

Neuroprotective effects of selegiline on rat neural stem cells treated with hydrogen peroxide

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Abstract. Oxidative stress and reactive oxygen species generation have been implicated in the pathogenesis of several neurological disorders including Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis and multiple sclerosis. In the present study, the neuroprotective effects of selegiline against hydrogen peroxide-induced oxidative stress in hippocampus-derived neural stem cells (NSCs) were evaluated. NSCs isolated from neonatal Wistar rats were pretreated with different doses of selegiline for 48 h and then exposed to 125 μM H_2O_2 for 30 min. Using MTT and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assays, acridine orange/ethidium bromide staining and reverse transcription-quantitative polymerase chain reaction, the effects of selegiline on cell survival, apoptosis and the expression of B-cell lymphoma 2 (Bcl-2) and heat shock protein 4 (Hspa4) in pretreated stem cells were assessed compared with a control group lacking pretreatment. The results indicated that the viability of cells pretreated with 20 μM selegiline was significantly increased compared with the control group ($P < 0.05$). Additionally, 20 μM selegiline increased the mRNA expression of Bcl-2 and Hspa4 ($P < 0.05$ vs. control) and suppressed oxidative stress-induced cell death (apoptosis and necrosis; $P < 0.05$ vs. control and 10 μM groups). From these findings, it was concluded that selegiline may be a therapeutic candidate for the treatment of neurological diseases mediated by oxidative stress.

Introduction

The most common neurodegenerative disorders, namely Alzheimer's disease (AD) and Parkinson's disease (PD), are

prevalent in ~1% of individuals aged 60 years and older (1). Their etiologies remain uncertain, though there are a number of established pathogenic factors, including oxidative stress, neural apoptosis, mitochondrial dysfunction, excitotoxicity, impairment of the ubiquitin-proteasome system and inflammation (2,3). Neuroprotective therapy has been suggested to prevent disease progression by inhibiting the action of pathogenic factors, for instance, by reducing the production of reactive oxygen species (ROS) (4). Overproduction of ROS may cause oxidative damage to biomolecules and subsequently DNA damage, ultimately leading to the development of neurodegenerative diseases. According to studies on PD, inhibitors of type B monoamine oxidase (MAO), including selegiline [also known as (-)-deprenyl] and rasagiline, are among the most promising neuroprotective agents identified to date (5-7). MAO exists in two forms, MAO-A and -B. The catalytic activity of these enzymes generates H_2O_2 and nitrogen species, which are toxic products that may cause oxidative damage to mitochondrial DNA (mtDNA) and thus have potential implications for apoptosis, aging and neurodegenerative processes (8). The MAO-B inhibitor, selegiline, is typically recommended as a first-line treatment for PD and has been demonstrated to possess neuroprotective functions (9); notably, this inhibitor protected neuronal cells against induced cell death in cellular and animal models (10,11). The neuroprotective functions of selegiline have been attributed to stabilization of the mitochondria, to the prevention of death signaling processes, and to upregulation of the anti-apoptotic B-cell lymphoma 2 (Bcl-2) protein family and neurotrophic factors (10,11). Previous studies have also indicated that MAO inhibitors suppress H_2O_2 -induced oxidative stress and attenuate the induced cell injury by promoting expression of the Bcl-2 family (10,11). Unlike other drugs with neuroprotective properties, selegiline and its metabolites are able to cross the blood-brain barrier, following which they exhibit highest accumulation in the thalamus, basal ganglia, mesencephalon and cingulate gyrus (12,13).

As a follow-up to previous research by our group (14,15), the present study investigated the *in vitro* therapeutic effects of selegiline on the apoptosis and survival of hippocampus-derived rat neural stem cells (NSCs) treated with hydrogen peroxide, namely through MTT and terminal deoxynucleotidyl

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transferase-mediated dUTP nick end labeling (TUNEL) assays and acridine orange/ethidium bromide staining, along with reverse transcription-quantitative polymerase chain reaction (RT-qPCR) to determine the expression of heat shock protein 4 (Hspa4) and Bcl-2.

Materials and methods

Isolation and expansion of NSCs. NSCs were isolated from the hippocampus of 5 neonatal Wistar rats (10 days old) purchased from the the Razi Vaccine and Serum Research Institute (Karaj, Iran) using a neurosphere assay as described previously (16). Prior to cell isolation, the rats were housed under a 12-h light/dark cycle at 24°C and 30-35% humidity with food and water available *ad libitum*. NSCs were collected from the hippocampus of the rats under anesthesia with ketamine (100 mg/kg) and xylazine (10 mg/kg; intraperitoneal injection). The dissected hippocampi were washed in phosphate-buffered saline (PBS) supplemented with 4.5 g/l glucose solution and then centrifuged for 5 min at 1,600 x g and 4°C. The collected tissues in the pellet were dissociated for 30 min at room temperature (RT) using a digestion mixture of 2.5 U/ml papain (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 40 U/ml dispase II (Sigma-Aldrich; Merck KGaA) and 400 U/ml accutase (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), with mixing of the solution every 10 min. Subsequently, the cell mixture was passed through a 70- μ m cell strainer and then centrifuged for 5 min at 800 x g and 4°C. Following centrifugation, the pellets were resuspended in 1 ml Dulbecco's modified Eagle's medium F-12 (DMEM/F12; Invitrogen; Thermo Fisher Scientific, Inc.). The isolated cells were seeded in a 25-cm² non-adherent plastic flask (10⁵ cells/ml) in DMEM/F12 supplemented with 2% B27 supplement (Gibco; Thermo Fisher Scientific, Inc.), 20 ng/ml basic fibroblast growth factor (bFGF; Invitrogen; Thermo Fisher Scientific, Inc.), 20 ng/ml epidermal growth factor (EGF; Invitrogen; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 mg/ml streptomycin and incubated at 37°C in 5% CO₂ for 1-2 weeks to enable neurosphere formation. The medium and growth factors were replenished every 2 days. Following this, floating neurospheres were collected by centrifugation at 300 x g for 5 min at RT. They were then dissociated enzymatically using trypsin-EDTA (0.25%) and mechanically (by pipetting) to single cells. The cells were then suspended in DMEM/F12 supplemented with 2% B27, 20 ng/ml bFGF, 20 ng/ml EGF and 5% fetal bovine serum (Sigma-Aldrich; Merck KGaA) for 1 week at 37°C and 5% CO₂ in 6-well adherent plates (5x10⁶ cells/well) coated with poly-L-lysine (Sigma-Aldrich; Merck KGaA) and passaged up to three times. Nestin as a neural stem/precursor cell marker (17) was evaluated immunocytochemically. The present study adhered to institutional guidelines for the care and use of laboratory animals, and all experimental procedures were reviewed and approved by the Ethics Committee for the Use of Experimental Animals at Tarbiat Modares University (Tehran, Iran).

Selegiline cell treatments. H₂O₂ was used to induce oxidative stress, and was prepared from 30% stock solution prior to each experiment. Third-passage NSCs were cultured in 96-well plates (10⁵ cells/well) in DMEM/F12 medium supplemented

with 2% B27, 20 ng/ml bFGF and 20 ng/ml EGF. The NSCs were then incubated at 37°C in 5% CO₂ with 125 μ M H₂O₂ for 30 min. To evaluate the neuroprotective effects of selegiline, cells were pretreated with different concentrations of selegiline (Sigma-Aldrich; Merck KGaA; 0, 10, 20, 30 and 40 μ M) 48 h prior to the H₂O₂ treatment. NSCs without selegiline treatment (0 μ M) were used as a control group. The control cells were cultured in the above DMEM/F12 medium for 48 h at 37°C, then treated with 125 μ M H₂O₂ for 30 min.

Immunostaining. The hippocampus-derived NSCs were cultured on cover slides (5x10³ cells/slide) in the supplemented DMEM/F12 (2% B27, 20 ng/ml bFGF and 20 ng/ml EGF) for 48 h at 37°C in 5% CO₂, then fixed in 3% paraformaldehyde for 20 min at RT. The cells were then permeabilized in 100% methanol for 30 min at RT to enable antibody-antigen interaction. For immunofluorescence, the cells were incubated with anti-nestin monoclonal antibodies, (1:200; ab6142; Abcam, Cambridge, UK) for 2 h at 4°C, then with fluorescein isothiocyanate-conjugated rabbit anti-mouse antibody (1:300; ab6724; Abcam) for 2 h at RT. Nuclei were counterstained with ethidium bromide for 1 min at RT to visualize the nuclei. Following staining, cells were examined using an inverted fluorescence microscope and the number of immunopositive cells were counted in a minimum of 100 cells per experiment. Corresponding negative controls were established using secondary antibodies without primary antibody incubation, to exclude nonspecific binding of secondary antibody to the sample.

Viability assay. Cell viability was evaluated using an MTT bromide assay, as described previously (18). Cells were treated with 125 μ M H₂O₂ and different concentrations of selegiline 48 h before the H₂O₂ treatment. The treated cells were incubated with 1 mg/ml MTT (Sigma Aldrich; Merck KGaA) for 4 h, after which the culture medium (DMEM/F12 with 2% B27, 20 ng/ml bFGF and 20 ng/ml EGF) was removed and 100 μ l dimethyl sulfoxide was added to each well to dissolve the formazan crystals. The quantity of formazan dissolved was quantified from absorbance at 570 nm (A570) using a microplate ELISA reader, and relative cell viability (%) was calculated as follows: (A570 of treated samples/A570 of controls) x100 (19,20).

TUNEL detection of apoptotic cells. Following treatment, the NSCs were fixed with 4% paraformaldehyde in PBS for 30 min at RT. The cells were subjected to a TUNEL assay using an *In-Situ* Cell Death Detection kit (Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions. TUNEL-positive cells were labeled using diaminobenzidine as the chromogen for 3-7 min at RT, and counterstained with hematoxylin for 5 min at RT. The percentage of TUNEL-positive cells was assessed using an Olympus phase contrast fluorescence microscope (Olympus Corporation, Tokyo, Japan) in five randomly selected fields for each well.

Acridine orange/ethidium bromide staining. Necrotic morphological changes in the treated cells were assessed by acridine orange/ethidium bromide staining. Following the selegiline and H₂O₂ treatments, the NSCs were washed with

Table I. Primer sequences.

Gene	GenBank accession no.	Forward, 5'-3'	Reverse, 5'-3'
B2M	NM_012512.2	CTTGCCATTCAGAAAACCTCC	CATCGGTCTCGGTGGGTG
Bcl-2	NM_016993	ACGGTGGTGGAGGAACCTCTCAGG	AATCAAACAGAGGTCGCATGCTGG
Hspa4	NM_153629	GAGTGC GAATGCTTCAGACCTCCC	CGTGTGGCTCCACCAACTATCTCC

Primers were designed with Gene Runner 3.05 software (produced by info@genfanavaran.com). B2M, β 2-microglobulin; Bcl-2, B-cell lymphoma 2; Hspa4, heat shock protein 4.

PBS buffer and fixed with 4% paraformaldehyde for 15 min at RT, then stained with acridine orange/ethidium bromide (Sigma-Aldrich; Merck KGaA; 100 μ g/ml of each) for 5 min at RT. The number of necrotic cells, identified by orange/yellow cytoplasmic staining and by non-condensed chromatin and/or non-fragmented nuclei (21), were counted in a total of 200 cells. The cells were observed using a fluorescence microscope.

RT-qPCR. RT-qPCR was performed with cDNA obtained from the 0 (control) and 20 μ M selegiline groups following induced oxidative stress. A total of 1,000 ng purified RNA obtained from cultured cells with TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) was used to synthesize 20 μ l cDNA using a RevertAid™ First Strand cDNA Synthesis kit (Fermentas, Germany) according to the manufacturer's instructions. The cDNA was used to quantify Bcl-2 and Hspa4 mRNA levels, with β 2-microglobulin (B2M) used as an internal control for normalization. The primer sequences of all primers used are listed in Table I. The PCR reaction was performed in a 25- μ l final reaction volume [containing forward and reverse primers (200 nM each), cDNA (0.5 μ l), SYBR®-Green I (12.5 μ l; Fermentas; Thermo Fisher Scientific, Inc.) and nuclease-free water up to final volume] for 40 cycles at 95°C for 15 sec followed by 60°C for 1 min. Relative changes in target mRNA levels were determined using the $\Delta\Delta C_q$ method (22).

Statistical analysis. Data analysis was performed using SPSS 15.0 software (SPSS, Inc., Chicago, IL, USA). All data are presented as the mean \pm standard error of the mean from 5 independent experiments. To compare differences between the means of multiple groups, one-way analysis of variance followed by Tukey's post hoc test was used. $P < 0.05$ was considered to indicate statistical significance.

Results

Generation of NSCs. The results of the primary culture of hippocampus-derived NSCs are presented in Fig. 1A-D. Following NSC isolation and culture, single cells were observed to form primary neurospheres by day 7 (Fig. 1A). Secondary neurospheres originated from the primary neurospheres, and a homogeneous adherent NSC population was obtained after 3 passages (Fig. 1B). The majority of isolated cells ($98.71 \pm 0.29\%$) exhibited positive staining for nestin marker (Fig. 1D), indicating successful isolation of NSCs, and negative controls incubated in the absence of primary antibody confirmed the specificity of labeling (data not shown).

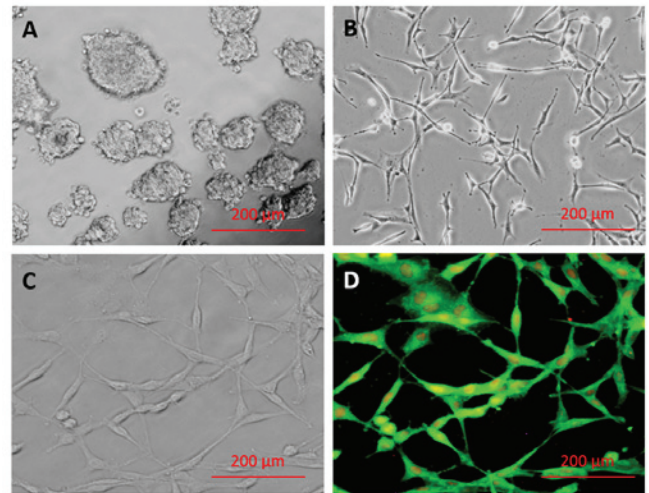


Figure 1. Representative photomicrographs of hippocampus-derived NSCs. (A) Floating neurospheres derived from rat hippocampus after culture for 7 days. (B) Hippocampus-derived NSCs at passage 3. (C) Phase contrast micrograph and (D) immunostaining of nestin (green) in the same field. Nuclei were counterstained with ethidium bromide (red). Magnification, x200. NSC, neural stem cell.

Neuroprotective effect of selegiline on NSC viability. An MTT assay was performed to determine the neuroprotective effect of increasing concentrations of selegiline (0-40 μ M) against oxidative stress induced by 125 μ M H_2O_2 . The attained values were normalized based on values of NSCs without any treatment (negative control group). As depicted in Fig. 2A, pretreatment with 20 μ M selegiline for 48 h caused an increase in the percentage of viable cells ($64.4 \pm 2.17\%$) compared with the 0 ($29.66 \pm 2.04\%$; $P < 0.05$) and 10 μ M ($58.44 \pm 6.60\%$) selegiline groups. However, further increases in selegiline concentration caused a decline in cell viability compared with the 20 μ M selegiline group, which was deemed to be significant for 40 μ M ($44.94 \pm 2.94\%$; $P < 0.05$).

Neuroprotective effect of selegiline on NSC survival. A TUNEL assay and acridine orange/ethidium bromide staining were performed to assess the pro-survival effects of selegiline in hippocampus-derived NSCs treated with increasing concentrations of selegiline for 48 h followed by H_2O_2 exposure. The TUNEL and necrotic cell staining indicated that H_2O_2 induced apoptotic and necrotic cell death (Fig. 2A-E). As depicted in Fig. 2A, pretreatment with 20 μ M selegiline caused significant decreases in the percentages of apoptotic ($30.10 \pm 1.48\%$) and necrotic ($27.32 \pm 2.68\%$) cells compared

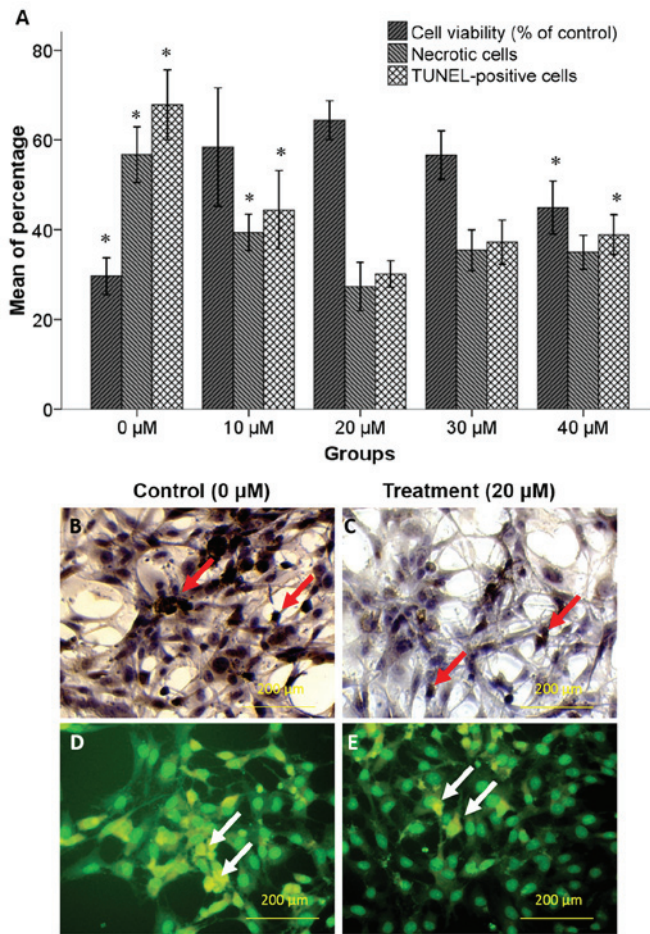


Figure 2. Effect of selegiline on cell viability, apoptosis and necrosis. (A) Mean percentages of viable, apoptotic and necrotic cells (relative to untreated cells) determined by MTT assay, TUNEL and acridine orange/ethidium bromide staining, respectively, following 125 μM H_2O_2 exposure with selegiline pretreatment. (B-E) Representative TUNEL and acridine orange staining images of NSCs at (B and D) 0 and (C and E) 20 μM selegiline followed by 125 μM H_2O_2 exposure. Red arrows indicate DNA fragmentation. Late necrotic NSCs exhibited orange/red staining in non-condensed, non-fragmented nuclei (white arrows). Counterstaining with hematoxylin is shown in blue. Magnification, x200. The bars indicate the mean \pm standard error of the mean. * $P < 0.05$ vs. 20 μM group. NSC, neural stem cell; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

with the 10 μM selegiline group (44.4 \pm 4.39 and 39.37 \pm 2.01%, respectively; $P < 0.05$) and the 0 μM (control) group (67.84 \pm 3.91 and 59.74 \pm 3.07%, respectively; $P < 0.05$). However, further increases in selegiline concentration caused increased rates of cell apoptosis and necrosis compared with the 20 μM selegiline group, which was deemed to be significant at 40 μM on cell apoptosis (33.89 \pm 2.21%; $P < 0.05$).

Effect of selegiline on Bcl-2 and Hspa4 expression. Increased levels of Bcl-2 and Hspa4 mRNA in H_2O_2 -induced NSCs pretreated with 20 μM selegiline for 48 h were confirmed by RT-qPCR (Fig. 3). Notably, following normalization to the expression of B2M, it was observed that the mRNA levels of Bcl-2 and Hspa4 were significantly increased in the 20 μM selegiline group (2.90 \pm 0.05 and 1.18 \pm 0.03, respectively) compared with the control group (1.01 \pm 0.11 and 0.77 \pm 0.02, respectively; $P < 0.05$).

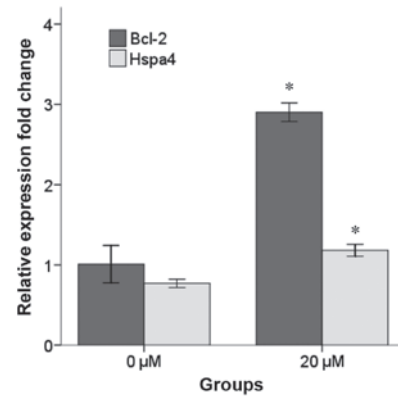


Figure 3. Fold changes in Bcl-2 and Hspa4 mRNA expression in H_2O_2 -induced NSCs treated with 20 μM selegiline. The results of polymerase chain reaction were presented as relative expression normalized to β 2-microglobulin mRNA amplification. The bars indicate the mean \pm standard error of the mean. * $P < 0.05$ vs. 20 μM group. Bcl-2, B-cell lymphoma 2; Hspa4, heat shock protein 4; NSC, neural stem cell.

Discussion

In the present study, it was demonstrated that selegiline increased Bcl-2 and Hspa4 mRNA expression in hippocampus-derived NSCs, and protected the cells against H_2O_2 -induced apoptosis and necrosis. It has previously been reported that free radicals serve a causal role in biomolecular damage and neuronal cell death following oxidative stress (2,23). In neurodegenerative disorders, a reduction of oxidative stress may attenuate apoptotic cell death and disease progression (24). MAO serves important roles in the generation of H_2O_2 and nitrogen species, and in apoptosis, aging and neurodegenerative processes (8,25). Furthermore, increased levels of MAO-B mRNA and enzymatic activity have been observed in neurodegenerative diseases including PD (26).

In vitro and *in vivo* experiments have demonstrated that selegiline is a potent inhibitor of MAO-B and also enhances the synthesis of neurotrophic factors including glial cell-derived neurotrophic factor and brain-derived neurotrophic factor (9,27,28). As a selective MAO-B inhibitor, selegiline may be used as an anti-PD drug to exert antioxidant and anti-apoptotic effects (29,30). It has also been indicated that selegiline decreases oxidative stress and cell death induced by 1-methyl-4-phenylpyridinium (MPP^+), an inducing agent of PD (31). However, the protective effect of selegiline against MPP^+ -induced neuronal cell degeneration may be opposing dependent on concentration; while micromolar to submillimolar doses of selegiline promoted increased cell viability, concentrations of selegiline greater than 1 mM induced a decrease in cell viability in the MPP^+ -treated cells (32). This opposing effect of selegiline regarding anti-apoptotic activity has also been demonstrated in A-2058 human melanoma cell culture, in which selegiline at a concentration range of 10^{-7} - 10^{-3} M caused significant inhibition of apoptosis, while treatment 10^{-3} M selegiline caused 50% apoptosis after treatment for 72 h (29). The current results are in accordance with these previous studies.

The MAO-B inhibitors, rasagiline and selegiline, protect neuronal cells through upregulation of the pro-survival protein Bcl-2 and neurotrophic factors (33). In the present study,

increases in the mRNA levels of Bcl-2 and Hspa4 were identified in hippocampal NSCs following treatment with 20 μ M selegiline for 48 h. The Bcl-2 protein family controls the release of cytochrome *c* (Cyt-*c*) from the mitochondria and consists of pro-apoptotic (Bcl-2-associated X protein, Bcl-2 homologous antagonist/killer, BH3 interacting-domain death agonist, Bcl-2-associated death promoter) and anti-apoptotic [Bcl-2, Bcl-extra large (Bcl-xL)] members (34). The primary function of anti-apoptotic Bcl-2 is to suppress mitochondrial Cyt-*c* release, by regulating physiological membrane permeability, Ca²⁺ release and oxidative stress in the mitochondria (35,36). Specific regions of Bcl-2, namely the Bcl-2 homology-3 and -4 domains, exert anti-apoptotic effects in *in vivo* and *in vitro* models (37,38). Additionally, overexpression of Bcl-2 protein has been documented to reduce tissue damage in animal models of PD (39,40) and ischemia (41,42), and protects cells against apoptosis (43). Furthermore, Bcl-2 overexpression prevented Cyt-*c* release following peroxynitrite-induced apoptotic signaling, indicating the potential of Bcl-2 as a neuroprotective agent (44). In a previous study, selegiline increased the mRNA and protein levels of Bcl-2 and Bcl-xL in SH-SY5Y cells (45), but not in MAO-B-containing Caco-2 and U118MG cells (46,47). These studies are consistent with present findings regarding the significant upregulation of Bcl-2 and improved survival of NSCs in response to 20 μ M selegiline.

Interestingly, it has been reported that Hspa4 may decrease the protein degradation and increase the stability of Bcl-2 during oxidative stress, and that an association may exist between Bcl-2 and the anti-apoptotic effect of Hspa4 (48). In consideration of the increases in Bcl-2 and Hspa4 mRNA and the potential role of Bcl-2 in the selegiline-treated group, the current study indicated that these factors may have been associated with the decreased percentages of necrotic and TUNEL-positive cells decreased in the 20 μ M selegiline group compared with the 0 (control) and 10 μ M groups.

In conclusion, the current data suggested that selegiline is effective in protecting NSCs against oxidative stress. Therefore, selegiline may be considered as a drug candidate for neurological disorders in which oxidative stress serves an important role in pathogenesis.

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