Alzheimer’s disease (AD) is a progressive neurodegenerative disease afflicting many people worldwide. Although the symptoms of AD can be ameliorated by the licensed treatments, there is a pressing need to develop disease-modifying treatments (1). So far, the pathogenesis of AD is still unclear, but it is approved that beta-amyloid (Aβ) is one of the main culprits due to the formation of senile plaques (2, 3). The neurotoxic Aβ is generated from the amyloid precursor protein (APP), which is a transmembrane glycoprotein in neurons and cleaved by secretases (4). At the early stage of AD, the generation of Aβ is increased in APP metabolism and the clearance is failed (5). The excessive monomeric Aβ will aggregate into soluble oligomers spontaneously and fibrils successively, and then deposit in senile plaques (6, 7). Meanwhile, the Aβ oligomers can induce oxidative stress through the formation of reactive oxygen species (ROS) and result in the oxidative damage for the lipid peroxidation and protein oxidation (8). As the main source of ROS, mitochondria serve as a pivotal role in the progress of AD and mediate the neuronal apoptosis through the intrinsic pathway (9, 10). In the discovery of effective therapy for the prevention of AD, natural products play an important part such as galantamine, huperzine A, piperine (11), ginsenoside Rd (12), vanillic acid (13) and cedrin (14). Esculetin (Fig. 1) is a coumarin found in many medicinal plants such as Perilla frutescens Britton var. crispa form viridis (15), Polygonum perfoliatum (16) and Artemisia capillaris (17). Previous pharmacological studies have shown there are many beneficial effects of esculetin such as antitumor (18, 19), hepatoprotection (20), aldose reductase inhibition (21), anti-anxiety and anti-depression (22), anti-inflammation (23) and so on. In our program to search bioactive natural compounds for the prevention of AD, we are interested in esculetin for its antioxidative activity and have carried out a series of investigations using SH-SY5Y cells to evaluate the protective effects in vitro and elucidate the associated mechanisms. At present, we report these findings herein.

ESCULETIN ATTENUATES NEUROTOXICITY INDUCED BY Aβ25-35 IN SH-SY5Y CELLS VIA INHIBITING OXIDATIVE STRESS AND MITOCHONDRIA-MEDIATED APOPTOSIS

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Abstract: Alzheimer’s disease (AD) is a neurodegenerative disease afflicting many people worldwide. As the specific biomarker, beta-amyloid (Aβ) is considered to serve as a central role in the progress of AD. To discover effective therapy for AD, the protective effects of esculetin on SH-SY5Y cells against the neurotoxicity induced by Aβ25-35 were evaluated. As a result, esculetin can improve the viability of SH-SY5Y cells injured by Aβ25-35 (58.0%, 66.1% and 82.1% at 0.1, 1 and 10 µM vs 49.5% at 0 µM). Further investigations have demonstrated the protective effects of esculetin at different concentrations of 0.1, 1 and 10 µM are associated with its inhibition of oxidative stress and apoptosis, which is more observable at both 1 and 10 µM. These observations can give evidences for the following investigation in vivo and discovery of novel preventive method for AD.

Keywords: esculetin, Alzheimer’s disease, SH-SY5Y cells, oxidative stress, apoptosis

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MATERIALS AND METHODS

Chemicals and reagents

Esculetin was purchased from Xi’an Lisen Biotechnology Co. Ltd (Xi’an China), and the purity is more than 95% analyzed by HPLC. Huperzine A was obtained from Dalian Meilun Biotech Co., Ltd (Dalian, China) and its purity was also higher than 95% by HPLC analysis. Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum were supplied by Invitrogen Gibco Co. (Grand Island, NY, USA). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), beta-amyloid 25-35 (Aβ25-35), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Reactive oxygen species (ROS) assay kit, lactic dehydrogenase (LDH) activity assay kit, superoxide dismutase (SOD) activity assay kit, mitochondrial membrane potential assay kit, malondialdehyde (MDA) assay kit, catalase (CAT) activity assay kit, bicinchoninic acid (BCA) protein concentration assay kit, Caspase-3 activity assay kit were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing China). Bcl-2, Bax, cytochrome c and β-actin antibodies, secondary antibody conjugated to horseradish peroxidase and enzyme-link chemiluminescence (ECL) detection kit were purchased from Beyotime Biotechnology Institute (Nantong, China).

Preparation of Aβ25-35

Aβ25-35 was employed instead of the most toxic peptide fragment Aβ1-42 for their similar pattern to induce neural injury (24). Aβ25-35 was dissolved in sterile deionized distilled water at room temperature to obtain a 1 mM stock solution. The Aβ25-35 solution was incubated at 37°C for 7 days to form the aggregation of Aβ25-35 and then stored at -20°C. The stock solution was diluted to the desired concentrations with the culture medium immediately before use.

Cells culture and treatment

The human neuroblastoma SH-SY5Y cells were obtained from the cell bank of Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM with 10% fetal bovine serum in a humid atmosphere containing 5% CO2 and 95% air at 37°C. After incubation for 48 h, cells were incubated with certain esculetin (0, 0.1, 1 and 10 µM) for 3 h and then exposed to the aggregated Aβ25-35 (50 µM) for additional 24 h.

Cell viability

To determine the cell viability, MTT method was used. SH-SY5Y cells were cultured in 96-well microplates and adjusted to 1 x 10^4 per well. After treatments and specific incubation, 10 µL MTT solution (5 mg/mL sterile stock solution) was added to each well and the incubation was carried out for 4 h at 37°C in a humidified atmosphere of 5% CO2. Then the culture medium was removed and 100 µL DMSO was added to dissolve the formazan crystals. After 30 min, the optical density (OD) of the wells was read at 570 nm in a microplate reader (Molecular Devices, CA, USA). Cell viability was expressed as a relative percentage of OD values compared with the control group. In addition, to evaluate the protective effects of esculetin, 10 µM huperzine A was chosen as the positive control.

Release of intracellular LDH

As a marker of cell degeneration, the activity of extracellular LDH was measured using LDH activity assay kit according to the manufacturer’s instructions. The SH-SY5Y cells were plated at a density of 1 x 10^4 per well and treated as above. The cells were precipitated by centrifugation at 500 x g for 5 min at room temperature and the supernatant was collected to measure the activity of released LDH. The OD values were detected on a microplate reader at 440 nm. The activity of released LDH was expressed as U/L from the OD values.

Activity of SOD

Xanthine oxidase method was used to determine the activity of SOD by the SOD activity assay kit. The cells were treated as above and washed with PBS. Then the cells were lysed on ice and the lysates were centrifuged at 12000 x g for 5 min. The supernatant was collected and the reagents were added into 20 µL supernatant. After incubation for 30 min at 37°C, the OD values were obtained from a microplate reader at 560 nm. The activity of SOD was derived from the OD values against the standard.

Activity of CAT

The CAT activity was estimated using the CAT activity assay kit according to the manufacturer’s instructions. After treated as above, 50 µL supernatant was collected as a sample and diluted in 1.95 mL PBS. Then 1 mL hydrogen peroxide (10%) was added into the mixture. The CAT activity was then quantified from the OD values at 240 nm versus the standard.

Content of MDA

The content of MDA was monitored by the MDA assay kit following the manufacturer’s
instructions. After treatment as above, ice-cold PBS was added into the culture medium and the cells were homogenized. Then the homogenate was centrifuged at 1600 × g for 10 min. The supernatant (100 µL) was sucked and mixed with the reagent (thiobarbituric acid). Then the sample was boiled for 40 min and subsequently cooled to room temperature. The OD value was recorded on a microplate reader at 532 nm and the concentration of MDA was calculated from the standard of the assay kit.

Generation of intracellular ROS

The intracellular ROS was derived from the fluorescence intensity of DCFH-DA according to the manufacturer’s protocol. In brief, after treatment as above, the culture medium was removed and the cells were rinsed with PBS. Then the DCFH-DA in DMEM was added into the microplates and incubated at 37°C for 30 min. The cells were washed with PBS again and the fluorescence intensity was recorded on the microplate reader at excitation wavelength of 485 nm and emission wavelength of 520 nm. The level of ROS was expressed as the percentage of the fluorescence intensity against the control group.

Mitochondrial membrane potential (∆∆ψm)

Mitochondrial membrane potential (∆∆ψm) was also determined through the fluorescence intensity of rhodamine-123. Rhodamine-123 can accumulate in mitochondria of normal cells. When the ∆∆ψm decreases in apoptotic cells, it will be released into the cytosol and emit fluorescence. Following the treatment, rhodamine-123 (2 mM) was added into the culture medium and incubated for 15 min. Then the fluorescence intensity was recorded at an excitation wavelength of 488 nm and an emission wavelength of 530 nm. ∆∆ψm was expressed as the percentage of fluorescence intensity compared to control group.

Caspase-3 activity

The activity of Caspase-3 was determined using the Caspase-3 activity assay kit through colorimetric method. The SH-SY5Y cells were treated as above and washed with PBS. Then the cells were lysed on ice for 15 min and centrifuged at 16000 × g and 4°C for 10 min. The supernatant was incubated with substrate (Ac-DEVD-pNA) at 37°C for 2 h. The OD values were measured on a microplate reader at 405 nm.

Expression of cytochrome c, Bcl-2 and Bax

Western blot analysis was employed to analyze the expression of cytochrome c, Bcl-2, and Bax. The pretreated SH-SY5Y cells were lysed with lysis buffer on ice for 30 min. Then the lysate was centrifuged at 12000 × g and 4°C for 15 min, and the

Figure 2. Effects of esculetin on the SH-SY5Y cell viability injured by Aβ25-35. ###p < 0.001 vs control group; *p < 0.05 and ***p < 0.001 vs 0 µM group
supernatant was sucked for the analysis of cytochrome c, Bcl-2, and Bax. Total protein in the supernatant was determined by a BCA assay kit. These samples were separated by electrophoresis on 15% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and transferred to PVDF membranes. After blocked with nonfat milk, the membranes were incubated overnight with primary antibodies of cytochrome c, Bcl-2, Bax, and β-actin at 4°C. The membranes were tested with secondary antibody conjugated to horseradish peroxidase at room temperature for 1 h and detected by enzyme-link chemiluminescence detection kit. β-actin was used as the internal control.

Figure 3. Effects of esculetin on the release of intracellular LDH on SH-SY5Y cells injured by Aβ25-35. ****p < 0.001 vs control group; *p < 0.05 and ***p < 0.001 vs 0 µM group

Figure 4. Effects of esculetin on the activity of SOD in SH-SY5Y cells injured by Aβ25-35. ****p < 0.001 vs control group; *p < 0.05 and ***p < 0.001 vs 0 µM group
Statistical analysis

The results were expressed as the means ± standard deviation and analyzed by GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Comparisons among different groups were implemented by one way analysis of variance (one way ANOVA) followed by Newman-Keuls test for multiple comparisons. And p < 0.05 was considered as statistical significance.

RESULTS

Esculetin attenuates the survival of SH-SY5Y cells injured by Aβ25-35

As shown in Figure 2, compared with the control group, the cell viability was decreased after the treatment of Aβ25-35. In the presence of esculetin, the decreased cell viability induced by Aβ25-35 was attenuated significantly. Similarly, huperzine A could also

Figure 5. Effects of esculetin on the activity of CAT in SH-SY5Y cells injured by Aβ25-35. ***p < 0.001 vs control group; *p < 0.05 and ###p < 0.001 vs 0 µM group

Figure 6. Effects of esculetin on the MDA content in SH-SY5Y cells injured by Aβ25-35. ###p < 0.001 vs control group; **p < 0.01 and ###p < 0.001 vs 0 µM group
improve the viability of SH-SY5Y cells injured by Aβ_{25-35} and there was no difference between 10 µM esculetin and huperzine A in statistics. Meanwhile, Aβ_{25-35} can result in the release of intracellular LDH (Fig. 3) according to the increased activity of LDH in a culture medium. But pretreated with esculetin, the extracellular LDH activity was inhibited apparently, which indicates esculetin can block the release of intracellular LDH. These results demonstrate the protective effects of esculetin against the neurotoxicity induced by Aβ_{25-35} in SH-SY5Y cells.

Esculetin improves the status of oxidative stress in SH-SY5Y cells injured by Aβ_{25-35}

As the major factor of Aβ-induced toxicity, oxidative stress in SH-SY5Y cells was further inves-

![Image](image1.png)

**Figure 7.** Effects of esculetin on the production of ROS in SH-SY5Y cells injured by Aβ_{25-35}. ###p < 0.001 vs control group; **p < 0.01 and ***p < 0.001 vs 0 µM group

![Image](image2.png)

**Figure 8.** Effects of esculetin on the mitochondrial membrane potential in SH-SY5Y cells injured by Aβ_{25-35}. *p < 0.05 and ***p < 0.001 vs 0 µM group
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As a result, Aβ25-35 can reduce the activities of two anti-oxidative enzymes, SOD (Fig. 4) and CAT (Fig. 5) in SH-SY5Y cells. With different concentrations of esculetin, the activities of both SOD and CAT were obviously elevated. Accordingly, the production of MDA was increased as the product of lipid peroxidation under the induction of Aβ25-35 (Fig. 6). Esculetin can inhibit the production of MDA significantly at 0.1, 1 and 10 µM. Since the excessive accumulation of ROS directly leads to lipid peroxidation, the levels of intracellular ROS were evaluated. As shown in Figure 7, the generation of ROS was increased in the presence of Aβ25-35. On the contrary, the excessive generation of ROS was ameliorated by esculetin. In addition, as the main site of ROS generation, mitochondria are susceptible to excessive ROS, which could give rise to depolarization of ∆∆ψm. In this investigation, the ∆∆ψm in SH-SY5Y cells declined sharply with the treatment of Aβ25-35 (Fig. 8). However, the reduced...
Δψm was reversed by esculetin. In general, these results revealed esculetin can improve the poor status of oxidative stress induced by Aβ25-35 in SH-SY5Y cells.

**Esculetin inhibits apoptosis of SH-SY5Y cells injured by Aβ25-35.**

Apoptosis is the final fate of neurons injured by Aβ. In the presence of Aβ25-35, the activity of Caspase-3 in SH-SY5Y cells was increased obviously (Fig. 9). Accordingly, western blot analysis revealed the expression of cytochrome c and Bax was up-regulated. Whereas, the expression of Bcl-2 was down-regulated (Fig. 10). These results indicated apoptosis occurred in SH-SY5Y cells. When different concentrations of esculetin appeared in the culture medium of SH-SY5Y cells, it was observed that the apoptosis was reversed. The increased activity of Caspase-3 was inhibited in different extents. At the same time, the up-regulation of cytochrome c and Bax, as well as down-regulation of Bcl-2, was attenuated. These observations implied esculetin could inhibit apoptosis of SH-SY5Y cells induced by Aβ25-35.

**DISCUSSION AND CONCLUSION**

AD is a major factor of dementia inflicted on people all over the world. As the specific biomarker of AD, Aβ is the direct cause of AD (25). Aβ derived from APP can exert neurotoxicity on neurons through oxidative damage and apoptosis (26, 27). Herein, we have evaluated the protective effects of esculetin on SH-SY5Y cells against neurotoxicity induced by Aβ25-35. The results showed esculetin could protect SH-SY5Y cells injured by Aβ25-35 from MTT assay and the release of intracellular LDH. Further studies have revealed the protective effects of esculetin were associated with inhibiting oxidative stress and apoptosis.

In the progress of AD, accumulation of Aβ oligomers can change the cellular redox status and bring about apoptotic cell death mediated by oxidative stress (28). Aβ can promote the overproduction of ROS derived from diverse cellular sources including enzymatic reactions, mitochondrial deterioration, and imbalance in redox transition metal ions (28). The excessive ROS accumulates and causes functional and structural changes in critical macromolecules leading to lipid peroxidation, protein oxidation, and DNA cleavage (8). As the end product of lipid peroxidation, the content of MDA represents the extent of oxidative stress and is closely related to the oxidative damage. SOD and CAT are important intrinsic antioxidants against free radicals and following lipid peroxidation, and they can scavenge excessive ROS (29). The production of ROS is in mitochondria and deleterious to mitochondria, which can lead to the depolarization of mitochondrial membrane potential (30). In this investigation, the activities of SOD and CAT in SH-SY5Y cells were inhibited by Aβ25-35 and reversed with the treatment of esculetin. Meanwhile, the excessive production of MDA and ROS was reduced by esculetin and the depolarization of mitochondrial membrane potential was relieved.

In the presence of excessive Aβ, neurons will undergo apoptosis finally without any prevention (31). As the effector enzyme, Caspase-3 directly affords the morphological changes in apoptosis (32). Cytochrome c releases from mitochondria in apoptotic cells and is involved in the formation of apoptosomes, which can activate downstream Caspase-3 (33). Bcl-2 and Bax are important members of Bcl-2 protein family regulating apoptosis. The former is anti-apoptotic and the latter is pro-apoptotic. The release of cytochrome c is regulated by Bcl-2 and Bax (34). In our investigation, the activity of Caspase-3 elevated by Aβ25-35 in SH-SY5Y cells was inhibited by esculetin. And the up-regulated expression of cytochrome c and Bax was down-regulated. By contrast, the down-regulation of Bcl-2 was up-regulated significantly.

Lipophilicity/hydrophilicity is an important factor affecting the ADME properties of drugs in vivo. And it is commonly expressed as logP, which usually falls into the range from 2 to 5 for the drugs easily delivered to the binding sites (35). Polar surface area (PSA) is a parameter to indicate the ability for a compound to permeate cells and the PSA of a compound with the good permeability to the cellular membrane is less than 140 angstroms^2 (36). The logP and PSA values of esculetin are 1.05 and 66.76 in theoretical calculation by ChemDraw, which implied its poor drug-like properties. But Sudhakar and Elizabeth have reported esculetin can cross the blood-brain barrier to exert the neuroprotective effects against MPTP-induced neurotoxicity in a mouse model of Parkinson’s disease (37). Hence, the protective effects of esculetin against neurotoxicity induced by Aβ in vivo should be further investigated in the future.

In conclusion, this investigation reveals esculetin can protect SH-SY5Y cells against the neurotoxicity induced by Aβ25-35 in vitro, and the mechanisms are closely associated with its inhibition of oxidative stress and apoptosis. These findings can give evidences for the discovery of new chemical molecules applied in the prevention of AD and further investigation in vivo.
REFERENCES


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