



Research Paper

Artemisinin protects PC12 cells against β -amyloid-induced apoptosis through activation of the ERK1/2 signaling pathway



Zhiwen Zeng¹, Jinying Xu¹, Wenhua Zheng^{*}

Faculty of Health Sciences, University of Macau, Taipa, Macau, China

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ABSTRACT

Accumulating evidence displays that an abnormal deposition of amyloid beta-peptide ($A\beta$) is the primary cause of the pathogenesis of Alzheimer's disease (AD). And therefore the elimination of $A\beta$ is regarded as an important strategy for AD treatment. The discovery of drug candidates using culture neuronal cells against $A\beta$ peptide toxicity is believed to be an effective approach to develop drug for the treatment of AD patients. We have previously showed that artemisinin, a FDA-approved anti-malaria drug, has neuroprotective effects recently. In the present study, we aimed to investigate the effects and potential mechanism of artemisinin in protecting neuronal PC12 cells from toxicity of β amyloid peptide. Our studies revealed that artemisinin, in clinical relevant concentration, protected and rescued PC12 cells from $A\beta_{25-35}$ -induced cell death. Further study showed that artemisinin significantly ameliorated cell death due to $A\beta_{25-35}$ insult by restoring abnormal changes in nuclear morphology, lactate dehydrogenase, intracellular ROS, mitochondrial membrane potential and activity of apoptotic caspase. Western blotting analysis demonstrated that artemisinin activated extracellular regulated kinase ERK1/2 but not Akt survival signaling. Consistent with the role of ERK1/2, preincubation of cells with ERK1/2 pathway inhibitor PD98059 blocked the effect of artemisinin while PI3K inhibitor LY294002 has no effect. Moreover, $A\beta_{1-42}$ also caused cells death of PC12 cells while artemisinin suppressed $A\beta_{1-42}$ cytotoxicity in PC12 cells. Taken together, these results, at the first time, suggest that artemisinin is a potential protectant against β amyloid insult through activation of the ERK1/2 pathway. Our finding provides a potential application of artemisinin in prevention and treatment of AD.

1. Introduction

Neurodegenerative diseases refer to a range of neuronal disabilities associated with massive neuronal loss at the late stages of the diseases [1]. These neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD), are major problems worldwide [2]. NDs can cause by various reasons, including oxidative stress, excitotoxicity, inflammation, and apoptosis [3]. Along with the raise of extended life-expectancy and rapidly increasing prevalence in aging society, AD, the most common forms of NDs, is becoming a major economic, social, and healthcare burden throughout the world, and has been identified as a public health priority by the World Health Organization [4].

AD is the most common cause of dementia as well as the leading lethal disease among the elderly. It is characterized by the deposition of β -amyloid ($A\beta$) plaques, neurofibrillary tangles (NFTs) and neuronal loss [5]. $A\beta$ is produced in all neurons with significant non-pathological activity [6]. However, its aggregates are believed to be the principal

component of senile plaques found in the brains of patients with AD [7,8]. Therefore, the abnormal deposition of $A\beta$ in plaques of brain tissue leads to a high diversity of toxic mechanisms, including oxidative stress, mitochondrial diffusion, and excitotoxicity through interactions with neurotransmitters receptors [9]. These effects could cause the loss of neurons, and synaptic lesions, all of which are considered to affect the memory [10]. Modulation of $A\beta$ -induced neurotoxicity has emerged as a possible therapeutic approach to ameliorate AD onset and progression [11]. Development of safe and effective therapies for AD is of the highest priority for our aging society. Results of clinical trials with neurotrophic factors, however, have been rather disappointing because of the difficulty in delivering therapeutic proteins to the central nervous system [12]. The development of small molecules acting on neuronal cells has been proposed as an alternative [13]. Thus, identification of potential protective candidates to prevent and eliminate of excessive accumulation of $A\beta$ is considered an important strategy for the treatment of AD.

Artemisinin, a sesquiterpene lactone, was originally isolated from

^{*} Correspondence to: Faculty of Health Sciences, University of Macau, Room 4021, Building E12, Avenida de Universidade, Taipa, Macau, China.

E-mail address: wenhuazheng@umac.mo (W. Zheng).

¹ Contribution is the same.

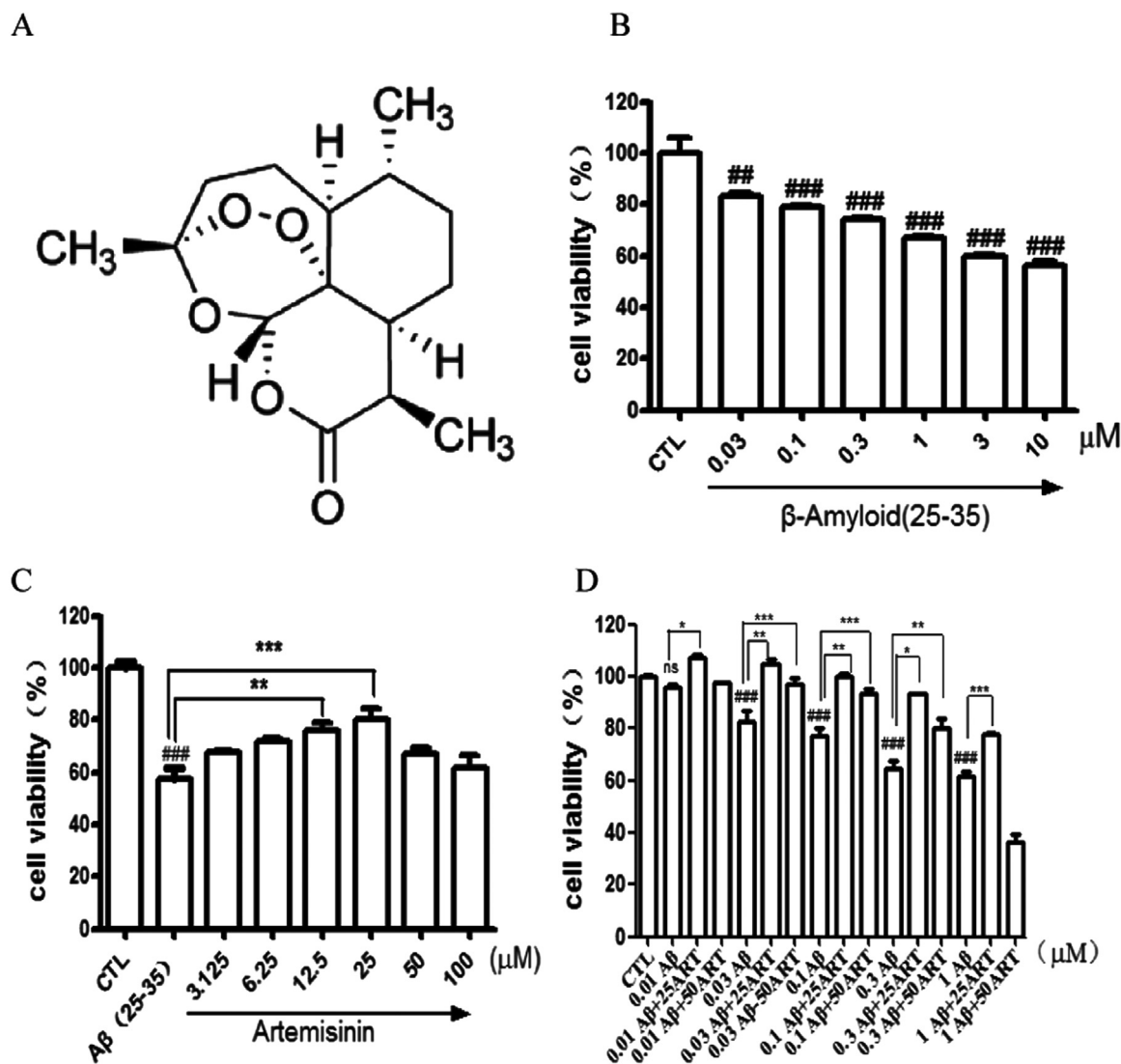


Fig. 1. Artemisinin concentration-dependently suppressed A β 25-35-induced cell viability loss in PC12 cells. (A) The structure of artemisinin. (B) Cells were treated with A β 25-35 (0.03–10 μ M) or 0.1% DMSO (vehicle control) for 24 h and cell viability was measured using the MTT assay (N=3). (C) Cells were pre-treated with artemisinin (3.125–100 μ M) or 0.1% DMSO (vehicle control) for 1 h and then incubated with or without 0.3 μ M A β 25-35 for a further 24 h and cell viability were measured by MTT assay (N=3). (D) PC12 cells were incubated with 0.1, 0.3, 1 μ M A β 25-35 for 30 min and post-treated with artemisinin (25 or 50 μ M) for 24 h and cell viability were measured by MTT assay (N=3). *P < 0.05, **P < 0.01, ***P < 0.005 versus the control or A β 25-35 treated group as indicated; ###P < 0.005 versus control group was considered statistically significant differences.

the qinghao (the Chinese name of plant *Artemisia annua* L.) by Chinese scientists [14]. At present, artemisinins are among the most effective therapies for malaria with great safety and the preferred first-line treatment for the disease [15]. Except these effects, accumulated studies indicate that artemisinin and its derivatives have neuroprotective effect, therefore be potential candidates for treatment of AD [16–18]. Artemisinin easily penetrate the blood–brain barrier and we have previously showed that artemisinin was able to protect PC12 cells and cortical neurons from free radical/oxidative stress induced by hydrogen peroxide and sodium nitroprusside (SNP) [17,19]. Free radical/oxidative stress is believed as a key player in A β peptide neuronal toxicity and it is most possible that artemisinin also can protect neuronal cells from A β induced injury. However, the action of artemisinins on A β -induced cell apoptosis which mimics the A β burden in vivo is not known.

Based on these observations, the purpose of this study was to assess whether artemisinins could protect against A β -induced cytotoxicity in PC12 cells and the underlying mechanisms. In the present study, we found that the protective effect of artemisinin against A β -induced damage in PC12 cells was via restoring abnormal changes in nuclear

morphology, intracellular ROS, mitochondrial membrane potential and caspase activation. We also investigated the roles of the ERK1/2 and Akt pathway in the protective effect of artemisinin. All of these may provide an interesting view of the potential application of artemisinins in future research for AD.

2. Materials and methods

2.1. Materials

Analytical grade artemisinin was purchased from Chengdu Kangbang Biotechnology Ltd. China. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolyl-carbocyanineiodide (JC-1), and Hoechst 33342 were purchased from Molecular Probes (Eugene, OR, USA). A β 25–35, A β 1–42 and DMSO were obtained from Sigma (Sigma, US). Horseradish peroxidase-conjugated anti-rabbit, anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-Akt473, anti-Akt, anti-GAPDH and anti-Tubulin were purchased from Cell Signaling Technology (Woburn, USA). PD98059 and LY294002 were obtained from Merck Millipore.

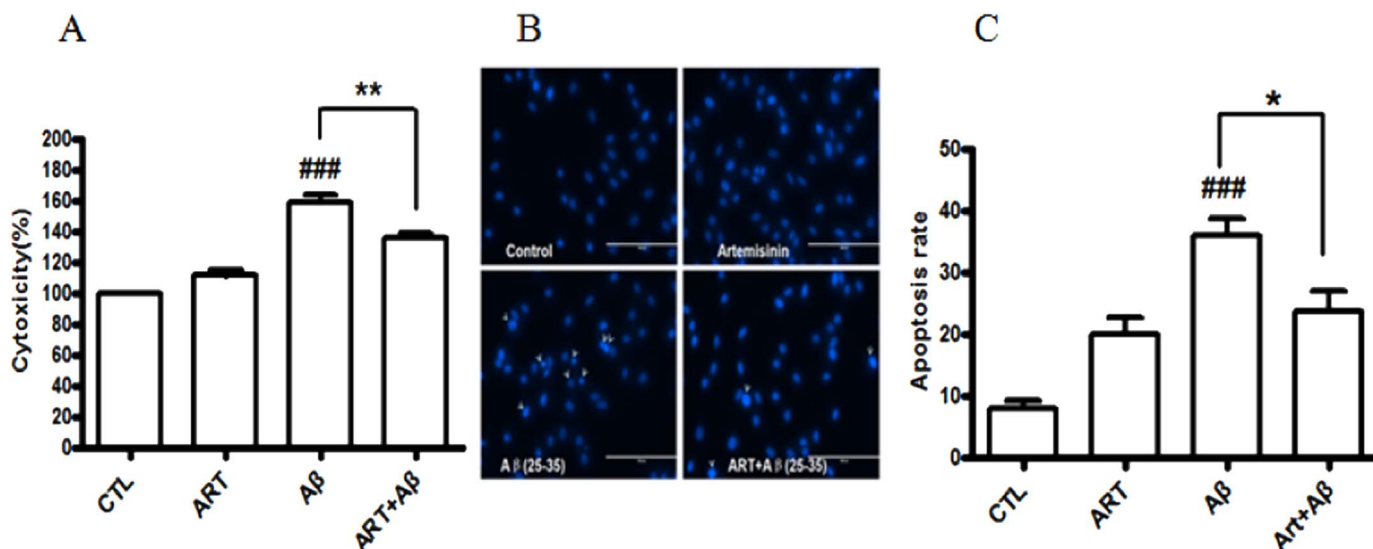


Fig. 2. Artemisinin suppressed A β 25-35-induced LDH release and apoptosis in PC12 cells. After pre-treatment with 25 μ M artemisinin or 0.1% DMSO (vehicle control) for 1 h, PC12 cells were incubated with or without 0.3 μ M A β 25-35 for another 24 h the release of LDH (A) was measured by LDH assay and the apoptosis cells (N=3) (B) was detected by staining with Hoechst 33342 and visualized by fluorescence microscopy. The number of apoptotic nuclei with condensed chromatin (C) was counted from the photomicrographs and presented as a percentage of the total number of nuclei (N=3). ###P < 0.005 versus control group; *P < 0.05, **P < 0.005 versus the A β -treated group were considered statistically significant differences.

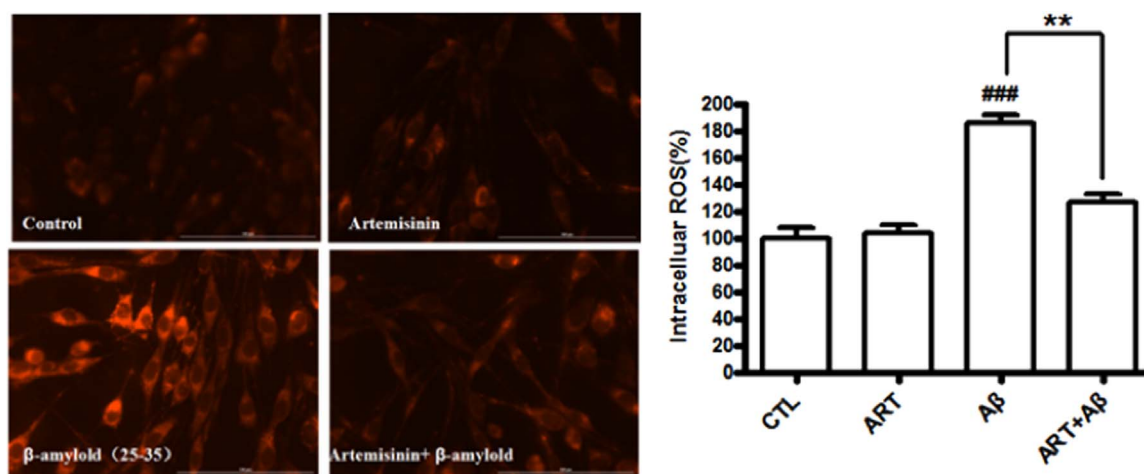


Fig. 3. Artemisinin reduced the increase of A β 25-35-induced oxidative stress in PC12 cells. After pre-treatment with 25 μ M artemisinin or 0.1% DMSO (vehicle control) for 1 h, PC12 cells were incubated with or without 0.3 μ M A β 25-35 for another 24 h. Intracellular ROS level was determined by the CellROXs Deep Red Reagent (N=3). ###P < 0.005 versus control group; **P < 0.01 versus A β -treated group was considered significantly different.

Super Signal West Pico chemiluminescent substrate was purchased from Thermo Scientific (Rockford, IL, USA). Gibco fetal bovine serum (FBS) and penicillin-streptomycin (PS) were purchased from Life Technologies (Grand Island, NY, USA).

2.2. Cell culture

PC12 cells were kindly provided by Dr. Gordon Guroff (National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD). Cells were cultured in 75-cm² flasks in DMEM supplemented with 5% fetal bovine serum (FBS), 5% horse serum, 100 μ g/ml streptomycin, 100 U/ml penicillin and incubated at 37 °C with 5% CO₂ humidified atmosphere. The medium was replaced every 2–3 days, and cells were sub-cultured by trypsin treatment twice a week, at a split of 1:4. Cells were seeded into 96-well, 48-well, or 6-well plates coated with 10 μ g/ml poly-D-lysine and allowed to grow for at least 24 h.

2.3. MTT assay

PC12 cells were seeded in 96-well plates (coated with 10 μ g/ml poly-D-lysine) at a density of 4–8 \times 10⁵ cells/well in 1% serum medium for 24 h as previously described [20]. After serum starvation, the cultures were incubated with drugs or inhibitors for appropriate time, and then treated with A β ; 24 h later MTT assay was performed. Then the cells were incubated with MTT (0.5 mg/ml) for additional 3 h. The medium was aspirated from each well and DMSO (200 μ l) (Sigma, USA) was added. The absorbance of each well solution was obtained with a Multiskan Ascent Revelation Plate Reader (Thermo, USA) and the data are presented as Optical Density (OD) at wavelength of 570 nm. Assays were repeated at least three to six times in quadruplicate.

2.4. LDH assay

Cell cytotoxicity was measured by lactate dehydrogenase (LDH) released into the incubation medium when cellular membranes were destroyed. PC12 cells were seeded into 96-well plates (5 \times 10³ cells/-

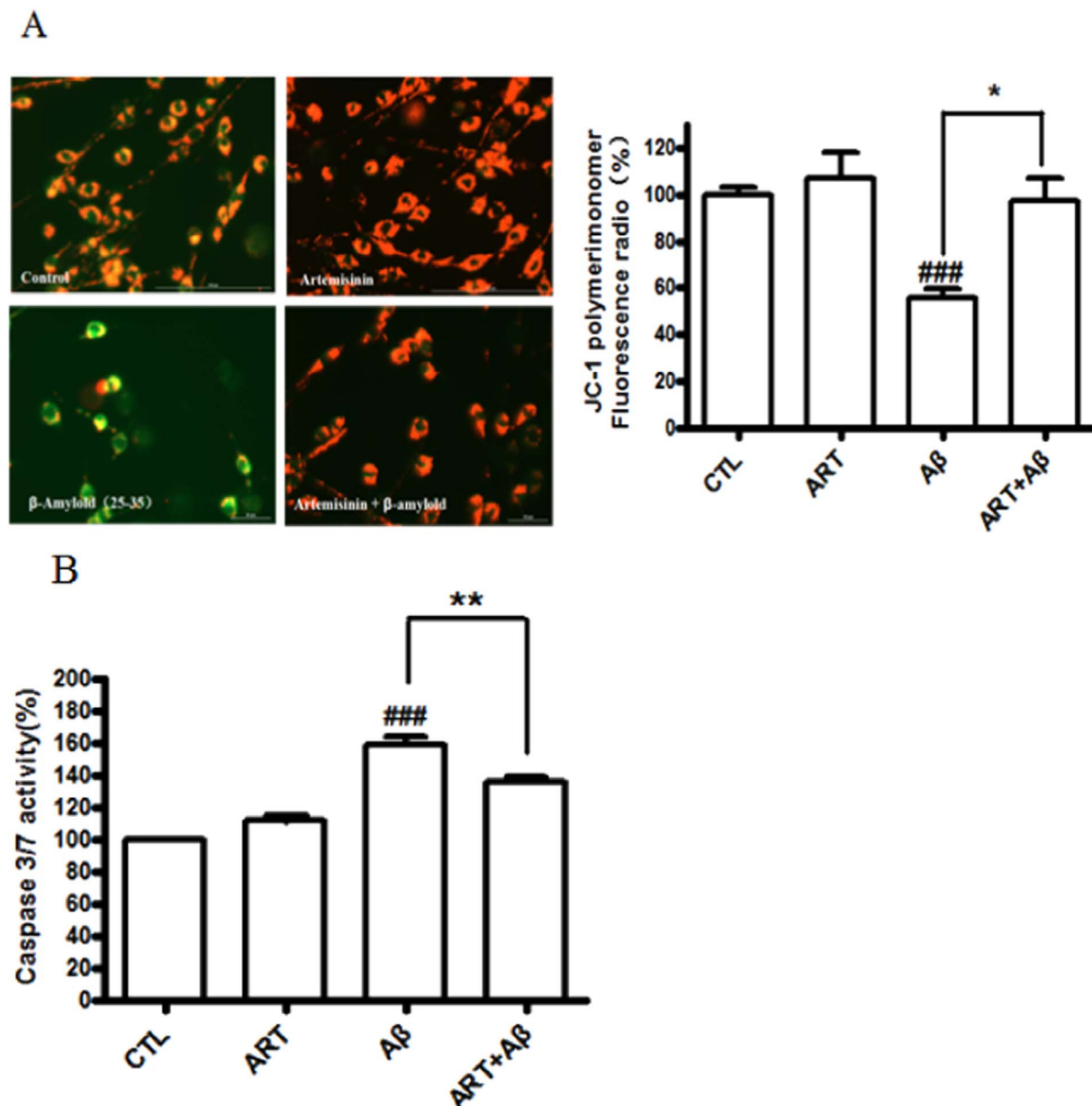


Fig. 4. Artemisinin attenuated Aβ-induced mitochondrial membrane potential ($\Delta\psi_m$) loss and caspase 3/7 activity increase in PC12 cells. After pre-treatment with 25 μ M artemisinin or 0.1% DMSO (vehicle control) for 1 h, PC12 cells were incubated with or without 0.3 μ M A β 25-35 for another 24 h, $\Delta\psi_m$ was determined by the JC-1 assay (N=3) (Fig. 4A) and quantification of caspase 3/7 activity was determined by caspase 3/7 activity assay (N=3) (Fig. 4B). ###P < 0.005 versus control group; *P < 0.05, **P < 0.01 versus A β -treated group were considered significantly different.

well). After appropriate treatment, the activity of released LDH in the medium was determined according to instructions of CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega, USA). The fluorescent intensity was measured using Infinite M200 PRO Multimode Microplate at an excitation wavelength of 560 nm and emission at 590 nm. All values of % LDH released were normalized to the control group.

2.5. Detection of apoptotic nuclei by Hoechst 33342 staining

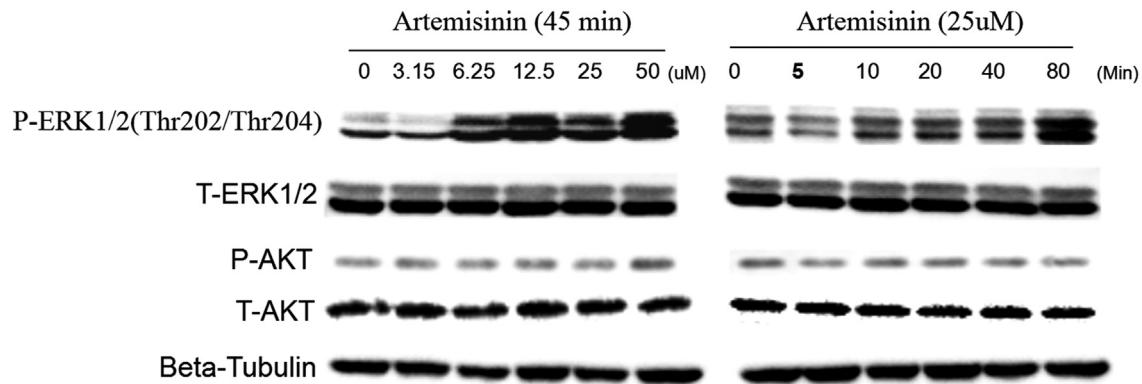
Apoptosis of cells was examined by staining with the DNA binding dye, Hoechst 33342 as previously described [21]. PC12 cell cultures were fixed with 4% formaldehyde in PBS for 10 min at 4 °C. Cells were then incubated with 10 μ g/ml of Hoechst 33342 for 20 min to stain the nuclei. After washing with PBS for two times, the apoptotic cells were observed under a Fluorescence microscope (Olympus, Japan). Cells

exhibiting condensed chromatin or fragmented nuclei were scored as apoptotic cells. For each Hoechst staining experiment, at least 200 cells in 5 random fields were collected and quantified. The data is presented as percentage of apoptotic cells (apoptotic cells/total cells \times 100).

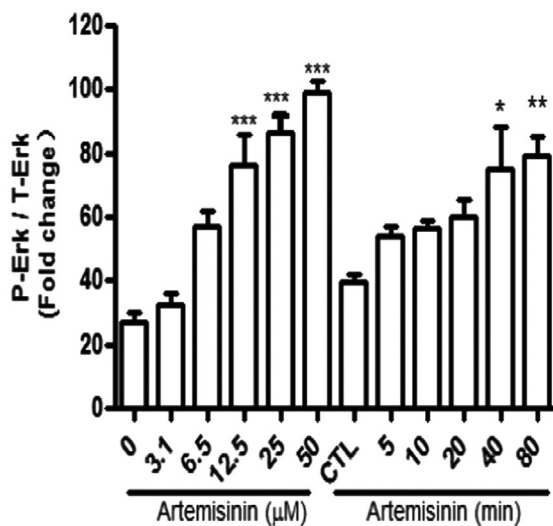
2.6. Measurement of intracellular ROS levels

Intracellular ROS generation was assessed using CellROXs Deep Red Reagent (Thermo Fisher Scientific, USA). After appropriate treatment, the PC12 cells were incubated with CellROXs Deep Red Reagent (5 mM) in DMEM for 1 h in the dark, rinsed twice with 1x PBS solution and the fluorescence was observed and recorded using a fluorescent microscope at an excitation wavelength of 640 nm and an emission wavelength of 665 nm. Semiquantification of ROS level was evaluated by using Image-J software. All values of % ROS level were normalized to the control group.

A



B



C

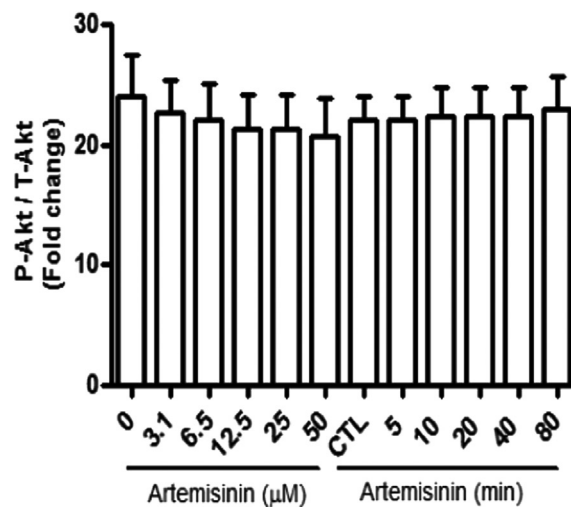


Fig. 5. Artemisinin stimulated the phosphorylation of ERK1/2 in a time- and concentration-dependent manner in PC12 cells. (A) The PC12 cells were collected with artemisinin treatment for different times (0, 5, 10, 20, 40 and 80 min) at 25 μ M, or at different concentrations (3.15, 6.25, 12.5, 25 and 50 μ M) for 45 min. The expression of phosphorylated ERK1/2, total ERK1/2, phosphorylated Akt, total Akt and beta-tubulin (A) were detected by Western blotting with specific antibodies. Quantification of representative protein band from Western blotting (B and C $N=3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$ versus the control group was considered significantly different.

2.7. Measurement of mitochondrial membrane potential ($\Delta\psi_m$)

JC-1 dye was used to monitor mitochondrial integrity. In brief, PC12 cells were seeded into black 96-well plates (1×10^4 cells/well). After appropriate treatment, the PC12 cells were incubated with JC-1 (10 μ g/ml in medium) at 37 $^{\circ}$ C for 15 min and then washed twice with PBS. For signal quantification, the intensity of red fluorescence (excitation 560 nm, emission 595 nm) and green fluorescence (excitation 485 nm, emission 535 nm) were measured using a Infinite M200 PRO Multimode Microplate. Mitochondrial membrane potential ($\Delta\psi_m$) was calculated as the ratio of JC-1 red/green fluorescence intensity and the value was normalized to the control group. The fluorescent signal in the cells was also observed and recorded with a fluorescent microscope.

2.8. Caspase 3/7 activity assay

After treatment, the activity of caspase 3/7 was measured using the commercially available Caspase-Glo^{3/7} Assay (Invitrogen, USA) according to the manufacturer's protocol. Briefly, PC12 cells were lysed in lysis buffer and centrifuged at 12,500 \times g for 5 min. 15 ml of cell lysate was incubated with 50 ml of 2X substrate working solution at room temperature for 30 min in 96-well plates. The fluorescence

intensity was then determined by Infinite M200 PRO Multimode Microplate at an excitation wavelength of 490 nm and emission at 520 nm. The fluorescence intensity of each sample was normalized to the protein concentration of sample. All values of % caspase 3/7 activities were normalized to the control group.

2.9. Western blotting

Western blotting was performed as previously described [22]. Cells from different experimental conditions were lysed with ice-cold RIPA lysis buffer. Protein concentration was measured with a BCA protein assay kit according to the manufacturer's instructions. Samples with equal amounts of proteins were separated on 10% polyacrylamide gels, then were transferred to PVDF membrane, and probed with selective antibody respectively. The intensity of the bands was quantified using ImageJ software.

2.10. Data analysis and statistics

Statistical analysis was performed using GraphPad Prism 5.0 statistical software (GraphPad software, Inc., San Diego, CA, USA). All experiments were performed in triplicate. Data are expressed as

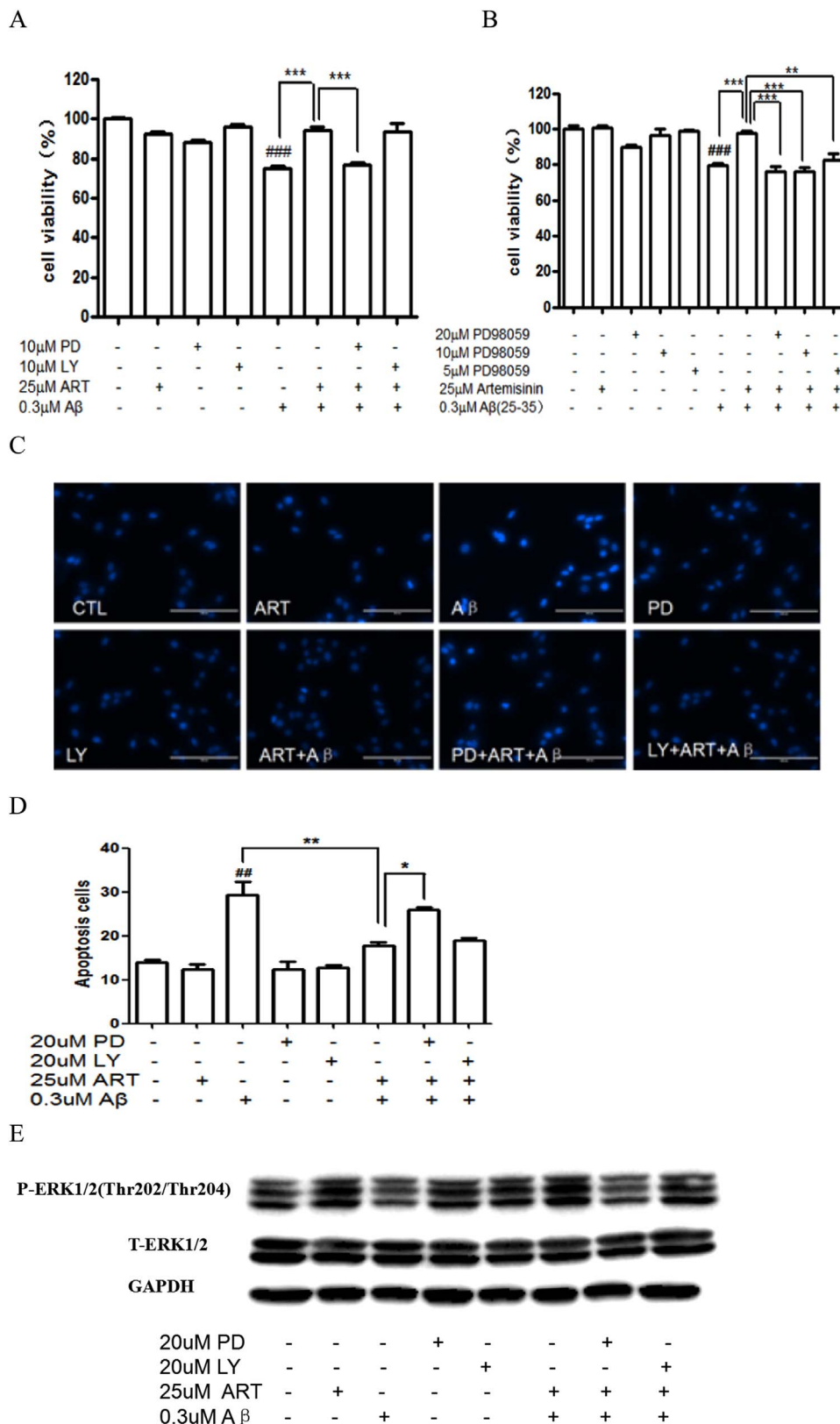


Fig. 6. ERK 1/2 pathway mediated the protect effects of artemisinin in PC12 cells. PC12 cells were pre-treated with 10 μM PD98059 or 10 μM LY294002 (A), or 5, 10, 20 μM PD98059 (B) for 30 min, and treated with 25 μM artemisinin for 1 h, then incubated with or without 0.3 μM Aβ25-35 for a further 24 h, and the cell viability was determined by MTT assay (N = 3). PC12 cells were pre-treated with the ERK1/2 inhibitor PD98059 (20 μM) or PI3K inhibitor LY294002 (20 μM) for 30 min, and afterward treated with 25 μM artemisinin for 1 h, then incubated with or without 0.3 μM Aβ25-35 for a further 24 h; the apoptosis cells was detected by staining with Hoechst 33342 and visualized by fluorescence microscopy (C). The number of apoptotic nuclei with condensed chromatin was counted from the photomicrographs and presented as a percentage of the total number of nuclei (N = 3) (D). PC12 cells were pre-treated with the ERK1/2 inhibitor PD98059 (20 μM) or PI3K inhibitor LY294002 (20 μM) for 30 min, and afterward treated with 25 μM artemisinin for 1 h, then incubated with or without 0.3 μM Aβ25-35, The expression of phosphorylated ERK1/2, total ERK1/2; and GAPDH were detected by Western blotting with specific antibodies (N = 3). ###P < 0.005 versus control group; **P < 0.01, ***P < 0.005 versus the artemisinin or artemisinin plus Aβ25-35-treated group as indicated were considered significantly different.

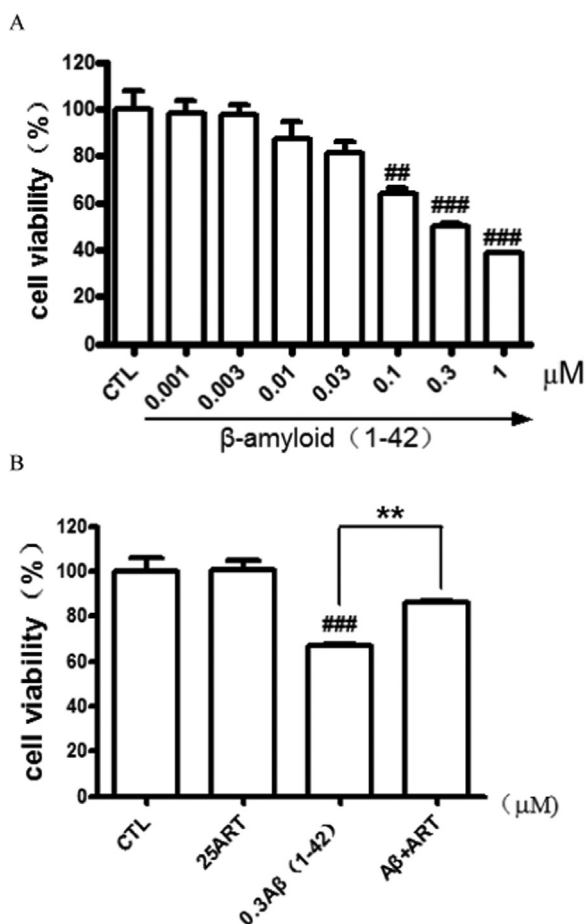


Fig. 7. Artemisinin suppressed A β 1-42-induced cell viability loss in PC12 cells. (A) Cells were treated with A β 1-42 (0.001–1 μ M) or 0.1% DMSO (vehicle control) for 24 h and cell viability was measured using the MTT assay. (B) Cells were pre-treated with artemisinin (25 μ M) or 0.1% DMSO (vehicle control) for 1 h and then incubated with or without 0.3 μ M A β 1-42 for a further 24 h and cell viability were measured by MTT assay (N = 3). ###P < 0.005 versus control group; **P < 0.01 versus the A β 1-42 treated group was considered statistically differences.

mean \pm standard deviation (SD). Statistical analysis was carried out using one-way ANOVA followed by Tukey's multiple comparison, with $p < 0.05$ considered statistically significant.

3. Results

3.1. Artemisinin concentration-dependently suppressed A β 25-35 induced cytotoxicity in PC12 cells

A β -induced apoptosis in PC12 cells was a common and reliable cellular toxicity model for AD related studies in vitro [23–25]. We first tested the cytotoxicity of the most usual used peptide A β 25–35 (active component of A β peptides) on PC12 cells by MTT assay in our laboratory conditions. As shown in Fig. 1B, exposure of cells to different concentrations of A β 25–35 for 24 h resulted in a notable decrease of the cell viability in a concentration-dependent manner, which indicated that A β 25–35 could induce toxicity in PC12 cells. The toxicity was observed in the lowest dose of 0.03 μ M and maximal in 3–10 μ M. 0.3 μ M A β 25–35 was chosen in following experiment because of the 30–40% cell death was observed.

To evaluate the protective effects of artemisinin, PC12 cells were treated with artemisinin for 1 h before exposure to A β 25-35 for 24 h. The result of MTT assay (Fig. 1C) revealed that the treatment of 0.3 μ M A β 25-35 resulted in dominant cell death (43%), whereas pretreatment with 12.5 and 25 μ M of artemisinin significantly attenuated A β 25-35-

induced cell death. To clarify whether artemisinin could rescue cell from toxicity of A β 25-35, PC12 cells were incubated with 0.1, 0.3, 1 μ M A β 25-35 for 30 min and post-treated with artemisinin (25 or 50 μ M) for 24 h. The results (Fig. 1D) demonstrated that artemisinin not only can protected but also rescue PC12 cells against A β 25-35-induced cell death. The protective activity of artemisinin was also confirmed by the lactate dehydrogenase (LDH) assay. As shown in Fig. 2A, pre-treated cells with 25 μ M of artemisinin for 1 h significantly reduced A β 25-35-induced LDH leakage (from 160% to 135%). Nuclei condensation was observed in PC12 cells after exposure to A β 25-35 in Hoechst 33342 staining assay (Fig. 2B). However, a pretreatment of 25 μ M artemisinin definitely improved these changes (from 36% to 23%) (Fig. 2C).

3.2. Artemisinin prevented A β 25-35 induced ROS production in PC12 cells

Previous studies reported that the toxicity of A β 25-35 was mediated through the generation of ROS and our group also reported artemisinin can quench the ROS production [26–28]. Therefore, we investigated whether artemisinin blocked A β 25-35-induced oxidative stress in PC12 cells. Cellular oxidative stress was determined by the CellROXs Deep Red Reagent. PC12 cells were pretreated with or without 25 μ M artemisinin for 1 h and then treated with A β 25-35 for 24 h. As expected (Fig. 3), artemisinin significantly reduced the intracellular production of ROS induced A β 25-35 (from 185.4% to 126.6%).

3.3. Artemisinin restored A β 25-35 induced change of mitochondrial membrane potential and caspase 3/7 activity

Mitochondrial inhibition lead to the loss of mitochondrial membrane potential ($\Delta\psi_m$) involved in the progression of AD and neuron apoptosis caused by A β 25-35 [29–31]. We elucidated whether artemisinin could reduce A β 25-35-induced $\Delta\psi_m$ loss in further study. The $\Delta\psi_m$ in PC12 cells was assessed by analyzing the red/green fluorescent intensity ratio of JC-1 staining. The results (Fig. 4A) revealed that 25 μ M artemisinin pretreatment significantly prevented the declined of mitochondrial membrane potential induced by A β 25-35 (from 55.4% to 97.1%). Caspase 3/7 activation is a main biomarker in the apoptosis progress of neuronal cells [32]. As shown in Fig. 4B, treatment of cells with 0.3 μ M A β 25-35 for 24 h increased caspase 3/7 activity to 159% compared to the control group. In contrast, pre-treatment with artemisinin significantly reduced caspase 3/7 activation to 135%.

3.4. Artemisinin stimulated the phosphorylation of ERK1/2 in a time- and concentration-dependent manner in PC12 cells

It was widely accepted that ERK pathway phosphorylation is a key biochemical event responsible for cell survival and apoptosis [33,34]. Previous studies indicated that artemisinin was able to activate ERK/CREB signaling in various cells [17,18,28,35,36]. We therefore tested whether ERK and Akt pathways are involved in artemisinin-induced neuroprotective effects in PC12 cells. As shown in Fig. 5A–C, phosphorylation of ERK1/2 but not Akt, gradually increased after the addition of artemisinin in a time- and dose-dependent fashion.

3.5. ERK 1/2 pathway mediated the protect effects of artemisinin in PC12 cells

We have previously reported that ERK1/2 pathway rather than PI3K pathway mediated the neuroprotective effects of artemisinin in cells against H₂O₂ or NO insult [18,28]. In order to examine whether the up regulation of ERK1/2 phosphorylation is involved in the survival promoting effect of artemisinin on cell apoptosis induced by A β 25-35, we investigated the effects of specific inhibitors for the ERK1/2 and PI3K pathways (the ERK1/2 pathway inhibitor PD98059 and PI3K inhibitor LY294002) and we found that the ERK1/2 pathway inhibitor blocked the effects of artemisinin while LY294002, a PI3K kinase

inhibitor had no effect (Fig. 6A). To further confirm the effects of ERK/1/2 pathway inhibitor PD98059 in more detail, the cultures preincubated with different concentrations of the ERK inhibitor PD98059 (5, 10, 20 μM) for 30 min were treated with artemisinin (25 μM) for 1 h, and the viability of cells was determined by the MTT assay after another 24 h. As shown in Fig. 6B, the protective effect of artemisinin was blocked in the presence of appropriate concentration of ERK inhibitor PD98059. Nuclei condensation was observed in PC12 cells treated with A β 25–35 along in Hoechst 33342 staining assay, while pretreatment of cells with artemisinin (25 μM) for 1 h reversed the effect of A β 25–35 (Fig. 6C). However, a pretreatment of PD98059 definitely abolished the effect of artemisinin (Fig. 6D). Western blot in Fig. 6E showed that A β 25–35 was able to significantly down regulation the phosphorylation of ERK1/2, and artemisinin reversed decreased phosphorylation of ERK1/2, while PD98059 pretreatment blocked the reversed effects of artemisinin. The concentrations of inhibitors themselves used in the current experiments, had no effect on the cell death as shown. Thus these data put together suggested that the ERK1/2 pathway rather than the Akt pathway is mediating the protective effect of artemisinin.

Because A β 25–35 is a shorter toxic fragment (active component of A β 1–42), people will desire to see the effect of A β 1–42 itself on the PC12 cells and the protection of artemisinin on the toxic effect of A β 1–42. Similarly, as shown in Fig. 7A, cells exposed to A β 1–42 for 24 h resulted in a remarkable cell viability reduction, and as shown in Fig. 7B, artemisinin significantly protected PC12 cells from toxicity of A β 1–42.

4. Discussion

At present study, we investigated protective effect of artemisinin on the toxic effect of beta amyloid on neuronal PC12 cells. Our results at the first time, showed that artemisinin, at therapeutic relevant concentration, protected and rescued neuronal cells from toxicity of beta amyloid peptides. Further experiments with various kinase inhibitors showed that the effect of artemisinin was mediated with the ERK1/2 pathway.

AD is a common and devastating neurodegenerative disorder, and its hallmark pathologic characteristics are β -amyloid plaques, neurofibrillary tangles and neuron progressive death [37]. Currently, due to rapidly aging populations, the prevalence of AD is growing exponentially and it becomes a huge burden on society and patients' families [38,39]. However, the pathogenesis of AD has not yet clearly been explained despite great progress in basic research, and there remains a dearth of effective treatments and cures. It is widely accepted that the formation and deposition of A β is one of the main typical pathological characteristics of AD brain [40]. A β is an amino acid peptide fragment derived by proteolysis from the integral membrane protein known as A β precursor protein [41]. The neurotoxicity of A β , including different A β fragments, has been widely reported [6]. Therefore how to inhibit the cell apoptosis induced by A β was attracting considerable and increasing concern of the public.

Recent reports indicate that artemisinin has neurotrophic and neuroprotective effects in PC12 cells [17,18]. Firstly, artemether, a lipid-soluble derivative of artemisinin has been reported to possess anti-inflammatory properties by activated Nrf2 activity [16]. As we know, neuroinflammation played a major role in the formation of A β ; besides, activated Nrf2 activity also shows benefits to AD in reports [42]. In addition, the reports have showed that artemisinin decreased BACE1 expression, and suppressed NF- κ B activity and NALP3 activation [43]. Furthermore, it just reported that artemisinin was able to modulate GABA (A) receptor signaling, while the GABA(A) receptor signaling was seriously altered in aging and AD [44,45]. In our present experiment, we explored whether artemisinin treatment could attenuate PC12 cells apoptosis due to A β 25–35 exposure. In line with other reports, we found that A β 25–35 was cytotoxicity to PC12 cells and showed a concentration-dependent manner [27]. In addition, data from Hoechst

33342 staining assay showed that the A β 25–35 also induced the apoptosis of PC12 cells. When pretreated with artemisinin, the cell viability of PC12 cell incubated with A β 25–35 was strikingly increased (Fig. 1). Additionally, artemisinin also attenuated the apoptosis in PC12 cells induced by A β 25–35, which further implies that artemisinin confer protective effects on PC12 cells insult with A β 25–35 (Fig. 2).

It's widely accepted that ROS was one of the indicators of oxidative stress and associated with a large array of neurological diseases and brain ageing itself [10]. Reports confirmed that the presence of diffuse ROS resulted in oxidative stress of the entire brain in AD cases [46]. Our previous study showed that H₂O₂ increased the oxidative stress injury through elevating ROS production and artemisinin significantly attenuated H₂O₂-induced ROS accumulation [28]. Here, we found that A β 25–35 resulted in the collapse of the $\Delta\psi\text{m}$ and increase of ROS in PC12 cells while pretreatment of artemisinin significantly suppress these abnormal changes in PC12 cells (Fig. 3). The decrease of cell viability and the increase of LDH release caused by A β 25–35 were significantly suppressed by artemisinin, suggesting that antioxidant activity of artemisinin contributes to its protective effects.

Increased mitochondrial DNA damage and protein loss of electron transport chain in neuron have been found in AD patients, suggesting that mitochondrial dysfunction plays a key role in the process of AD [10,29]. Mitochondrial apoptotic pathway due to mitochondria deficiency plays a crucial role in the pathogenesis of AD [47]. It has been reported that strategies to block mitochondrial apoptotic pathway are potential therapeutics to prevent cells death. In this study, our results showed that A β 25–35-induced $\Delta\psi\text{m}$ loss could be suppressed by pretreatment with artemisinin. The increase of intracellular ROS induced by A β 25–35, leads to damage to the mitochondrial membrane and results in the collapse of the $\Delta\psi\text{m}$ and activation of the apoptotic cascade. Pre-treatment with artemisinin was able to suppress the activation of caspase 3/7 (Fig. 4).

ERK1/2 pathway is a key signaling component that plays a crucial role in the initiation and regulation of most of stimulated cellular processes such as proliferation, survival and apoptosis [34]. This pathway is reported as a key signaling to induce cellular antioxidant mechanism [48]. Our data suggest that activation of ERK 1/2 pathway plays a critical role in artemisinin-induced neuron protective effects against A β 25–35 insult; artemisinin stimulated phosphorylation of the ERK1/2 (Fig. 5), which is consistent with activation of the ERK1/2 classes, artemisinin triggered neuroprotective effects against cell viability lose and cell apoptosis was attenuated by specific pharmacological inhibitors of MEK, an upstream kinase of ERK1/2 kinase (Fig. 6). Therefore, these results provide mechanistic evidence to support the notion that artemisinin-regulated protective effects against A β 25–35 injury occur via ERK 1/2 activation.

In summary, our findings demonstrate that artemisinin is able to reduce A β -induced insult in PC12 cells through the regulation of multiple mechanisms including (1) suppressing the LDH release; (2) restraining the production of intracellular ROS; (3) modulating $\Delta\psi\text{m}$ and caspase 3/7 dependent pathway; (4) activating ERK1/2 signaling. The protective effects of artemisinin to attenuate A β -mediated intrinsic mitochondrial apoptotic pathway are, at least in part, mediated via the activation of ERK1/2 kinase signaling rather than Akt signaling. Our results offer support for the potential development of artemisinin to prevent neuronal cells death in the process of AD, although the effects of artemisinin in vivo required further investigations.

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