Neuroprotective effects of *Paeonia lactiflora* and its active compound paeoniflorin against Aβ_{25-35}-induced neurotoxicity in SH-SY5Y cells

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**Abstract** Excessive accumulation of the amyloid beta (Aβ) peptide has been implicated in the pathogenesis of Alzheimer’s disease (AD). *Paeonia lactiflora* (PL) has been used in treatments of several conditions such as inflammation, arthritis, and cognitive impairment. The purpose of this study was to investigate the neuroprotective effect and mechanisms of PL and its active compound, paeoniflorin (PF), on Aβ_{25-35}-induced neurotoxicity in SH-SY5Y cells. We evaluated cell viability, lactate dehydrogenase (LDH) release and reactive oxygen species (ROS) production. Furthermore, underlying mechanism of PL and PF on the regulation of amyloidogenic pathway was analyzed by Western blotting. In our results, Aβ_{25-35}-induced neuronal cell loss was observed, whereas treatment with PL (10, 50, and 100 μg/mL) and PF (1, 5, and 10 μg/mL) significantly elevated the cell viability, and decreased LDH release and ROS production. In addition, exposure of SH-SY5Y cells to Aβ_{25-35} significantly increased the protein levels of amyloid precursor protein (APP)-C-terminal fragment β, β-site APP-cleaving enzyme, and presenilin-1 and -2. However, treatment with PL and PF inhibited the amyloidogenic pathway via the down-regulation of those protein expressions. Taken together, our results indicate that PL, and its active compound PF, could protect SH-SY5Y cells against Aβ_{25-35}-induced cell neurotoxicity by attenuating LDH release and ROS production, and these effects may be attributed to regulation of amyloidogenic pathway-related protein expression. In conclusion, PL and PF could be a potential to prevent neurodegenerative disorders such as AD.

**Keywords** Alzheimer’s disease · Amyloid beta · Neuronal · Paeonia · Paeoniflorin

**Introduction**

Alzheimer’s disease (AD) is one of the most common causes of dementia in the elderly people, and is characterized by a decline in memory ability. AD is also causing loss of language abnormalities and disturbances of motor function [1,2]. Qiu et al. [3] reported that approximately 20% of the global population aged >60 years are diagnosed with AD. It is estimated that this number will almost double every 20 years and will reach 74.7 million in 2030 and 131.5 million in 2050 [4]. In this respect, the prevention and treatment of the disease have become of vital importance to AD patients in recent years. Although many synthetic chemical treatments have been developed, the treatments are associated with several side effects and there is not yet a definitive cure for AD [5].

The composition of the brain is 10% of neurons responsible for the main functions of the nervous system and 90% of glial cells responsible for maintaining and nourishing the nerve cells. Recent research suggests that neuronal cell damage in the brain of AD patients is associated with free radicals (such as reactive oxygen species [ROS]) [6], oxidative stress [7], inflammation [8], and neurotoxic amyloid beta (Aβ) peptide [9]. In particular, hypothetical deposition of Aβ peptides as a senile plaque is one of the most
well-known pathological hallmarks of memory disturbances and instrumental signs in AD [10,11]. Amyloid precursor protein (APP) can be generated via at least two major proteolytic processing pathways: (A) the non-amyloidogenic, in which γ-secretase cleaves the middle of the Aβ sequence precluding formation of full length Aβ; and (B) the potentially amyloidogenic pathway in which β-secretase cleaves APP, resulting in secretion of soluble APP β (sAPPβ), and a second membrane-bound C-terminal fragment of APP (CTFβ). Further cleavage of CTFβ by γ-secretase generates Aβ [12]. In the normal brain, low levels of Aβ fibrils have various synaptic activities such as neuronal growth [13], synaptogenesis [14], and cell adhesion [15]. However, Aβ-induced oxidative stress and neurotoxicity have been reported to cause neurodegeneration, since Aβ accumulation leads to neuronal lipid peroxidation and DNA oxidation [16]. In addition, high concentrations of Aβ lead to neurotoxicity and result in cell death [17].

Paeonia lactiflora (PL) is a medicinal plant that is cultivated worldwide, including in Korea, China, Japan, and its root has also been used for medicinal purposes in various diseases [18,19]. PL has been reported as a beneficial agent for the treatment of inflammation [20], arthritis [21], and cognitive impairment [22]. Paeoniflorin (PF), the highest contained monoterpenes glycoside in PL, is known to elicit various pharmacological effects such as anti-oxidant [23], anti-inflammatory [24], and neuroprotective effects [25]. However, the protective effects of PL and PF against Aβ toxicity and the underlying mechanisms of APP processing in SH-SYSY neuronal cells have not yet been evaluated. Therefore, we aim to determine whether PL and the active compound, PF, exert neuroprotective effects and affect APP processing in a cellular system.

Materials and Methods

Preparation of samples
PL used in this research was contributed by the Gyeongnam Oriental Anti-aging Institute (Sancheong, Korea). PL was dried by hot air and stored at −5°C until extraction. PL was added to 20 times of purified water and heated for 3 h at 90°C for extraction. The extract is filtered by using No. 2 filter paper (Whatman, Kent, UK) and the purified water was evaporated at 40°C in a refrigerator. PF (purity >98%) were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Prior to use, PL and PF were dissolved in dimethyl sulfoxide (DMSO) as the stock solution, and further diluted with Dulbecco’s modified eagle medium (DMEM) for experiment.

Instruments and reagents
DMEM, penicillin/streptomycin, fetal bovine serum (FBS) were supplied from Welgene (Daegu, Korea). 3-(4,5-Dimethylthiazol-2-yl)-2,3-diphenyl tetrazolium bromide (MTT), DMSO and dichlorofluorescin diacetate were purchased from Sigma Chemical Co. (St Louis, MO, USA). Lactate dehydrogenase (LDH) cytotoxicity detection kit was supplied from Takara Bio Co. (Shiga, Japan). Radioimmunoprecipitation assay (RIPA) buffer and 30% acrylamide bis solution were obtained from Elpis Biotech (Dajeon, Korea). Pre-stained protein size marker was obtained from GenDOPOT Inc. (Katy, TX, USA).

Preparation of Aβ25-35
Aβ25-35 peptide (Sigma Chemical Co.) was dissolved in sterile distilled water to achieve a concentration of 1 mM. Aβ25-35 stock solution was incubated to induce aggregation at 37°C for 3 days, and then stored at −20°C until use. For cell experiments, it was further diluted to 25 μM in culture medium.

Cell culture
SH-SYSY cells were obtained from KCLB (Korea Cell Line Bank, Seoul, Korea). The cells were maintained at 37°C in 5% CO2 incubator (Thermo Fisher Scientific Inc., Waltham, MA, USA) with DMEM containing 1% penicillin/streptomycin and 10% FBS. Cells were sub-cultured with 0.05% trypsin-EDTA in phosphate buffered saline (PBS). Cells were divided into the following groups: ‘Normal’ group represents the non-treated cells, ‘Control’ group represents the Aβ25-35-treated cells, ‘PL10’, ‘PL50’, or ‘PL100’ groups represent the three concentrations of Paeonia lactiflora treatment (10, 50, 100 μg/mL) in Aβ25-35-treated cells. ‘PF1’, ‘PF5’, or ‘PF10’ groups represent the three concentrations of paeoniflorin treatment (1, 5, 10 μg/mL) in Aβ25-35-treated cells.

MTT assay
After the cells approached confluence, the cells were seeded at 5 × 104 cells/well into 96-well plate for 24 h incubation. The cells were treated with various concentrations of PL (10, 50, and 100 μg/mL) and PF (1, 5, and 10 μg/mL) for 4 h. After then, the cells were stimulated with 25 μM of Aβ25-35 for 24 h. The MTT solution was added to each 96-well plate and incubated for 4 h at 37°C. After incubation, medium containing MTT was removed. The intracellular formazan product was dissolved in 200 μL of DMSO and absorbance was measured at 540 nm using a microplate reader (Thermo Fisher Scientific) [26].

LDH release assay
The LDH release assay was performed according to the manufacturer’s protocol using LDH cytotoxicity detection kit. When SH-SYSY cells reached 80-90% confluence, the cells were plated in 96-well plate at 5 × 104 cells/well and incubated for 24 h. The cells were pretreated with various concentrations of PL (10, 50, and 100 μg/mL) and PF (1, 5, and 10 μg/mL) for 4 h, and Aβ25-35 (25 μM) was added. After incubation 24 h, the supernatant (100 μL) and reaction mix (100 μL) were added to 96-well plate and incubated for 30 min at room temperature. The absorbance of
each well was read at 490 nm using a microplate reader (Thermo Fisher Scientific) [27].

Reactive oxygen species (ROS) production
The ROS scavenging activity was measured using DCFH-DA [28]. SH-SY5Y cells were seeded at 5×10^4 cells/well in 96-well plate and incubated for 24 h. The cells were pretreated with various concentrations of PL (10, 50, and 100 μg/mL) and PF (1, 5, and 10 μg/mL). After incubating 4 h, the cells were treated with 25 μM of Aβ_{25-35} for 24 h. The cells then incubated with 80 μM DCFH-DA for 30 min at 37°C. DCFH-DA was read by FLUOstar OPTIMA (BMG Labtech., Ortenberg, Germany) at the excitation absorbance of 480 nm and the emission absorbance of 535 nm.

Western Blot Analysis
SH-SY5Y cells were lysed according to the manufacturer’s instructions using RIPA buffer supplemented with 1× protease inhibitor cocktail (Sigma Chemical Co.). Proteins were separated by electrophoresis in a precast 10-13% SDS-PAGE and blotted onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The PVDF membranes were incubated with 5% skim milk dissolved in PBS-Tween® for 60 min at room temperature. The membrane was incubated overnight at 4°C for 60 min at room temperature. The membrane was incubated overnight at 4°C for 60 min at room temperature. The membrane was incubated with primary antibodies (PS-1; 1:1000, Cell Signaling); presenilin-2 (PS-2; 1:1000, Cell Signaling); CTFβ (1:1000, Sigma Chemical Co.); and β-actin (1:1000, Cell Signaling). The membrane was washed and incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies. Western bands were visualized using a chemiluminescent imaging system (Davinci Chemi, Seoul, Korea).

Statistical analysis
Statistical significance was verified by performing Duncan’s multiple range test using the program IBM SPSS version 23 (IBM Corporation, Armonk, NY, USA). Significance was set at p < 0.05. Results are expressed as mean ± standard deviation (SD).

Results
Effects of PL and PF on cell viability in Aβ_{25-35}-treated SH-SY5Y cells
We evaluated the protective activity of the PL and PF against oxidative stress induced by Aβ_{25-35}. The control group decreased to 41.49% compared to 100% of normal group, however cell viability was increased to 51.79% and 51.58%, in the presence of PL 100 μg/mL and PF 10 μg/mL, respectively (Fig. 1). This result suggests that PL and PF have the neuroprotective effect from Aβ_{25-35}-induced cell damage.

Effects of PL and PF on LDH release in Aβ_{25-35}-treated SH-SY5Y cells
When SH-SY5Y cells were exposed to 25 μM Aβ_{25-35} aggregates for 24 h, LDH release was increased from 92.53 to 100%. However, treatment with PL and PF reduced the LDH release in a concentration-dependent manner as illustrated in Fig. 2. In particular, at PL concentration of 100 μg/mL, LDH release was reduced as much as normal group (92.21%), showing that PF
protected neuronal cells from Aβ_{25-35} aggregates-induced neurotoxicity.

Effects of PL and PF on ROS production in Aβ_{25-35}-treated SH-SY5Y cells

We examined whether PL and PF inhibited ROS production induced by Aβ_{25-35} in SH-SY5Y cells. Fig. 3A and Fig. 3B show that the treatment of SH-SY5Y cells with Aβ_{25-35} significantly increased ROS levels in a time-dependent manner. As compared to control group (100%), the treatment with PL (100 μg/mL) and PF (10 μg/mL) significantly inhibited the excessive ROS production, by decreasing it to 91.75 and 91.71%, respectively. These results demonstrate that PL and PF were able to diminish oxidative stress by inhibiting ROS production (Fig. 3C), indicating that PL and PF have protective effect against Aβ_{25-35}-induced oxidative damage.

Effects of PL and PF on amyloidogenic pathway in Aβ_{25-35}-treated SH-SY5Y cells

We investigated the effect of PL and PF on the amyloidogenic pathway in SH-SY5Y cells by using the Western blotting. Our results showed that SH-SY5Y cells treated with Aβ_{25-35} increased BACE (Fig. 4A), CTFβ (Fig. 4B), PS-1 (Fig. 4C), and PS-2 (Fig. 4D) protein expression levels. However, the results indicated that the expression of these proteins was significantly inhibited after PL and PF treatment, compared with the Aβ_{25-35}-treated control group (Fig. 4). In particular, treatment of PF (5 and 10 μg/mL) significantly down-regulated the protein levels of CTFβ. Moreover, treatment of 10 μg/mL PF showed remarkable decrease in PS-1 protein expression compared with control group. These findings suggest that the inhibition of the amyloidogenic pathway in response to PF may provide the protective effect against Aβ neurotoxicity.

Results and Discussion

The Aβ peptide is a 38- to 48-amino acid residue peptide generated from APP (695- to 770-amino acids) cleaved by β-secretase and γ-secretase [29]. Overproduction of toxic Aβ leads to synaptic dysfunction and neuronal loss in the brain [30,31]. Several pieces of evidence demonstrated that accumulation of Aβ in the brain took an important role in oxidative stress causing the neuronal cell death [32]. Accumulation of oxidative stress in the brain of AD patients results in protein oxidation, lipid peroxidation, and free radical formation, which lead to cognitive impairment with memory and language dysfunction [7]. Here, we investigated protective effects of PL and PF against Aβ_{25-35}-induced neuronal apoptosis in SH-SY5Y cells.

A water/ethanol extract of PL is known as total glucosides of
**Fig. 4** Effects of PL and PF on the levels of BACE (A), CTF-β (B), PS-1 (C), and PS-2 (D) protein expression in Aβ\textsubscript{25-35}-treated SH-SY5Y cells. Values are mean ± SD; Means with the different letters are significantly different (p < 0.05) by Duncan’s multiple range test. β-actin was used as a loading control. ‘Normal’ group represents the non-treated cells, ‘Control’ group represents the Aβ\textsubscript{25-35}-treated cells, ‘PL10’, ‘PL50’, or ‘PL100’ groups represent the three concentrations of *Paeonia lactiflora* treatment (10, 50, 100 μg/mL) in Aβ\textsubscript{25-35}-treated cells. ‘PF1’, ‘PF5’, or ‘PF10’ groups represent the three concentrations of paeoniflorin treatment (1, 5, 10 μg/mL) in Aβ\textsubscript{25-35}-treated cells.
peony (TGP), which contains more than 15 components, including PF, albiflorin, and benzoxylpeacontiflorin, etc. Among them, PF is most abundant compound and accounts for over 90% of TGP, thus concentration of PF is used for standardization of dose of PL [33]. The content of PF in PL is reported to possess ranging from 0.16 to 6.91% [34,35]. Based on these studies, we decided to use the concentration range 10-100 μg/mL of PL and 1-10 μg/mL of PF, respectively.

SH-SY5Y cell is considered as a widely used model system in neuronal research, hence its high sensitivity to environmental stimulation and importance for functional biomaterial [36,37]. In the SH-SY5Y cells, the treatment of Aβ25-35 group showed neuronal cell loss, whereas PL- and PF-treated groups significantly elevated the neuronal cell viability. This protective effect of PL and PF was also distinguished by LDH assay. LDH could be released when cell membrane permeability was increased in damaged cell. In addition, a study reported that the Aβ25-35 peptide was related to increase of LDH release [38]. As shown by our results, there is decreased LDH release at the treatment of PL and PF in Aβ25-35-treated SH-SY5Y cells. Furthermore, we also investigated ROS production. From our data, the treatment with Aβ25-35 in SH-SY5Y cells significantly increased ROS production compared with normal group, whereas the treatment of PL and PF significantly inhibited in ROS production. Butterfield et al. [39] demonstrated that neurotoxic Aβ25-35 is mediated an oxidative stress in neuronal cells, and these toxic fibrils can be attenuated by antioxidants and free radical scavengers [40]. PL has been used to treat dementia [41] and was known for neuroprotective effect of PL and PF is reported ranging from 0.16 to 6.91% [34,35]. Based on these studies, we decided to use the concentration range 10-100 μg/mL of PL and 1-10 μg/mL of PF, respectively.

To maintain the homeostasis in the body, APP is divided into two different pathways, named amyloidogenic pathway and non-amyloidogenic pathway. The APP clearance (non-amyloidogenic pathway) started with sequential degrade of full-length APP, is cleaved by α-secretase and generates non-toxic sAPPα and CTFα [46]. In contrast, Aβ accumulation (amyloidogenic pathway) is occurred with cleavage of the large molecular APP by β- and γ-secretase, sequentially. A major β-secretase in the brain, BACE, produces sAPPβ and CTFβ [47]. Next, γ-secretase, which consisting of four components that is PS-1, PS-2, nicastrin, and anterior pharynx-defective-1, produces Aβ and amino-terminal APP intracellular domain from CTFβ [48,49]. Toxic Aβ oligomers and fibrils generated through the APP processing lead to the overproduction of ROS and NO. As a result, these oxidative stress and neurotoxicity can damage to DNA, leading to neuronal cell loss [50]. Thus, regulating of these APP processing may be promising interventions in treatment of AD. In this regard, the inhibitory effect of PL and PF on amyloidogenic pathway was analyzed by Western blotting. Our results determined that PL and PF inactivate the amyloidogenic pathway by down-regulation of BACE, CTFβ, PS-1, and PS-2 protein expressions. Especially, PF strongly decreased in levels of CTFβ and PS-1 protein. CTFβ is further cleaved by γ-secretase to generate Aβ, and the overproduction of CTFβ took a crucial role in AD [51,52]. Additionally, several researchers reported that increasing levels of CTFβ can induce AD-like endosome dysfunction in various cell lines and mouse cortical neurons [53,54]. PS proteins are famous for the key enzymes in central hypothesis for the cause of AD due to its constituting the active site in γ-secretase [55]. Plus, PS-1 is involved in APP processing in neuronal cultures derived from PS-1-deficient mouse embryos, and this protein appears to facilitate a proteolytic activity that cleaves the integral membrane domain of APP [56]. Borchelt et al. [57] demonstrated that one pathogenic mechanism by which PS-1 causes AD is to accelerate the rate of Aβ deposition in the brain of transgenic mice. Also, they proved that lack of PS-1 showed a marked (70-80%) decrease of Aβ and their precursor protein, CTFβ. In our results, PL and PF, especially PF, significantly diminished the PS-1 protein as much as normal cells, and decreased CTFβ production. Therefore, our results suggest that protective effect of PL and PF on cell viability, LDH, and ROS production was correlated with regulation of amyloidogenic signaling pathway, particularly, PF inactivates the γ-secretase by downregulating the PS-1 protein and thereby decreasing the production of CTFβ. In summary, our results showed that PL- and PF-treated SH-SY5Y neuronal cells increase in cell viability, and decrease in LDH release as well as ROS production. In addition, PL and PF significantly down-regulated BACE, CTFβ, PS-1, and -2 protein expressions. Especially, the treatment of PF showed tendency of decrease of CTFβ production by inhibition of BACE. Moreover, PF inhibited PS-1 protein expression, which constitutes the γ-secretase that initiates the final step of Aβ production. These present findings indicate protective effects of PL and PF on oxidative stress and amyloidogenic pathway against Aβ deposition in the brain of transgenic mice. Also, they proved that lack of PS-1 showed a marked (70-80%) decrease of Aβ and their precursor protein, CTFβ. In conclusion, the results indicate that PF, an active compound of PL, may contribute to prevention and treatment of neurodegenerative diseases, especially AD.

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