DNA, fats, and proteins [3]. Neurodegenerative disorders are long-term illnesses marked by gradual loss of neuron function and cell death[4, 5]. Selegiline (L-deprenyl) is a blocker of monoamine oxidase type B (MAO-B) originally used to treat Parkinson's disease [6]. Monoamine oxidase is a mitochondrial enzyme that speeds up the oxidative breakdown of monoamines like dopamine, norepinephrine, and serotonin. This process yields hydrogen peroxide, a major source of cellular reactive oxygen species (ROS). Thus, by adjusting the activity of this enzyme, selegiline not only boosts the beneficial effects of dopamine given in clinical settings but also helps lower ROS levels [7-9]. Multiple studies have also verified the cell death–preventing properties of selegiline, though the precise way it works is still unknown. Selegiline provides neuroprotective effects by controlling antioxidant enzymes and genes that prevent cell death. More specifically, selegiline increases the levels of Bcl-2 and Bcl-xL while decreasing BAX, thereby reducing damage caused by oxidative stress [10, 11].

PGC-1α is broadly viewed as a central regulator of genes necessary for mitochondrial biogenesis [12, 13]. Mitochondrial dysfunction or impaired mitochondrial renewal is a characteristic of most neurodegenerative diseases and is governed by a complex network of transcription factors. Nuclear respiratory factors (Nrfs), peroxisome proliferator-activated receptors (PPARs), and estrogen-related receptors (ERRs) are considered among these factors that turn on target genes involved in the expression of oxidases [14, 15]. A great deal of research has focused on understanding the role of Nrf2 in defending against oxidative stress. The Nrf2–Keap1 communication pathway, along with the resulting activation of protective proteins, is essential for shielding cells from oxidative injury [16]. Earlier work has shown that lacking Nrf2 in genetically

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modified mice raises their vulnerability to many types of oxidative stress–induced harmful and neurodegenerative conditions [17]. Enhanced Nrf2 function, brought about by chemopreventive substances, has been shown to offer stronger defense against oxidative harm in animal models [18, 19]. Consequently, cross-talk between Nrf2 and other signaling factors affects the effectiveness of cellular stress responses. ROS-triggered activation of Nrf2 starts the transcription of phase II detoxifying genes, strengthening cellular protective systems [3]. Neural Stem/Progenitor Cells (NSPCs) are a specific type of cell in the nervous system with the unique abilities to self-renew and generate other cell types. Because of these properties, they are a major focus of research aiming to repair damaged brains and spinal cords [20].

While selegiline is known for its neuroprotective properties, its direct effects on NSPCs and the specific transcriptional pathways involved remain poorly understood. Unlike previous studies that have primarily examined selegiline in differentiated neuronal models or focused on its MAO-B inhibitory activity, this work sought to determine whether selegiline could modulate antioxidant and anti-apoptotic gene expression in neural progenitor cells, thereby offering a novel mechanistic perspective on its cytoprotective actions. This study aimed to investigate whether selegiline exerts neuroprotective effects against oxidative stress-induced injury in hippocampal-derived neural stem/progenitor cells (HD-NSPCs), and to explore the underlying molecular mechanisms, with particular focus on the potential involvement of PGC-1α, Nrf2, and Bcl-2 gene expression.

2. Materials and methods

2.1. Isolation and Expansion of HD-NSPCs

HD-NSPCs were obtained from the hippocampal region of 10-day-old newborn Wistar rats (obtained from the Razi Institute), following a previously described method [21]. In brief, after deep sedation induced by intraperitoneal injections of ketamine (100 mg/kg) and xylazine (10 mg/kg), the brain was quickly taken out, and the hippocampus was separated and placed in a 4 g/L glucose solution. The tissue pieces were chopped with scissors and blades, then broken down chemically in a mixture containing papain (2.5 U/ml, Sigma-Aldrich), dispase II (40 U/ml, Sigma-Aldrich), and accutase (1 ml, Invitrogen; Thermo Fisher Scientific) for half an hour at room temperature. The resulting cell mixture was passed through a 70 μm cell sieve (Falcon) and spun at 1000 × g for 10 minutes at 4°C. The sediment was rinsed with PBS and re-suspended in neural stem cell culture medium (DMEM/F12) containing 2% B27 (Gibco), 20 ng/ml bFGF (Invitrogen), 20 ng/ml EGF (Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich; Merck KGaA). Cells were placed in non-sticking 25 cm^2 plastic containers and kept at 37°C with 5% CO_2 for one week to allow neurospheres to form, with the medium changed every two days.

Neurospheres were then broken apart into individual cells using 0.25% trypsin-EDTA (Sigma-Aldrich) and gentle pipetting. HD-NSPCs (10^5 cells per well) were subsequently grown in DMEM/F12 supplemented with 2% B27, 20 ng/ml bFGF, 20 ng/ml EGF, and 3% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific) for one week at 37°C with 5% CO_2. Cells were transferred up to three times in 6-well plates covered with poly-L-lysine.

For cell type confirmation, immunocytochemistry was carried out using an anti-nestin monoclonal antibody (1:200, Abcam), followed by a fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse secondary antibody (1:200, Millipore). Cells were grown on glass coverslips, fixed with 3% paraformaldehyde for 20 minutes at room temperature, and made permeable with 100% methanol for half an hour. After an overnight incubation with the primary antibody at 4°C, the secondary FITC-linked antibody was added for 4 hours. Cell nuclei were stained with ethidium bromide for 1 minute at room temperature.

2.2. Dose-Response Effect of Selegiline on Oxidative Stress

Passage 3 HD-NSPCs were placed into 96-well plates at a density of 5 × 10^4 cells per well in DMEM/F12 medium containing 2.7% B27, 20 ng/ml bFGF, and 20 ng/ml EGF, then grown for 48 hours. To evaluate the neuroprotective effects of selegiline, cells were exposed to different amounts of selegiline (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} M) over 48 hours, with the culture medium changed every 12 hours, before being subjected to H_2O_2. Oxidative injury was triggered by exposing HD-NSPCs to 125 μM H_2O_2 for half an hour at 37°C in a 5% CO_2 environment[22]. HD-NSPCs that did not receive selegiline treatment (0 μM) were used as controls, and another control group was maintained in DMEM/F12 medium for 48 hours followed by H_2O_2 exposure. Cell survival was assessed using the MTT assay. Cells were kept with 1 mg/ml MTT solution (Sigma-Aldrich) for 4 hours, after which the liquid was removed, and 100 μl of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. Absorbance was read at 570 nm using a microplate ELISA reader. The percentage of viable cells was calculated as: Relative viability (%) = (A570 of treated samples / A570 of untreated samples) × 100[23].

2.3. Detection of Necrotic and Apoptotic Cells

Following treatment, HD-NSPCs were preserved in PBS with 4% paraformaldehyde for 15 minutes at room temperature (RT). Cell death was identified using the TUNEL method with the In Situ Cell Death Detection Kit (Roche, Germany), following the kit's recommended protocol. Cells showing TUNEL positivity were made visible using diaminobenzidine (DAB) staining for 3–7 minutes at RT, followed by a 5-minute counterstain with hematoxylin.

Necrosis was assessed via acridine orange/ethidium bromide staining. Fixed HD-NSPCs were treated with acridine orange and ethidium bromide (100 μg/ml each; Sigma-Aldrich; Merck KGaA) for 5 minutes at RT. Necrotic cells were recognized by their orange to yellow cytoplasmic glow and examined under a fluorescence microscope (BX61, Olympus, Tokyo, Japan). The proportions of TUNEL-positive and necrotic cells were determined by tallying cells in five randomly chosen fields per well (200 cells total per sample).

2.4. Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

After oxidative injury was induced, real-time RT-PCR was carried out on cDNA generated from the control group (0 M) and the 10^{-7} M selegiline-treated group. Total RNA (1000 ng) from HD-NSPCs was isolated using TRIzol (In-

Table-1. Primer Sequences and PCR Parameters.

Gene	Gene Accession no.	Sense 5 → 3	Anti-sense 5 → 3
B2M	NM_012512.2	CTTGCCATTCAGAAAACTCC	CATCGGTCTCGGTGGGTG
Nrf2	NM_031789.2	ATTCCCGAGTTACAGTGTCT	CGTGGAGAGGATGCTGCT
Bcl-2	NM_016993.1	AGGATAACGGAGGCTGGGATG	GGTCTGCTGACCTCACTTGTGG
PGC-1α	NM_031347.1	GACAGAACTGAGAGACCGCT	CTGAGTTTGAATCTAGGTCTGC

vitrogen, Thermo Fisher Scientific) and reverse-transcribed into 20 µl of cDNA with the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Germany), following the kit's guidelines. The obtained cDNA was then used to measure the mRNA levels of PGC-1α, Nrf2, and Bcl-2, with beta-2-microglobulin (B2M) employed as an internal reference.

Each PCR mixture (12.5 µl total volume) contained 200 nM of forward and reverse primers (Shown in Table 1), 0.5 µl cDNA, 6.5 µl SYBR® Green I (Fermentas, Thermo Fisher Scientific), and nuclease-free water. Amplification was conducted with an initial denaturation step at 95°C for 15 seconds, followed by 40 cycles at 60°C for 1 minute. Relative mRNA expression levels were determined using the $2^{-\Delta\Delta Ct}$ method.

2.5. Statistics

The statistical evaluation was carried out with SPSS software (version 15). Results are shown as the mean ± standard error of the mean (SEM) from five independent experiments. Comparisons among groups were performed using one-way ANOVA, followed by Tukey's post hoc test for individual group comparisons. A P value below 0.05 was regarded as indicating statistical significance.

3. Results

3.1. Neural stem cells culture

HD-NSC primary culture results are shown in Figures 1A-D. After several hours, self-renewing neurons with multipolar processes and growth cone-like features were observed. Small spheroids formed after 3 days (Figure 1A). Intact neurospheres were cultured on poly-L-lysine-coated 6-well plates without dissociation, forming rosette-like structures (Figure 1B). The purity of HD-NSPCs at passage 3 was assessed using the progenitor cell marker nestin, which showed strong positive immunostaining (97.51 ± 0.48) (Figure 1C, D). A negative control without the primary antibody confirmed the specificity of the labeling.

3.2. Dose-Response Effect of Selegiline

Using the MTT assay, we demonstrated a dose-dependent neuroprotective effect of selegiline on HD-NSPCs exposed to 125 µM H_2O_2 (Figure 2). The results showed that increasing concentrations of selegiline (0–10^{-7} M) protected against oxidative stress induced by H_2O_2. However, high concentrations of selegiline significantly decreased HD-NSC viability in a dose-dependent manner. Scores were normalized to the control group (untreated HD-NSPCs). The NSH group (10^{-7} M selegiline for 48 hours followed by 125 µM H_2O_2 exposure) exhibited a significant increase in viable cells (64.95±3.4) compared to lower concentrations of selegiline (0, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-9} M), which had viability scores of 27.12±1.84, 38.23±1.42, 33.95±2.62, 42.47±2.26, and 46.01±2.24, respectively.

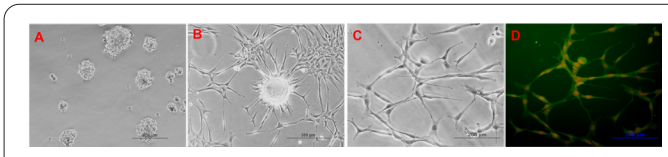


Fig. 1. Isolation and Culture of HD-NSPCs. A) Phase contrast microscopy of neurons in suspension culture for 3 days. B) Appearance of an asterisk-like structure after 7 days of culture. C & D) Phase contrast image and Nestin-positive immunohistochemistry of HD-NSPCs, respectively. Nestin is labeled green (FITC-conjugated secondary antibody) and the nucleus is red (ethidium bromide).

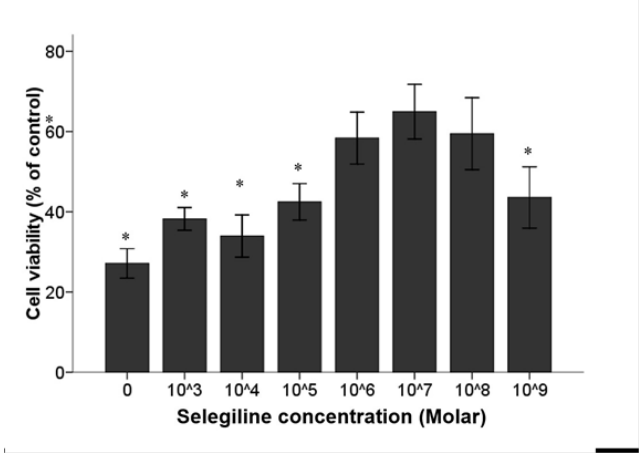


Fig. 2. Protective Effect of Selegiline on Cell Viability. Dose-response curve showing the viability of HD-NSPCs at different concentrations of selegiline, treated with 125 µM H_2O_2, as assessed by the MTT assay. Results are presented as the average percentage of viable cells relative to untreated HD-NSPCs. Each bar represents the mean of 5 replicates. Bars indicate mean ± SEM. $P < 0.05$ (compared to 10^{-7} M).

3.3. Detection of Apoptotic and Necrotic Cells

Using the TUNEL assay and acridine orange/ethidium bromide staining, we investigated the protective and anti-apoptotic effects of selegiline on HD-NSPCs treated with 10^{-7} M selegiline for 48 hours, followed by exposure to 125 µM H_2O_2. As shown in Figure 3 (A-D), TUNEL-positive nuclei and necrotic cells revealed that H_2O_2 induced both apoptotic and necrotic cell death. The percentage of TUNEL-positive cells was significantly lower in the selegiline-treated group compared to the control group (0 µM selegiline) ($P < 0.05$). Figures 3A, B and Figure 4 demonstrate a significant reduction in apoptotic cells in the NSH group compared to the NH group (49.42 ± 0.9% vs. 27.66 ± 1.51%; $P < 0.05$). Similarly, Figures 3C and 3D, and Figure 4 show a significant decrease in necrotic cells in the NSH group compared to the NH group (67.45 ± 1.41% vs. 34.15 ± 0.82%; $P < 0.05$).

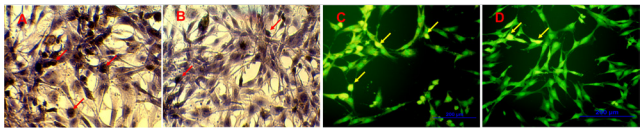


Fig. 3. Represent TUNEL and acridine orange staining, respectively. A & C) Represent NH, TUNEL, and acridine orange staining, respectively. B & D) Represent NSH, TUNEL, and acridine orange staining, respectively. Red arrows indicate DNA fragmentation, and late necrotic HD-NSPCs are shown with an orange-red nucleus (yellow arrow). Hematoxylin counterstaining is shown in blue in the TUNEL assay. Magnification: 200X.

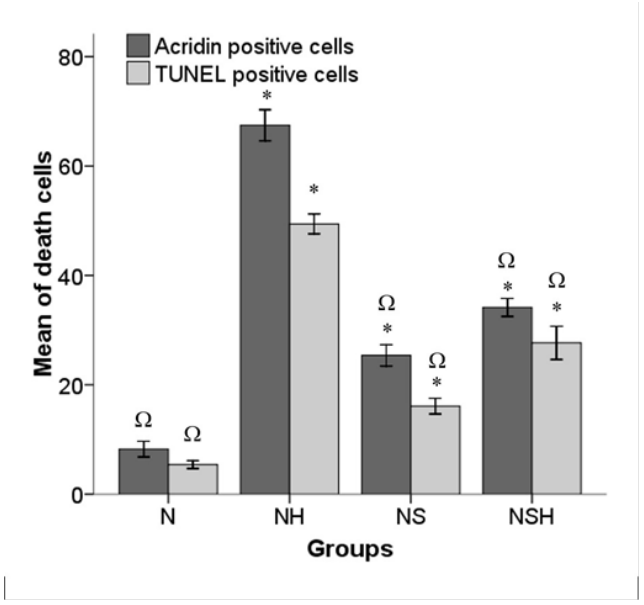


Fig. 4. Detection of Programmed Cell Death and Necrosis. Histogram plots show the mean percentages of programmed cell death and necrosis in the N, NH, NS, and NSH experimental groups. Bars represent the mean ± SEM. $P < 0.05$ (compared to group N), Ω (compared to group NH).

3.4. Gene Expression Results

The q-PCR results are shown in Figure 5. Real-time quantitative RT-PCR confirmed elevated mRNA levels of *PGC-1α*, *Nrf2*, and *Bcl-2* in the NSH group (HD-NSPCs treated with 10^{-7} M selegiline for 48 hours). Significant increases were observed in the mRNA levels of *PGC-1α*, *Nrf2*, and *Bcl-2* in the NSH group (2.63 ± 0.31, 5.00 ± 0.20, and 2.32 ± 0.16, respectively) compared to the NH group (0.52 ± 0.04, 3.26 ± 0.18, 1.62 ± 0.10, respectively) and the NS group (1.13 ± 0.10, 1.77 ± 0.19, 0.64 ± 0.07, respectively) ($P < 0.05$).

4. Discussion

In the present work, we showed that selegiline reduces apoptotic and necrotic cell death in HD-NSPCs subjected to oxidative injury by increasing the mRNA expression of PGC-1α, Nrf2, and Bcl-2. Earlier studies have found that free radicals can directly or indirectly harm essential biomolecules, thereby leading to neuronal cell death under oxidative stress conditions [1, 24]. Lowering oxidative stress in neurodegenerative illnesses has been shown to block neuronal apoptosis and delay disease progression. By easing oxidative damage, nerve cells can be shielded from degeneration, potentially enhancing outcomes in disorders such as Alzheimer's disease, Parkinson's disease, and other neurodegenerative conditions [1, 25]. Monoamine oxidase inhibitors (MAOIs), including selegiline and rasagiline, serve a key function in suppressing apoptosis [26]. According to both laboratory and animal studies, selegiline blocks MAO-B and boosts the production of neurotrophic factors [27, 28]. Additionally, it is an anti-Parkinson medication known for its antioxidant and anti-apoptotic characteristics [29]. Nevertheless, the exact protective mechanism of selegiline at the cellular level remains not completely understood [30]. In this study, we describe a distinct cellular defense mechanism of selegiline involving Nrf2- and PGC-1α–mediated stimulation of oxidative stress–responsive proteins. Prior research has shown that mitochondrial transcription factor A (mtTFA) helps regulate antioxidant signaling, and its levels rise when Nrf2 is activated[31]. We also report a new cytoprotective pathway of n-deprenyl involving the elevated expression of Nrf2, PGC-1α, and Bcl-2 genes. It is well known that PGC-1α is the primary controller of mitochondrial biogenesis and has shown neuroprotective effects in models of Parkinson's disease. Liu and colleagues have reported that increasing PGC-1α expression protects neurons from alcohol-induced harm [32]. Recent studies have shown that elevated PGC-1α levels trigger the transcription of Nrf1 and Nrf2, which in turn boosts the expression of mitochondrial transcription factor A (mtTFA) [33]. Mitochondrial transcription specificity factors are controlled by Nrf1, Nrf2, and the PGC-1α family of coactivators. Keap1 acts as a suppressor by trapping Nrf2 in the cytoplasm and promoting its breakdown through the Cul3/Rbx1 complex. Activation of Nrf2 and the subsequent induction of downstream antioxidant enzymes initiate the cellular antioxidant stress response [34, 35]. We performed assays to assess the genomic protection and antioxidant responses mediated by PGC-1α and Nrf2. There is strong evidence supporting the idea that PGC-1α acts upstream of Nrf2 in regulating antioxidant defense mechanisms [36]. In a study by Pang et. al, they showed that T-2 toxin triggers

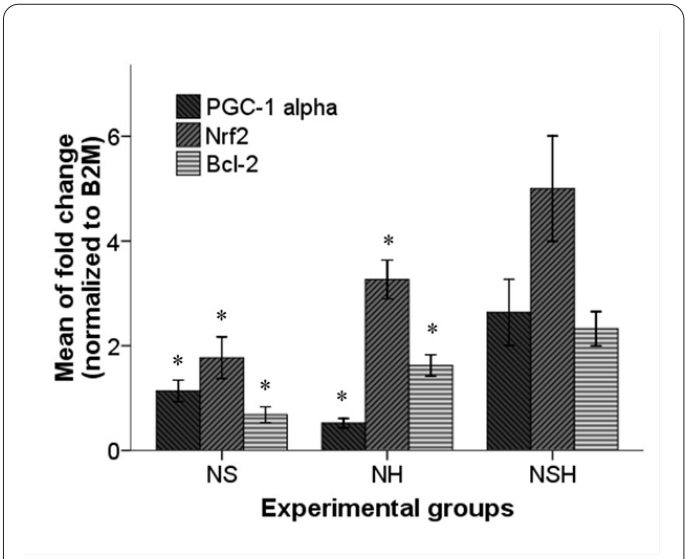


Fig. 5. Quantitative Real-Time RT-PCR Results. The fold change in mRNA levels of PGC-1α, Nrf2, and Bcl-2 is shown, normalized to B2M mRNA expression. Amplification of all genes from the NS, NH, and NSH groups revealed a significant increase in the expression of PGC-1α, Nrf2, and Bcl-2 following 10^{-7} M selegiline treatment. Bars represent the mean ± SEM. $P < 0.05$, compared to the NSH group.

oxidative damage and impairs mitochondrial function in SH-SY5Y cells, acting at least partially through the NRF2/PGC-1α axis to disrupt mitochondrial biogenesis [37]. The cooperative action of NRF2 and PGC-1α serves as central hubs that coordinate a range of vital cellular functions, including adaptive stress responses, mitochondrial turnover, and both antioxidant and neuroinflammatory defense systems [38]. Supporting the existence of this crosstalk, Li and colleagues demonstrated that in cortical neurons subjected to oxidative stress, PGC-1α confers both antioxidant and anti-apoptotic protection by stimulating the NRF1/NRF2/TFAM signaling cascade[39].

The outcomes of this study highlight the gene- and cell-protective effects of n-deprenyl, as evidenced by a lower percentage of TUNEL-positive cells in the NSH group compared to the NH group. Furthermore, Nrf2 helps prevent p53-dependent apoptosis and improves cell survival by directly promoting the transcription of anti-apoptotic genes, including Bcl-2 and Bcl-xL [40]. Our results showed that Nrf2 raises the levels of the anti-apoptotic protein Bcl-2, leading to reduced apoptotic cell death and improved cell survival after H_2O_2 exposure. These observations align with earlier reports. Selegiline has been found to activate Nrf2/ARE signaling in PC12 cells, a process that occurs independently of its MAO-B blocking activity [41]. Nrf2 serves as a regulatory protein that governs the activity of numerous genes involved in antioxidant defense [42]. Cytochrome C release from mitochondria is regulated by the Bcl-2 family, which includes both pro-apoptotic members (e.g., Bax, Bak, Bid, and Bad) and anti-apoptotic members (e.g., Bcl-2 and Bcl-xL). The main role of anti-apoptotic Bcl-2 is to regulate mitochondrial Ca^{2+} release, manage oxidative stress, and prevent cytochrome C release by preserving normal mitochondrial membrane permeability [13, 43]. According to a study by Jiangsu et al., the anti-apoptotic protein Bcl-2 is increased by the Nrf2 protein [44]. Moreover, when oxidative stress occurs, the protein Nrf2 enhances the production of multiple defensive genes, leading to increased levels of various antioxidant enzymes [45, 46]. Although the protective effects of selegiline have not been fully established, the current results suggest that its anti-apoptotic actions on neural stem cells are mediated through Nrf2 and PGC-1α. Additionally, Nrf2 controls the production of the mitochondrial protein UCP3, thereby decreasing reactive oxygen species (ROS) levels and protecting cells from death [47].

5. Conclusion

In summary, this work demonstrates the protective effects of (L)-deprenyl (selegiline) in HD-NSPCs during H_2O_2-induced stress. The results indicated that Nrf2, PGC-1α, and Bcl-2 could play central roles in selegiline's anti-apoptotic activity. Hence, it can be proposed that selegiline may somehow promote mitochondrial renewal and thereby contribute to the survival of NSPCs. While preliminary, these in vitro findings indicate that selegiline could offer a therapeutic avenue for various neurodegenerative disorders, though further validation is needed.

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Human and animal rights

In this in vitro investigation, no human study was conducted and just one rat was used to obtain HD-NSPCs. The protocol for the study conforms to the provisions of the WMA Declaration of Helsinki and also the ethical standards of the faculty of medicine, Zanjan University of Medical Sciences, Iran.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Funding

The majority of the experimental procedures were conducted at the laboratory of the Islamic Azad University, Ardabil Branch, while supplementary experimental procedures were performed at Zanjan University of Medical Sciences.

Conflict of interest

The authors declare that they have no conflict of interest.

Author's Contributions

A. Abdanipour contributed to the interpretation and observations and oversaw the project progress; A. Nikfar contributed to data collection; H. Feizi contributed to data analysis and wrote the paper.

Use of Artificial Intelligence Tools

Some sentences in this manuscript were revised with the assistance of artificial intelligence, using language models to improve clarity and readability. All intellectual content, interpretations, and conclusions are solely the responsibility of the authors.

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