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Acorus tatarinowii Schott extract protects PC12 cells from amyloid-β induced neurotoxicity

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Amyloid-β induced neurotoxicity has been identified as a major cause of Alzheimer's disease. *Acorus tatarinowii* Schott is one of the most frequently used Chinese herbs for Alzheimer's disease treatment. However, the effects of *Acorus tatarinowii* Schott on amyloid-β mediated nerve cell damage remains unknown. In the present study, neuronal differentiated PC12 cells were used as a model to evaluate the effects of *A. tatarinowii* Schott extract (ATSE) against $A\beta_{25–35}$ induced neurotoxicity. The results showed pretreatment with ATSE significantly protected PC12 cells from $A\beta_{25–35}$ induced cell death, lactate dehydrogenase release, DNA damage, mitochondrial dysfunction and cytochrome c release from mitochondria. In addition, pretreatment with ATSE also significantly inhibited $A\beta_{25–35}$ induced caspase-3 activation and reactive oxygen species generation in PC12 cells. These observations suggested that ATSE protects PC12 cells from amyloid-β induced neurotoxicity.

1. Introduction

Alzheimer's disease (AD), the leading cause of dementia, is an age-related, progressive neurodegenerative disorder characterized by decline in cognitive functions that usually begins with memory loss. The prevalence and incidence rate of AD are increasing exponentially with age (Reitz et al. 2011). Accumulated evidence suggests that amyloid-β (Aβ) induced neurotoxicity contributes to the pathogenesis of AD (Yankner and Lu 2009; Butterfield 2002). Aβ induced neuronal cell damage has been widely used as a model to investigate neuroprotective agents. Currently approved drugs, including donepezil, galantamine, rivastigmine and memantine, only showed marginal therapeutic benefits for AD (Tayeb et al. 2012). Thus, there is a great need to explore novel approaches for AD prevention and treatment.

Traditional Chinese medicine (TCM) has played a positive role in AD treatment. TCM treatment based on syndrome differentiation, or empirical herbal formulae, or specific TCM therapeutic principles, have been demonstrated to effectively improve cognitive function and brain function, and ameliorate clinical symptoms in patients with AD (Huo et al. 2008; Yu et al. 2012; Miao et al. 2012; Gang et al. 2005). TCM also showed synergistic effects in combination with donepezil (Gao et al. 2008). Excremental studies revealed that the effects of TCM in AD treatment involved multiple mechanisms, such as inhibiting β-amyloid deposition and Tau phosphorylation, regulate neurotransmitters, protect nerve cells from injury and apoptosis, and anti-oxidative stress (Gao et al. 2011; Li 2008). The top five most frequently used herbs in AD treatment are *Acorus tatarinowii* Schott (Shi Chang Pu), *Ligusticum chuanxiong* (Chuan Xiong), *Polygala tenuifolia* Willd (Yuan Zhi), *Salvia miltiorrhiza* Bge (Dan Shen), and prepared *Rehmannia glutinosa (Gdertn)* Iibosch (Shu Di) (Zhou et al. 2005).

The traditional TCM efficacy of *A. tatarinowii* Schott is inducing resuscitation, removing dampness to restore normal function of stomach, soothing nerves and benefiting intelligence. The effect of enhancing intelligence of *A. tatarinowii* Schott was first recorded in Shen-Nong's Classic of Materia Medica (AD 102–200). Currently, *A. tatarinowii* Schott has been widely used for neurological diseases treatment, such as AD, Parkinson's disease and epilepsy. It is a principal herb both for pared-herbs and herbal formula in the treatment of AD (Zhou et al. 2005; Zhang et al. 2012; Huang et al. 2009). It has been reported that *A. tatarinowii* Schott extract may ameliorate learning and memory deficits induced by Aβ (Tian et al. 2012). However, its effect on nerve cell damage remains unknown. In the present study, we evaluated the potential neuroprotective effects of *A. tatarinowii* Schott extract (ATSE) against Aβ induced toxicity in differentiated PC12 cells as a model neuronal cell.

2. Investigations and results

2.1. Effect of ATSE on the cell viability in Aβ-treated PC12 cells

$A\beta_{25–35}$, the highly toxic peptide of amyloid-β protein (Millucci et al. 2010), was used as a neurotoxicant. The effect of ATSE on the cell viability in $A\beta_{25–35}$ treated PC12 cells was observed by CCK-8 assay. As shown in Fig. 1A, ATSE stimu-

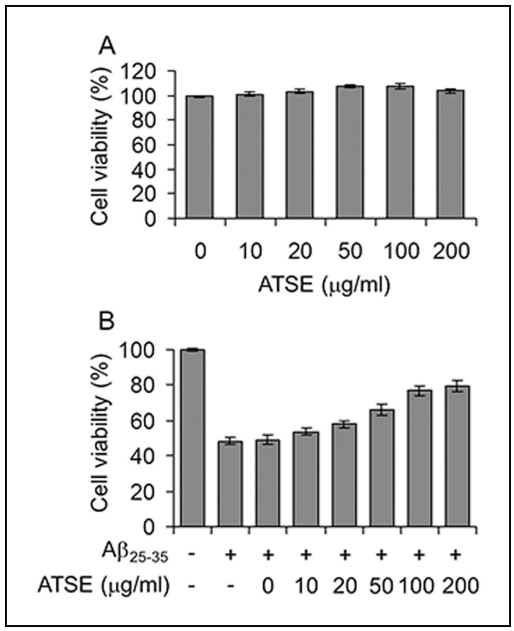

Fig. 1: Effect of ATSE on the cell viability in Aβ-treated PC12 cells A, differentiated PC12 cells were treated with different concentrations of ATSE for 24 h, cell viability was evaluated by CCK-8 assay. B, differentiated PC12 cells were pretreated with different concentrations of ATSE for 1 h, followed by $A\beta_{25-35}$ treatment for 24 h, cell viability was evaluated by CCK-8 assay. Data shown are representative of three independent experiments.

lated cell growth in PC12 cell to certain extent. Upon exposed to $A\beta_{25-35}$ for 24 h, the cell viability of PC12 cells was significantly inhibited ($P < 0.01$). Preincubation with 10–200 μg/ml of ATSE significantly increased cell viability in $A\beta_{25-35}$ treated PC12 cells in a dose-dependent manner ($P < 0.01$) (Fig. 1B). We selected 20–100 μg/ml concentration of ATSE for subsequent experiments.

2.2. Effect of ATSE on the LDH release in Aβ-treated PC12 cells

LDH, a soluble cytosolic enzyme, is released into the culture medium following loss of membrane integrity due to cell death. LDH release was used as an indicator for $A\beta_{25-35}$ induced cytotoxicity. As shown in Fig. 2, after treatment with $A\beta_{25-35}$ for 24 h, the LDH leakage was significantly increased in PC12 cells ($P < 0.01$). Pretreatment with 20–100 μg/ml of ATSE significantly decreased $A\beta_{25-35}$ elicited LDH release in PC12 cells in a dose-dependent manner ($P < 0.01$). These results suggested that ATSE protected PC12 cells from $A\beta_{25-35}$ induced cytotoxicity.

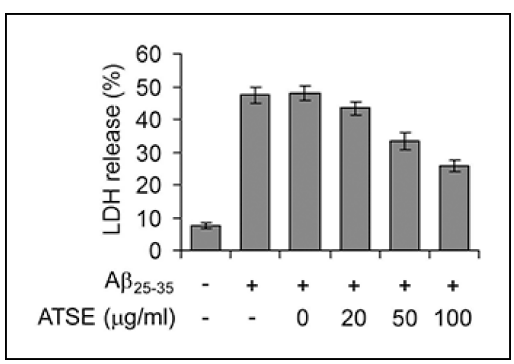

Fig. 2: Effect of ATSE on the LDH release in Aβ-treated PC12 cells Differentiated PC12 cells were pretreated with different concentrations of ATSE for 1 h, followed by $A\beta_{25-35}$ treatment for 24 h. LDH release was determined by LDH Cytotoxicity Assay Kit, and expressed as percentage of Triton X-100 induced LDH release. Data represented are from three independent experiments.

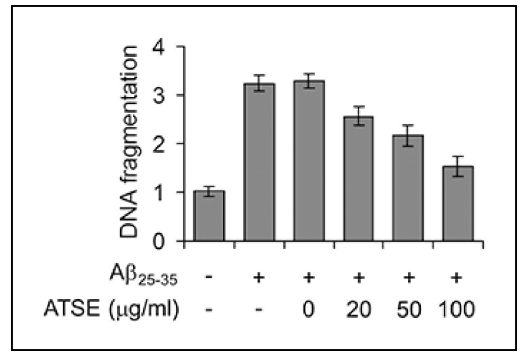

Fig. 3: Effect of ATSE on Aβ induced DNA damage in PC12 cells Differentiated PC12 cells were pretreated with different concentrations of ATSE for 1 h, followed by Aβ25–35 treatment for 24 h. DNA fragmentation was detected by photometric enzyme-linked immunoassay. Results were expressed as fold of non-treated control. Data illustrated are from three separate experiments.

2.3. Effect of ATSE on Aβ induced DNA damage in PC12 cells

The effect of ATSE on the DNA fragmentation in $A\beta_{25-35}$ treated PC12 cells is shown in Fig. 3. After treatment with $A\beta_{25-35}$ for 24 h, the DNA fragmentation in PC12 cells was significantly increased ($P < 0.01$). Pretreatment with 20–100 μg/ml of ATSE significantly decreased $A\beta_{25-35}$ induced DNA fragmentation in PC12 cells in a dose-dependent manner ($P < 0.01$). Since DNA fragmentation is indicative of apoptosis, these results suggested ATSE protected PC12 cells from $A\beta_{25-35}$ induced apoptosis.

2.4. ATSE prevented Aβ induced mitochondrial dysfunction in PC12 cells

Mitochondrial dysfunction and subsequently cytochrome c (Cty-C) release is a central event in the process of apoptosis (Favaloro et al. 2012). In the present study, JC-1 was used to detect mitochondrial membrane potential (MMP) as a parameter of mitochondrial function. JC-1 is a lipophilic cationic dye that remains in monomeric form in cytoplasm and yields green fluorescence in apoptotic cells with low MMP. As shown in Fig. 4A and B, after treatment of PC12 cells with $A\beta_{25-35}$ for 24 h, the MMP was significantly reduced ($P < 0.01$). Pretreatment with 20–100 μg/ml of ATSE significantly countered the lowering of MMP induced by Aβ25–35 ($P < 0.01$).
Meanwhile, the Cty-C concentration in cytosol was measured by a commercial ELISA kit. PC12 cells treated with $A\beta_{25-35}$ for 24 h showed an increase in cytosolic Cty-C levels ($P < 0.01$). Pretreatment with 20–100 μg/ml of ATSE significantly attenuated $A\beta_{25-35}$ induced Cty-C release in a dose-dependent manner ($P < 0.01$) (Fig. 4C). These observations suggested ATSE prevented $A\beta_{25-35}$ induced mitochondrial dysfunction in PC12 cells.

2.5. Effect of ATSE on caspase-3 activity in Aβ25–35 treated PC12 cells

Cell apoptosis is executed by a caspase cascade, caspase-3 is the final executor of apoptosis, and its activation has been recognized as hallmark of apoptosis (Mazumder et al. 2008). The effect of ATSE on caspase-3 activity in $A\beta_{25-35}$ treated PC12 cells is shown in Fig. 5. $A\beta_{25-35}$ activated caspase-3 in PC12 cells ($P < 0.01$). Pretreatment with 20–100 μg/ml of ATSE significantly inhibited $A\beta_{25-35}$ induced caspase-3 activation in PC12 cells in a dose-dependent manner ($P < 0.01$).

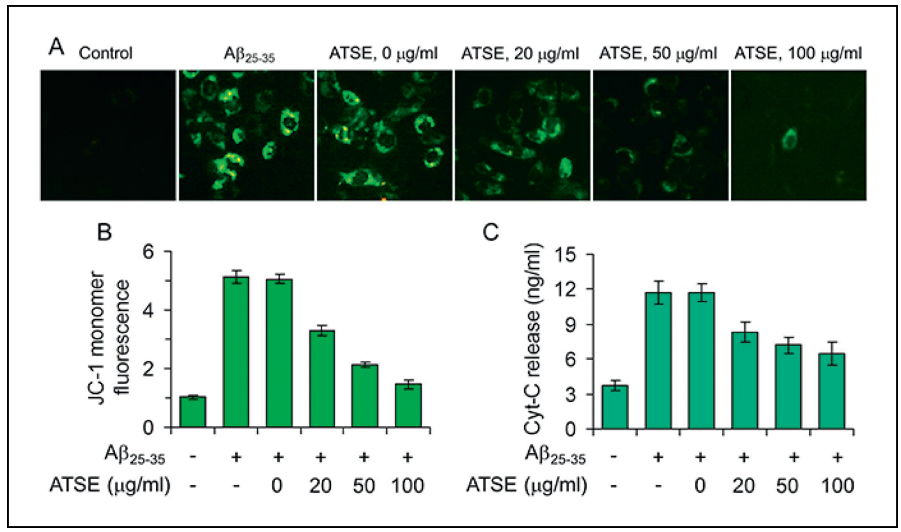

Fig. 4: Effects of ATSE on Aβ induced mitochondrial dysfunction Differentiated PC12 cells were pretreated with different concentrations of ATSE for 1 h, followed by Aβ25–35 treatment for 24 h. MMP was detected by JC-1 staining, and observed under fluorescence microscope (×200) (A), and measure by fluorescence microplate reader at excitation wavelength of 490 nm and emission wavelength of 530 nm, and expressed as fold of non-treated control. C, after treatment, the Cty-C concentration in cytosol was determined by ELISA. Data presented are from three separate experiments.

2.6. Effect of ATSE on ROS generation in Aβ25–35 treated PC12 cells

It has been reported that β-amyloid mediated ROS generation contributed to AD pathogenesis, and has been suggested as a therapeutic target for AD (Pagani and Eckert 2011; Dumont and Beal 2011) We also observed the effects of ATSE on ROS generation in $A\beta_{25–35}$ treated PC12 cells. As shown in Figure 6, exposure of PC12 cells to $A\beta_{25–35}$ for 24 h resulted in a significant increase in ROS generation ($P<0.01$). PC12 cells pretreated with ATSE showed a significant reduction in the ROS levels ($P<0.01$). These results suggested that ATSE inhibited $A\beta_{25–35}$ induced ROS production in PC12 cells.

3. Discussion

Amyloid-β peptide has been identified as one of the major causes of AD. The mechanisms of amyloid-β toxicity on neurons includes mitochondrial dysfunction, apoptosis and ROS generation (Yankner et al. 2009; Mazumder et al. 2008; Wang et al. 2007). In the present study we observed that $A\beta_{25–35}$ caused a significant decrease in cell viability and increase in LDH release due to membrane damage in differentiated PC12 cells that confirmed $A\beta_{25–35}$ neurotoxicity on neuronal differentiated PC12 cells. In this *in vitro* model, ATSE significantly reversed these changes induced by $A\beta_{25–35}$, suggested neuroprotective effects of ATSE against $A\beta_{25–35}$ induced toxicity that may be beneficial for the treatment of AD.

It has been demonstrated that amyloid-β peptide may activate the mitochondrial apoptosis pathway. Amyloid-β peptide induces mitochondrial dysfunction, which leads to the release of Cyt-C, forming an apoptosome that activates the initiating protease caspase-9 which in turn activates executioner caspases-3, causing the cell to undergo apoptosis (Wang et al. 2007; Meier and Vousden 2007). In the present study, we found that $A\beta_{25–35}$ treatment down-regulated MMP, increased mitochondrial Cyt-C release and activated caspase-3 accompanied by DNA fragmentation in neuronal differentiated PC12 cells. These observations suggested that $A\beta_{25–35}$ induced apoptosis in neuronal differentiated PC12 cells. Pretreatment with ATSE inhibited these changes, suggested ATSE protected PC12 cells from $A\beta_{25–35}$ induced apoptosis.

Amyloid-β peptide may also increase ROS generation (Viña et al. 2011). High levels of ROS may promote cell death *via* mitochondrial pathway and/or death receptor pathway (Circu and Aw, 2010). Natural products, such as walnut extract, resveratrol and *Poria cocos* water extract may inhibit amyloid-β induced ROS generation (Muthaiyah et al. 2011; Jang et al. 2007; Park

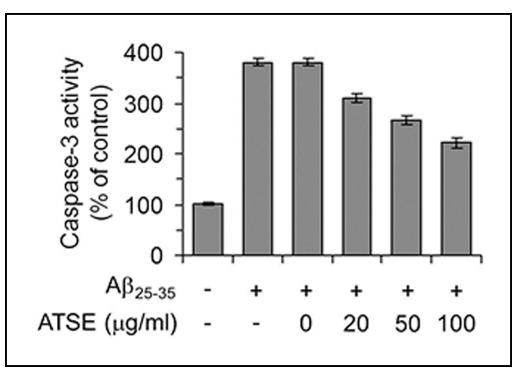

Fig. 5: Effect of ATSE on caspase-3 activity in Aβ25–35 treated PC12 cells Differentiated PC12 cells were pretreated with different concentrations of ATSE for 1 h, followed by Aβ25–35 treatment for 24 h. After treatment, caspase-3 activity was detected as described in the Experimental section. Caspase-3 activities were expressed as percentage of control. Data shown are representative of three independent experiments.

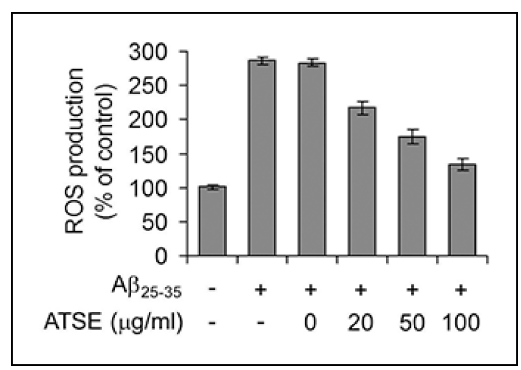

Fig. 6: Effect of ATSE on ROS generation in Aβ25–35 treated PC12 cells Differentiated PC12 cells were pretreated with different concentrations of ATSE for 1 h, followed by Aβ25–35 treatment for 24 h. The production of intracellular ROS was detected by DCFH-DA staining and quantified with fluorescence microplate reader at wave lengths of 488 nm for excitation and 525 nm for emission. ROS production was expressed as percentage of control. Data shown are representative of three independent experiments.

et al. 2009). In the present study, we found that ATSE attenuated amyloid-β induced ROS production in neuronal differentiated PC12 cells, suggesting that ROS inhibition may contribute to ATSE mediated neuroprotective effects.

In summary, our results demonstrated that ATSE protected PC12 cells from $A\beta_{25–35}$ mediated cell death by inhibiting the mitochondrial apoptotic pathway and reducing ROS generation. The present study provides new insight into the application of a Chinese herb for AD treatment worth to be further studied.

4. Experimental

4.1. Chemicals and reagents

Amyloid-β protein fragment 25–35 ($A\beta_{25–35}$) was from Sigma-Aldrich (St. Louis, MO). Colorimetric CaspACE^{TM} Assay System was the product of Promega (Madison, WI). Recombinant Rat NGF and Rat/mouse cytochrome c ELISA kit were from R&D Systems (Minneapolis, MN). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo (Kumamoto, Japan). Cell Death Detection ELISA^{Plus} kit was purchased from Roche Applied Sciences (Basel, Switzerland). Lactate dehydrogenase (LDH) Cytotoxicity Assay Kit was obtained from Cayman Chemical (Ann Arbor, MI). BCA Protein Assay kit and 2',7'-dichlorofluorescin diacetate (DCFH-DA) was provided by Beyotime Institute of Biotechnology (Jiangsu, China).

4.2. Preparation of Acorus tatarinowii Schott extract

Acorus tatarinowii Schott extract (ATSE) was prepared as a lyophilized-dry powder of hot water extracts as described previously (Hu et al. 2012). Authentic herb materials were provided by Longhua Hospital herb store. *A. tatarinowii* Schott (300 g) was soaked for 30 min, and decocted twice with the 8-fold volume of distilled water for 30 min. The decoction was filtered and centrifuged twice at 12,000 rpm for 30 min to remove insoluble ingredients. The supernatants were mixed with an equal volume of ethanol and kept at 4 °C overnight, and centrifuged at 12,000 rpm for 30 min to remove insoluble ingredients. The resultant supernatants were lyophilized, weighed, dissolved in RPMI1640 medium and adjusted to a concentration of 100 mg/ml, sequentially passed through 0.45 μm and 0.22 μm filters sterilization and stored at 4 °C until use.

4.3. Preparation of β-amyloid

$A\beta_{25–35}$ aggregation was performed as described previously (Park et al. 2008). Lyophilized $A\beta_{25–35}$ was dissolved in RPMI1640 media and incubated at 37 °C with constant oscillation for 3 days to induce aggregation. The aggregated $A\beta_{25–35}$ was then diluted to 100 μg/ml (100 μM) and stored at -20 °C until use.

4.4. Cell culture

PC12 cells were obtained from Cell Bank of Type Culture Collection of Chinese Academy of Sciences. PC12 cells were grown in RPMI1640 medium with 10% horse serum and 5% FBS and 1% Pen-Strep, and maintained at a 37 °C in a humidified incubator with a 5% CO_2 atmosphere. For neuronal differentiation, PC12 cells were treated with 50 ng/mL NGF for 2 days and examined under a microscope (Xing et al. 2011; Kumar et al. 2010). Differentiated PC12 cells were used in all experiments.

4.5. Cell viability assay

Differentiated PC12 cells were seeded into 96-well plate (8×10^3 cells/well). After 24 h cells were pretreated with various doses of ATSE or same volume of RPMI1640 for 1 h, followed by 20 μM aggregated $A\beta_{25–35}$ treatment for 24 h. At the end of treatment, cell viability was evaluated by CCK-8 assay according to the manufacturer's instructions. The cell viability rate was calculated as follows: cell viability (%) = (experimental OD value / control OD value) × 100%.

4.6. Determination of cytotoxicity

Differentiated PC12 cells were preincubated with or without ATSE for 1 h, and followed by exposure to 20 μM aggregated $A\beta_{25–35}$ for 24 h. At the end of treatment, $A\beta_{25–35}$ induced cytotoxicity was evaluated by using the LDH release assay according to the manufacturer's instructions. For total cellular LDH activity determining, PC12 cells were exposed to 0.2% Triton X-100. Results were expressed as percentage of Triton X-100 induced LDH release.

4.7. Quantification of DNA fragmentation for apoptosis

Apoptosis was analyzed by using the Cell Death Detection ELISA^{PLUS} kit. This assay is a photometric enzyme-linked immunoassay, which detects cytoplasmic histone-associated-DNA-fragments (mono- and oligonucleosomes) as an indicator of apoptosis. Differentiated PC12 cells were seeded into 96-well plate (8×10^3 cells/well). Differentiated PC12 cells were preincubated with or without ATSE for 1 h, and followed by exposure to 20 μM aggregated $A\beta_{25–35}$ for 24 h. At the end of treatment, PC12 cells were collected for quantification of DNA fragmentation according to the manufacturer's protocol. Results were expressed as fold of non-treated control.

4.8. Measurement of mitochondrial membrane potential (MMP)

Differentiated PC12 cells were preincubated with or without ATSE for 1 h, and followed by exposure to 20 μM aggregated $A\beta_{25–35}$ for 24 h. MMP changes were determined by JC-1 staining according to the manufacturer's manual. The presence of JC-1 monomeric green fluorescence was monitored under a fluorescence microscope, and quantitated with a fluorescence microplate reader at excitation wavelength of 490 nm and emission wavelength of 530 nm.

4.9. Mitochondrial cytochrome c release assay

The release of cytochrome c (Cyt-C) from mitochondria into the cytosol was assessed by enzyme-linked immunosorbent assay (ELISA). Cytosolic proteins of PC12 cells were prepared as described (Yim et al. 2007). In brief, differentiated PC12 cells were pretreated with or without ATSE for 1 h, and followed by exposure to 20 μM aggregated $A\beta_{25–35}$ for 24 h. After the treatment, PC12 cells were collected and suspended in buffer (20 mM HEPES-KOH at pH 7.5, 250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.5 mM dithiothreitol, and 0.1 mM PMSF) for 15 min on ice and homogenized by successive passage through a syringe needle for five times, and the cytoplasmic fraction was then obtained by centrifugation at 12,000 rpm for 30 min. Protein concentrations were determined using the BCA Protein Assay kit according to the protocol provided by the manufacturer. Cytosolic cytochrome c was measured by ELISA according to the manufacturer's protocol. The results were expressed as ng/ml by reference to the standard curve.

4.10. Measurement of intracellular ROS generation

Intracellular reactive oxygen species (ROS) production was detected by DCFH-DA staining. DCFH-DA is cleaved intracellularly by nonspecific esterases to form DCFH, which is further oxidized by ROS to form the fluorescent compound DCF. Differentiated PC12 cells were preincubated with or without ATSE for 1 h, and followed by exposure to 20 μM aggregated $A\beta_{25–35}$ for 24 h, and stained with DCFH-DA at 37 °C for 20 min in the dark. The presence of DCF fluorescence was quantitated with a fluorescence microplate reader at excitation wavelength of 488 nm and emission wavelength of 525 nm.

4.11. Statistical analysis

Results are expressed as means ± standard deviation of at least two independent experiments, each conducted in triplicate. Differences between control and treatment were analyzed by one-way ANOVA. Differences were considered significant at $P < 0.05$.

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