



## Protective effect of pyrroloquinoline quinone against A $\beta$ -induced neurotoxicity in human neuroblastoma SH-SY5Y cells

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### ABSTRACT

The neurotoxicity of aggregated  $\beta$ -amyloid (A $\beta$ ) has been implicated as a critical cause in the pathogenesis of Alzheimer's disease (AD). It can cause neurotoxicity in AD by evoking a cascade of oxidative damage-dependent apoptosis to neurons. In the present study, we for the first time investigated the protective effect of pyrroloquinoline quinone (PQQ), an anionic, water soluble compound that acts as a redox cofactor of bacterial dehydrogenases, on A $\beta$ -induced SH-SY5Y cytotoxicity. A $\beta_{25-35}$  significantly reduced cell viability, increased the number of apoptotic-like cells, and increased ROS production. All of these phenotypes induced by A $\beta_{25-35}$  were markedly reversed by PQQ. PQQ pretreatment recovered cells from A $\beta_{25-35}$ -induced cell death, prevented A $\beta_{25-35}$ -induced apoptosis, and decreased ROS production. PQQ strikingly decreased Bax/Bcl-2 ratio, and suppressed the cleavage of caspase-3. These results indicated that PQQ could protect SH-SY5Y cells against  $\beta$ -amyloid induced neurotoxicity.

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Alzheimer's disease (AD) is neuropathologically characterized by deposition of  $\beta$ -amyloid (A $\beta$ ) 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 A $\beta$ -induced oxidative stress plays an important role in the pathogenesis or progression of AD. Excessive accumulation of A $\beta$  in the brain is a possible cause of neurodegeneration [12], although the precise mechanism by which A $\beta$  induces neuronal death are not well understood [15]. One putative mechanism implicates the production of reactive oxygen species (ROS) [5]. Indeed, several studies have shown that oxidative stress is involved in apoptotic mechanisms where excessive ROS production can lead to neuronal apoptosis, as in neurodegenerative disorders such as AD [9,19]. And now it is generally accepted that A $\beta$  can contribute to neurodegeneration and neuronal loss during the course of AD [17].

Thus, one promising preventive or therapeutic intervention in AD may attenuate A $\beta$ -mediated neurotoxicity. Recently, considerable attention has been focused on identifying naturally occurring antioxidants that are able to protect against A $\beta$ -mediated cytotoxicity. Many antioxidants, including ginkgo biloba [24], huperzine A [36], curcumin [28], and Ginsenoside Rg1 [35] have been demonstrated to inhibit A $\beta$ -induced neurotoxicity.

Pyrroloquinoline quinone (PQQ), an anionic, water soluble compound, was initially isolated from cultures of methylotrophic bacteria as a crystalline acetone adduct and was proposed to be a

cofactor of many bacterial primary alcohol dehydrogenases [29]. Since the discovery of PQQ, it has been identified in various vegetables, fruits, milk and even tissues of mammalian animal [21,22]. As an essential antioxidant or nutrient, PQQ has been drawing attention from both the nutritional and the pharmacological viewpoint. Recently, it has been proposed that PQQ can be classified as a new B vitamin [20].

Previous studies have shown that PQQ possesses many pharmacological activities, including anti-inflammatory [10], hepatoprotective [34], cardioprotective [42], and antioxidative activities [14,27,31,40]. Of note, recent studies have showed that PQQ has neuroprotective activity. PQQ has been reported to prevent neuronal cell death in a stroke model [2,14,18,40,41]. PQQ was also reported to be effective in an animal model of epilepsy [30]. PQQ has been demonstrated to be neuroprotective *in vitro* against NMDA-mediated neurotoxic injury [1], and against methylmercury-induced neurotoxicity [39]. Moreover, it has been reported that PQQ is a potent neuroprotective nutrient against 6-hydroxydopamine-induced neurotoxicity [11].

Although PQQ exhibited neuroprotective activity, the protective effect of PQQ against A $\beta$ -induced neuronal cell death has not been reported. This study investigated the possible protective effects of PQQ on the A $\beta$ -induced apoptotic cell death in cultured SH-SY5Y cells.

Hoechst 33258 and ECL detection kit were purchased from Beyotime (Beijing, China). Modified Dulbecco's Eagle's medium (DMEM) supplement was from Gibco. Complete protease inhibitor was from Roche. Antibodies against Bcl-2 and Bax were from Santa Cruz. Antibodies against cleaved caspase-3 were from Calbiochem

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(San Diego, CA, USA). All the other chemicals used were purchased from Sigma, unless otherwise stated.

Human neuroblastoma SH-SY5Y cells were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin at 37 °C and 5% CO<sub>2</sub> [16]. To induce cell injury, cells were incubated with 25  $\mu$ M A $\beta$ <sub>25–35</sub> for 24 h. To study the effects of PQQ, cells were pre-incubated with PQQ for 1.0 h, and then A $\beta$ <sub>25–35</sub> was added to the medium for an additional 24 h. A $\beta$ <sub>25–35</sub> 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 and stored frozen at –20 °C until use [8].

Cell viability was assessed using MTT reduction assay. Apoptosis of SH-SY5Y cells was analyzed by Hoechst staining [8]. Nuclear morphology was viewed using a fluorescence microscope. The number of cells with apoptotic morphology appearing condensed or fragmented nuclei was counted. The intracellular ROS was analyzed by H<sub>2</sub>DCF-DA assay [23]. Briefly, following treatment, the cells were loaded with H<sub>2</sub>DCF-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. Changes in the expression levels of Bcl-2 and Bax, and the induction of caspase-3 were assessed by western blotting.

All data were presented as the mean  $\pm$  S.E.M. Data were subjected to statistical analysis via one-way ANOVA followed by Student's *t*-test. Mean values were considered to be statistically significant at *P* < 0.05.

MTT assay was used to test the toxicity of PQQ to SH-SY5Y cells. PQQ at each of these concentrations (1–20  $\mu$ M) alone did not cause any apparent cytotoxicity (Fig. 1A). The A $\beta$ <sub>25–35</sub>-induced cytotoxicity was first evaluated by the MTT reduction assay. As shown in Fig. 1B, A $\beta$ <sub>25–35</sub> significantly decreased the cell viability. However, the cytotoxic effects were attenuated by the pretreatment with PQQ, 10 and 20  $\mu$ M PQQ significantly blocked cytotoxic effects of A $\beta$ <sub>25–35</sub> on cell viability.

Apoptotic nuclei are characterized by condensed nuclear and apoptotic bodies. Antiapoptotic properties of PQQ were verified by quantitation of Hoechst stained apoptotic nuclei from three independent experimental sets. Hoechst staining showed increased number of apoptotic cells following A $\beta$ <sub>25–35</sub> exposure for 24 h, which was few in the control group (Fig. 2A). Preincubation with 10  $\mu$ M PQQ decreased the number of apoptotic cells compared to cells treated with A $\beta$ <sub>25–35</sub> alone (Fig. 2B). These results indicated that PQQ suppresses A $\beta$ <sub>25–35</sub>-induced DNA damage in SH-SY5Y cells.

To determine the generation of intracellular ROS induced by 25  $\mu$ M A $\beta$ <sub>25–35</sub> for 24 h, we performed flow cytometry analysis using the ROS-sensitive fluorescence probe, DCF. As shown in Fig. 3, exposure to A $\beta$ <sub>25–35</sub> caused an elevation of the intracellular ROS levels which was about 2.0-fold relative to that of control cells. Pretreatment with PQQ suppressed the intracellular ROS elevation. These results indicated that PQQ has the ability to scavenge ROS induced by A $\beta$ <sub>25–35</sub>.

The Bcl-2 family includes antiapoptotic members such as Bcl-2, and proapoptotic members such as Bax. The ratio of Bax to Bcl-2 has been reported to be correlated with apoptosis. Our results showed that exposure cells to A $\beta$ <sub>25–35</sub> induced a robust increase in the protein level of Bax, and a strong change in the protein level of Bcl-2, and the ratio of Bax/Bcl-2 expression increased approximate 2.3-fold in A $\beta$ <sub>25–35</sub> treatment alone compared with the control using western blot analysis (Fig. 4A and B). While, PQQ pretreatment could prevent the A $\beta$ <sub>25–35</sub>-induced increase of the Bax/Bcl-2 ratio (Fig. 4A and B). The effect of PQQ on A $\beta$ <sub>25–35</sub>-induced apoptosis may be, at least in part, mediated by regulating of Bcl-2 and Bax expression.

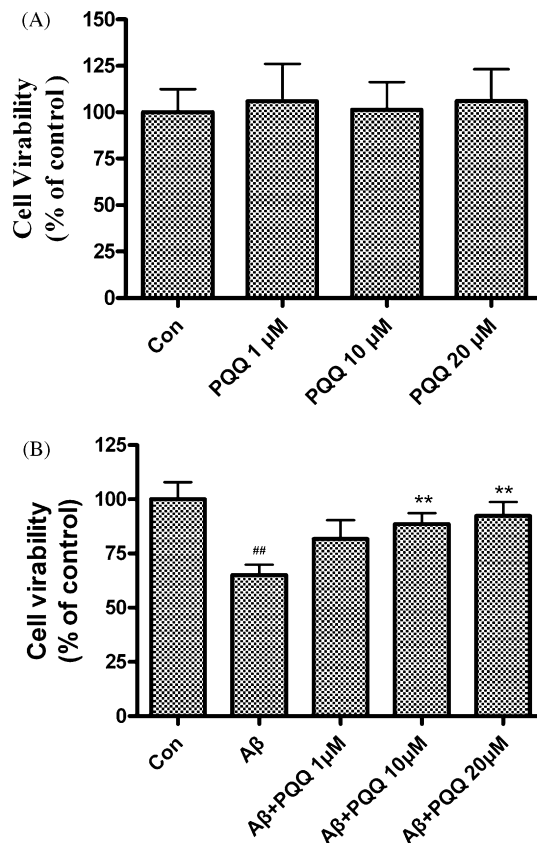
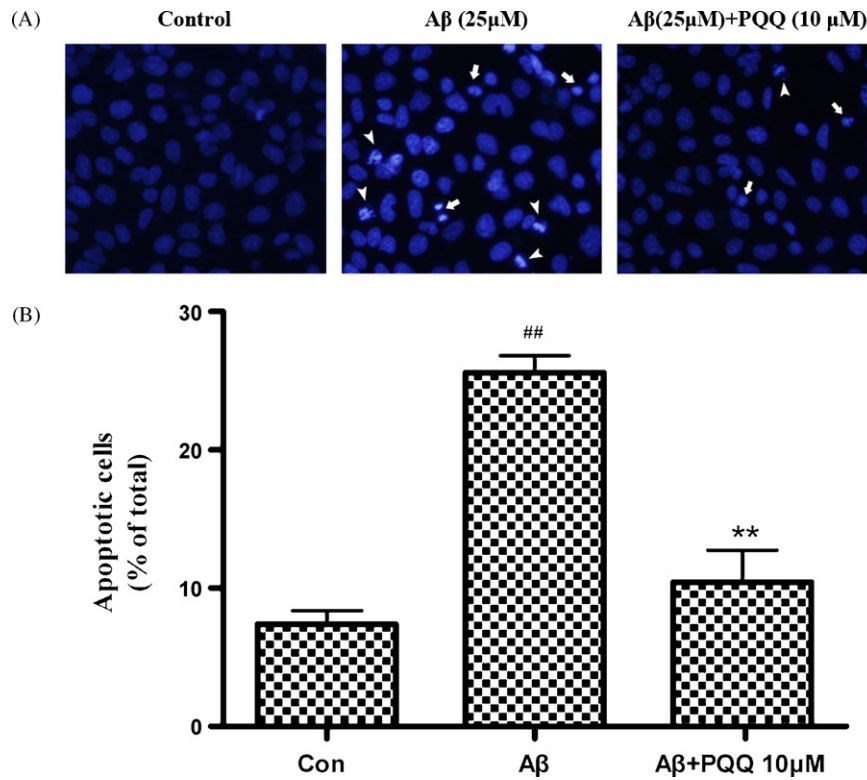


Fig. 1. PQQ inhibits A $\beta$ <sub>25–35</sub>-induced neurotoxicity. SH-SY5Y cells were incubated without or with PQQ (1, 10, 20  $\mu$ M) for 1.0 h, followed by incubation with A $\beta$ <sub>25–35</sub> for another 24 h. After this incubation, cell viability was determined with the MTT assay. Results are shown as the mean  $\pm$  S.E.M. and represent three independent experiments. ### *P* < 0.001 vs. control group; \*\* *P* < 0.01 vs. A $\beta$ <sub>25–35</sub>-treated-alone group.

Next we detected the induction 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 has been suggested to play an important role in A $\beta$ -induced apoptosis [3]. As shown in Fig. 5, the expression level of cleaved caspase-3 was increased after exposed to A $\beta$ <sub>25–35</sub>, more than 2.6-fold increase compared with control group, but the activated caspase-3 protein level was declined in PQQ pretreatment groups. This result indicated that PQQ can inhibit the induction of caspase-3, mirroring the antiapoptotic effect of PQQ against A $\beta$ <sub>25–35</sub>-induced apoptosis by Hoechst staining.

Substantial evidence indicates that excess of A $\beta$ , which aggregates into toxic fibrillar deposits, plays a central role in the aetiology of Alzheimer's disease [32]. In support of this hypothesis, numerous *in vitro* and *in vivo* studies have reported on the neurotoxic effects of A $\beta$ -related fragments in neurons derived from regions severely affected in AD [4,7]. Although the precise mechanisms mediating the toxic properties of A $\beta$  have yet to be fully understood, it has been proposed that they are associated with oxidative stress-dependent apoptosis [25]. And many researchers have demonstrated that A $\beta$  triggered apoptotic degeneration in *in vitro* neuronal experiments [13,37]. Considerable attention has recently been focused on identifying naturally occurring antioxidants that are able to protect against A $\beta$ -mediated cytotoxicity.

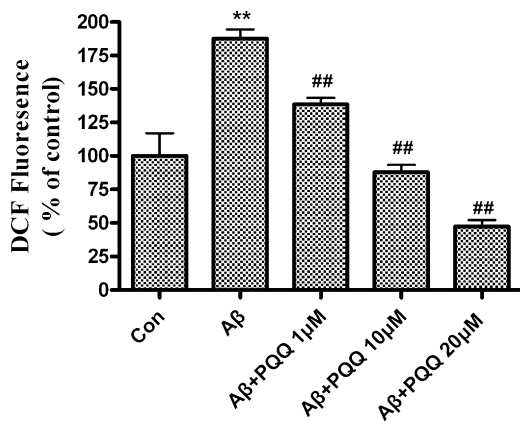
Based on the fact that PQQ has the neuroprotective activity, and PQQ has neuroprotective effect *in vitro* against NMDA-, methylmercury-, glutamate, and 6-hydroxydopamine-induced neurotoxicity [1,11,31,39], it is reasonable to speculate that whether PQQ has the neuroprotective activity against A $\beta$ -induced neurotoxicity. As expect, in the present study, we have shown that



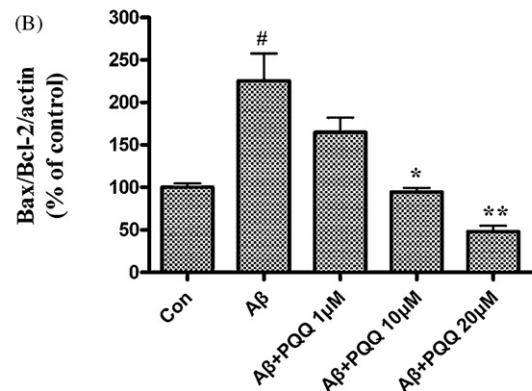
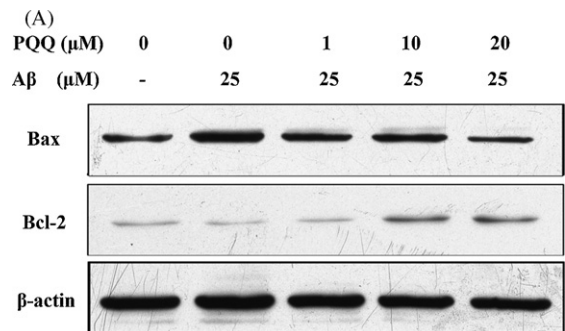
**Fig. 2.** PQQ prevents Aβ<sub>25–35</sub>-induced apoptosis. (A) SH-SY5Y cells, with or without pretreated PQQ for 1.0 h, were exposed to Aβ<sub>25–35</sub> for 24 h, and then were subjected to Hoechst 33258 staining and viewed under a fluorescence microscope. Arrows indicated condensed nuclei and arrowheads indicated fragmented nuclei. (B) Quantification of abnormal nuclei after exposure to Aβ<sub>25–35</sub> in the presence or absence of PQQ. The results are representative of three independent experiments. Data are mean ± S.E.M. ## *P* < 0.01 vs. control; \*\* *P* < 0.01 vs. Aβ<sub>25–35</sub>-treated-alone group.

PQQ can protect human neuroblastoma SH-SY5Y cells against Aβ-induced toxicity. The MTT assay indicated that 10, 20 μM PQQ significantly protects SH-SY5Y cells from Aβ toxicity (Fig. 1). The neuroprotective effects were also confirmed by analysis of morphological nuclear changes (Fig. 2). All these observations are consistent with previously reported results.

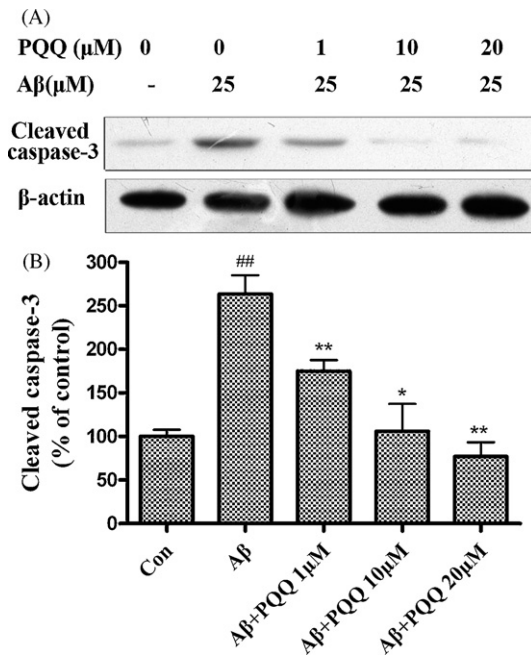
Compelling evidence showed that oxidative stress is extensive in the AD brains, and plays a key role in Aβ induced neuronal cell death [26]. Many reports have demonstrated the involvement of ROS formation in Aβ-induced neurotoxicity [6]. PQQ inhibited the Aβ<sub>25–35</sub>-induced overproduction of intracellular ROS in SH-SY5Y



**Fig. 3.** PQQ inhibits Aβ<sub>25–35</sub>-induced ROS generation. ROS formation, evaluated by the oxidation of H<sub>2</sub>DCF to DCF, was assessed 24 h after incubation with Aβ<sub>25–35</sub>. PQQ was pretreated before Aβ<sub>25–35</sub> for 1.0 h. Intracellular ROS were determined by flow cytometry. Relative fluorescence intensity obtained from three independent experiments, and the amount of intracellular ROS is expressed relative to that in controls. \*\* *P* < 0.01 vs. control; ## *P* < 0.01 vs. Aβ<sub>25–35</sub>-treated-alone group.



**Fig. 4.** Effect of PQQ on the expression of Bcl-2 family proteins in Aβ<sub>25–35</sub>-induced SH-SY5Y cells. The cells were pretreated with PQQ for 1.0 h prior to exposure to 25 μM Aβ for 24 h. (A) Analysis of Bcl-2 and Bax protein levels in SH-SY5Y cells by western blotting. (B) Effects of PQQ on the ratio of values of Bax/Bcl-2/actin. Densitometric analysis is mean ± S.E.M. of three independent experiments. # *P* < 0.05 vs. control; \* *P* < 0.05 and \*\* *P* < 0.01 vs. Aβ<sub>25–35</sub>-treated-alone group.



**Fig. 5.** PQQ inhibits  $A\beta_{25-35}$ -induced induction of cleaved caspase-3 protein. The cells were pretreated with PQQ for 1.0 h prior to exposure to 25  $\mu$ M  $A\beta$  for 24 h. The amount of cleaved caspase-3 was determined using immunoblots analysis. (A) Representative image of immunoblots for cleaved caspase-3. (B) Densitometric analysis of changes in levels of cleaved caspase-3. Data are means  $\pm$  S.E.M. for three independent experiments. ## $P$ <0.01 vs. control; \* $P$ <0.05 and \*\* $P$ <0.01 vs.  $A\beta_{25-35}$ -treated-alone group.

cells. Thus, these findings suggest that PQQ protects SH-SY5Y cells against  $A\beta_{25-35}$ -induced apoptosis by attenuating the intracellular ROS level.

The subsequent experiments explored the mechanism of the neuroprotection of PQQ on  $A\beta_{25-35}$ -induced cell death in SH-SY5Y cells. We next examined the effect of PQQ on the  $A\beta_{25-35}$ -induced expression of Bax and Bcl-2 protein levels, and the induction of cleaved caspase-3 protein levels in SH-SY5Y cultured cells by western blot analysis.

Bcl-2 family members are major regulators of mitochondrial integrity and mitochondria-initiated cytochrome *c* release and caspase activation. The Bcl-2 family includes antiapoptotic 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 [38]. The ratio of Bax to Bcl-2 has been reported to be correlated with apoptosis. Our results showed that treatment of cells with  $A\beta_{25-35}$  induced a robust increase in the protein level of Bax, and a strong change in the protein level of Bcl-2, and there was an approximate 2.3-fold increase in the ratio of Bax/Bcl-2 expression in  $A\beta_{25-35}$  treatment compared with the control. While, PQQ pretreatment could prevent the  $A\beta_{25-35}$ -induced increase of the Bax/Bcl-2 ratio (Fig. 4). The effect of PQQ on  $A\beta_{25-35}$ -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 PQQ, suggesting a mitochondria-dependent apoptosis-inhibiting effect of PQQ on  $A\beta_{25-35}$  insult.

Caspase-3 has been suggested to play an important role in  $A\beta$ -induced apoptosis [3]. An increase in expression of activated caspase-3 has been detected in AD brains [33]. The activation of caspase-3 may be a downstream event following exposure to  $A\beta_{25-35}$ . In the present study, a marked increase of caspase-3 p17-activated caspase-3 subunit in  $A\beta_{25-35}$  treatment was observed. However, a significant decrease in the PQQ pretreatment compared with  $A\beta_{25-35}$  treatment (Fig. 5).

In summary, we document the neuroprotective effects of PQQ in attenuating  $A\beta$ -induced neurotoxicity. Our study demonstrates that PQQ reduces the toxicity induced by  $A\beta_{25-35}$  in SH-SY5Y cells by limiting the induction of death signaling proteins, Bax, Bcl-2, and caspase-3 enzymes. Our findings suggest that PQQ holds potential for neuroprotection and therefore, may be promising for the treatment of Alzheimer's disease.

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