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Genistein exerts a cell-protective effect via Nrf2/HO-1//PI3K signaling in $A\beta_{25-35}$-induced Alzheimer's disease models *in vitro*

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Abstract

Introduction. Alzheimer's disease (AD), a very common neurodegenerative disorder, is mainly characterized by the deposition of β-amyloid protein ($A\beta$) and extensive neuronal cell death. Currently, there are no satisfactory therapeutic approaches for AD. Although neuroprotective effects of genistein against $A\beta$-induced toxicity have been reported, the underlying molecular mechanisms remain unclear. Furthermore, the PI3K/Akt/Nrf2 signaling pathway is associated with AD. The aim of the study was to investigate whether genistein can modulate Nrf2/HO-1/PI3K signaling to treat AD.
Materials and methods. Cell viability assay, the measurement of heme oxygenase-1 (HO-1) expression by reverse transcription-polymerase chain reaction (RT-qPCR), and western blot were performed on the SH-SY5Y cells induced by $A\beta_{25-35}$ in response to the treatment with genistein. Moreover, PI3K p85 phosphorylation was measured.
Results. Genistein enhanced the HO-1expression at both the mRNA and protein levels, as well as the PI3K p85 phosphorylation level. In addition, genistein increased the survival of SH-SY5Y cells treated with $A\beta_{25-35}$*via* HO-1 signaling. However, following transfection with Nrf2 small interfering RNA (siRNA) and treatment with LY294002, an inhibitor of PI3K p85, genistein could not upregulate HO-1 to exert neuroprotective effects on SH-SY5Y cells treated with $A\beta_{25-35}$.
Conclusions. These results suggest that genistein exerts a neuroprotective effect on SH-SY5Y cells *in vitro via* Nrf2//HO-1/PI3K signaling, providing a foundation for the application of genistein in the treatment of neurodegenerative diseases related to Nrf2/HO-1/PI3K signaling. *(Folia Histochemica et Cytobiologica 2021, Vol. 59, No. 1, 49–56)*

Key words: Alzheimer's disease; amyloid β_{25-35}; *in vitro*; SH-SY5Y cells; genistein; heme oxygenase-1; Nrf2; siRNA; Nrf2/HO-1/PI3K pathway

Introduction

Alzheimer's disease (AD), a progressive neurodegenerative disease, affects the aging population around the world [1] and accounts for approximately 60–80% of dementia cases [2]. AD is characterized by the accumulation of β-amyloid peptide ($A\beta$), neurofibrillary tangles (NFTs) and neuronal loss [3, 4]. The deposition of $A\beta$ may serve as the key step in the initiation of the AD pathological process, and other downstream events, including neuroinflammation, oxidative stress and tau protein accumulation, may be the main causes of neurodegeneration [5]. Currently, despite large improvements in understanding the pathogenesis of AD, existing drugs can only alleviate the symptoms and slow the progression of cognitive declines; there

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are no effective strategies for the treatment of AD [6–8]. As a consequence, identifying the pathological molecular mechanisms is a very important research target related to the treatment of AD.

The present study aimed to focus on the natural products with cost-effective and fewer toxic properties. It has been widely acknowledged that phytochemicals, including genistein, curcumin, resveratrol, quercetin and catechins, are promising therapeutics for the treatment of AD due to their functions in inhibiting oxidative stress, neuroinflammation and mitochondrial dysfunction [9]. Genistein, a natural isoflavone constituent found in soybean extract, can cross the blood-brain barrier in mice [10] and it possesses a variety of pharmacological activities, including anticancer, anti-fibrotic, anti-inflammatory and anti-oxidative activities [11, 12]. Additionally, genistein is a cell-permeable, reversible, substrate competitive inhibitor of protein tyrosine kinases, including autophosphorylation of epidermal growth factor receptor kinase, and regulates diverse intracellular signal transductions [13]. Genistein downregulates the production of TNF-α and the activation of NF-κB in endothelial cells [14, 15], and reduces the production of TLR4 in lipopolysaccharide (LPS)-induced BV2 microglia cell line [16]. Genistein has also been reported to improve learning and memory in numerous diseases [17–19], as well as ameliorate astrogliosis in AD [20, 21].

Since multiple and interdependent mechanisms are involved in the pathological process of AD, the present study searched for other targets relating to genistein that could ameliorate AD. Therefore, nuclear factor erythroid 2-related factor 2(Nrf2)//heme oxygenase-1 (HO-1) signaling was selected as a target of the present study. In the physiological state, induction of HO-1 may serve as a beneficial or adaptive response to a number of stimuli, indicating a protective role in numerous disorders [22]. It has been reported that the agents can exert essential protective roles against oxidative stress and inflammation *via* modulating Nrf2/HO-1 [23]. HO-1 has been found to exhibit anti-inflammatory, immunomodulatory and cytoprotective properties, the therapeutic potential of HO-1 can be harnessed by the use of phytochemicals and novel HO-1 inducers [24]. In addition, genistein can upregulate HO-1 expression in mice with doxorubicin-induced cardiotoxicity [25] and in PC12 neuronal cells incubated with amyloid β_{25-35} [26].

Taken together, the aim of the present study was to evaluate the effects and underlying mechanisms of genistein in SH-SY5Y cells treated with Aβ_{25-35}, a peptide applied to mimic the neuropathological conditions of AD. It was revealed that genistein may exert a cell-protective effect against Aβ_{25-35}-induced neurotoxicity in SH-SY5Y cells *via* Nrf2/HO-1/phosphatidylinositol-3 kinase (PI3K) signaling.

Materials and methods

Genistein. Genistein (cat.345834, SigmaAldrich, St. Louis, MO, USA), dissolved in 0.1% DMSO as a stock solution of 3 mM, was further diluted in culture medium and added to SH-SY5Ycells at the indicated final concentration.

Preparation of Aβ peptide. A$\beta_{25-35}$was purchased from Shanghai Strong Biotechnology Co., Ltd. (Shanghai, China) and prepared as described by Kreutz *et al.* [27]. Before the treatment of SH-SY5Y cells, aliquots dissolved in sterilized ddH_2O (1 mg/ml) and stored at −20°C. Then aliquots of Aβ_{25-35} were incubated for 96 h at 37°C to obtain the aggregated Aβ.

Nrf2 small interfering RNA (siRNA). The Nrf2 siRNA was purchased from Shanghai Sangon Co., Ltd. (Shanghai, China). The Nrf2 siRNA sequences were sense, 5'-GGUUGA GAC UAC CAU GGU UTT-3' and anti-sense, 5'-AAC CAU GGU AGU CUC AAC CTT-3'. The control siRNA sequences were sense 5'-UUC UCC GAA CGU GUC ACG UTT-3' and anti-sense, 5'-ACG UGA CAC GUU CGG AGA ATT-3'. After cells were washed in PBS, Lipofectamine®2000 reagent (Solarbio Science & Technology Co.) was used for siRNA transfection. The transfection was performed for 4 h.

Cell culture and treatments. SH-SY5Y cells were cultured as described by He *et al.* [28]. A total of 1 × 10^4 SH-SY5Y cells were seeded into 96-well cell culture plates (for the cell viability assay) or 24-well cell culture plates (for reverse transcription-quantitative PCR (RT-qPCR), and western blot analysis) and treated as follows: (i) Cells were pretreated with genistein (10, 30 or 50 μM) for 90 min prior to co-culture with Aβ_{25-35} at 20 mM for 24 h; (ii) cells were pretreated with ZnPP (Zinc Protoporphyrin, an inhibitor of the HO-1, 10 μM) and genistein (10, 30 or 50 μM) for 90 min prior to a 24-h co-culture with Aβ_{25-35} at 20 μM; (iii) cells were pretreated with Nrf2 siRNA (100 nM) and genistein (10, 30 or 50 μM) for 90 min prior to a 24-h co-culture with Aβ_{25-35} at 20 μM; and (iv) cells were pretreated with LY294002 (10 or 20 μM) and genistein (10, 30 or 50 μM) for 90 min prior to a 24-h co-culture with Aβ_{25-35} at 20 μM. Subsequently, a cell viability assay, RT-qPCR and western blot were performed.

Cell viability assay. The cell viability assay was performed as described previously [29]. At the indicated time-points, SH-SY5Y cells were incubated with the culture medium supplemented with 10 μL of 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide (MTT, at a concentration of 500 μg/ml) (M1020, Solarbio, Beijing, China) for 4 h. After aspirating the culture medium, 100 μL DMSO was then added. Following incubation at 37°C for 30 min, the optical density was measured spectrophotometrically at 410 nm.

Reverse transcription-polymerase chain reaction (RT-qPCR). RT-qPCR was performed according to the standard protocols and as described previously [30]. Quantitative real-time PCR was performed using SYBR Green Kit (Takara) in an iCycler iQTM (Bio-Rad, Hercules, CA, USA).The primer sequences used for qPCR were as follows: HO-1, 5'-CAT CCT GCG TCT GGA CCT GG' (sense) and 5'-TAA TGT CAC GCA GAT TTC C-3' (antisense); and GAPDH, 5'-ATG GCC TCC CTG TAC CAC ATC-3' (sense) and 5'-TGT TGC GCT CAA TCT CCT CCT-3' (antisense).

Western blot. Western blot was performed as described previously [31]. Protein samples heated at 95°C were separated via 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto polyvinylidene difluoride (PVDF) membranes (EMD Millipore) at 300 mA for 3 h. The membranes were blocked with 5% non-fat dry milk or BSA dissolved in Tris-HCl saline buffer containing 0.1% Tween-20 (TBST, PH 7.4). Subsequently, the blots were incubated overnight at 4°C with one of the following antibodies: Rabbit anti-HO-1 (1:1000; ab13248, Abcam, Cambridge, UK), rabbit anti-Nrf2 (1:1000; ab137550, Abcam), rabbit anti-PI3K p85 (1:1000; ab191606, Abcam) and rabbit anti-β-actin (1:500; ab8227, Abcam). After washing three times for 5 min each in TBST, the membranes were incubated with HRP-coupled goat anti-rabbit secondary antibodies (1:1000; Boster, Wuhan, China) diluted in TBST for 1 h. Membranes were washed three times in TBST for 5 min each at room temperature. The immunoreactive signals were then visualized with enhanced chemiluminescence solution (Bio-Rad). The signal intensity was quantified by densitometry using ImageJ 5.0 software (Dental Diagnosis Science, San Antonio, TX, USA).

Statistical analysis. Data are presented as the mean ± SD. Comparisons between groups were performed using ANOVA followed by Bonferroni's *post hoc* test using GraphPad Prism 6 software. Statistical significance was considered at $P < 0.05$.

Results

Genistein increased the HO-1 expression in SH-SY5Y cells treated with Aβ_{25-35}

To investigate the effects of genistein on the SH-SY5Y cells induced by Aβ_{25-35}, RT-qPCR and western blot analyses were performed after the cells were pretreated with genistein and co-cultured with Aβ_{25-35}. It was observed that, in comparison with the vehicle control, the HO-1 mRNA level was increased in response to Aβ_{25-35} treatment. Compared with the Aβ_{25-35}-treated group, genistein (10, 30 and 50 μM) significantly increased the HO-1 mRNA level of Aβ_{25-35}-treated SH-SY5Y cells (Fig. 1A).

Similar pattern of HO-1 response to Aβ_{25-35} and genistein treatment was observed at the protein levels (Fig. 1B–C).

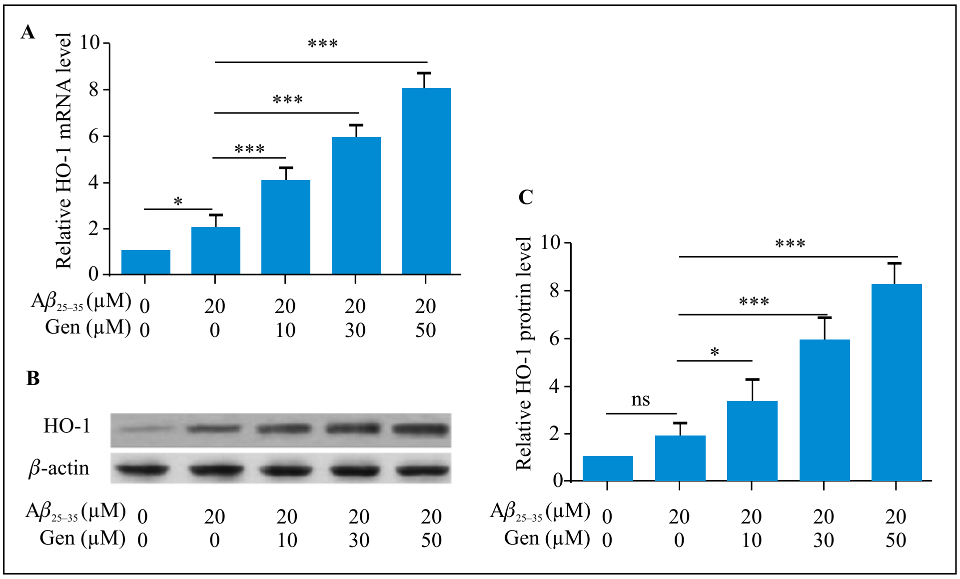

Figure 1. Determination of the effects of genistein on HO-1 level in SH-SY5Y cells induced by A$\beta_{25\text{-}35}$. A total of 1 × 10^4SH--SY5Y cells were pretreated with genistein at the concentrations of 10, 30 and 50 μM for 90 min prior to a 24-h co-culture with A$\beta_{25\text{-}35}$ (20 mM). Subsequently, RT-qPCR and western blot were performed as described in methods. **A.** HO-1 mRNA level. **B** and **C.** RelativeHO-1 protein content was assessed with western blot in cells treated with A$\beta_{25\text{-}35}$ and without or with various concentrations of genistein (Gen). *$p < 0.05$, ***$p < 0.001$ from five independent experiments.

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Genistein reduced the death of SH-SY5Y cells treated with Aβ$_{25-35}$ via upregulating HO-1

To investigate the effect of genistein on Aβ$_{25-35}$-inducedapoptosis of SH-SY5Y cells, a MTT assay was performed.

A cell viability assay revealed that, in comparison with the vehicle control, the cell survival rate was decreased in response to Aβ$_{25-35}$ treatment. Genistein (10, 30 and 50 μM) significantly increased the survival rate of Aβ$_{25-35}$-treated SH-SY5Y cells. Additionally, inhibition of HO-1 by ZnPP (Zinc Protoporphyrin, an inhibitor of the HO-1) reduced the effects of genistein on the cell survival rate of SH-SY5Y cells treated with Aβ$_{25-35}$ (Fig. 2).

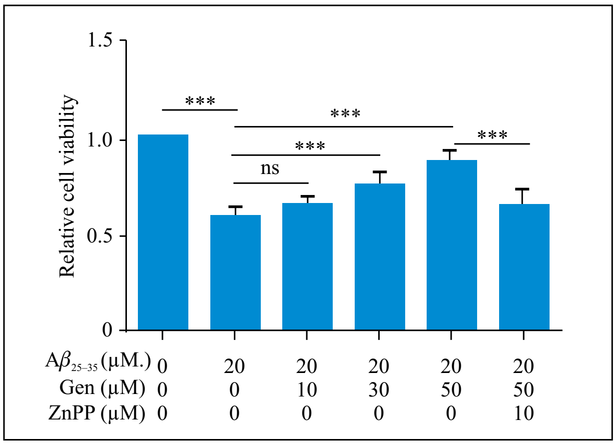

Figure 2. Determination of the effects of genistein on the survival of SH-SY5Y cells induced by Aβ$_{25-35}$ after inhibiting the HO-1. A total of 1 × 10^4 SH-SY5Y cells were co-pretreated with ZnPP (Zinc Protoporphyrin, an inhibitor of the HO-1) and genistein at the concentrations of 10, 30 and 50 μM for 90 min prior to a 24-h co-culture with Aβ$_{25-35}$ (20 mM). A cell viability assay was then performed. ***$p < 0.01$ from five independent experiments.

Inhibiting Nrf2 signaling reverses the neuroprotective effect of genistein on upregulating HO-1 in Aβ$_{25-35}$-treated SH-SY5Y cells

To investigate the effects of the Nrf2 signaling pathway on the neuroprotective role of genistein on upregulating HO-1 in SH-SY5Y cells induced by Aβ$_{25-35}$, HO-1 protein level was evaluated by western blot after the cells were pretreated with Nrf2 siRNA and genistein.

It was observed that, following inhibition of Nrf2 signaling by Nrf2 siRNA, the effect of genistein on the upregulation of HO-1 protein level in Aβ$_{25-35}$-treated SH-SY5Y cells was partially abolished (Fig. 3A, B).

Inhibiting PI3K signaling reverses the effect of genistein on upregulating HO-1 in Aβ$_{25-35}$-treated SH-SY5Y cells

To investigate the effects of the PI3K signaling pathway on the neuroprotective role of genistein on upregulating HO-1 in SH-SY5Y cells induced by Aβ$_{25-35}$, PI3K p85 phosphorylation level and HO-1 protein level were evaluated by western blot after the cells were pretreated with LY294002 (an inhibitor of PI3K p85) and genistein, and co-cultured with Aβ$_{25-35}$. It was observed that in comparison with the vehicle control, the P85 phosphorylation level was decreased in response to Aβ$_{25-35}$ treatment. Compared with the

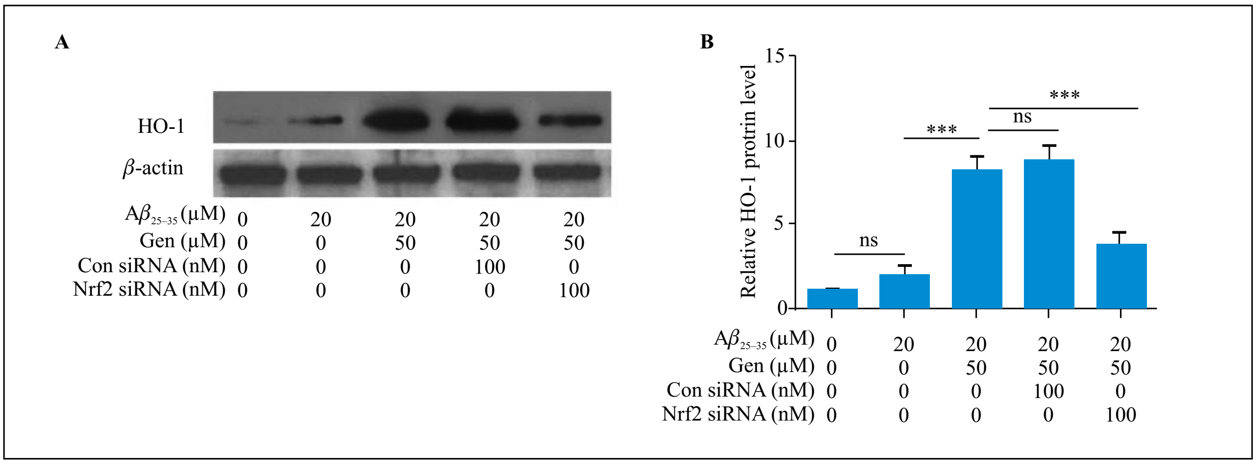

Figure 3. Determination of the effects of genistein on the HO-1 in SH-SY5Y cells induced by Aβ$_{25-35}$ after inhibiting Nrf2. 1 × 10^4 SH-SY5Y cells were co-pretreated with Nrf2 siRNA and 50 μM genistein for 90 min prior to a 24-h co-culture with Aβ$_{25-35}$, followed by western blot. **A–B.** HO-1 protein level was upregulated after the treatment with genistein. Abbreviations: con siRNA — control siRNA; ***$p < 0.001$ from five independent experiments.

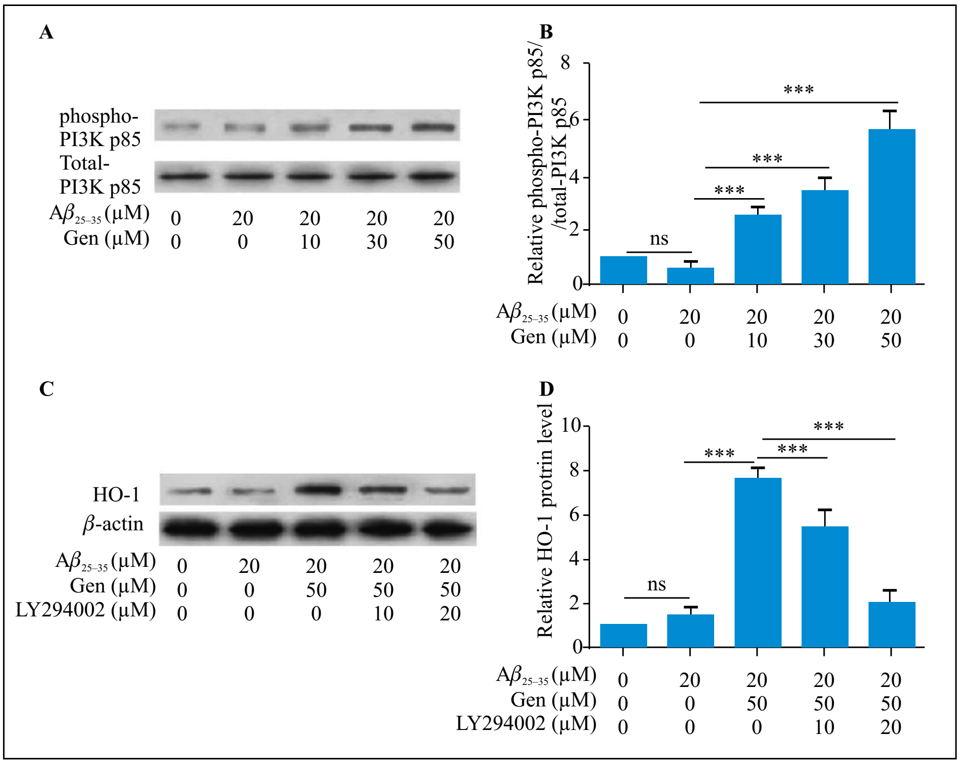

Figure 4. Determination of the effects of genistein on the HO-1 levels in SH-SY5Y cells induced by Aβ_{25-35} after inhibiting the PI3K. A total of 1 × 10^4 SH-SY5Y cells were co-pretreated with LY294002 and genistein at a concentration of 10, 30 or 50 μM for 90 min prior to a 24-h co-culture with Aβ_{25-35}, followed by western blot. **A–B.** The PI3K p85 phosphorylation level in the nucleus was upregulated in SH-SY5Y cells in a dose-dependent manner. **C–D.** The HO-1 protein level in the nucleus was downregulated. ***$p < 0.001$ from five independent experiments.

Aβ_{25-35}-treated group, genistein (10, 30 and 50 μM) significantly increased the PI3K p85 phosphorylation level of Aβ_{25-35}-treated SH-SY5Y cells (Fig. 4A, B). It was also observed that, after inhibiting PI3K signaling, genistein did not upregulate HO-1 protein level in Aβ_{25-35}-treated SH-SY5Y cells (Fig. 4C, D).

Discussion

Previous studies have examined the potential use of genistein as a treatment for AD [32]; genistein has been shown to exert a protective effect in AD *in vitro via* the Nrf2 signaling pathway [33–35]. In the present study, genistein treatment increased cell survival in SH-SY5Y cells treated with Aβ_{25-35}. Furthermore, following inhibition of the Nrf2 and PI3K p85 signaling pathways, genistein was unable to exert these cell-protective roles. These findings suggested that genistein treatment may protect SH-SY5Y cells from the neurotoxicity induced by Aβ_{25-35} treatment *via* the Nrf2/HO-1/PI3K signaling pathway.

Aβ peptide fragments can induce neuronal cell death directly or indirectly [36], and oligomeric Aβ peptides have been identified as a key factor in the multiple pathogenic changes in AD and, more generally, in dementia [37]. Deposition of Aβ_{25-35} in the brain triggers tau protein phosphorylation and formation of intracellular NFTs, subsequently leading to mitochondrial dysfunction and membrane rupture, which then proceeds to necrosis or apoptosis [38]. It has been reported in previous *in vitro* studies that genistein protects against cell death [39, 40]. Genistein protects against Aβ-induced toxicity in SH-SY5Y cells by regulation of Akt and Tau phosphorylation [41]. Genistein and galantamine combinations decrease A$\beta_{(1-42)}$-induced genotoxicity and cell death in SH-SY5Y Cell Line [42]. The present study used SH-SY5Y cells to generate an *in vitro* model to investigate the effect of genistein on the neurotoxicity induced by Aβ_{25-35}.

Increased oxidative stress occurs in response to increased Aβ levels [43]. Oxidative stress has generally been implicated in neurodegenerative disorders and, more specifically, in the onset and development of AD [44]. HO-1 induction may indicate a pro-oxidative status since HO-1 is activated under oxidative stress. Zhai *et al.* demonstrated that genistein upregulated HO-1 and GCLC expression via the EKR1/2 and PKC/Nrf2 pathways during oxidative stress using

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a H_2O_2-induced cell model [45]. Genistein can exert neuroprotective effects against Aβ-induced oxidative stress *via* activating α7nAChR and its downstream phosphatidylinositol 3-kinase (PI3K)/Akt/Nrf2 signaling cascades [46]. The present study observed that genistein could promote the survival of SH-SY5Y cells treated with Aβ_{25-35} *via* HO-1 signaling.

Nrf2 is considered a "master regulator" of the antioxidant response, and it is also a regulator of maintaining the body's redox homeostasis [47]. Under an oxidative stimulus, Nrf2 is translocated to the nucleus where it interacts with small proteins and binds to ARE to activate the transcription of antioxidant genes, such as the nicotinamide adenine dinucleotide phosphate oxidase complex: quinone oxidoreductase 1, glutathione S-transferases, γ-glutamylcysteine ligase and heme oxygenase 1 [48]. It has been reported that genistein treatment can activate the Nrf2 pathway to augment the antioxidative system *in vitro* and *in vivo* [49]. The present study revealed that genistein could upregulate Nrf2 to increase HO-1 in SH-SY5Y cells treated with Aβ_{25-35}.

Akt is a serine/threonine kinase that regulates a wide range of processes, including cell survival, cell growth and apoptosis [50]. Previous studies have reported that Aβ peptide may decrease Akt phosphorylation, thus inhibiting its activation [51]. Reduced activation of Akt is known to induce tau protein hyperphosphorylation and cell death [50]. Genistein can stimulate the PI3K/Akt pathway and thereby the release of NO [52]. The present study revealed that genistein could upregulate PI3K phosphorylation to increase HO-1 in SH-SY5Y cells treated with Aβ_{25-35}.

In conclusion, the present study demonstrated that genistein could alleviate the neurotoxicity of Aβ_{25-35} in SH-SY5Y cells by improving the cell survival and anti-oxidative response. These effects may be reversed by inhibiting the Nrf2 and PI3K signaling pathways. These findings suggest that a novel strategy for the treatment of AD may involve genistein.

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Declaration of competing interest

All authors declare no competing interests.

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