Environmental Toxicology and Pharmacology 27 (2009) 103–110

Contents lists available at ScienceDirect

Environmental Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/etap

In vitro protective effects of pyrroloquinoline quinone on methylmercury-induced neurotoxicity

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ARTICLE INFO

Article history:
Received 27 April 2008
Received in revised form 24 August 2008
Accepted 27 August 2008
Available online 7 September 2008

Keywords:
Methylmercury
Pyrroloquinoline quinone
Apoptosis
Oxidative stress
Neuroprotection

ABSTRACT

Methylmercury (MeHg), as a well-known neurotoxicant, has been implicated to induce massive neurodegeneration. Pyrroloquinoline quinone (PQQ) is a novel redox cofactor and also exists in various plants and animal tissues. In vivo as well as in vitro experimental studies have shown that PQQ functions as an essential nutrient or antioxidant. In this study, we demonstrated the protective effects of PQQ on MeHg-induced neurotoxicity in PC12 cells. The results showed that after pretreatment of PC12 cells with PQQ prior to MeHg exposure, the MeHg-induced cytotoxicity was significantly attenuated, and then DNA fragmentation was correspondingly reduced. PQQ prevented the disruption of mitochondrial membrane potential, up-regulated the level of Bcl-2, and consequently inhibited the activation of caspase-3. Moreover, PQQ also decreased the production of ROS and maintained the GSH levels in PC12 cells exposed to MeHg. Thus, these data indicate that PQQ can protect neurons against MeHg-induced apoptosis and oxidative stress via ameliorating the mitochondrial dysfunction. Data from this study provide a new useful strategy for the treatment of neuronal toxicity induced by mercury toxins.

1. Introduction

Mercury is a ubiquitous environmental contaminant. Methylmercury (MeHg), one organic form of mercury, can easily cross the blood–brain and placental barriers and cause central nervous system (CNS) damage in both the adult and developing brain (Clarkson, 1997; Lapham et al., 1995). Over the last decade, extensive research has been conducted to elucidate the cellular events associated with MeHg-induced neurotoxicity. Investigators have presented work on induction of apoptosis by MeHg in multiple cell types in vitro (Kunimoto, 1994). On the other hand, previous studies on the mechanism of MeHg neurotoxicity have implicated the generation of reactive oxygen species (ROS) and depletion of intracellular glutathione (GSH) as important contributors to observed MeHg-induced cytotoxicity (Sanfeliu et al., 2001). Depletion of GSH associated with MeHg exposure may reduce the cellular ability to destroy free radicals and ROS, so that it eventually triggered the apoptotic cell death.

Pyrroloquinoline quinone (PQQ), a noncovalently bound redox cofactor of bacterial dehydrogenases, was initially isolated from cultures of methylotropic bacteria (Salisbury et al., 1979). As designated in earlier literature, this bacterially synthesized quinone is highly soluble, heat-stable and capable of continuous redox cycling. Following the discovery of PQQ, it has been identified in various fruits, vegetables, milk and even tissues of mammalian animal at pico- or nano-molar levels (Kumazawa et al., 1992, 1995). As an essential nutrient or antioxidant, PQQ has been drawing attention from both the nutritional and the pharmacological viewpoint. Mice fed chemically defined diets devoid of PQQ that are otherwise nutritionally adequate have impaired neonatal growth and abnormal features, including friable skin, evidence of hemorrhage and diverticuli, reduction in general fitness, and a hunched posture (Steinberg et al., 1994). Decreased fertility and defects in immune function also occurs with PQQ deficiency (Steinberg et al., 1994). Recently, it has been proposed that PQQ can be classified as a new B vitamin (Kasahara and Kato, 2003). Moreover, it has been reported that PQQ is a potent neuroprotective nutrient against 6-hydroxydopamine-induced neurotoxicity (Hara et al., 2007).

In epidemiological studies, the general population is exposed to MeHg primarily through ingestion from a diet. Because animal and human body systems do not appear to synthesize PQQ, the diet is also assumed to be the major source (Smidt et al., 1991).

Abbreviations: PQQ, pyrroloquinoline quinone; MeHg, methylmercury; CNS, central nervous system; LDH, lactate dehydrogenase; TUNEL, Tdt-mediated dUTP nick end labeling; Rh123r, hodamine 123; MMP, mitochondrial membrane potential; ROS, reactive oxygen species; DCFH-DA, 2,7-dichlorofluorescein diacetate; DCF, dichlorofluorscein; GSH, glutathione; FBS, fetal bovine serum; PBS, phosphate-buffered saline.

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1382-6689/$ – see front matter.
doi:10.1016/j.etap.2008.08.010

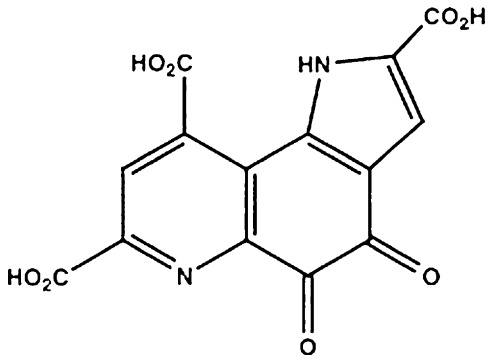

Fig. 1. The chemical structure of pyrroloquinoline quinone (PQQ), shown here as 4,5-dioxo-H-pyrrolo(2,3-*f*) quinoline-2,7,9-tricarboxylic acid.

However, little is known about the potential protective effect of PQQ against MeHg neurotoxicity. Due to its inherent properties mentioned above, it is possible that PQQ protects neurons from MeHg-induced neurotoxicity.

Dopamine neurons are implicated in a wide variety of functions such as attention, cognition, motor, and reward-related behaviours. Moreover, a number of neurological pathologies, including Parkinson's disease, schizophrenia, mood disorders, as well as deficits in attention, motor control, and perception have been associated to dysfunctions of the dopaminergic system. The toxic effects of MeHg on the developing dopaminergic system might predispose to the onset of pathological conditions later in life (Cernichiari et al., 1995). PC12 cell line was established from rat adrenal pheochromocytoma cell (Greene and Tischler, 1976). The membrane receptors and synthesized transmitters in PC12 cells are similar to dopaminergic neurons located in midbrain. The current investigation capitalized on the fact that undifferentiated PC12 cells represents immature neurons that are most vulnerable to the effects of MeHg (Igata, 1993; Harada, 1995). Therefore, we focused on the effects of PQQ on MeHg-induced neurotoxicity in undifferentiated PC12 cells in order to provide experimental basis for the treatment of neuronal toxicity induced by mercury toxins.

2. Materials and methods

2.1. Reagents

Pyrroloquinoline quinone (PQQ, Fig. 1) was obtained from Shanghai Medical Life Science (Shanghai, China). Methylmercury chloride (MeHg) and 2,7-dichlorofluorescein diacetate (DCFH-DA) and Rhodamine 123 (Rh123) were purchased from Sigma Chemical (St. Louis, MO, USA). RPMI 1640 and equine serum were purchased from Hyclone (Logan, UT, USA). Fetal bovine serum (FBS) was purchased from Hangzhou Sijiqing Company (Hangzhou, China). The monoclonal antibodies to Bcl-2 and Bax were purchased from Beijing Zhongshan Biotechnology Co. Ltd. (Beijing, China). The fluorescein isocyanate (FITC)-conjugated secondary antibody was obtained from Sigma Chemical (St. Louis, MO, USA). Lactate dehydrogenase (LDH) assay kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). GSH assay kit was purchased from Beyotime Institute of Biotechnology (Haimen, China). Caspase-3 activity kit was purchased from Nanjing Keygen Biotech. Co. Ltd. (Nanjing, China).

2.2. Cell culture

PC12 cells were obtained from Peking Union Medical College (Beijing, China) and cultured in RPMI 1640 medium supplemented with 10% (v/v) equine serum and 5% (v/v) fetal bovine serum. Cells were maintained at 37 °C in an air and 5% CO_2 atmosphere.

2.3. Cytotoxicity assay

Cytotoxicity was quantitatively assessed by measuring the activity of LDH released from the damaged cells into the culture medium (Vian et al., 1995). Briefly, PC12 cells were plated at density of 8.0×10^5 cells per well in 6-well plates. After 24 h, cells were exposed to various concentrations of MeHg (2, 4, 6, 8 and 10 μM) for 4 h, or pretreated with different concentrations of PQQ (3, 30, 300, 3000 and 30,000 nM) for 30 min, and then were exposed to 6 μM MeHg for 4 h. To test the toxicity of PQQ to PC12 cells, we also treated cells with PQQ for 4 h. At the end of treatments, the cell suspension was centrifuged at 4000 × *g* at 4 °C for 5 min, and then the supernatants were collected, whereas the cell pellets were lysed with cell lysis buffer containing 1% Trition X-100. LDH assays in supernatant aliquots and lysates were performed by using the cytotoxicity assay kit according to the manufacturer's protocol. The wavelength to measure absorbance was 440 nm and LDH expressed cytotoxicity (%) was calculated using the formula: (supernatant value − blank value)/[(supernatant value − blank value) + (lysates value − blank value)] × 100%.

2.4. Quantification of DNA fragmentation in apoptosis

DNA fragmentation was determined by using the Tdt-mediated dUTP nick end labeling (TUNEL) assay according to the manufacturer's protocol. Briefly, PC12 cells were plated in 6-well plates (8×10^5 per well). After 24 h, cells were treated with different concentrations of MeHg (2, 4, 6 and 8 μM) for 4 h, or pretreated with PQQ (3, 30 and 300 nM) for 30 min, and then were exposed to 6 μM MeHg for 4 h. Cells were washed with cold PBS, fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.1% Triton X-100 on ice for 2 min, and then incubated with 50 μl TUNEL

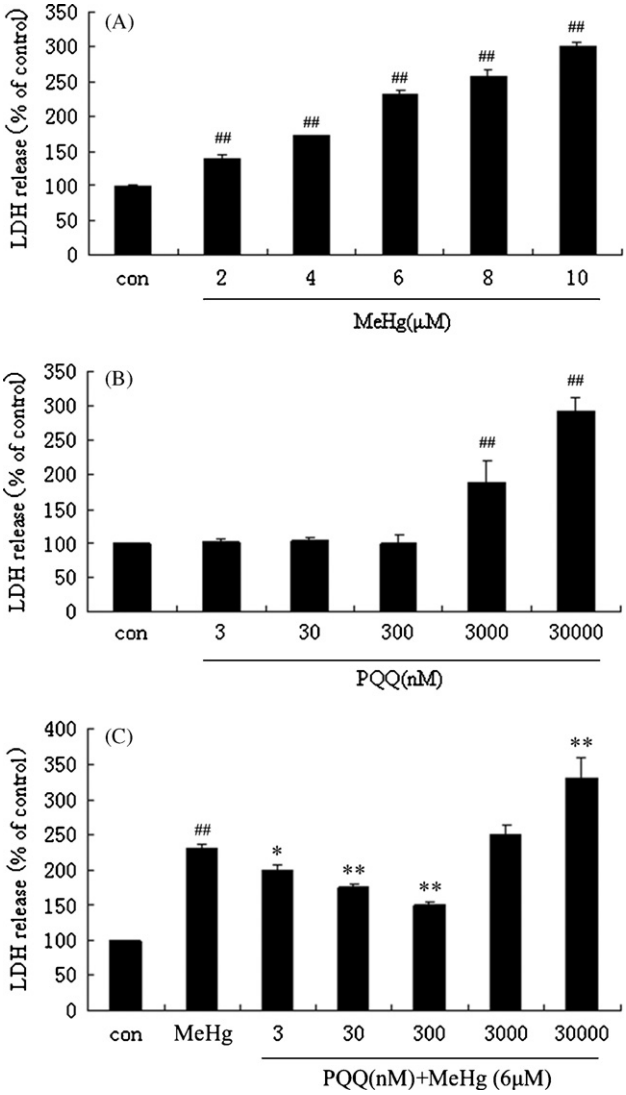

Fig. 2. (A) Effect of MeHg on LDH release in PC12 cells. PC12 cells were treated with MeHg for 4 h. (B) Effect of PQQ on LDH release in PC12 cells. PC12 cells were treated with PQQ for 4 h. (C) Effect of PQQ on LDH release in PC12 cells exposed to MeHg. PC12 cells were pretreated with PQQ for 30 min, and then were exposed to 6 μM of MeHg for 4 h. After the treatment, LDH release in cell suspensions and the total LDH were measured. Values are means ± S.D. of triplicate independently experiments. $^{\#}p < 0.05$ or $^{\#\#}p < 0.01$, compared with control, $^{*}p < 0.05$ or $^{**}p < 0.01$, compared with 6 μM MeHg treatment group.

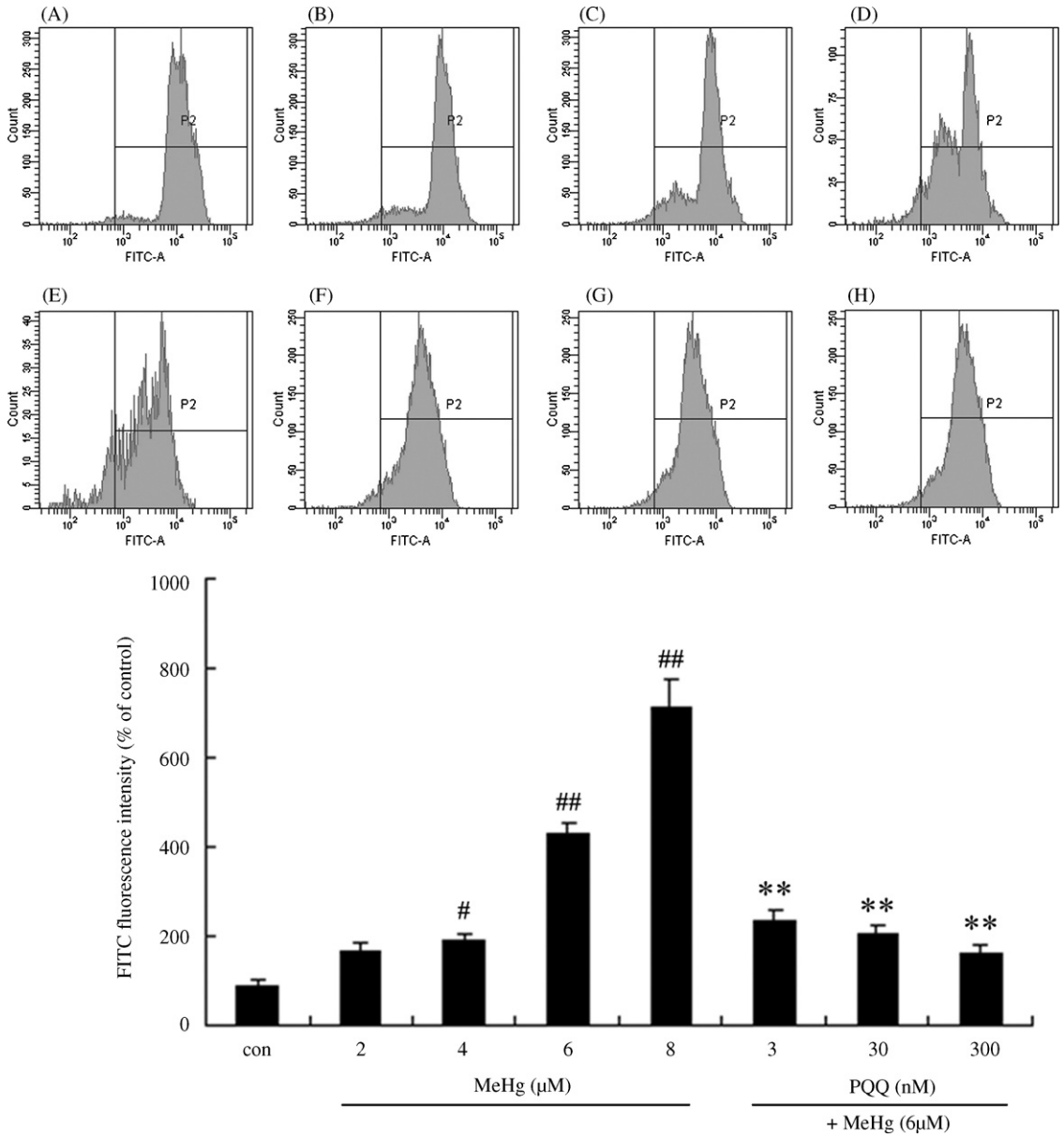

Fig. 3. Effect of PQQ on MeHg-induced DNA fragmentation in PC12 cells. (A) Control. (B–E) PC12 cells were treated with 2, 4, 6 and 8 μM MeHg for 4 h, respectively. (F–H) Cells were pretreated with PQQ (3, 30 and 300 nM) for 30 min, and then were exposed to 6 μM of MeHg for 4 h. After the treatment, flow cytometric analyses were performed to examine the cell numbers of DNA strand breaks according to fluorescence intensity. Values are means ± S.D. of triplicate independently experiments. $^{\#}p < 0.05$ or $^{\#\#}p < 0.01$, compared with control, $^{*}p < 0.05$ or $^{**}p < 0.01$, compared with 6 μM MeHg treatment group.

assay solution for 1 h at 37 °C in the dark. A minimum of 10,000 cells were analyzed per sample for FITC fluorescence by a FACScan flow cytometer (BD Biosciences Pharmingen, USA).

2.5. Measurement of mitochondrial membrane potential (MMP)

Mitochondrial membrane was monitored using the fluorescent dye Rhodamine 123 (Rh-123), a cell permeable cationic dye, which preferentially enters into mitochondria based on the highly negative mitochondrial membrane potential (MMP). Depolarization of MMP results in the loss of Rh-123 from the mitochondria and a decrease in intracellular fluorescence (Satoh et al., 1997). Briefly, harvested cells were washed twice with PBS and coincubated with Rh-123 (10 μg/ml) at 37 °C for 30 min in the dark. The stained cells were resuspended in PBS and analyzed directly by flow cytometry.

2.6. Analysis of Bcl-2 and Bax protein

The levels of Bcl-2 and Bax protein was measured by flow cytometry as described (Liu and Zhu, 1999). Briefly, PC12 cells were collected and washed with PBS. After fixation with 2% paraformaldehyde for 20 min and permeabilization with 0.5% Triton-X 100, then cells were incubated with primary antibodies against Bcl-2 or Bax for 30 min, respectively, followed by incubation with corresponding FITC-conjugated secondary antibodies for 30 min at room temperature in the dark. After the cells were washed with PBS, the antigen density was analyzed by the flow cytometry.

2.7. Analysis of caspase-3 activity

The activity of caspase-3 was evaluated using caspase-3 assay kit. The assay is based on spectrophotometric detection of the chromophore *p*-nitroaniline (*p*NA) after its cleavage by caspase-3 from the labeled substrate, Ac-DEVD-*p*NA. Briefly, cell lysates were prepared after their respective treatment. Assays were performed on 96-well plates by incubating 50 μl protein of cell lysate per sample in 50 μl reaction buffer containing 5 μl caspase-3 substrate (Ac-DEVD-*p*NA). Lysates were incubated at 37 °C for 4 h. Samples were measured with a microplate reader at an absorbance of 405 nm. The detailed analysis procedure was described in the manufacturer's protocol.

2.8. Measurement of ROS production

Intracellular ROS production was detected by means of an oxidation-sensitive fluorescent probe dye, 2,7-dichlorofluorescein diacetate (DCFH-DA). DCFH-DA was

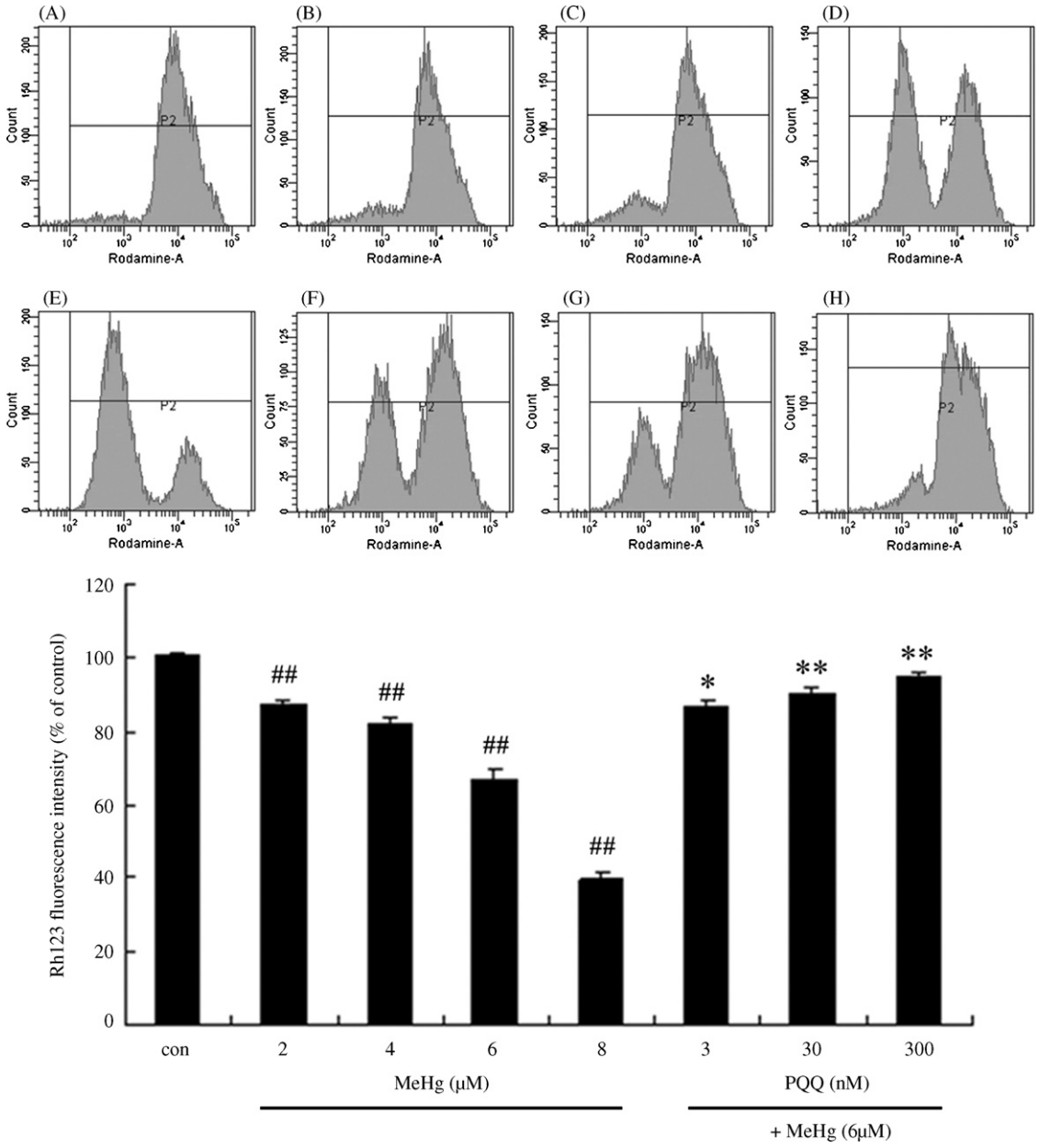

Fig. 4. Effect of PQQ on MMP in PC12 cells exposed to MeHg. (A) Control. (B–E) PC12 cells were treated with 2, 4, 6 and 8 μM MeHg for 4 h, respectively. (F–H) Cells were pretreated with PQQ (3, 30 and 300 nM) for 30 min, and then were exposed to 6 μM of MeHg for 4 h. After the treatment, flow cytometric analysis was performed to measure the fluorescence intensity. Values are means ± S.D. of triplicate independently experiments. $^{\#}p < 0.05$ or $^{\#\#}p < 0.01$, compared with control, $^{*}p < 0.05$ or $^{**}p < 0.01$, compared with 6 μM MeHg treatment group.

deacetylated intracellularly by nonspecific esterase, which was further oxidized to the highly fluorescent compound dichlorofluorscein (DCF) in the presence of cellular peroxides (Wang and Joseph, 1999). After treatment, harvested cells were washed twice with PBS and incubated with DCFH-DA (20 μM) at 37 °C for 30 min in the dark. DCF fluorescence intensity was detected by flow cytometer.

2.9. Assessment of GSH levels

Cellular GSH levels were assessed using GSH assay kit. DTNB (5,5'-dithiobis(2-nitrobenzoic acid)), known as Ellman's reagent, was developed for the detection of thiol compounds. Since DTNB and GSH react to generate 2-nitro-5-thiobenzoic acid that is a yellow colored product, GSH concentration can be determined by the measurement at 412 nm absorbance. After treatment, harvested cells were deproteinated and centrifuged at 10,000 × g for 10 min. Supernatants were added to a 96-well plate and assay was then performed according to the manufacturer's instructions.

2.10. Statistics analysis

All results were expressed by mean ± S.D. The significance of difference was evaluated with one-way analysis of variance (ANOVA) procedures followed by Dunnett's 2-sided test. A probability level of $p < 0.05$ or $p < 0.01$ was considered statistically significant. All experiments were performed at least three times.

3. Results

3.1. Effect of PQQ on MeHg-induced LDH release in PC12 cells

LDH assay was performed to investigate the cytotoxicity. As shown in Fig. 2A, compared with control group, treatment with MeHg resulted in an increase of LDH release (2 μM: 141 ± 2.7%

of control; 4 μM: 172 ± 1.4% of control; 6 μM: 232 ± 5.7% of control; 8 μM: 258 ± 9.5% of control; 10 μM: 302 ± 5.7% of control). Nanomolar levels of PQQ had no effect on PC12 cells, whereas micromolar levels of PQQ significantly increased LDH release (Fig. 2B). Preincubation with 3, 30 and 300 nM PQQ blocked LDH release in PC12 cells exposed to 6 μM of MeHg to 201 ± 7.6%, 175 ± 4.6% and 150 ± 3.1%, respectively. However, micromolar levels of PQQ clearly enhanced the toxicity of MeHg, which increased LDH release to 250.8 ± 14.2% and 330 ± 28.6%, respectively (Fig. 2C). Based on the above reasons, we decided to use the treatment concentration of PQQ (3, 30 and 300 nM) in all subsequent experiments.

3.2. Effect of PQQ on MeHg-induced DNA fragmentation in PC12 cells

The effect of PQQ on MeHg-induced DNA fragmentation of PC12 cells was examined with TUNEL assay (Fig. 3). Compared with control group, DNA fragmentation caused by MeHg at 4 μM and above was found to be statistically significant (4 μM: 192 ± 11.2% of control; 6 μM: 433 ± 20.2% of control; 8 μM: 713 ± 63% of control). When cells were pretreated with PQQ for 30 min, then exposed to 6 μM of MeHg for 4 h, DNA fragmentation was significantly attenuated by PQQ in a dose-dependent manner (3 nM: 236 ± 20.5% of control; 30 nM: 209 ± 13.3% of control; 300 nM: 164 ± 15.8% of control).

3.3. Effect of PQQ on MeHg-induced disruption of MMP

MMP in MeHg-induced PC12 cells pretreated with PQQ was evaluated by flow cytometric analysis with the fluorescent dye Rh123 (Fig. 4). Exposure of PC12 cells to various doses of MeHg showed a noticeable decrease in the fluorescent intensity (2 μM: 87 ± 1.1% of control; 4 μM: 82 ± 1.9% of control; 6 μM: 67 ± 2.9% of control; 8 μM: 40 ± 1.6% of control). When cells were pretreated with PQQ, then exposed to 6 μM of MeHg for 4 h, disruption of MMP was evidently inhibited by different concentrations of PQQ to 87 ± 1.7% of 3 nM, 90 ± 1.8% of 30 nM and 95 ± 0.7% of 300 nM, respectively.

3.4. Effect of PQQ on the levels of Bcl-2 and Bax in MeHg-treated PC12 cells

Bcl-2 gene family has been associated with mitochondrial function during apoptosis. We therefore examined the levels of Bcl-2 and Bax proteins by flow cytometric analysis. The results revealed that MeHg treatment caused the decrease of Bcl-2 protein (2 μM: 91 ± 6.7% of control; 4 μM: 80 ± 5.8% of control; 6 μM: 67 ± 2.8% of control; 8 μM: 47 ± 5.9% of control), but the levels of Bax protein were unchanged. PC12 cells which were pretreated with PQQ showed up-regulation for the levels of Bcl-2 protein in a dose-dependent manner (3 nM: 72 ± 5.8% of control; 30 nM: 81 ± 3.9% of control; 300 nM: 90 ± 3.9% of control), whereas the levels of Bax protein were still unaffected (Fig. 5).

3.5. Effect of PQQ on MeHg-induced caspase-3 activation

Caspase-3 has been shown to play a pivotal role in the execution phase of apoptosis induced by diverse stimuli. As shown in Fig. 6, PC12 cells treated with various doses of MeHg for 4 h, showed a significant increase in caspase-3 activity (2 μM: 194 ± 3% of control; 4 μM: 237 ± 25% of control; 6 μM: 270 ± 26.2% of control; 8 μM: 267 ± 25.8% of control). When cells were pretreated with PQQ, then exposed to 6 μM of MeHg, the caspase-3 activity was noticeably inhibited by PQQ in a dose-dependent manner (3 nM: 251 ± 5.1%

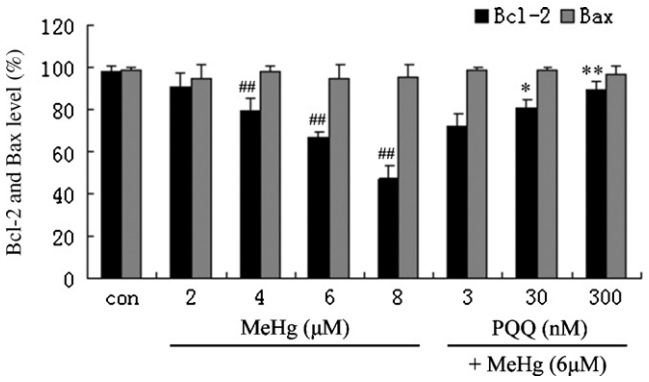

Fig. 5. Effect of PQQ on the levels of Bcl-2 and Bax in PC12 cells exposed to MeHg. PC12 cells were treated with 2, 4, 6 and 8 μM MeHg for 4 h, or pretreated with PQQ (3, 30 and 300 nM) for 30 min, and then were exposed to 6 μM of MeHg for 4 h. After the treatment, the levels of Bcl-2 and Bax were analyzed by the flow cytometry. Values are means ± S.D. of triplicate independently experiments. $^{\#}p < 0.05$ or $^{\#\#}p < 0.01$, compared with control, $^{*}p < 0.05$ or $^{**}p < 0.01$, compared with 6 μM MeHg treatment group.

of control; 30 nM: 216 ± 11.1% of control; 300 nM: 158 ± 3.9% of control).

3.6. Effect of PQQ on MeHg-induced overproduction of ROS in PC12 cells

The effect of PQQ on MeHg-induced ROS generation was detected by flow cytometer (Fig. 7). Compared with control group, there was a dose-dependent increase in ROS generation in PC12 cells treated with 2 μM (150 ± 7.9% of control), 4 μM (190 ± 16.9% of control), 6 μM (283 ± 71.5% of control) and 8 μM (380 ± 59.9% of control) MeHg. When PC12 cells were pretreated with PQQ (3, 30 and 300 nm) prior to 6 μM MeHg treatment, the production of ROS was evidently reduced from 283 ± 71.5% to 235 ± 20.8%, 209 ± 23% and 139 ± 5.8%, respectively.

3.7. Effect of PQQ on MeHg-induced depletion of GSH

As shown in Fig. 8, when PC12 cells were exposed to various concentrations of MeHg for 4 h, the levels of GSH extremely decreased (2 μM: 74 ± 1.3% of control; 4 μM: 53 ± 2.6% of control; 6 μM: 29 ± 2.5% of control; 8 μM: 27 ± 1% of control). Compared

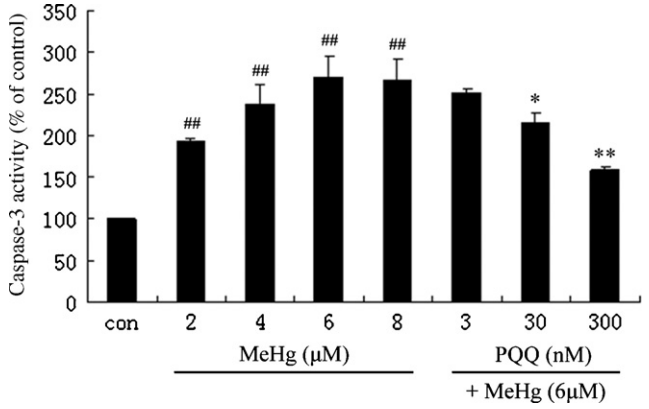

Fig. 6. Effect of PQQ on MeHg-induced caspase-3 activation. PC12 cells were treated with 2, 4, 6 and 8 μM MeHg for 4 h, or pretreated with PQQ (3, 30 and 300 nM) for 30 min, and then were exposed to 6 μM of MeHg for 4 h. The caspase-3 activity was measured with a microplate reader. Values are means ± S.D. of triplicate independently experiments. $^{\#}p < 0.05$ or $^{\#\#}p < 0.01$, compared with control, $^{*}p < 0.05$ or $^{**}p < 0.01$, compared with 6 μM MeHg treatment group.

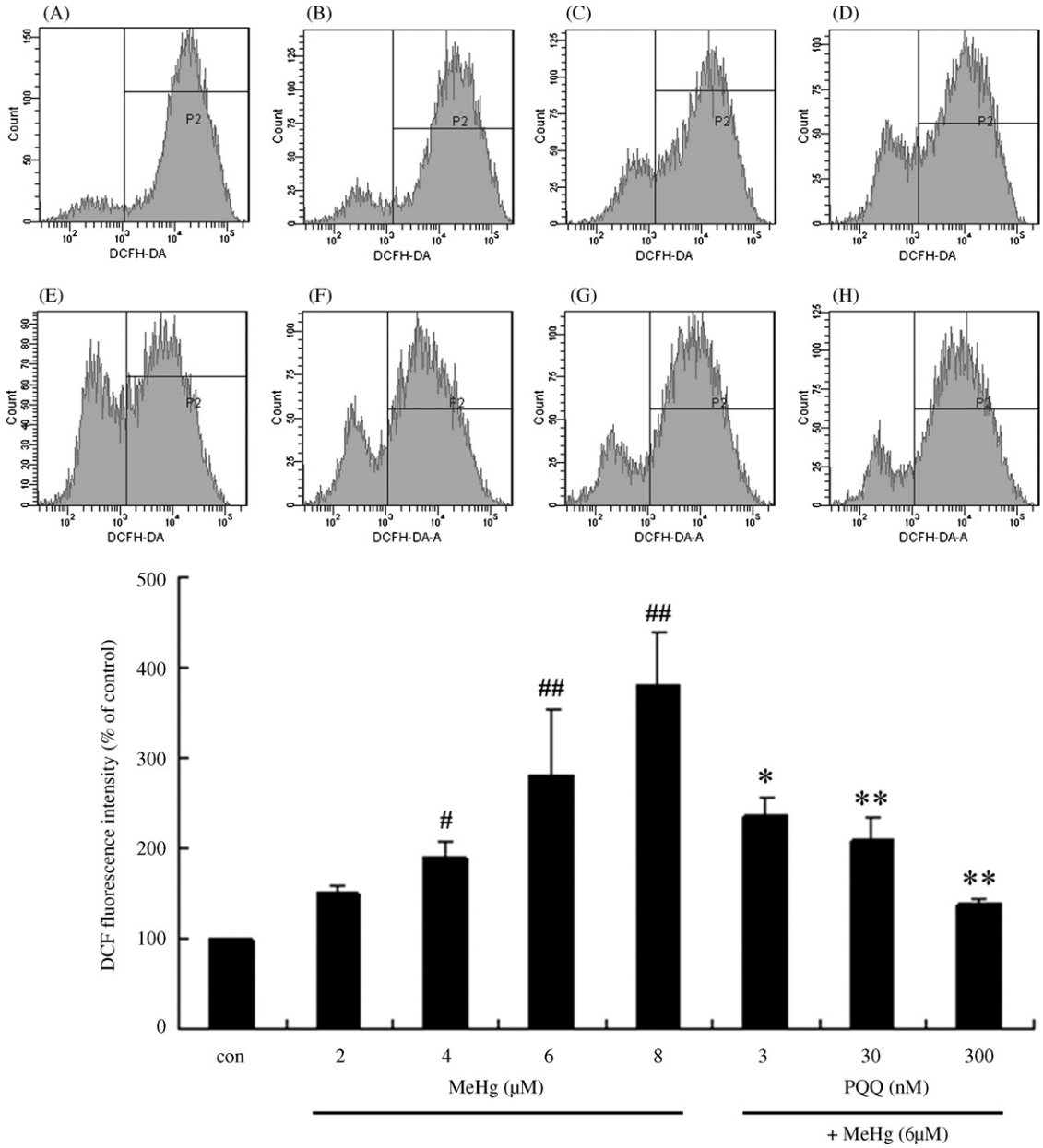

Fig. 7. Effect of PQQ on MeHg-induced overproduction of ROS in PC12 cells. (A) Control. (B–E) PC12 cells were treated with 2, 4, 6 and 8 μM MeHg for 4 h, respectively. (F–H) Cells were pretreated with PQQ for 30 min, and then were exposed to 6 μM of MeHg for 4 h. After the treatment, flow cytometric analyses were performed to examine DCF fluorescence intensity. Values are means ± S.D. of triplicate independently experiments. $^{\#}p < 0.05$ or $^{\#\#}p < 0.01$, compared with control, $^{*}p < 0.05$ or $^{**}p < 0.01$, compared with 6 μM MeHg treatment group.

with 6 μM MeHg treatment group, the levels of GSH was mildly elevated by different concentrations of PQQ (3 nM: 32 ± 1.2% of control; 30 nM: 37 ± 1.5% of control; 300 nM: 39 ± 1.9% of control).

4. Discussion

LDH is a cytosolic enzyme present within all mammalian cells. The normal plasma membrane is impermeable to LDH, but damage to the cell membrane results in a change in the membrane permeability and subsequent leakage of LDH into the extracellular fluid (Koh and Choi, 1987). Therefore, LDH release is a marker of cell injury. The present study indicated that exposure to MeHg resulted in the PC12 cells injury. The nanomolar levels of PQQ significantly blocked the LDH release in a dose-dependently manner. Unexpectedly, when we extended the concentration of PQQ to micromolar levels, we found that combination of PQQ and MeHg showed the synergistic toxicity effects. Moreover, micromolar levels of PQQ exerted its cytotoxicity, whereas nanomolar levels of PQQ had no effect on PC12 cells. It is very important to note that compounds that engage in redox cycling can also be effective free radical initiators. Due to its antioxidant and pro-oxidant properties, PQQ has also been demonstrated to initiate DNA damage in cell-free systems (Hiraku and Kawanishi, 1996), induce cell death (He et al., 2003), and result in the nephrotoxicity (Watanabe et al., 1989). It may be therefore regarded as a janus-faced molecule. The ultimate effect of PQQ is strongly dependent on its concentrations, redox system and microenvironment. The current investigations demonstrated that nanomolar levels of PQQ could protect PC12 cells against MeHg-induced neurotoxicity by maintaining the plasma membrane integrity and may be worth further research to deter-

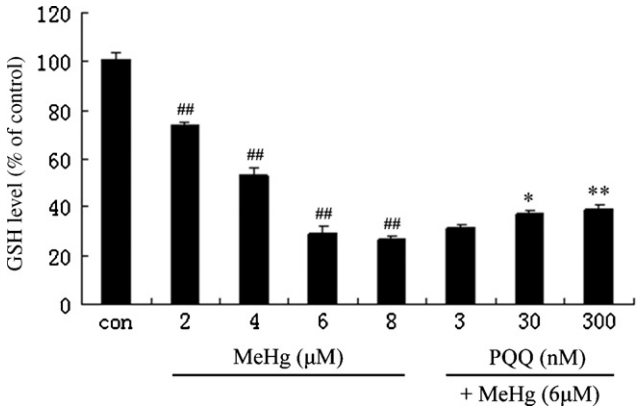

Fig. 8. Effect of PQQ on MeHg-induced depletion of GSH. PC12 cells were treated with 2, 4, 6 and 8 μM MeHg for 4 h, or pretreated with PQQ (3, 30 and 300 nM) for 30 min, and then were exposed to 6 μM of MeHg for 4 h. The GSH levels were measured with a microplate reader. Values are means ± S.D. of triplicate independently experiments. $^{\#}p < 0.05$ or $^{\#\#}p < 0.01$, compared with control, $^{*}p < 0.05$ or $^{**}p < 0.01$, compared with 6 μM MeHg treatment group.

mine its therapeutic potential. To our knowledge, this is the first report describing the in vitro protective effect of PQQ on MeHg-induced neurotoxicity.

DNA damage is a common feature of MeHg toxicity reported in a variety of studies, and evidence has been supplied that MeHg activates apoptosis-related endonucleases that cause regular chromatin cleavage in neuronal models (Miura et al., 1999). TUNEL assay provided sufficient evidence that exposure of 6 μM and 8 μM of MeHg to PC12 cells lead to a significant increase of DNA fragmentation (4.3-fold and 7.1-fold, respectively), while exposure of 2 μM and 4 μM of MeHg lead to a relatively minor increase (nearly 2-fold). Pretreatment with PQQ (3, 30 and 300 nm) evidently decreased the degree of MeHg-caused DNA strand breaks (2.3-fold, 2.0-fold and 1.6-fold, respectively) compared with 6 μM MeHg treatment group. Since DNA fragmentation is a biochemical hallmark of apoptosis in the majority of cells, these results indicate that PQQ can effectively protect PC12 cells against MeHg-induced apoptosis.

Mitochondria play a central role in amplifying and mediating extrinsic apoptotic pathways. The idea that mitochondria may be a site of action of MeHg developed from both in vivo and in vitro studies (Yoshino et al., 1966; Sone et al., 1977). The loss of MMP is an early event in mitochondria-mediated apoptosis (Takahashi et al., 2004). Data from this study vertified that the MMP significantly decreased in PC12 cells after exposure to MeHg. This confirms that MeHg causes damage of the mitochondria, as observed previously in other neural cell models exposed to this toxic agent, such as primary culture of rat astrocytes and rat cerebellar granule neurons (Yee and Choi, 1996; Castoldi et al., 2000). There is preliminary evidence that it is a dissociable cofactor of mitochondrial respiratory complex I (Gallop et al., 1993). In vitro treatment of isolated mitochondria from rat liver with PQQ effectively protects against the oxidative stress-induced inactivation of mitochondrial respiratory chain (He et al., 2003). Recently, further researches demonstrated that dietary PQQ could influence mitochondrial amount and function (Stites et al., 2006) and modulate mitochondrial DNA content (Bauerly et al., 2006). Data from this study showed that pretreatment of PC12 cells with PQQ effectively inhibited the MeHg-induced disruption of MMP.

After the disruption of MMP, mitochondrial cytochrome *c* was released, which ultimately cleave pro-caspase-3 to form active caspase-3. Activation of caspase-3 is an important step in the execution phase of apoptosis and its inhibition blocks cell apoptosis (Budihardjo et al., 1999). Numerous studies have shown that Bcl-2, as a negative regulator of cell death in the Bcl-2 family members, protects cells against suffering from apoptosis induced by various stimuli in a wide variety of cell types (Korsmeyer, 1992), whereas Bax as a positive regulator of cell death promote or accelerate cell death. Moreover, over expression of Bcl-2 disrupts the pro-apoptotic proteins of Bax and prevents the mitochondrial release of cytochrome *c*, thereby inhibiting the activation of caspases cascade and apoptosis (Solange and Martinou, 2000). Other studies have also proofed that Bcl-2 inhibits apoptosis via regulating ion transport and preventing the collapse of MMP (Shimizu et al., 1996). Our results demonstrated that PQQ markedly reduced MeHg-induced cell death in PC12 cells by the up-regulation of Bcl-2, inhibited the loss of MMP which causing the release of apoptogenic substances such as cytochrome c from mitochondria into cytosol (Nicholls and Budd, 2000), and subsequently inactivating caspase-3, although the anticipant reduction of Bax level was invisible after simultaneous treatment with PQQ. With the aforementioned results, we can describe that PQQ exerted its protection against MeHg-induced apoptosis by partially inhibiting the mitochondrial apoptotic pathway.

To further understand the underlying protective mechanism of PQQ, we examined the changes of endogenous GSH and ROS production. The high thiol reactivity of MeHg has been suggested to be the basis of its harmful biological effects (Sanfeliu et al., 2003). GSH is the most abundant low-molecular-weight thiol compound in cells, and plays an important role in antioxidant defense. A purported mechanism of MeHg-induced neurotoxicity is thought to involve a decrease in GSH levels (Choi et al., 1996). Further, ROS are known to mediate MeHg-induced neurotoxicity in multiple experimental models (Gasso et al., 2001; Park et al., 1996; Sarafian, 1999). It has been suggested that depleted GSH levels lead to impairment of mitochondrial functions, including energy metabolism, increased ROS production and eventual cell death (Choi et al., 1996; Shenker et al., 1999; Swamy and Huat, 2003). Our studies indicated that MeHg extremely decreased GSH levels, and this probably occurs by binding to free thiol groups and by inhibiting its regeneration. The overproduction of ROS caused by MeHg was also observed and the apoptotic effect of MeHg on PC12 cells may be mediated by oxidative stress. PQQ mildly elevated GSH levels and significantly reduced the ROS production in PC12 cells exposed to MeHg. Previous studies have indicated that PQQ can scavenge $O_2^{\bullet -}$ and $^{\bullet}OH$ efficiently (Urakami et al., 1997), inhibit lipid peroxidation (Hamagishi et al., 1990), and protect the isolated heart from reoxygenation injury (Xu et al., 1993). Therefore, PQQ is thought to be an excellent antioxidant. Moreover, PQQ can decrease the cataract formation in chicks received injections of hydrocortisone by stabilizing GSH levels in both lens and livers (Nishigori et al., 1989). These observations permitted us to propose that excessive ROS and GSH depletion lead to an amplifying cycle of oxidative stress. The end result of this damaging cellular reactions initiated by MeHg will lead to dysfunction of PC12 cells and contribute to their inability to maintain normal intracellular milieu, thus, indirectly leading to death. Whereas, pretreatment with PQQ can effectively suppress an additional increase of intracellular ROS and to some extent maintain the GSH levels, and thereby protect PC12 cells against MeHg-induced apoptotic cell death.

In summary, altogether our findings demonstrated that PQQ can protect PC12 cells against MeHg-induced neurotoxicity. Specifically, the underlying mechanisms may be involved in maintaining the plasma membrane integrity, inhibiting the DNA fragmentation, preventing the disruption of mitochondrial membrane potential, up-regulating the level of Bcl-2, and consequently inhibiting the activation of caspase-3. Moreover, suppressing the overproduction of intracellular ROS and stabilizing the GSH levels may also con-

tribute to the protective effect of PQQ on MeHg-induced apoptosis and oxidative stress. MeHg represents a significant environmental contaminant with established risk to human health, especially to neurons. Therefore, the results obtained highlighted the potential of PQQ in offering protection against MeHg-induced neuronal toxicity.

Conflicts of interest statement

None declared.

Acknowledgements

This work was supported by grants from National Outstanding Youth Foundation of China (30125034) and National Natural Science Foundation of China (30371053). This work was also partially supported by scientific and Technological Plan Project of Dalian city (2003B3NS024).

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