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Edaravone reduces Aβ-induced oxidative damage in SH-SY5Y cells by activating the Nrf2/ARE signaling pathway

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ABSTRACT

Aims: Edaravone potentially alleviates cognitive deficits in a mouse model of Alzheimer's disease (AD). However, the mechanism of edaravone in suppressing AD progression remains unclear. We aim to investigate the mechanism of edaravone in suppressing oxidative stress-mediated AD progression in vitro.

Main methods: Human neuroblastoma SH-SY5Y cells were pretreated with different concentrations of edaravone prior to the induction by $A\beta_{25-35}$. Cell viability, apoptosis, reactive oxygen species, and expression of anti-oxidative response elements (ARE) including Nrf2, SOD, and HO-1 were assessed.

Key findings: The results showed that apoptosis and reactive oxygen species levels significantly increased in $A\beta_{25-35}$-treated cells, whereas the mRNA and protein levels of Nrf2, SOD and HO-1 decreased. The opposite changes were observed in cells that were pre-treated with edaravone, particularly at a concentration of 40 μM. $A\beta_{25-35}$-treatment suppressed Nrf2 expression and nuclear translocation were rescued by Edaravone. Genetic inhibition of Nrf2 greatly decreased the protective effect of edaravone against cell apoptosis and cytotoxicity induced by $A\beta_{25-35}$, accompanied by decreases in SOD and HO-1 expression.

Significance: Activation of the Nrf2/ARE signaling pathway may underlie the protective effects of edaravone against the oxidative damage associated with Alzheimer's disease.

1. Introduction

Alzheimer's disease (AD) is a common neurodegenerative disorder that causes progressive cognitive impairment, such as deficits in memory, executive functions, and visuospatial skills. Approximately 35.6 million people were exposed to AD insults in 2010, and this number is expected to increase to 115 million by 2050 [1]. Discovering new diagnostic methods and treatment strategies are considered main challenges for AD research. Generally, the typical pathological features of AD include extracellular fibrillation of Aβ to so-called senile plaques and intracellular accumulation of hyper-phosphorylated neurofibrillary Tau tangles, which have toxic effects on neurons and speed progression of AD [2]. Oxidative stress is another critical contributor to the onset and progression of AD. Compared to other cells, brain neurons are more susceptible to injury caused by oxidative stress [3]. Therefore, anti-oxidant therapy may be useful for treating AD.

Edaravone is a free-radical scavenger that readily crosses the blood-brain-barrier [4]. A phase-I clinical study in healthy Chinese volunteers shows it to be a safe and well tolerated drug [5]. Edaravone is clinically applied in the treatment of cerebral ischemic stroke [6,7], acute cerebral large vessel occlusion [8] and amyotrophic lateral sclerosis [9]. It is also confirmed to be effective in preventing degeneration of dopamine neurons in the midbrain in rotenone-induced rat model of Parkinson's disease [10]. Compelling evidence shows that edaravone has a potent capacity to inhibit amyloid aggregation, to attenuate amyloid-induced oxidation in vitro, and to alleviate cognitive-deficit-related behavior in a mouse model of AD [11,12]. However, the underlying mechanisms of edaravone in alleviating AD symptoms and pathogenesis remain unclear.

Nuclear factor (erythroid-derived 2)-like 2, or Nrf2, is a major transcriptional regulator of cellular antioxidant defense enzymes. Activation of Nrf2 is thus considered an effective strategy to resist cellular oxidative damage. Genetic inhibition of Nrf2 exacerbates cognitive deficits in a mouse model of AD [13]. Nrf2 activation improves glutathione levels and neuron viability in AD mice [14]. However, whether Nrf2 is involved in the protective effects of edaravone in AD

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pathogenesis is currently unrevealed. In this study, we report that edaravone exerts its antioxidant effects by activating the Nrf2 signaling pathway in the human neuroblastoma SH-SY5Y cell line, an in vitro model of AD.

2. Materials and methods

2.1. Drug and reagents

Edaravone was purchased from Jida Pharmaceutical (Kunming, China). $A\beta_{25–35}$ was obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle's medium with nutrient mixture F-12 (DMEM/F-12) was purchased from Gibco (New York, CA, USA) and fetal bovine serum (FBS) from HyClone (Logan City, UT, USA). A reactive oxygen species (ROS) detection kit was obtained from Beyotime (Shanghai, China). Anti-Nrf2, anti-superoxide dismutase, and horseradish peroxidase goat anti-rabbit IgG were obtained from ABCAM Biotechnology (Cambridge, UK). Anti-β-actin and anti-GAPDH were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A heme oxygenase-1 (HO-1) detection kit was obtained from Shanghai Enzyme-linked Biotechnology Co., Ltd. (Shanghai, China), an enhanced chemiluminescence detection kit from Solarbio (Beijing, China), and an RNA fast200 kit from Pioneer (Shaanxi, China). All chemical reagents used in this study were of analytical grade.

2.2. Cell culture and treatments

Human neuroblastoma SH-SY5Y cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM/F12 containing 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL) in an incubator at 37 °C with 5% CO_2. $A\beta_{25–35}$ was dissolved in water and then incubated for 7 days to form aggregates before use.

A preliminary experiment was performed to examine the effect of edaravone on cell viability. SH-SY5Y cells were incubated with 20 μM, 40 μM, or 80 μM concentrations of edaravone. Cells were cultured in different plates until cell densities reached 70% to 80% and then divided into five groups: control, model group (Model), edaravone 20 μM group (E20), edaravone 40 μM group (E40), and edaravone 80 μM group (E80). The control group cells were incubated with only the culture medium for 24 h without any further treatment. Cells in the Model group were incubated with the medium for 3 h, followed by treatment with $A\beta_{25–35}$ (25 μM) for 24 h. Based on our pre-experiment data, the relative cell viability of SH-SY5Y cells decreased with increasing concentration of $A\beta_{25–35}$, and the statistical correlation coefficient between them was -0.907 ($p < .001$). The SH-SY5Y cell viability had a 50% decrease when treated with 25 μM of $A\beta_{25–35}$. Therefore, this concentration was used in all subsequent experiments. Cells of the E20, E40, and E80 groups were pretreated for 3 h with edaravone at a final concentration of 20 μM, 40 μM, or 80 μM, respectively, and then treated for an additional 24 h with $A\beta_{25–35}$ at a final concentration of 25 μM [15].

Interfering RNAs against human Nrf2 (si-Nrf2) and the matched negative control siRNA (NC) were purchased from Invitrogen (CA, USA). SH-SY5Y cells at a density of 2.5×10^6 per well were collected, washed and resuspended in 100 μL of serum-free medium, and were then transfected with si-Nrf2 (Nrf2-624, Nrf2-934 and Nrf2-1498) or NC at a final concentration of 150 pmol/L with the assistance of Lipofectamine RNAi Mix. The transfected cells were incubated for 24 h at 37 °C followed by centrifugation and resuspension in culture medium for further examination or treatments with edaravone and/or $A\beta_{25–35}$.

2.3. MTT assay of cell viability

Cell viability was assessed using a conventional MTT assay. Cells were cultured in 96-well plates (2.0×10^5 cells/well). After treatment, 20 μL of MTT stock solution (5 mg/mL) was added, and cells were incubated at 37 °C for another 4 h. The resulting MTT formazan was extracted with 150 μL of dimethyl sulfoxide. Absorbance was recorded with a microtiter plate reader at 490 nm. The cell viability was calculated as a percentage based on the following equation: (absorbance in treated sample/absorbance in control) × 100%.

2.4. Flow cytometry measurement of cell apoptosis and intracellular reactive oxygen species

The apoptosis of SH-SY5Y cells was examined by double staining with Annexin V-FITC (KeyGen Biotech, Nanjing, China) and propidium iodide (PI). After treatment, cells were washed twice with phosphate-buffered saline, centrifuged at 2000 rpm for 5 min, and then harvested. Cells were suspended in 500 μL of binding buffer and mixed with 5 μL of Annexin V-FITC and 5 μL of PI. Cells were then incubated at room temperature in the dark for 15 min. Flow cytometry (FACS Calibur, Becton-Dickinson, Franklin Lakes, NJ, USA) was performed to detect apoptotic cells and the results were analyzed using the CellQuest software (BD Bioscience). For this measurement, the Annexin V-PI stained cells can be differentiated into healthy cells (Annexin V−/PI−), early apoptotic cells (Annexin V+/PI−) and late necrotic cells (Annexin V+/PI+). In this study, the percentage of apoptotic cells was calculated by the sum of the percentage of early apoptotic and late necrotic cells.

Intracellular ROS were detected using an oxidation-sensitive fluorescent probe (DCFH-DA). DCFH-DA was deacetylated intracellularly by nonspecific esterase, which was further oxidized by ROS to the fluorescent compound 2, 7-dichloro-fluorescein (DCF). After treatment, the cells were exposed to serum-free medium containing DCFH-DA (10 μM) at room temperature for 20 min, then washed twice in phosphate-buffered saline. The resultant cells were collected and adjusted to 1.0×10^7/mL. The fluorescence intensity of DCF was detected by FACS Calibur flow cytometry at an excitation wavelength of 488 nm and an emission wavelength of 535 nm. The ROSup chemical mixture containing H_2O_2 in the detection kit was referred as a positive control to stimulate ROS production, which was added into the non-treated SH-SY5Y cells (100 μM) for 1 h before the determination of ROS.

2.5. ELISA for detecting heme oxygenase-1 concentration

The HO-1 concentration in SH-SY5Y cells was examined using an ELISA kit purchased from Shanghai Enzyme-linked Biotechnology Co., Ltd. (Shanghai, China). The cells were cultured in 6-well plates (2.0×10^7 cells/well) for different treatments. An HO-1 detection kit was used following the manufacturer's instructions.

2.6. Western blot analysis of Nrf2 and superoxide dismutase (SOD) protein

The treated SH-SY5Y cells were collected and the whole cell lysates were used to detect SOD expression and the cell cytosol and nuclear extracts were extracted to detect Nrf2 expression in each fractions using western blot. A Nuclear/Cytosol Fractionation Kit (Biovision, Milpitas, CA, USA) was used to extract the cytosolic and nuclear fractions of treated cells. Cells were washed with cold PBS and then centrifuged at 600 ×*g* for 5 min at 4°. The collected cells were suspended in ice-cold hypotonic buffer A containing DTT and protease inhibitors and incubated in an ice bath for 15 min followed by another centrifugation. The supernatant was incubated with cytosol extraction buffer B and then collected as a cytosolic fraction. Meanwhile, the cell pellet (contains nuclei) was resuspended in 100 μL of nuclear extraction buffer C and incubated at 0 °C for 2 h. After vortex mixing on ice, the supernatant was centrifuged at 16,000 ×*g* for 10 min and the resultant supernatant was collected as a nuclear extract. The extract protein concentration was measured using the Bradford method.

Afterwards, approximately 50 μg of total protein from cell lysates or fractions was separated using 12% SDS-PAGE and transferred to

polyvinylidene difluoride membranes (Millipore, Temecula, CA, USA), followed by blocking with 5% skim milk powder in phosphate-buffered saline containing 0.1% Tween-20 at room temperature for 1 h. The immunoblots were incubated at 4 °C overnight with the primary antibodies rabbit polyclonal anti-Nrf2 antibody (1:500), rabbit polyclonal anti-SOD1(superoxide dismutase) (1:1500), anti-β-actin (1:1000), and anti-GAPDH (1:500). After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies at 37 °C for 1 h. Antibody binding was visualized using an enhanced chemiluminescence detection kit. The immunoblotted bands were quantitatively analyzed using Image-Pro Plus software.

2.7. qRT–PCR detection of Nrf2, superoxide dismutase, and heme oxygenase-1 mRNA

SH-SY5Y cells were cultured in 6-well plates (2.0×10^7 cells/well). After treatment, total RNA was extracted using an RNA fast200 kit. RNA concentration was determined using NanoDrop 2000 (Thermo Scientific, Rockford, IL, USA). For each sample, 2 μg of total RNA was reverse-transcribed to single-stranded cDNA using PrimeScript™ RT Master Mix (Takara, Tokyo, Japan). Then, qRT-PCR was performed using SYBR® Premix Ex Taq™ II (Takara) to quantitatively detect the gene expression of Nrf2, HO-1, SOD, and β-actin (the internal control) on an Applied ABI FastStepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, USA). The primer pairs were designed using the Primer Quest Oligo Design and synthesized by Dingguo Changsheng (Beijing, China) and their sequences were as follows: NRF2 (forward) 5′-CAG CTT TTG GCG CAG ACA TT-3′, (reverse) 5′-GAC TGG GCT CTC GAT GTG AC-3′; HO-1: (forward) 5′-GCC ATG AAC TTT GTC CGG TG-3′, (reverse) 5′- TTT CGT TGG GGA AGA TGC CA-3′; SOD (forward) 5′-CTA GCG AGT TAT GGC GAC GA -3′, (reverse) 5′-TCT TCA TCC TTT GGC CCA CC-3′ and β-actin (forward) 5′-ATC ATG TTT GAG ACC TTC AAC A -3′, (reverse) 5′-CAT CTC TTG CTC GAA GTC CA-3′. All assays were subjected to melting curve analysis to confirm the presence of a single PCR product. The level of each gene was calculated based on the $2^{-\triangle\triangle CT}$ method.

2.8. Statistical analysis

Statistical analyses were performed using SPSS statistical software version 22.0 (SPSS Inc., Chicago, IL, USA). All data are expressed as the mean ± SD of at least three ($n = 3$) independent experiments. Data were compared among multiple treatment groups using a one-way analysis of variance (ANOVA) and Bonferroni post hoc comparison where one variable was indicated. A two-way multivariate analysis of variance (MANOVA) followed by post-hoc Bonferroni test was performed where two or more variable factors were applied. All *F* statistics are reported using Pillai's Trace. All statistical tests were two-sided and evaluated at the 0.05 level of statistical significance.

3. Results

3.1. Edaravone pretreatment enhanced resistance to $A\beta_{25–35}$-induced cytotoxicity in SH-SY5Y cells

To investigate whether edaravone adversely affected cell viability, SH-SY5Y cells were incubated with edaravone (20 μM, 40 μM, or 80 μM) for 24 h. Treatment with edaravone at various concentrations did not significantly affect cell viability, which exceeded 95%, compared to that of the control (Fig. 1A). Afterwards, we observed the effect of edaravone on cell viability when the cells were exposed to $A\beta_{25–35}$, a pathogenic inducer of AD. $A\beta_{25–35}$ dramatically suppressed cell viability, which was rescued by pretreatment with edaravone, especially at a concentration of 40 μM (Fig. 1B). Under a light microscope (200× magnification), SH-SY5Y cells showed a bulky spindle or triangle shape with many mesh-like, dendritic protrusions. After exposure to $A\beta_{25–35}$, cell bodies transformed, appearing rounded and shrunken with aggregated and fragmented nuclei. It was estimated that 20% of the cells showed morphologic changes after $A\beta_{25–35}$ stimulation. All edaravone treatment concentrations (20 μM, 40 μM, and 80 μM) improved these phenomena, with the highest improvement at the 40 μM dose with less than 10% of the cells presenting morphologic changes (Fig. 1C–D). Only 7.2% of cells in the control group underwent apoptosis. Apoptosis increased significantly to 17.8% in the presence of $A\beta_{25–35}$. Pretreatment with edaravone reduced the ratio of apoptotic cells in response to $A\beta_{25–35}$ stimulation, with the lowest levels observed at a concentration of 40 μM ($p < .05$). As shown in Fig. 1E and F, the rates of apoptosis in the three edaravone groups were significantly lower than the rate in the $A\beta_{25–35}$ group ($p < .05$).

3.2. Edaravone preconditioning alleviated $A\beta_{25–35}$-induced oxidative stress in SH-SY5Y cells

ROS levels strikingly increased in $A\beta_{25–35}$-treated cells, compared with that of the control group (346.40 vs. 183.77, $p < .05$). With edaravone pretreatment, especially at the concentration of 40 μM, ROS levels were significantly lower than in the sole $A\beta_{25–35}$-treatment group ($p < .05$, Fig. 2A and B). As shown in Fig. 2C and D, SOD expression also substantially decreased in SH-SY5Y cells that were exposed to $A\beta_{25–35}$. Compared with the sole $A\beta_{25–35}$ group, SOD levels significantly increased after edaravone preconditioning. The protective effects of edaravone peaked at a concentration of 40 μM.

3.3. Edaravone pretreatment activated Nrf2 and HO-1 signals in SH-SY5Y cells exposed to $A\beta_{25–35}$

Based on the inhibitory effects of edaravone on $A\beta_{25–35}$-induced oxidative stress, we investigated the mechanism of edaravone in resisting oxidative injury. Nrf2 is a critical regulator in maintaining the oxidation-reduction hemostasis. Our data showed that mRNA levels of Nrf2 and its downstream gene, HO-1, decreased after exposure to $A\beta_{25–35}$. Preconditioning with 20 μM and higher concentrations of edaravone neutralized the inhibitory effect of $A\beta_{25–35}$ on Nrf2 and HO-1 mRNA expression, with the most improvement at the 40 μM concentration (Fig. 3A). ELISA also showed similar changes in HO-1 concentrations in response to edaravone treatment (Fig. 3B). Western blot showed that $A\beta_{25–35}$ exposure also suppressed cytosolic Nrf2 expression and nuclear translocation. Edaravone treatment elevated nuclear Nrf2 by approximately 5 to 8 folds (Fig. 3C and D).

3.4. Nrf2 silencing diminished the protective effects of edaravone on cytotoxicity induced by $A\beta_{25–35}$

To further determine whether edaravone depends on Nrf-2/HO-1 signaling, SH-SY5Y cells were transfected with Nrf2 siRNA and cell viability were then determined. The transfection efficiency of Nrf2 siRNA into the cells was confirmed by evaluating the Nrf2 mRNA level and Nrf2 protein level in cytosolic and nuclear fractions. All three Nrf2 siRNA candidates exhibited inhibitory effects on Nrf2 expression, compared to the negative control. Nrf2-1498 showed optimal inhibitory effects, and Nrf2-934 also had comparable role with Nrf2-1498 in reducing Nrf2 expression (Fig. 4A–C). We then determined changes of SH-SY5Y cell viability in response to drug treatments and/or siRNA interferences using MANOVA statistical analysis. Analyses were conducted using drug treatments (3 levels: Aβ, E40, and Aβ + E40) and siRNA interferences (4 levels: control, NC, Nrf2-1498 and Nrf2-934) as within-groups and between-groups factors. It was showed that the 3 treatments had significant effects on cell viabilities of SH-SY5Y cells [$F = 18.25$, $p < .001$] and si-Nrf2 interferences also significantly affected cell viabilities [$F = 9.87$, $p < .001$]. Significant interactive effects were seen for the MANOVA between treatments and siRNA silencing on cell viabilities [$F = 6.12$, $p = .002$] (Fig. 4D). Furthermore, MANOVA

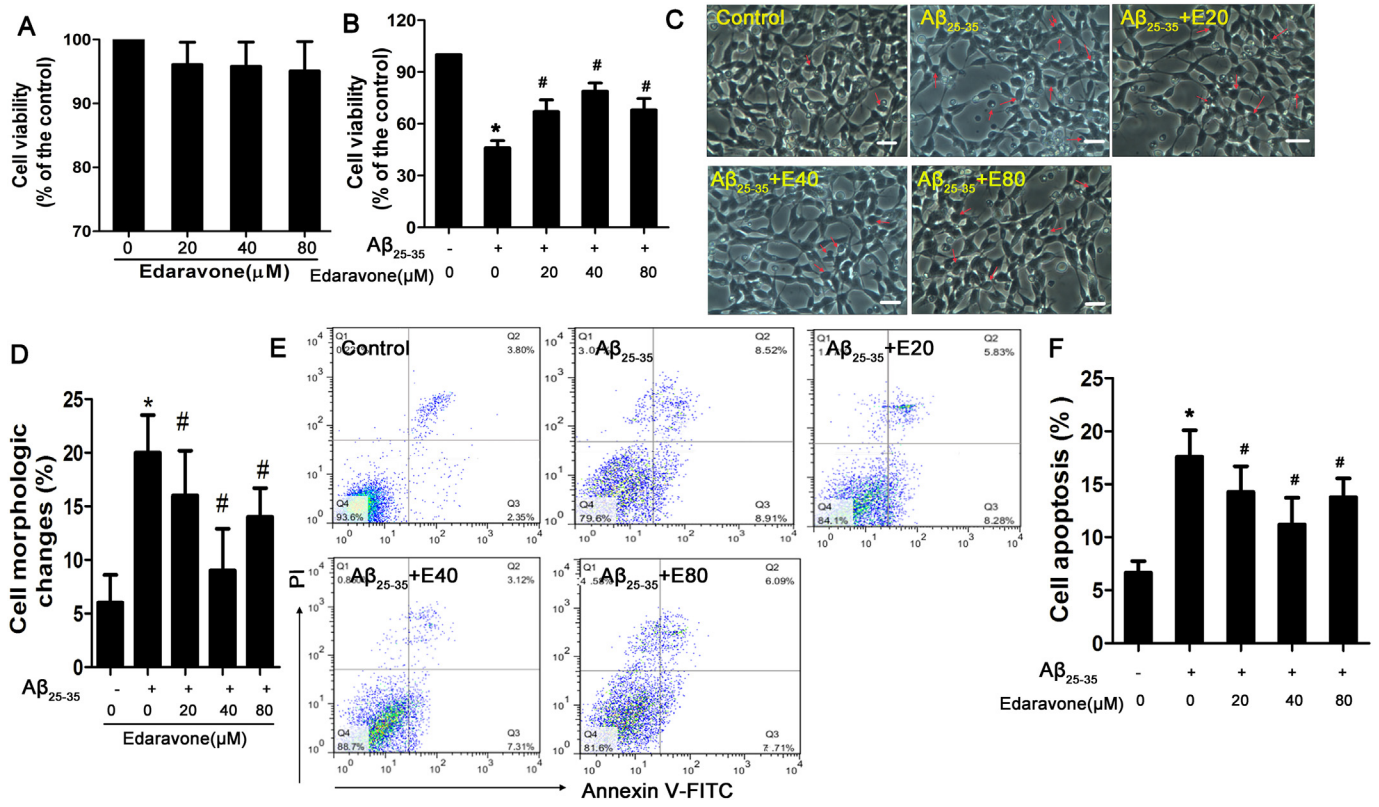

Fig. 1. Effect of edaravone on $A\beta_{25-35}$-induced cytotoxicity of SH-SY5Y cells. (A) Cells were incubated with edaravone at a concentration of 0 μM, 20 μM, 40 μM, or 80 μM for 24 h. Cell viability was examined using MTT assay by measuring the absorbance at 490 nm. (B) Effect of edaravone on $A\beta_{25-35}$-induced damage in SH-SY5Y cells. Cells were incubated with 0 μM, 20 μM, 40 μM, or 80 μM concentrations of edaravone for 3 h, followed by incubation with a 25-μM concentration of $A\beta_{25-35}$ for another 24 h. Cell viability was then determined by MTT assay. (C–D) Morphology of cells in response to each treatment. E20, E40, and E80 represent edaravone concentrations of 20 μM, 40 μM, and 80 μM, respectively. Scale bar: 20 μm. The red arrows showed the damaged cells with morphologic changes. Mean cell counts underwent abnormal morphologic change in each treatment were compared. (E) Representative images of flow cytometry evaluation of cell apoptosis in the presence of edaravone and $A\beta_{25-35}$. (F) Quantitative percentage of apoptotic cells in each treatment group. $^{*}p < .05$, compared with the control. $^{\#}p < .05$, compared with the $A\beta_{25-35}$ group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

analysis showed that E40 and/or Aβ treatments as well as Nrf-2 siRNA significantly affected apoptosis of SH-SY5Y cells [$F = 8.89$, $p < .001$; $F = 5.35$, $p = .007$] with significant interactive effects between these two factors [$F = 5.58$, $p = .023$] (Fig. 4E and F). These results indicated that the E40 and/or Aβ treatments may affect cell viability and apoptosis via Nrf2 signaling. In addition, Nrf2-934 also had comparable effects with Nrf2-1498 on cell viability and apoptosis, which ruling out the potential off-target effects of Nrf2-1498.

3.5. Nrf2 silencing diminished the inhibitory effect of edaravone on oxidative stress in SHSY-5Y cells exposed to $A\beta_{25-35}$

We further determined the effect of Nrf2 silencing using Nrf2-1498 on oxidative stress in cells treated with Aβ and E40, alone and in combination. MANOVA analyses were performed using drug treatments (3 levels: Aβ, E40, and Aβ + E40) and siRNA interferences (3 levels: control, NC, Nrf2-1498) as within-groups and between-groups factors. It was showed that the 3 treatments had significant effects on ROS production of SH-SY5Y cells [$F = 10.58$, $p < .001$], while si-Nrf2 interference failed to significantly affect ROS level [$F = 1.05$, $p = .121$]. No significant interactive effects were observed between treatments and siRNA silencing on ROS production [$F = 0.77$, $p = .442$] (Fig. 5A and B). Furthermore, MANOVA analysis showed that E40 and/or Aβ treatments as well as Nrf-2 siRNA significantly affected HO-1 concentration in the supernatant of SH-SY5Y culture [$F = 12.36$, $p < .001$; $F = 19.32$, $p < .001$] with significant interactive effects between these two factors [$F = 7.68$, $p < .001$] (Fig. 5C). Similarly, both E40 and/or Aβ treatments as well as Nrf-2 siRNA had notable effect on the mRNA level and cytosolic and nuclear expression of Nrf2, accompanied by the changes of its antioxidative response elements SOD and HO-1. All the three molecules showed similar change patterns after the various treatments. Nrf2 silencing greatly reduced the transcription of SOD and HO-1 in the presence of edaravone and $A\beta_{25-35}$. Likewise, edaravone's preventive effect against oxidative stress on HO-1 and SOD expression were diminished after Nrf2 silencing. Cytosolic Nrf2 and nuclear Nrf2 were enriched in response to edaravone treatment, regardless of exposure to $A\beta_{25-35}$, while these effects of edaravone ceased after Nrf2 inhibition (Fig. 5D and E).

4. Discussion

Edaravone plays a neuroprotective role in AD, either by reducing Aβ production and aggregation [12,16,17] or by attenuating $A\beta_{1-40}$-induced enhancement of voltage-gated calcium channel currents [18]. In the current study, we verified the protective effects of edaravone pretreatment against $A\beta_{25-35}$-induced oxidative damage in SH-SY5Y cells. We also explored the potential signaling pathway responsible for its effects. We showed that pretreating SH-SY5Y cells with edaravone prior to $A\beta_{25-35}$ exposure significantly reduced $A\beta_{25-35}$ cytotoxicity and decreased oxidative stress by activating Nrf2/ARE signaling.

Aβ is produced by the amyloidogenic pathway via the consecutive actions of β-secretases and γ-secretases on the β-amyloid precursor protein. $A\beta_{1-42}$ is the predominant form of Aβ in the brains of patients with AD. $A\beta_{25-35}$ is the smaller, 11-amino acid fragment of the full-length peptide. $A\beta_{25-35}$ has been used in many laboratories as a convenient alternative in AD investigations, because it shows more rapid

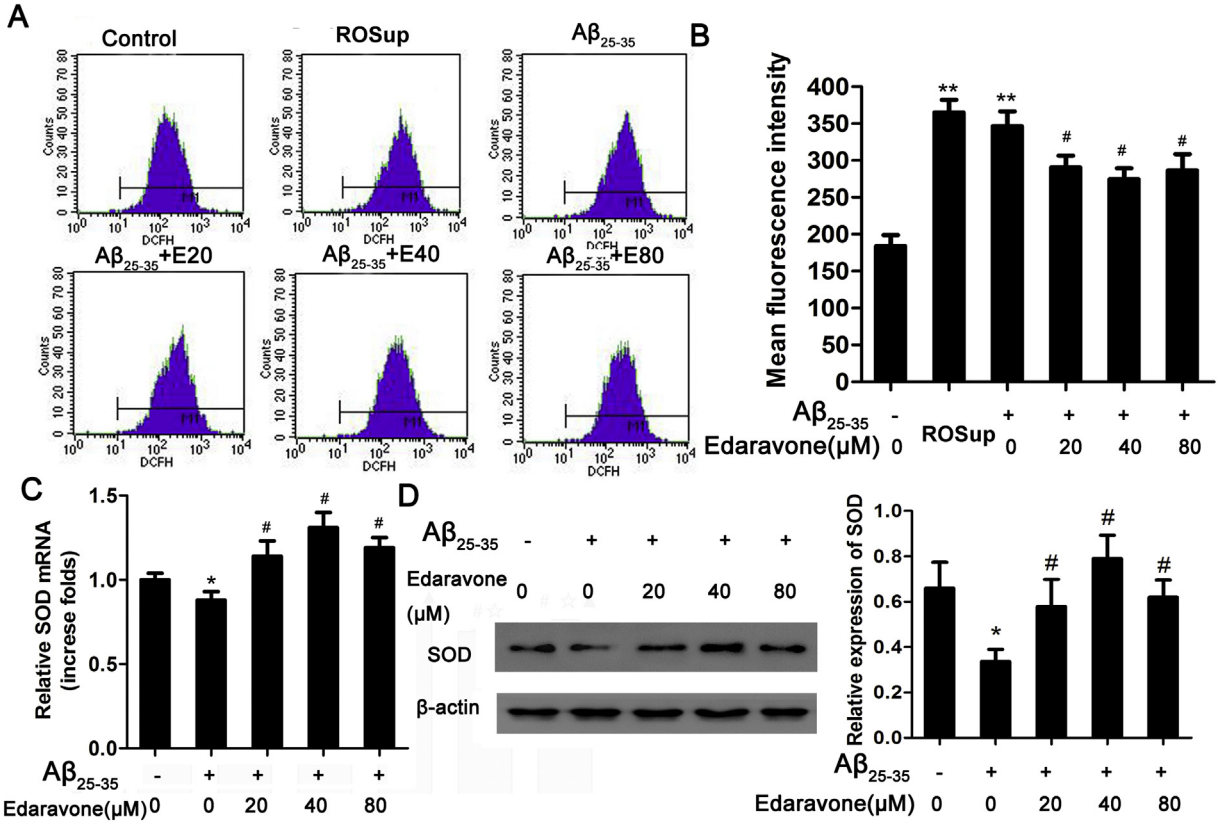

Fig. 2. Effect of edaravone on $A\beta_{25-35}$-induced oxidative stress in SH-SY5Y cells. Cells were incubated with 0 μM, 20 μM, 40 μM, or 80 μM concentrations of edaravone for 3 h, followed by incubation with a 25-μM concentration of $A\beta_{25-35}$ for another 24 h (A) Representative images of flow cytometry evaluation of ROS levels in the presence of edaravone and $A\beta_{25-35}$. The ROSup chemical mixture (100 μM) containing H_2O_2 was used as a positive control to stimulate ROS production and the cells received no other treatments. (B) Quantitative mean fluorescence intensity in each treatment group. (C) SOD mRNA level determined by RT-PCR. (D) Western blot analysis and quantitative data of SOD protein levels for each treatment. $^{*}p < .05$, $^{**}p < .01$, compared with the control. $^{\#}p < .05$, compared with the $A\beta_{25-35}$ group.

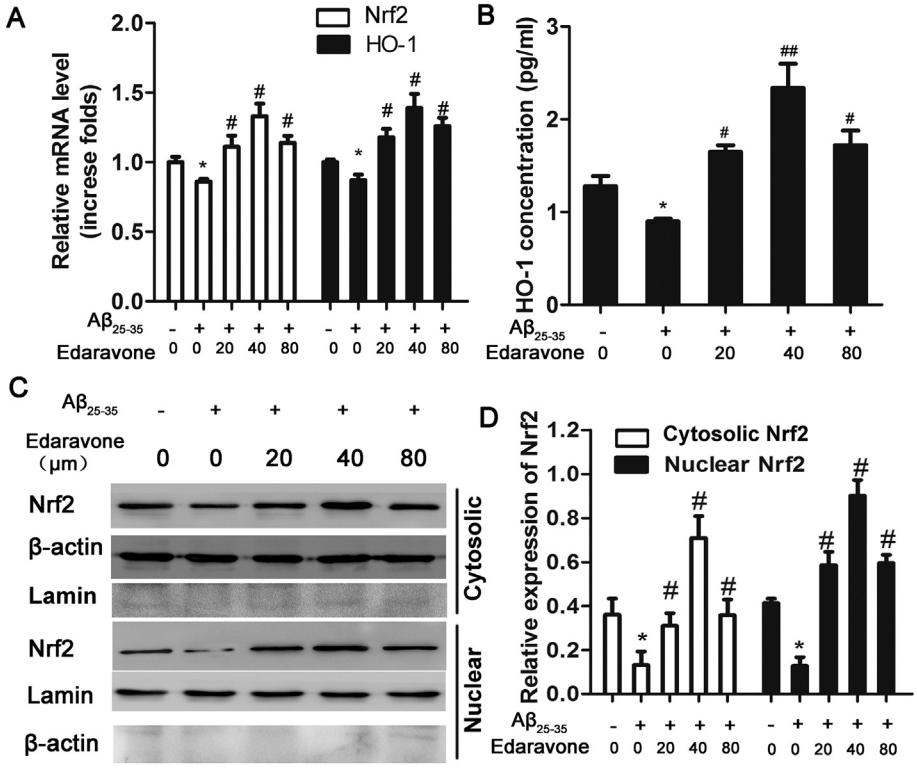

Fig. 3. Effect of edaravone on Nrf2 and HO-1 levels in SH-SY5Y cells. Cells were incubated with 0 μM, 20 μM, 40 μM, or 80 μM concentrations of edaravone for 3 h, followed by incubation with a 25-μM concentration of $A\beta_{25-35}$ for another 24 h. Then the Nrf2 and HO-1 levels were determined. (A) Nrf2 and HO-1 mRNA level determined by RT-PCR. (B) HO-1 concentration determined by ELISA. (C–D) Cytosolic and nuclear fractions of the cells were extracted and western blot was performed to analyze and quantify the expression of cytosolic Nrf2 and nuclear Nrf2 for each treatment. $^{*}p < .05$, compared with the control. $^{\#}p < .05$, $^{\#\#}p < .01$, compared with the $A\beta_{25-35}$ group.

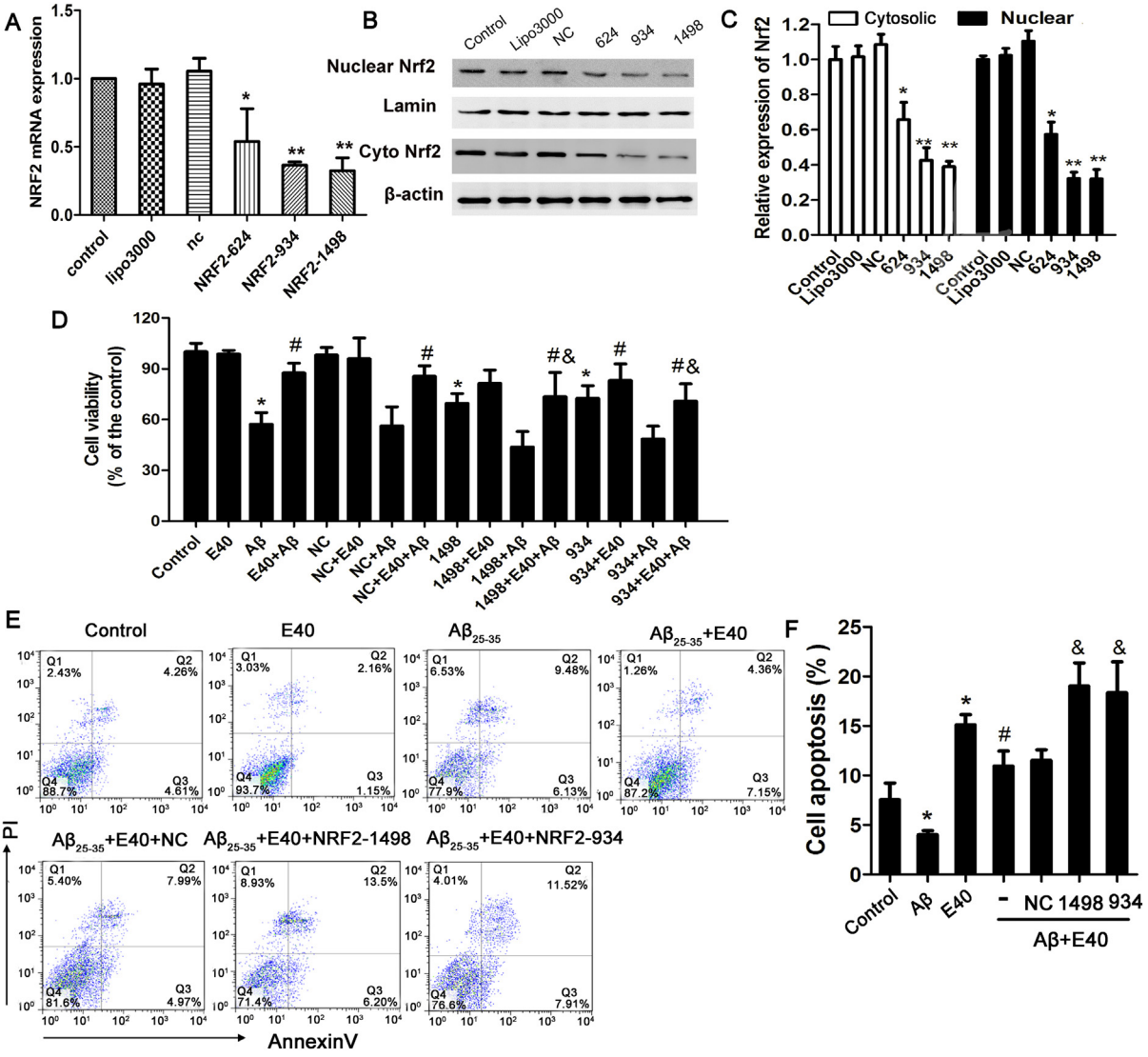

Fig. 4. Nrf2 inhibition weakened protective role of edaravone against cytotoxicity induced by $A\beta_{25-35}$. (A) 3 different siRNAs targeting Nrf2 were synthesized and were introduced to SH-SY5Y cells by Lipo3000 regent. After 24 h of transfection, the inhibition efficiency of specific siRNA on Nrf2 mRNA level was evaluated by RT-PCR. (B—C) Cytosolic and nuclear Nrf2 expression levels after the 3 siRNA transfection (D) After the transfection of si-Nrf2-934, si-Nrf2-1498 and NC for 24 h, the cells were then treated with 40 μM of edaravone and $A\beta_{25-35}$ (25 μM), alone or in combination. Cell viability changes were determined using MTT assay. (E) Representative images of flow cytometry evaluation of cell apoptosis after Nrf2 siRNA (1498 and 934) transfection in the presence of edaravone and $A\beta_{25-35}$. (F) Quantitative percentage of apoptotic cells in each treatment group. $^{*}p < .05$, $^{**}p < .01$, compared with the control, $^{\#}p < .05$, compared with the $A\beta_{25-35}$ group in control, NC or siRNA group, $^{\&}p < .05$, compared with the $A\beta_{25-35}$ + E40 + NC group.

toxicity and causes increased oxidative damage [17]. Oxidative stress is an important contributor to AD pathogenesis and progression, so $A\beta_{25-35}$ is useful for stimulating pathogenesis of AD in vitro. The human brain is more susceptible to oxidative stress insults than other organs due to its high oxygen consumption. Cerebral neurons are vulnerable to oxidative damage and have poor regeneration capability. In AD, the accumulation of Aβ induces oxidative stress, which in turn promotes Aβ deposition, tau hyperphosphorylation, and the subsequent loss of synapses and neurons. Therefore, antioxidants may be useful for AD treatment [19,20].

$A\beta_{25-35}$ induced significant cytotoxicity in SH-SY5Y cells. Our study demonstrated a protective effect of edaravone in these cells. Cell viability markedly increased and apoptosis decreased in cells that were incubated with edaravone for 3 h prior to $A\beta_{25-35}$ exposure. Edaravone thus may prevent $A\beta_{25-35}$-induced cell death. Meanwhile, edaravone at a concentration of 40 μM showed priority in preventing $A\beta_{25-35}$ induced cell damage than that of 80 μM. We consider that edaravone reacts with free radicals and changes into edaravone-radical intermediates and oxidation products, and excess accumulation of these intermediates or products in response to high dose of edaravone may discount the benefit of cells from this radical scavenger. Meanwhile, baseline oxidative stress should be essential to redox homeostasis and cell signaling transition, and the production and quenching of free radical need to be closely regulated to prevent cellular damage [21]. High dose of edaravone may disturb this basal homeostasis of the cells. Actually, this dose effect has been evidenced in several animal and clinical studies in neurological diseases. In a more recent animal study focusing its effect on spinal cord injury, moderate dose of edaravone minimized the negative consequences of spinal cord injury, facilitated functional recovery and exhibited better inhibitory effect on MDA than that of the high dose [22]. Besides, lower-dose intra-aortic edaravone injection prevented immediate and delayed neuronal injury by reducing neuronal cell damage. Furthermore, low dose of edaravone showed higher clinical efficacy and lower rate of adverse effects than the high-dose group in treating ischemic cerebral diseases [24]. However, mechanisms of the dose-effect of edaravone should be determined in further

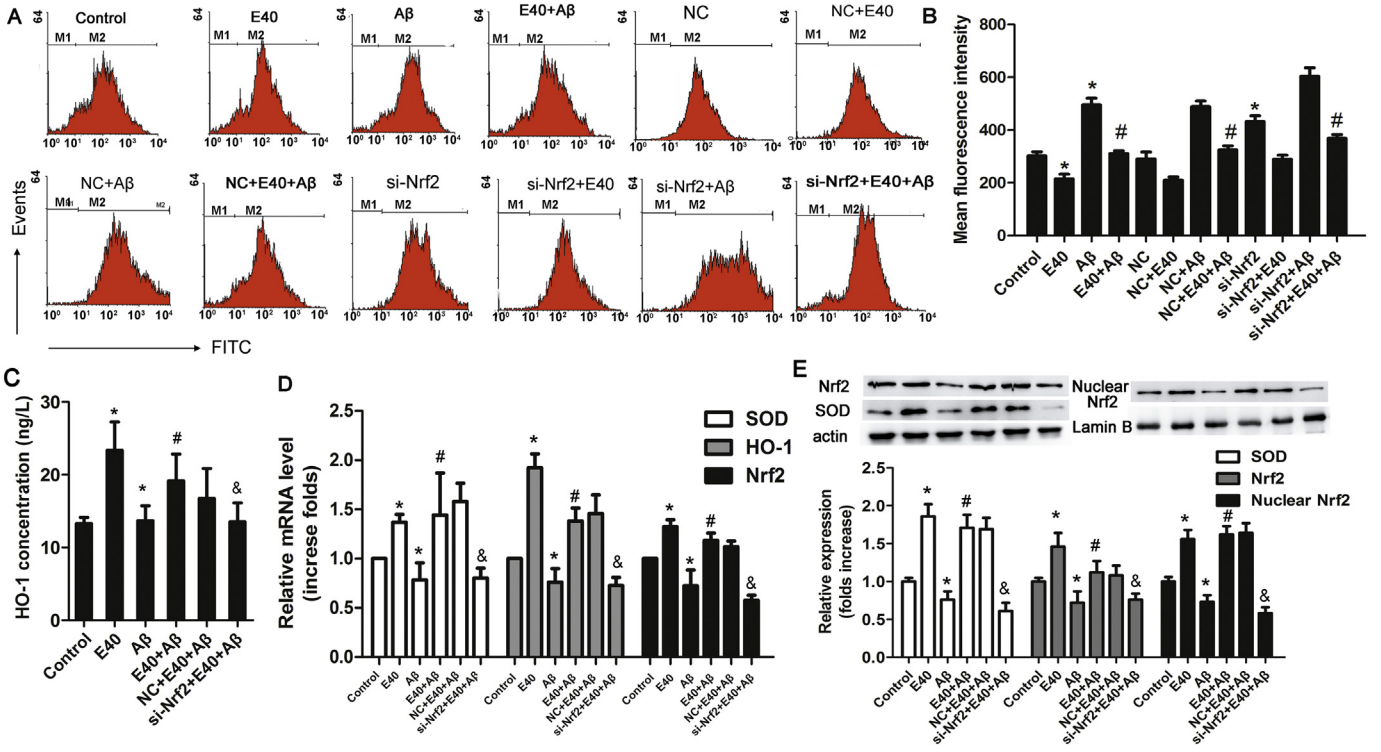

Fig. 5. Nrf2 inhibition impaired the antioxidative role of edaravone in SH-SY5Y cells exposed to $A\beta_{25–35}$. (A–B) After the transfection of si-Nrf2-1498 and NC for 24 h, the cells were then treated with 40 μM of edaravone and 25 μM of $A\beta_{25–35}$, alone or in combination, then ROS level was determined. Representative images of flow cytometry evaluation of ROS levels and quantitative mean fluorescence intensity in each treatment group. (C) Concentration of HO-1 by ELISA in different groups, edaravone at a concentration of 40 μM and $A\beta_{25–35}$ at 25 μM were used. (D) The mRNA levels of Nrf2, SOD, and HO-1. (E) Total protein of SOD, and cytosolic Nrf2 and nuclear Nrf2 were detected by western blot. $^{*}p < .05$, compared with the control, $^{\#}p < .05$, compared with the $A\beta_{25–35}$ group, $^{\&}p < .05$ compared with the $A\beta_{25–35}$ + E40 + NC group.

studies. Emerging evidence has confirmed the effects of edaravone in alleviating cognitive deficits and neurotoxicity in a mouse model of AD [11,12]. Our previous study demonstrated that edaravone ameliorated $A\beta_{25–35}$ induced oxidative stress and apoptosis in SH-SY5Y and PC12 cells [16,25]. However, the underlying mechanism of its protective effects on oxidative cell damage remains unclear. Li et al. [26] showed that edaravone's effects may be associated with Bcl-2 upregulation and Bax downregulation. We found that ROS levels significantly increased in SH-SY5Y cells that were exposed to $A\beta_{25–35}$ and decreased in cells that were pretreated with edaravone. An additional mechanism of edaravone thus may involve attenuating $A\beta_{25–35}$-induced mitochondrial dysfunction [19].

In this study, we found that intracellular Nrf2 mRNA and protein levels increased with edaravone pretreatment followed by $A\beta_{25–35}$ exposure. This finding is important, given that Nrf2 may play a central role in the transcriptional regulation of phase II enzymes [27]. Nrf2 also is involved in many disorders involving oxidative stress [28,29]. When cells are exposed to ROS or electrophilic reagents, Nrf2 disassociates from its cytosolic inhibitor, Keap-1, and translocates to the nucleus, where it combines with the cis-acting ARE (the promoter region of many phase II enzymes). To facilitate ROS removal, Nrf2 in the nucleus then upregulates numerous cytoprotective phase II detoxifying enzymes and antioxidant genes, such as HO-1, SOD, glutathione S-transferase, and γ-glutamylcysteine synthase [27,30,31]. Thus, the Nrf2/ARE signaling pathway plays a crucial role in oxidative stress. Our results show that pretreatment with edaravone increased HO-1 and SOD levels as well as the Nrf2 nuclear translocation either in non-treated or $A\beta_{25–35}$-injured SH-SY5Y cells, indicating that edaravone not only functions as a radical scavenger via their direct intereration, but also is able to activate the Nrf2/ARE signaling pathway. In the condition of $A\beta_{25}$ exposure, edaravone profoundly increases Nrf2/ARE proteins expression over basic non-treated control to resist cell damage by $A\beta_{25}$. These findings are consistent with the strong expression of Nrf2 and activation of the Nrf2/ARE pathway in cerebral ischemia and ischemia-reperfusion injury in rats [32,33]. Similar results also have been revealed in H_2O_2-induced Neuro-2A cells [34] and in H_2O_2-induced or MPP (+)-induced PC12 cells [35,36]. Overall, the data indicated that edaravone induced expression of the antioxidant enzymes SOD and HO-1 by upregulating Nrf2, which might enhance the removal of excess ROS during Aβ-induced oxidative damage. Our study also found that additional Nrf2 silencing did not substantially change the ROS level in SH-SY5Y cells treated with $A\beta_{25}$ + E40, while sole Nrf2 silencing significantly increased ROS. We considered that chemical removal function of edaravone may compensate the decrease of Nrf2 system in attenuating ROS production.

With regard to the antioxidant effects of the Nrf2/ARE pathway, in addition to increased Nrf2 transcription and translation, Nrf2 translocation from the cytosol to the nucleus is of great importance. Tsai et al. [37] found that diallyl trisulfide could upregulate the Nrf2 protein level and promote its translocation from the cytosol to the nucleus, where Nrf2 triggers the expression of HO-1 in H9c2 cells. Huang et al. [38] revealed that panaxatriol saponins, with the help of Nrf2 nuclear translocation, upregulate the expression of HO-1 in oxygen-glucose deprived PC12 cells. The current study revealed that nuclear Nrf2 protein in cells that were pretreated with edaravone before exposure to $A\beta_{25–35}$ was higher than that in cells that were not pretreated before exposure. These results indicate that edaravone protects damaged SH-SY5Y cells by regulating Nrf2 at the transcriptional and translational level and by translocation. However, the effect of edaravone on Nrf2 translocation needs further investigation.

5. Conclusion

We found that edaravone ameliorated the harmful effects of $A\beta_{25–35}$ in SH-SY5Y cells by decreasing cytotoxicity, reducing the generation of ROS, and increasing cell survival. The mechanism underlying these protective effects could be attributed to increased Nrf2 expression and translocation from the cytoplasm to the nucleus, which increases expression of the antioxidant enzymes SOD and HO-1. More work is needed to confirm these effects in primary cultured neuronal cells and AD animal models.

Declarations of interest

The authors declare that there are no conflicts of interest.

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