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Research Report

Acteoside protects human neuroblastoma SH-SY5Y cells against β-amyloid-induced cell injury

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ABSTRACT

Amyloid β-peptide (Aβ) has been implicated in the pathogenesis of AD. It can cause cell death in AD by evoking a cascade of oxidative damage to neurons. So antioxidant compounds may throw a light on the treatment of AD. In the present study, we investigated the protective effect of acteoside (AS), an antioxidative phenylethanoid glycoside, on $A\beta_{25-35}$-induced SH-SY5Y cell injury. Exposure of cells to 25 μM $A\beta_{25-35}$ for 24 h caused viability loss, apoptotic increase and reactive oxygen species (ROS) increase, pre-treatment with acteoside for 1.5 h significantly reduced the viability loss, apoptotic rate and attenuated Aβ-mediated ROS production. In addition, AS strikingly inhibited $A\beta_{25-35}$-induced mitochondrial dysfunctions, including lowered membrane potential, increased Bax/Bcl-2 ratio, cytochrome c release and the cleavage of caspase-3. Taken together, these results indicated that acteoside could protect SH-SY5Y cells against β-amyloid-induced cell injury by the attenuating ROS production and the modulating apoptotic signal pathway through Bcl-2 family, cytochrome c, and caspase-3.

1. Introduction

Alzheimer's disease (AD) is neuropathologically characterized by deposition of β-amyloid (Aβ) plaques and intracellular neurofibrillary tangles and loss of neurons in the brain. Although the cause of AD remains unclear, several lines of evidence suggest that Aβ-induced oxidative stress plays an important role in the pathogenesis or progression of AD (Butterfield et al., 2001). Aβ induces oxidative stress (OS) (Hensley et al., 1994), and OS promotes the production of Aβ (Tamagno et al., 2008). Recent studies showed that OS contributes to Aβ accumulation. Aβ, in turn, induces OS and HNE production resulting in increased levels of β- and γ-secretases, which further enhances Aβ production (Shen et al., 2008; Tamagno et al., 2008), thereby providing a vicious circle among Aβ production/accumulation, OS and β-/γ-secretase for the cleavage of APP. Thus, one promising preventive or therapeutic intervention in AD may attenuate or suppress the oxidative stress-dependent, Aβ-mediated cytotoxicity. Therefore, numerous antioxidants, such as huperzine A (Xiao et al., 2002), melatonin (Feng and Zhang, 2004), curcumin (Park et al., 2008), ginkgo biloba (Yao et al., 2001) and Ginsenoside Rg1 (Wei et al., 2008) have been demonstrated to inhibit Aβ-induced neurotoxicity.

Acteoside,2-(3,4-Dihydroxyphenyl)ethyl-O-R-L-rhamnopyranosyl-(1→3)-4-O-caffeoyl-β-D-glucopyranoside, was a phenylethanoid glycoside first extracted from *Verbascum sinuatum* and named "verbascoside". The chemical structure of this com-

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pound was elucidated by Birkofer, who also introduced the new name "acteoside" (Birkofer et al., 1968). Previous studies have shown that acteoside has various pharmacological activities, such as anti-inflammatory (Díaz et al., 2004), hepatoprotective (Xiong et al., 1999), anti-apoptotic (Pu et al., 2003) and antioxidative activities (Chiou et al., 2004).

Recently, it has been suggested that acteoside has neuroprotective activities. Acteoside inhibits neuronal death induced by 1-methyl-4-phenylpyridinium ion (MPP^+) (Li et al., 2008; Sheng et al., 2002) and glutamate (Koo et al., 2006). Although acteoside exhibited neuroprotective activity, the protective effect of acteoside against Aβ-induced cytotoxicity has not been reported. In the present study, we investigated the effects of acteoside on Aβ-induced neurotoxicity in SH-SY5Y cells. Our study verified that acteoside exerts neuroprotective effects against Aβ-induced cell injury.

2. Results

2.1. *Acteoside ameliorates aged $A\beta_{25-35}$-induced cell injury*

MTT assay was used to test the toxicity of acteoside to SH-SY5Y cells. As shown in Fig. 1A, acteoside at each of these concentrations (10–40 μg/ml) alone did not cause any apparent cytotoxicity. SH-SY5Y cells were incubated with 25 μM $A\beta_{25-35}$ for 24 h with/without different concentrations of acteoside (10–30 μg/ml). The $A\beta_{25-35}$-induced cytotoxicity was first evaluated by the MTT reduction assay. As shown in Fig. 1B, $A\beta_{25-35}$ significantly decreased the cell viability. However, the cytotoxic effects were attenuated by the pre-treatment with acteoside, 20 and 30 μg/ml acteoside significantly blocked

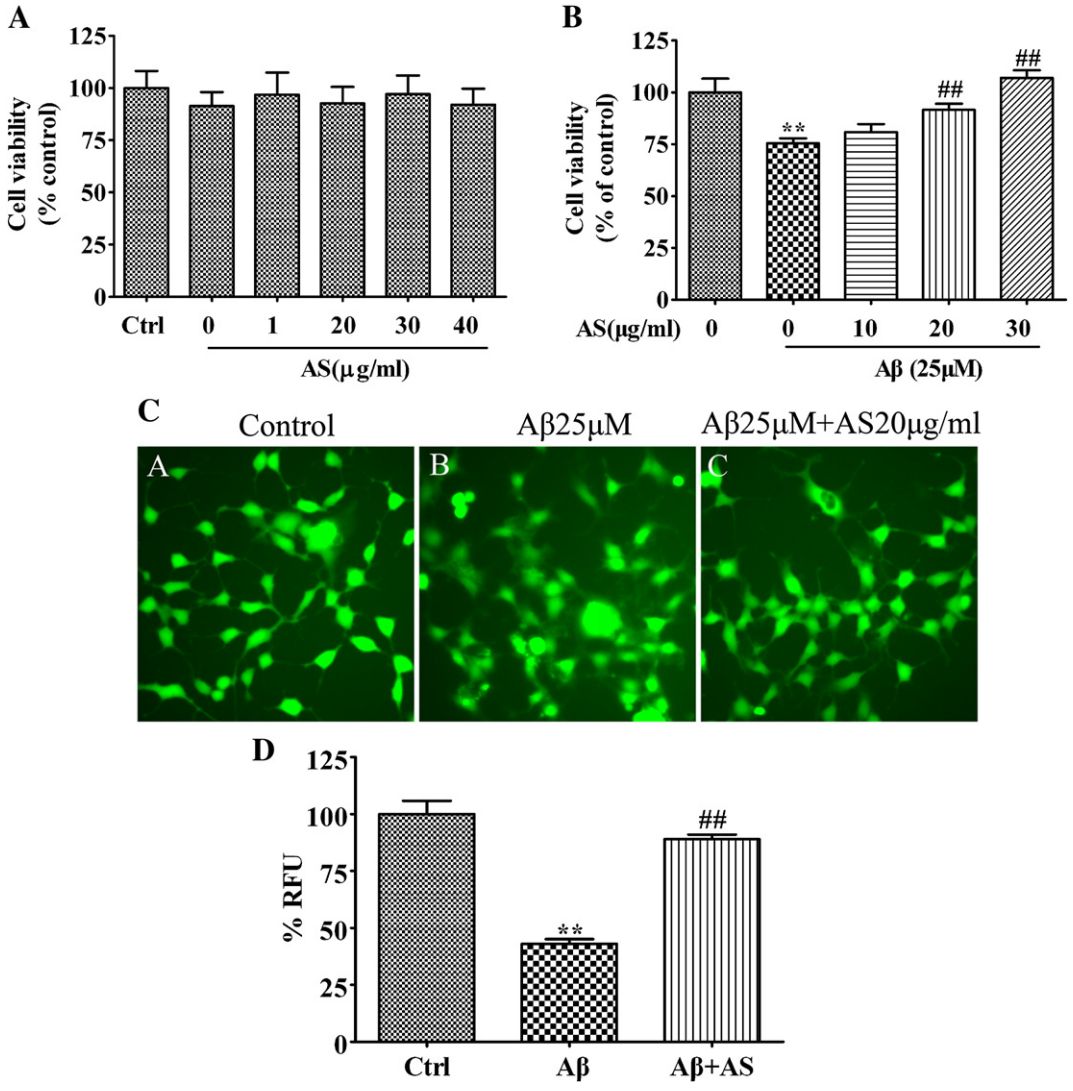

Fig. 1 – Acteoside (AS) ameliorated aged $A\beta_{25-35}$-induced cell injury. SH-SY5Y cells exposed to acteoside in the absence (A) or presence (B) of $A\beta_{25-35}$ in vitro. Cells were incubated without or with acteoside (10, 20, 30 μg/ml) for 1.5 h, followed by incubation with $A\beta_{25-35}$ for another 24 h. After this incubation, cell viability was determined with the MTT assay. (C) After 24 h of $A\beta_{25-35}$ treatment with or without acteoside, calcein stained cells were observed under a fluorescence microscope. (D) The fluorescence intensity of normal control cells was calculated as 100%, and all data were normalized to control cells and shown as mean ± S.E.M from three independent experiments. $^{**}P<0.01$ vs. control; $^{\#\#}P<0.01$ vs. $A\beta_{25-35}$-treated alone.

cytotoxic effects of $A\beta_{25-35}$ on cell viability. The protective effect of acteoside on $A\beta_{25-35}$-induced neurotoxicity was also analyzed by calcein-AM assay. Continuous 24 h exposure to $A\beta_{25-35}$ decreased the fluorescence intensity, while 20 μg/ml acteoside pretreatment significantly attenuated the effects of $A\beta_{25-35}$ (Fig. 1C). In addition, the effects were also visually confirmed under the fluorescence microscope. The number of cells exhibiting bright green fluorescence diminished after $A\beta_{25-35}$ treatment, and 20 μg/ml acteoside prevented the loss of fluorescence staining caused by $A\beta_{25-35}$ (Fig. 1D).

2.2. *Acteoside prevents $A\beta_{25-35}$-induced apoptosis*

Apoptotic nuclei are characterized by condensed nuclear and apoptotic bodies. Anti-apoptotic properties of acteoside were verified by quantitation of Hoechst stained apoptotic nuclei from three independent experimental sets. Hoechst staining showed increased number of apoptotic cells following $A\beta_{25-35}$ treatment for 24 h, which was few in the control culture (Fig. 2A). Pretreatment with 20 μg/ml acteoside decreased the number of apoptotic cells compared to cells treated with $A\beta_{25-35}$ alone (Fig. 2B). These results indicated that acteoside suppresses $A\beta_{25-35}$-induced DNA damage in SH-SY5Y cells. We also examined the effect of acteoside on $A\beta_{25-35}$-induced DNA fragmentation. The exposure of SH-SY5Y cells to $A\beta_{25-35}$ for 24 h produced an internucleosomal DNA fragmentation, as shown by DNA ladder. In contrast, pretreatment with 20 μg/ml acteoside strongly reduced the degree of DNA fragmentation (Fig. 2C).

2.3. *Acteoside prevented $A\beta_{25-35}$-induced oxidative injury*

To determine the generation of intracellular ROS induced by 25 μM $A\beta_{25-35}$ for 24 h, we performed fluorescence images and flow cytometry analysis using the ROS-sensitive fluorescence probe, DCF. The fluorescence images were obtained under confocal microscopy (Fig. 3A). Cytometry assay showed that the exposure to $A\beta_{25-35}$ caused an elevation of the intracellular ROS levels which was about 1.9-fold relative to that of control cells (Fig. 3B). Pretreatment with acteoside suppressed the intracellular ROS elevation. These results indicated that acteoside has the ability to scavenge ROS induced by $A\beta_{25-35}$.

2.4. *Acteoside improved mitochondrial membrane potential in SH-SY5Y cells*

Change in mitochondrial membrane potential ($\Delta\Psi m$) is a key indicator in programmed cell death. Levels of $\Delta\Psi m$ in SH-SY5Y cells were determined using flow cytometry with the fluorescence probe rhodamine 123. Cells exposed to 25 μM $A\beta_{25-35}$ for 24 h markedly decreased Rh123 staining, indicating a drop in $\Delta\Psi m$ which is related to mitochondrial dysfunction. Acteoside (20, 30 μg/ml) significantly improved $A\beta_{25-35}$-induced impairments in MMP (Fig. 4). These results suggested that acteoside attenuates $A\beta_{25-35}$-induced mitochondrial dysfunction, and such effects could play an important role in protecting SH-SY5Y cells against $A\beta_{25-35}$-induced cytotoxicity.

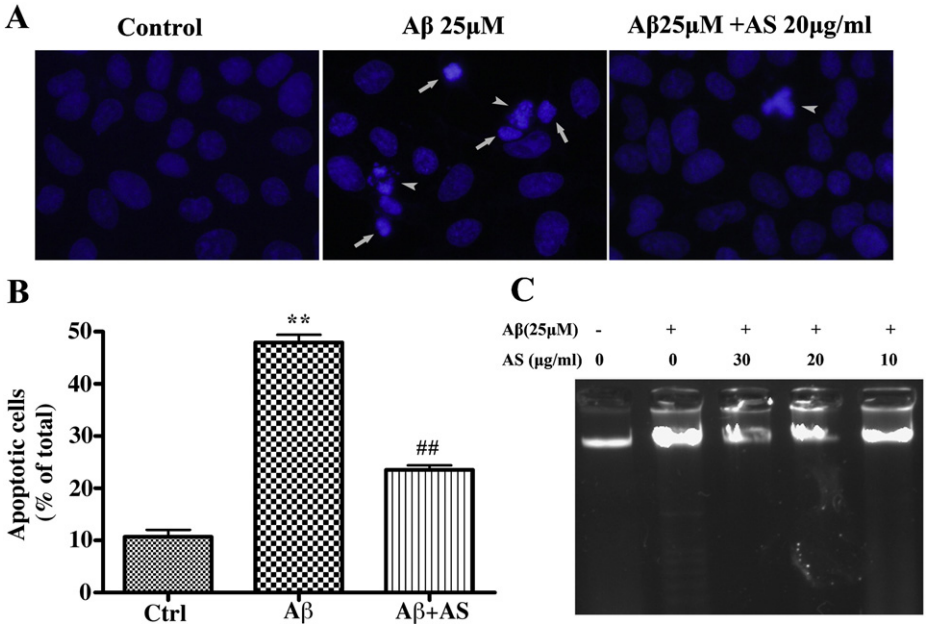

Fig. 2 – Acteoside (AS) prevents aged $A\beta_{25-35}$-induced apoptosis. (A) SH-SY5Y cells, with or without pretreated acteoside (AS) for 1.5 h, were exposed to $A\beta_{25-35}$ for 24 h, and then were subjected to Hoechst 33258 staining and viewed under a fluorescence microscope (magnification, ×400). Arrows indicated condensed nuclei and arrowheads indicated fragmented nuclei. (B) Quantification of abnormal nuclei after exposure to $A\beta_{25-35}$ in the presence or absence of acteoside. Results are taken from three independent experiments. (C) Acteoside suppressed $A\beta_{25-35}$-induced DNA fragmentation. SH-SY5Y cells were exposed to $A\beta_{25-35}$ (25 μM) for 24 h in the presence or absence of acteoside (10, 20, 30 μg/ml). After the treatment, the DNA was obtained from the cells and analyzed on 1.5% agarose gel. The results are representative of three independent experiments. Data are mean ± S.E.M. $^{**}P<0.01$, vs. control; $^{\#\#}P<0.01$, vs. $A\beta_{25-35}$-treated alone.

2.5. *Effect of acteoside on the expression of proapoptotic proteins in Aβ$_{25–35}$-induced SH-SY5Y cells*

Bcl-2 family members are major regulators of mitochondrial integrity and mitochondria-initiated cytochrome c release and caspase activation. The Bcl-2 family includes anti-apoptotic members such as Bcl-2, and proapoptotic members such as Bax. Bax is potent regulators of cytochrome c release from mitochondria under a variety of stress conditions. Bcl-2 prevents release of cytochrome c by heterodimerizing with Bax (Yang et al., 1997). The ratio of Bax to Bcl-2 has been reported to be correlated with apoptosis. Our results showed that treatment of cells with Aβ$_{25–35}$ induced a robust increase in the protein level of Bax, and a slight change in the protein level of Bcl-2, and there was an approximate 2.4-fold increase in the ratio of Bax/Bcl-2 expression in Aβ$_{25–35}$ treatment compared with the control using western blot analysis. While, acteoside pretreatment could prevent the Aβ$_{25–35}$-induced increase of the Bax/Bcl-2 ratio (Fig. 5). The effect of acteoside on Aβ$_{25–35}$ -induced apoptosis may be, at least in part, mediated by regulating of Bcl-2 and Bax expression. The ratio of Bax/Bcl-2 was down-regulated by acteoside, suggesting a mitochondria-dependent apoptosis-inhibiting effect of acteoside on Aβ$_{25–35}$ insult.

2.6. *Effect of acteoside on Aβ$_{25–35}$-induced cytochrome c release*

After the disruption of MMP, mitochondrial cytochrome c was released, which ultimately cleave pro-caspase-3 to form active caspase-3 (Bras et al., 2005). Next, we investigated the effect of acteoside on Aβ$_{25–35}$-induced cytochrome c release. As shown in Fig. 6, Aβ$_{25–35}$ significantly increased the release of cytochrome c from mitochondria to the cytosol, and acteoside (10, 20, 30 μg/ml) pretreatment inhibited the release of cytochrome c.

2.7. *Effect of acteoside on the expression level of caspase-3*

Caspase-3 has been shown to play a pivotal role in the execution phase of apoptosis induced by diverse stimuli. Caspase-3

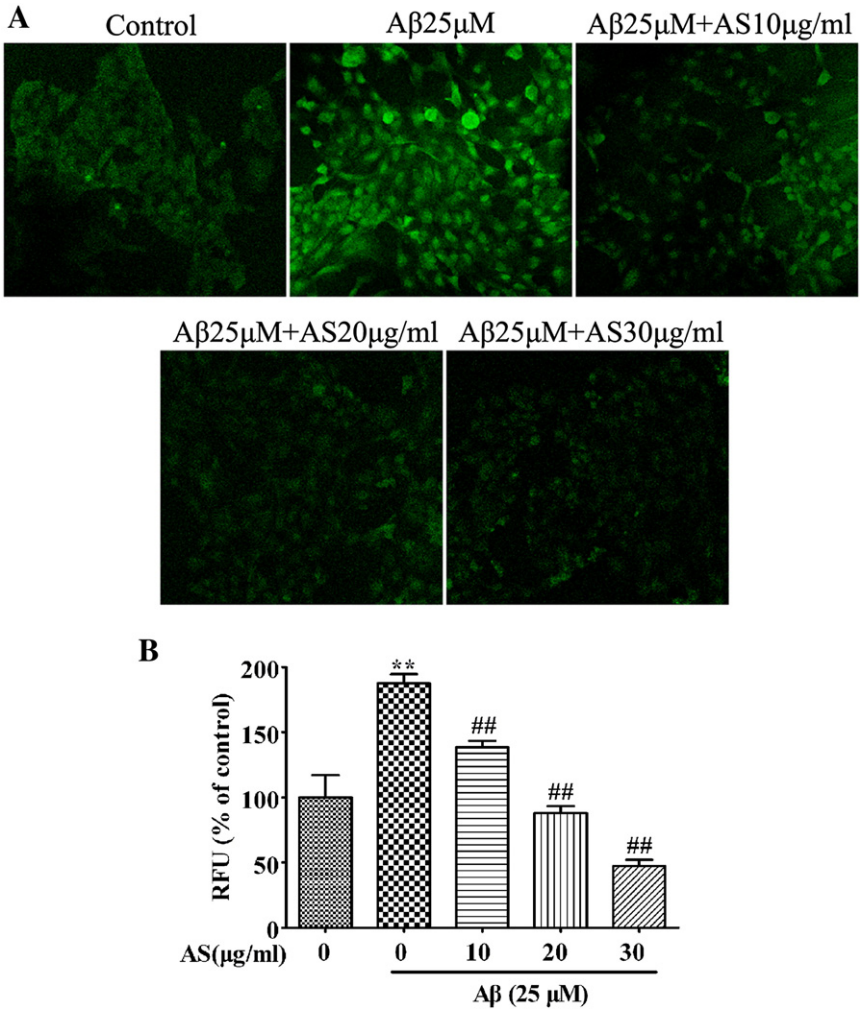

Fig. 3 – Acteoside (AS) prevented Aβ$_{25–35}$-induced oxidative stress. (A) ROS formation, evaluated by the oxidation of H_2DCF to DCF, was assessed 24 h after incubation with Aβ$_{25–35}$. AS was pretreated before Aβ$_{25–35}$ for 1.5 h. Intracellular ROS in 25 μM Aβ$_{25–35}$-treated cells with or without acteoside were imaged on confocal laser microscope. (B) Intracellular ROS were determined by flow cytometry. The amount of intracellular ROS is expressed relative to that in controls. $^{**}P<0.01$ vs. control; $^{\#\#}P<0.01$, vs. Aβ$_{25–35}$-treated alone.

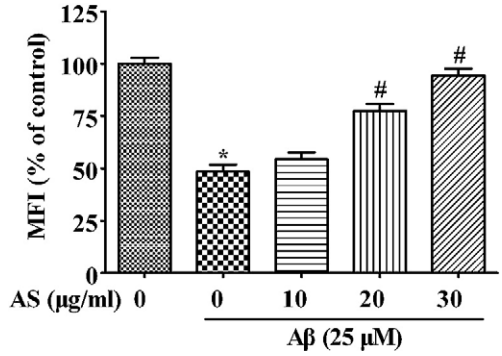

Fig. 4 – Effect of acteoside (AS) on loss of MMP induced by $A\beta_{25-35}$. MMP was monitored using the fluorescent dye Rh123. Mean relative fluorescent density (MFI) of Rh123 was calculated and normalized with that from control. Data were presented as mean ± S.E.M. of three independent experiments. $^{*}P<0.05$ vs. control; $^{\#}P<0.05$, vs. $A\beta_{25-35}$-treated alone.

activation led to DNA breakage, nuclear chromatin condensation and cell apoptosis. As shown in Fig. 7, the expression level of cleaved caspase-3 was increased after exposed to $A\beta_{25-35}$, more than 2.5-fold increase compared with control group, but the

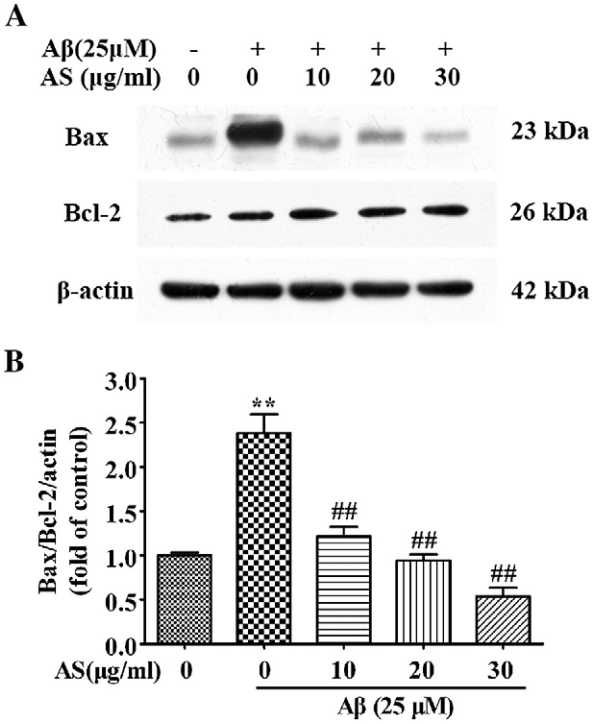

Fig. 5 – Effect of acteoside (AS) on the expression of Bcl-2 family proteins in $A\beta_{25-35}$-induced SH-SY5Y cells. The cells were pretreated with acteoside for 1.5 h prior to exposure to 25 μM Aβ for 24 h. (A) Assessment of Bcl-2 and Bax protein levels in SH-SY5Y cells by western blotting. (B) Effects of acteoside on the ratio of values of Bax/Bcl-2. Densitometric analysis is mean ± S.E.M. of three independent experiments. $^{**}P<0.01$ vs. control; $^{\#\#}P<0.01$, vs. $A\beta_{25-35}$-treated alone.

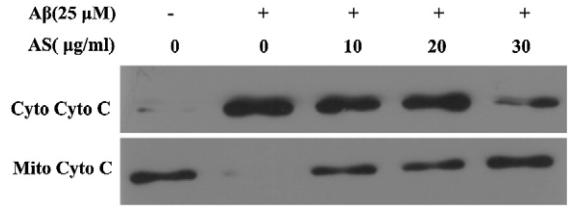

Fig. 6 – Acteoside (AS) inhibits $A\beta_{25-35}$-induced cytochrome c release in SH-SY5Y cultured cells. The cells were pretreated with acteoside for 1.5 h prior to exposure to 25 μM Aβ for 24 h. The amount of cytosolic cytochrome c (Cyto Cyto C) and mitochondrial cytochrome c (Mito Cyto C) were determined by western blot analyses.

activated caspase-3 protein level was declined in acteoside pretreatment groups.

3. Discussion

There is abundant evidence suggesting that Aβ contributes to the pathogenesis of AD. It is now well known that Aβ is neurotoxic to neuronal cells via an oxidative stress-dependant apoptotic process. The neurotoxicity of Aβ has been reported to be mediated with oxygen free radicals and attenuated by antioxidants and free radical scavengers (Xiao et al., 2002). The present study demonstrated that acteoside, which is an antioxidative phenylethanoid glycoside extracted from plants, significantly

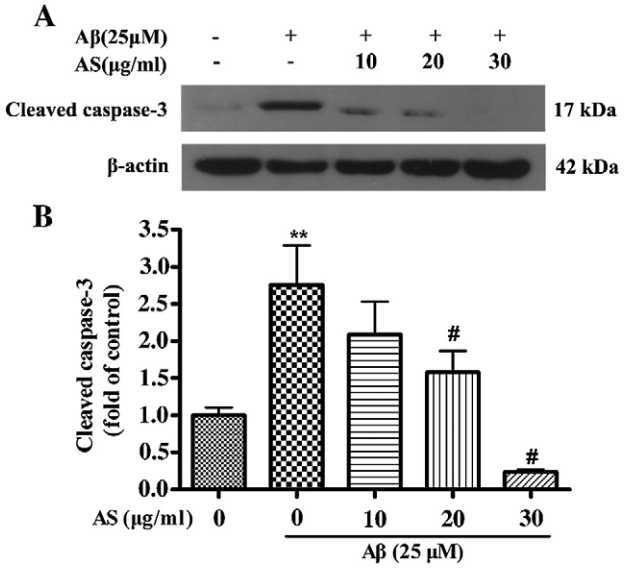

Fig. 7 – Acteoside(AS) inhibits $A\beta_{25-35}$-induced induction of cleaved caspase-3 protein levels in SH-SY5Y cultured cells. The cells were pretreated with acteoside for 1.5 h prior to exposure to 25 μM Aβ for 24 h. The amount of cleaved caspase-3 was determined using western blot analysis. (A) Representative image of immunoblots for cleaved caspase-3. (B) Densitometric analysis of changes in levels of cleaved caspase-3. Data are means ± SEM for three independent experiments. $^{**}P<0.01$ vs. control; $^{\#}P<0.05$, vs. $A\beta_{25-35}$-treated alone.

prevented SH-SY5Y cells from Aβ-induced neuronal cell injury dose-dependently and the neuroprotective effect of acteoside against $A\beta_{25-35}$-induced neuronal cell injury may be through antioxidant and anti-apoptotic mechanisms. Acteoside has been demonstrated to display a potent antioxidant property and its antioxidant effects were extensively studied recently. In vitro, there are several studies of neuroprotective effects of acteoside against neuronal injury. Acteoside inhibits neuronal death induced by 1-methyl-4-phenylpyridinium ion (MPP^+) (Li et al., 2008; Sheng et al., 2002) and glutamate (Koo et al., 2006). Present results showed that acteoside is also neuroprotective against Aβ-induced cell injury. The results of MTT assay indicated that 20, 30 μg/ml acteoside significantly protects SH-SY5Y cells from Aβ toxicity (Fig. 1B). The neuroprotection effects were also confirmed by calcein-AM staining assay, analysis of morphological nuclear changes and DNA fragmentation. All these observations are consistent with previously reported results.

Compelling evidence showed that OS is extensive in the AD brains, and plays a key role in Aβ induced neuronal cell death (Markesbery, 1997; Miranda et al., 2000). In agreement with these notions, this study demonstrated that $A\beta_{25-35}$ induces ROS generation, and the intracellular ROS scavenging activity of acteoside was in a dose dependent pattern. Acteoside attenuates $A\beta_{25-35}$-induced mitochondrial membrane potential, and such effects could play an important role in protecting SH-SY5Y cells against $A\beta_{25-35}$-induced cytotoxicity. Since acteoside significantly decreased ROS and restored MMP level that had been altered as a result of $A\beta_{25-35}$ treatment. ROS, which was predominantly produced in the mitochondria, led to the free radical attack of membrane phospholipids and loss of mitochondrial membrane potential, which caused the intermembrane protein cytochrome *c* release out of the mitochondria and ultimately activated caspase-3 (Bras et al., 2005). The subsequent experiments explored the mechanism of the neuroprotection of acteoside on $A\beta_{25-35}$-induced cell death in SH-SY5Y cells. We next examined the effect of acteoside on the $A\beta_{25-35}$-induced induction of Bax and Bcl-2 protein levels, cytochrome *c* release and induction of cleaved caspase-3 protein levels in SH-SY5Y cultured cells by western blot analysis.

It is well known that ROS-induced oxidative DNA damage can cause cell apoptosis (Datta et al., 2002). Furthermore, several studies have demonstrated that ROS are involved in the apoptotic mechanisms triggered by Aβ-mediated neurotoxicity and may therefore contribute to the regulation of apoptosis proteins in some AD models (Behl et al., 1994; Fukui et al., 2005). Bcl-2 family plays a key role in the mitochondrial apoptotic pathway. The Bcl-2 family of proteins, an important endogenous regulator in cellular activity after a variety of physiological and pathological insults, has been suggested to be directly dependent on the elevation of Bax and its translocation to the mitochondrial membrane (Murphy et al., 2000). After translocated to the mitochondrial membrane, Bax can homodimerize and activate the terminal caspases by alteration of mitochondrial functions, resulting in the release of apoptosis promoting factors into the cytoplasm (Adams and Cory, 1998). Conversely, Bcl-2/Bax heterodimer formation may prevent or reduce some of these downstream events. Bcl-2 appeared to inhibit the mitochondria depolarization and ROS production, while Bax-induced mitochondrial depolarization and ROS production. With the mitochondrial membrane's potential depletion by ROS, the permeability transition pore (PTP) opened and intermembrane proteins were released out of the mitochondrial, which in turn activated a downstream executive caspase-3 and cell death (Gross et al., 1999). Bcl-2 family members such as Bax and Bcl-2 are implicated in the process of apoptotic cell death induced by ROS-generating agents such as $A\beta_{25-35}$ (Xiao et al., 2002). Thus, scavenging of ROS, inhibition of Bax/Bcl-2 ratio by acteoside could be related to acteoside's antioxidant effect.

Because the release of cytochrome *c* from mitochondria triggers caspase activation, blocking this critical step should interfere with the cell death program thereby rescue dying neurons. The release of cytochrome *c* from mitochondria to the cytosol is essential for caspase-3 activation and activates downstream cell death pathways (Skulachev, 1998). In the present study, we have shown that AS inhibited $A\beta_{25-35}$-induced cytochrome *c* release and the cleavage of caspase 3. The activation of caspase-3 is also believed to be important for commitment to or execution of neuronal apoptosis (Harada and Sugimoto, 1999). The suppressive effect of acteoside on caspase-3 activity further suggests that the protective effect of acteoside on cell death is related to its antioxidant effects.

In conclusion, our study demonstrates that acteoside reduces the toxicity induced by $A\beta_{25-35}$ in SH-SY5Y cells by limiting the induction of death signaling proteins, Bax and caspase enzymes. We document the neuroprotective effects of acteoside in attenuating Aβ-induced induction of cell death signaling cascades and in preserving cell viability, and suggest a neuroprotective effect of acteoside on $A\beta_{25-35}$ insult.

4. Experimental procedures

4.1. *Materials*

Acteoside, obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), was dissolved in sterile distilled water. Calcein-AM, rhodamine 123 (Rh123), dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Inc. Hoechst 33258 and ECL detection kit were purchased from Beyotime (Beijing, China). Modified Dulbecco's Eagle's medium (DMEM) supplement was obtained from Gibco Invitrogen Corporation. The fluorescent dyes 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) were purchased from Invitrogen. Complete Protease Inhibitor was from Roche, Molecular Biochemicals. Antibodies against cytochrome *c* were from Cell Signaling (Beverly, MA, USA). Antibodies against cleaved caspase-3 were from Calbiochem (San Diego, CA, USA). Antibodies against Bcl-2 and Bax were from Santa Cruz Biotechnology Inc. All the other chemicals used were of the high grade available commercially.

4.2. *Preparation of aged $A\beta_{25-35}$*

$A\beta_{25-35}$ was solubilized in distilled water at a concentration of 5 mM, incubated in a capped vial at 37 °C for 3 days to form aggregated form (Feng and Zhang, 2004) and stored frozen at −20 °C until use.

4.3. Cell culture and treatment

Human neuroblastoma SH-SY5Y cells were obtained from the American Tissue Culture Collection. All cells were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C and 5% CO_2 (Huang and May, 2006). To induce cell injury, cells were incubated with 25 μM $A\beta_{25–35}$ for 24 h. To study the effects of acteoside, cells were pre-incubated with acteoside for 1.5 h, and then $A\beta_{25–35}$ was added to the medium for an additional 24 h.

4.4. Determination of cell viability

Cell viability was assessed using conventional MTT reduction assay. The cultured cells in 96-well plates were pre-incubated with acteoside for 1.5 h and exposed to 25 μM $A\beta_{25–35}$ for 24 h, then 20 μl of MTT stock solution (5 mg/ml) was added to the culture medium for treating another 4 h at 37 °C. The resulted MTT formazan was extracted with 150 μl DMSO and the absorbance was recorded with a microtiter plate reader. Cell viability was also analyzed by calcein-AM assay. After 25 μM $A\beta_{25–35}$ treatment, the cells were incubated with 2 μM calcein-AM at 37 °C for 30 min (Leskiewicz et al., 2008). The fluorescence was measured with a fluorescence plate reader. The fluorescence intensity is proportional to the viability of cells. The fluorescence intensity of control cells was calculated as 100%, and all data were normalized to control cells from three independent experiments. In an independent experiment, calcein stained cells also were observed under a fluorescence microscope as described previously (Kajta et al., 2007).

4.5. Measurement of apoptotic cell death

Apoptosis of SH-SY5Y cells was analyzed by Hoechst staining. After exposed to $A\beta_{25–35}$ with or without acteoside as described above, the cells on coverslips were fixed in 4% paraformaldehyde for 20 min, and then stained with Hoechst 33258 for 15 min (Feng and Zhang, 2004). Nuclear morphology was viewed using a fluorescence microscope. The number of cells with apoptotic morphology appearing condensed or fragmented nuclei was counted.

4.6. DNA fragmentation

Fragmented DNA was isolated by DNA extraction kit (Beyotime, China) according to the manufacturer's instructions. The eluants containing DNA pellets were electrophoresed on a 1.5% agarose gel at 80 V for 1.5 h. The gel was examined and photographed by ultraviolet gel documentation system.

4.7. Intracellular ROS generation detection

The intracellular ROS was analyzed by H_2DCF-DA assay (Lin et al., 2006). Briefly, following treatment, the cells were loaded with H_2DCF-DA for 15 min at 37 °C. Cells were washed with PBS, harvested and pelleted by centrifugation and then resuspended in 0.5 ml PBS. Fluorescence intensity was then monitored using flow cytometer. Data were analyzed and expressed as a percentage of the control. DCF labeled cells were observed under confocal microscopy (Jung et al., 2008).

4.8. Measurement of mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi m$) was monitored using the Rh123 (Satoh et al., 1997). Rh123 was added to media (at a final concentration of 10 μg/ml) after the cells exposed to $A\beta_{25–35}$ with or without acteoside pretreatment. After incubation at 37 °C for 30 min, cells were washed and resuspended in PBS for cytometry assay with the excitation wavelength at 488 nm and the emission wavelength at 523 nm using flow cytometer. Analyses were performed on 10,000 cells.

4.9. Cytochrome c assay

For measurement of cytochrome c release, the cytosol fractions were prepared as previously reported (Unkila et al., 2001). Briefly, cells were washed twice with 10 ml of cold PBS, resuspended in 500 ×*g* of fresh cytosolic extract buffer (250 mM sucrose, 20 mM Hepes pH 7.4, 10 mM KCl, 1 mM EGTA, 1 mM EDTA, 1 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride, 1 mM benzamidine, 1 mM pepstatin A, 10 mg/ml leupeptin, and 2 mg/ml aprotonin) and incubated for 30 min on ice with frequent tube tapping. Cells were lysed with 50 strokes of a Dounce Homogenizer (2 ml, tight pestle) on ice, and then nuclei, unbroken cells, and cell debris were pelleted at 2500 rpm for 10 min at 4 °C. The supernatant was spun again at 13,000 rpm for 20 min at 4 °C. The supernatant (now containing the cytosolic extract) was carefully transferred and the supernatants were stored at −80 °C, and the final pellet was used as the mitochondrial fraction. The cytochrome c levels were determined using a monoclonal antibody to cytochrome c by western blotting as described below.

4.10. Western blot analysis

Western blot analysis was used for Bcl-2, Bax, and p17 subunit of caspase-3. After exposure to acteoside, and/or $A\beta_{25–35}$ for the indicated dosages and times, approximately 5×10^6 cells were washed twice with cold PBS, and lysed in ice-cold lyse buffer (50 mM Tris–HCl, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EGTA) with a mixture of protease inhibitors (Complete Protease Inhibitor, Roche, Molecular Biochemicals) for 30–40 min with frequent vortexing on ice, and the protein concentrations of the supernatants were determined using the Bradford method. Cell lysates were boiled for 10 min in 2× sample buffer and separated by SDS-polyacrylamide gel electrophoresis, which was followed by transfer to a nitrocellulose membrane. Nonspecific binding sites were blocked for 1 h with 5% nonfat milk in Tris-buffered saline. Membranes were incubated for 1 h at room temperature with primary or secondary antibodies diluted in 5% milk. Blots were washed extensively with Tris-buffered saline and developed using the ECL detection system and quantified by densitometry. The following antibodies were employed in experiments described in the text: anti-Bcl-2, anti-Bax, anti-caspase-3 p17 antibody, anti-β-actin antibody, anti-cytochrome c antibody.

4.11. *Statistical analysis*

All data were presented as the mean±S.E.M. Data were subjected to statistical analysis via one-way ANOVA followed by student's *t*-test with GraphPad Prism 4.0 software (GraphPad Software, Inc., San Diego, CA). Mean values were considered to be statistically significant at $P<0.05$.

Acknowledgments

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REFERENCES

Adams, J., Cory, S., 1998. The Bcl-2 protein family: arbiters of cell survival. Science 281, 1322–1326.

Behl, C., Davis, J., Lesley, R., Schubert, D., 1994. Hydrogen peroxide mediates amyloid beta protein toxicity. Cell 77, 817–827.

Birkofer, L., Kaiser, C., Thomas, U., 1968. Acteoside and neoacteoside: sugar esters from *Syringa vulgaris* (L.). Z. Naturforsch. B. 23, 1051–1058.

Bras, M., Queenan, B., Susin, S., 2005. Programmed cell death via mitochondria: different modes of dying. Biochemistry (Mosc) 70, 231–239.

Butterfield, D., Drake, J., Pocernich, C., Castegna, A., 2001. Evidence of oxidative damage in Alzheimer's disease brain: central role for amyloid beta-peptide. Trends Mol. Med. 7, 548–554.

Chiou, W., Lin, L., Chen, C., 2004. Acteoside protects endothelial cells against free radical-induced oxidative stress. J. Pharm. Pharmacol. 56, 743–748.

Datta, K., Babbar, P., Srivastava, T., Sinha, S., Chattopadhyay, P., 2002. p53 dependent apoptosis in glioma cell lines in response to hydrogen peroxide induced oxidative stress. Int. J. Biochem. Cell Biol. 34, 148–157.

Díaz, A., Abad, M., Fernández, L., Silván, A., De Santos, J., Bermejo, P., 2004. Phenylpropanoid glycosides from *Scrophularia scorodonia*: in vitro anti-inflammatory activity. Life Sci. 74, 2515–2526.

Feng, Z., Zhang, J., 2004. Melatonin reduces amyloid beta-induced apoptosis in pheochromocytoma (PC12) cells. J. Pineal. Res. 37, 257–266.

Fukui, K., Takatsu, H., Shinkai, T., Suzuki, S., Abe, K., Urano, S., 2005. Appearance of amyloid beta-like substances and delayed-type apoptosis in rat hippocampus CA1 region through aging and oxidative stress. J. Alzheimers Dis. 8, 299–309.

Gross, A., McDonnell, J., Korsmeyer, S., 1999. BCL-2 family members and the mitochondria in apoptosis. Genes Dev. 13, 1899–1911.

Harada, J., Sugimoto, M., 1999. Activation of caspase-3 in beta-amyloid-induced apoptosis of cultured rat cortical neurons. Brain Res. 842, 311–323.

Hensley, K., Carney, J., Mattson, M., Aksenova, M., Harris, M., Wu, J., Floyd, R., Butterfield, D., 1994. A model for beta-amyloid aggregation and neurotoxicity based on free radical generation by the peptide: relevance to Alzheimer disease. Proc. Natl. Acad. Sci. U. S. A. 91, 3270–3274.

Huang, J., May, J., 2006. Ascorbic acid protects SH-SY5Y neuroblastoma cells from apoptosis and death induced by beta-amyloid. Brain Res. 1097, 52–58.

Jung, J., Roh, K., Jeong, Y., Kim, S., Lee, E., Kim, M., Oh, W., Oh, H., Kim, W., 2008. Estradiol protects PC12 cells against CoCl2-induced apoptosis. Brain Res. Bull. 76, 579–585.

Kajta, M., Domin, H., Grynkiewicz, G., Lason, W., 2007. Genistein inhibits glutamate-induced apoptotic processes in primary neuronal cell cultures: an involvement of aryl hydrocarbon receptor and estrogen receptor/glycogen synthase kinase-3beta intracellular signaling pathway. Neuroscience 145, 592–604.

Koo, K., Kim, S., Oh, T., Kim, Y., 2006. Acteoside and its aglycones protect primary cultures of rat cortical cells from glutamate-induced excitotoxicity. Life Sci. 79, 709–716.

Leskiewicz, M., Regulska, M., Budziszewska, B., Jantas, D., Jaworska-Feil, L., Basta-Kaim, A., Kubera, M., Jagla, G., Nowak, W., Lason, W., 2008. Effects of neurosteroids on hydrogen peroxide- and staurosporine-induced damage of human neuroblastoma SH-SY5Y cells. J. Neurosci. Res. 86, 1361–1370.

Li, Y., Lu, J., Li, Q., Zhao, Y., Pu, X., 2008. Pedicularioside A from *Buddleia lindleyana* inhibits cell death induced by 1-methyl-4-phenylpyridinium ions (MPP^+) in primary cultures of rat mesencephalic neurons. Eur. J. Pharmacol. 579, 134–140.

Lin, Y., Liu, A., Wu, H., Westenbroek, C., Song, Q., Yu, H., Ter Horst, G., Li, X., 2006. Salvianolic acid B, an antioxidant from *Salvia miltiorrhiza*, prevents Abeta(25–35)-induced reduction in BPRP in PC12 cells. Biochem. Biophys. Res. Commun. 348, 593–599.

Markesbery, W., 1997. Oxidative stress hypothesis in Alzheimer's disease. Free Radic. Biol. Med. 23, 134–147.

Miranda, S., Opazo, C., Larrondo, L., Muñoz, F., Ruiz, F., Leighton, F., Inestrosa, N., 2000. The role of oxidative stress in the toxicity induced by amyloid beta-peptide in Alzheimer's disease. Prog. Neurobiol. 62, 633–648.

Murphy, K., Ranganathan, V., Farnsworth, M., Kavallaris, M., Lock, R., 2000. Bcl-2 inhibits Bax translocation from cytosol to mitochondria during drug-induced apoptosis of human tumor cells. Cell Death Differ. 7, 102–111.

Park, S., Kim, H., Cho, E., Kwon, B., Phark, S., Hwang, K., Sul, D., 2008. Curcumin protected PC12 cells against beta-amyloid-induced toxicity through the inhibition of oxidative damage and tau hyperphosphorylation. Food Chem. Toxicol. 46, 2881–2887.

Pu, X., Song, Z., Li, Y., Tu, P., Li, H., 2003. Acteoside from *Cistanche salsa* inhibits apoptosis by 1-methyl-4-phenylpyridinium ion in cerebellar granule neurons. Planta Med. 69, 65–66.

Satoh, T., Enokido, Y., Aoshima, H., Uchiyama, Y., Hatanaka, H., 1997. Changes in mitochondrial membrane potential during oxidative stress-induced apoptosis in PC12 cells. J. Neurosci. Res. 50, 413–420.

Shen, C., Chen, Y., Liu, H., Zhang, K., Zhang, T., Lin, A., Jing, N., 2008. Hydrogen peroxide promotes Abeta production through JNK-dependent activation of gamma-secretase. J. Biol. Chem. 283, 17721–17730.

Sheng, G., Zhang, J., Pu, X., Ma, J., Li, C., 2002. Protective effect of verbascoside on 1-methyl-4-phenylpyridinium ion-induced neurotoxicity in PC12 cells. Eur. J. Pharmacol. 451, 119–124.

Skulachev, V., 1998. Cytochrome *c* in the apoptotic and antioxidant cascades. FEBS Lett. 423, 275–280.

Tamagno, E., Guglielmotto, M., Aragno, M., Borghi, R., Autelli, R., Giliberto, L., Muraca, G., Danni, O., Zhu, X., Smith, M., Perry, G., Jo, D., Mattson, M., Tabaton, M., 2008. Oxidative stress activates a positive feedback between the gamma- and beta-secretase cleavages of the beta-amyloid precursor protein. J. Neurochem. 104, 683–695.

Unkila, M., McColl, K., Thomenius, M., Heiskanen, K., Distelhorst, C., 2001. Unreliability of the cytochrome *c*-enhanced green fluorescent fusion protein as a marker of cytochrome *c* release in cells that overexpress Bcl-2. J. Biol. Chem. 276, 39132–39137.

Wei, C., Jia, J., Liang, P., Guan, Y., 2008. Ginsenoside Rg1 attenuates beta-amyloid-induced apoptosis in mutant PS1 M146L cells. Neurosci. Lett. 443, 145–149.

Xiao, X., Zhang, H., Tang, X., 2002. Huperzine A attenuates amyloid beta-peptide fragment 25–35-induced apoptosis in rat cortical neurons via inhibiting reactive oxygen species formation and caspase-3 activation. J. Neurosci. Res. 67, 30–36.

Xiong, Q., Hase, K., Tezuka, Y., Namba, T., Kadota, S., 1999. Acteoside inhibits apoptosis in D-galactosamine and lipopolysaccharide-induced liver injury. Life Sci. 65, 421–430.

Yang, J., Liu, X., Bhalla, K., Kim, C., Ibrado, A., Cai, J., Peng, T., Jones, D., Wang, X., 1997. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science 275, 1129–1132.

Yao, Z., Drieu, K., Papadopoulos, V., 2001. The Ginkgo biloba extract EGb 761 rescues the PC12 neuronal cells from beta-amyloid-induced cell death by inhibiting the formation of beta-amyloid-derived diffusible neurotoxic ligands. Brain Res. 889, 181–190.