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Protection of pyrroloquinoline quinone against methylmercury-induced neurotoxicity via reducing oxidative stress

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Abstract
Pyrroloquinoline quinone (PQQ) is a novel redox cofactor and also exists in various foods. *In vivo* as well as *in vitro* experimental studies have shown that PQQ functions as an essential nutrient or antioxidant. Methylmercury (MeHg), as a highly toxic environmental pollutant, could elicit central nervous system (CNS) damage. Considering the antioxidant properties of PQQ, this study was aimed to evaluate the effect of PQQ on MeHg-induced neurotoxicity in the PC12 cells. The results showed that, after pre-treatment of PC12 cells with PQQ prior to MeHg exposure, the MeHg-induced cytotoxicity was significantly attenuated and then the percentage of apoptotic cells and the arrest of S-phase in cell cycle were correspondingly reduced. Moreover, PQQ significantly decreased the production of ROS, suppressed the lipid peroxidation and increased the antioxidant enzyme activities in PC12 cells exposed to MeHg. These observations highlighted the potential of PQQ in offering protection against MeHg-induced neuronal toxicity.

Keywords: *Methylmercury, apoptosis, oxidative stress, pyrroloquinoline quinone, neuroprotection, antioxidant*

Introduction

Methylmercury (MeHg) is a worldwide contaminant. It is a well-established neurotoxicant that can have serious adverse effects on both the adult and developing brain. The general population is exposed to MeHg primarily through excessive ingestion from a diet that can lead to a dangerous toxicant burden with clinical or sub-clinical implications [1–3]. For example, Minamata disease (MD) was a mass neurotoxicity disaster, in Minamata and Niigata, Japan, in the 1950s and in the 1960s, respectively, which developed among the inhabitants long ingesting large quantities of fish and shellfish polluted with MeHg from local industrial discharge [4,5]. 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* [6]. On the other hand, previous studies on the mechanism of MeHg neurotoxicity have implicated the generation of reactive oxygen species (ROS) [7]. Accumulation of ROS associated with MeHg exposure may disturb intracellular redox balance, so that it eventually triggered the apoptotic cell death [8]. This effect can be partly attenuated by exogenous antioxidants [9–11]. Similar results have been observed in cell lines engineered to overexpress endogenous antioxidant defense systems, such as Mn-superoxide dismutase [12]. The capability of antioxidants to prevent MeHg-induced neuronal death have confirmed the key role of oxidative stress in MeHg toxicity, suggesting that elevation of ROS is upstream in the cell death cascade.

Pyrroloquinoline quinone (PQQ), 4,5-dioxo-H-pyrrolo (2,3-f) quinoline-2,7,9-tricarboxylic acid (Figure 1), is an anionic, water-soluble and heat-

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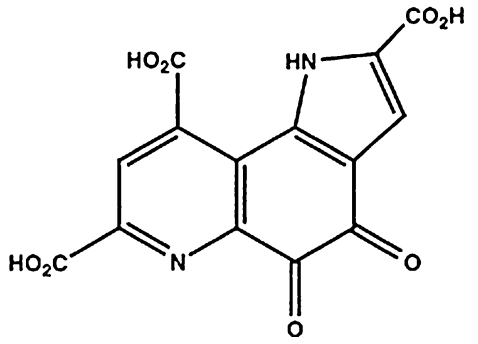

Figure 1. The chemical structure of pyrroloquinoline quinine.

stable compound that acts as a redox cofactor of bacterial dehydrogenases [13]. As designated in earlier literature, PQQ was initially isolated from cultures of methylotropic bacteria [14]. Its precise structure was deduced from X-ray diffraction data [15] and confirmed by organic synthesis [16]. PQQ has an orthoquinone structure that is directly responsible for the oxidoreduction and thus it has been evaluated as the third coenzyme following pyridine nucleotide and flavin in biological oxidoreduction. Rather soon after the discovery of PQQ, it has been identified in various plants and animal tissues at pico- or nano-molar levels [17,18]. Due to its inherent antioxidative properties and redox regulative functions, 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 haemorrhage and diverticuli, reduction in general fitness and a hunched posture [19]. Decreased fertility and defects in immune function also occur with PQQ deficiency [19]. Recently, it has been proposed that PQQ can be classified as a new B vitamin [20]. Moreover, pharmacological researches have demonstrated that PQQ has excellent neuroprotective efficacy in *in vivo* and *in vitro* models [21–26].

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 [27]. 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. PC12 cell line was established from rat adrenal pheochromocytoma cells [28]. The membrane receptors and synthesized transmitters in PC12 cells are similar to dopaminergic neurons located in the mid-brain. The current investigation capitalized on the fact that undifferentiated PC12 cells represent immature neurons that are most vulnerable to the effects of MeHg [29,30]. Therefore, we focused on the effects of PQQ on MeHg-induced neurotoxicity in undifferentiated PC12 cells in order to provide an experimental basis for the treatment of neuronal toxicity induced by mercury toxins.

Materials and methods

Reagents

Pyrroloquinoline quinone (PQQ) was purchased from Shanghai Medical Life Sciences (China). Methylmercury chloride (MeHg), Hoechst 33258, propidium iodide (PI), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl diphenyltet-razolium bromide (MTT) and nitroblue tetrazolium (NBT) were purchased from Sigma (Sigma-Aldrich Biotechnology, USA). Annexin V-fluorescein isothiocyanate (FITC) was purchased from BD pharmingen (BD Biosciences Pharmingen, USA). RPMI 1640 and equine serum were purchased from Hyclone (Hyclone Company, USA). Foetal bovine serum (FBS) was purchased from Hangzhou Sijiqing Company (China). Hydroxyl free radical (·OH), Malondialdehyde (MDA), Superoxide dismutase (SOD), Catalase (CAT) and Glutathione peroxidase (GSH-Px) assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (China). Hydrogen Peroxide (H_2O_2) assay kit was purchased from Beyotime Institute of Biotechnology (China).

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) foetal bovine serum. Cells were maintained at 37°C in an air and 5% CO_2 atmosphere.

Cell viability assay

Cell viability was measured by using the MTT assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases [31]. Briefly, PC12 cells were plated at a density of 2.0×10^4 cells per well in 96-well plates. After 24 h, the cells were exposed to various doses of MeHg for 4 h or pre-treated with different concentrations of PQQ for 30 min and then were exposed to MeHg for 4 h. To test the toxicity of PQQ to PC12 cells, we also treated cells with PQQ for 4 and 24 h. After the end of treatment time, the medium was carefully removed and then fresh medium containing MTT (final concentration 0.5 mg/ml) was added to the cells, followed by incubation for 2 h at 37°C. After that time, the formazan crystals were solubilized by adding 200 μl of DMSO. Absorbance was measured at 570 nm (630 nm as a reference) on a microplate reader (TECAN, Sunrise, Austria).

Cell cycle and sub-G_1 analysis

Cell cycle and sub-G_1 distribution were determined by staining DNA with propidium iodide (PI), as described previously [32]. Briefly, PC12 cells were plated in 6-well plates (8×10^5 cells per well). After 24 h, cells were pre-treated with PQQ (3, 30 and 300 nM) for 30 min and then were exposed to 6 μm MeHg for 4 h. After treatment, cells were pelleted, washed with cold PBS and fixed in 70% ethanol in PBS at -20°C for 12 h. The fixed samples were washed with cold PBS and dissolved in 100 μl of PBS containing 50 μg/ml RNase, 50 μg/ml PI, then incubated at 37°C for 30 min. The percentages of cells in the different phases of the cell cycle or having the sub-G_1 DNA content were measured with a FACScan flow cytometer (BD Biosciences Pharmingen, USA) to evaluate the cell cycle components.

Apoptosis detection

Apoptotic morphological changes in the nuclear chromatin of cells were detected by Hoechst 33258 staining, as described previously [33]. After treatment, cells were washed with cold PBS, fixed with 4% paraformaldehyde for 30 min and then incubated with 5 μg/ml Hoechst 33258 staining solution for 10 min. The staining cells were viewed and photographed under a fluorescence microscope (Olympus, Japan).

Apoptosis was quantified by staining cells with annexin V-fluorescein isothiocyanate (FITC) and PI labeling [34]. Briefly, harvested cells were washed twice with cold PBS and resuspended in 100 μl 1 × binding buffer. Then 5 μl of Annexin V-FITC and 5 μl of PI were added to cells and incubated at room temperature in the dark and 400 μl 1 × binding buffer was added to each sample. The flow cytometric analysis was performed immediately.

$O_2{\cdot}^-$, H_2O_2, ·OH and MDA assays

The $O_2{\cdot}^-$ production was evaluated by assessing the activity of PC12 cells to reduce NBT using the modified method [35]. At the end of treatment time, the medium was carefully removed and then fresh medium containing NBT (1 mg/ml) was added to the cells. Following incubation for 2 h at 37°C, the medium was removed. Cells were fixed with 100% methanol and solubilized in 120 μl 2 M KOH and 140 μl dimethyl sulphoxide. The optical densities at 570 nm were measured by a microplate reader.

H_2O_2 assay were carried out using an Hydrogen peroxide assay kit. After that time, cells were lysed with 200 μl schizolysis solution supplied by the kit, then the supernatants were gathered by centrifuging at 12 000 g at 4°C for 5 min. The 96-well plates containing 50 μl of supernatants and 100 μl of test solutions were placed at room temperature for 30 min and measured instantly with a microplate reader at a wavelength of 560 nm.

The ability of scavenging ·OH of PQQ was measured by means of colorimetry. Being produced in the Fenton reaction, the quantity of ·OH was proportional to that of H_2O_2. In the presence of Griess reagent, the red colour could develop and was proportional to the quantity of ·OH. Therefore, the ability of scavenging ·OH could be assessed by means of this principle. In the experiment, the operating liquid was prepared according to requirements of ·OH assay kit, following the directions of the manufacturer; 200 μl of harvested supernatants and 600 μl test reagent were mixed and reacted for 1 min at 37°C. Results were expressed as U/ml, in which one unit represented for scavenging 1.0 μmol/ml of H_2O_2 per minute at 37°C per ml of sample.

MDA, production of lipid peroxidation, was assayed by an improved colorimetric method. Briefly, 200 μl of harvested supernatants were mixed with solution supplied by the kit. Reaction mixture was heated at 95°C for 60 min. The pink-coloured chromogen formed by the reaction of 2-thiobarbituric acid with MDA was spectrophotometrically measured at 532 nm. The detailed analysis procedure was described in the manufacturer's protocol. The results were expressed as MDA nmol/ml.

SOD,CAT and GSH-Px assays

SOD, CAT and GSH-Px activities were measured using the corresponding assay kits. After treatment, harvested cells were washed with PBS, lysed with cell lysis buffer on ice and then centrifuged at 3500 × g for 10 min according to the manufacturer's instructions. The supernatants were subjected to enzyme activity assays. Total protein concentration of all samples was measured spectrophotometrically at 562 nm by BCA method with bovine serum albumin as a standard.

SOD activity was assayed spectrophotometrically at 550 nm by use of a xanthine and xanthine oxidase system. The rate of the reduction with $O_2{\cdot}^-$ is linearly related to the xanthine oxidase activity and is inhibited by SOD. Therefore, the 50% inhibition activity of SOD can be measured at 550 nm of absorbance. One unit of SOD activity was defined as the amount of SOD required for 50% inhibition of the xanthine and xanthine oxidase system reaction in 1 ml enzyme extraction of per milligram of protein. The enzyme activity was expressed as U/mg protein.

CAT can decompose the H_2O_2. This reaction can be terminated by the addition of ammonium octamolybdate. The yellow complex was formed by the reaction of rest H_2O_2 with ammonium octamolybdate. CAT activity was assayed spectrophotometrically at 405 nm by measuring the initial rate of the disappearance of H_2O_2. One unit of CAT activity was defined as 1 μmol H_2O_2 decomposed per milligram of protein

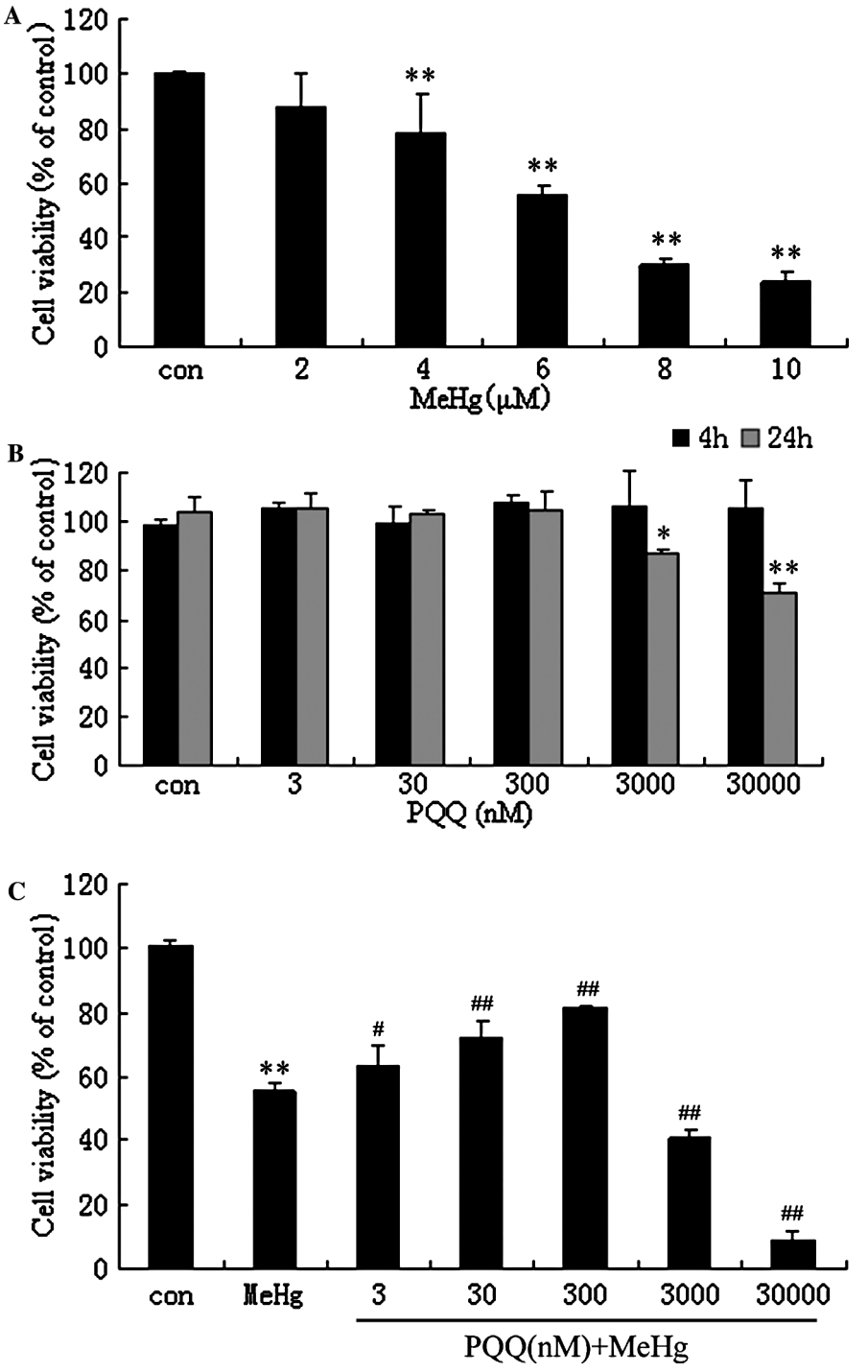

Figure 2. (A) Effect of MeHg on PC12 cell viability. (B) Effect of PQQ on PC12 cell viability. (C) Effect of PQQ on cell viability loss in PC12 cells exposed to MeHg. $^{\star}p<0.05$ or $^{\star\star}p<0.01$, compared with control, $^{\#}p<0.05$ or $^{\#\#}p<0.01$, compared with MeHg treatment group.

per second. The enzyme activity was expressed as U/mg protein.

GSH-Px was assayed spectrophotometrically by use of glutathione as a substrate by measurement of the decrease of enzymatic reaction of glutathione (except the effect of non-enzymatic reaction) at 412 nm in a system of enzymatic reaction of per milligram of protein per minute. One unit of GSH-Px activity was defined as the decreased amount of 1 μmol/L GSH (except the effect of non-enzymatic reaction) in systems of enzymatic reaction of 1 mg protein per minute. The enzyme activity was expressed as U/mg protein.

Statistical analysis

All the experiments were performed in triplicate and data were expressed by mean ± SD. 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.

Results

Effect of PQQ on cell viability loss in PC12 cells exposed to MeHg

As shown in Figure 2A, MeHg decreased the PC12 cell viability in a dose-dependent manner. PQQ had no effect on cell viability for 4 h. However, after 24 h, cell viability significantly decreased in the 3 and 30 μM PQQ treatment groups (Figure 2B). Compared with the control group, treatment with 6 μM MeHg for 4 h had been shown to cause nearly 50% viability loss in PC12 cells ($55.65 \pm 3.43\%$). For this reason, we decided to use the treatment concentration of 6 μM for 4 h in all subsequent experiments. The effect of PQQ on MeHg-induced cell viability loss is shown in Figure 2C. The MeHg-induced loss of cell viability was significantly attenuated by nanomolar levels of PQQ in a dose-dependent manner (3 nM: $63.6 \pm 6.05\%$; 30 nM: $72.4 \pm 4.86\%$; 300 nM: $81.4 \pm 0.9\%$). However, micromolar levels of PQQ clearly enhanced the toxicity of MeHg, which decreased the cell viability to $40.8 \pm 2.16\%$ and $8.8 \pm 2.87\%$, respectively.

Effect of PQQ on MeHg-induced changes in cell cycle

The effect of PQQ on MeHg-induced cell cycle progression of PC12 cells was studied by PI staining. The sub-G_1 area, appearing left to the G_1 peak (2n) in DNA distribution, represents the cell population undergoing apoptosis. Compared with the control group, treatment of cells with MeHg for 4 h led to a significant appearance of sub-G_1 area with concomitant accumulation in S-phase (Figure 3B). When cells were pre-treated with PQQ, then exposed to MeHg, the proportion of S-phase and sub-G_1 area remarkably decreased by PQQ in a dose-dependent manner (Figure 3C–E).

Effect of PQQ on MeHg-induced apoptosis progression of PC12 cells

To explore the protective effects of PQQ on MeHg-induced PC12 cells apoptosis, we observed the morphological changes by using Hoechst 33258 staining. After cells were treated with 6 μM of MeHg for 4 h, the number of shrunken or fragmented cells with condensed chromatin clearly increased (Figure 4B), while almost no apoptotic nuclei were observed in control cells (Figure 4A). When cells were pre-treated with PQQ for 30 min, then exposed to 6 μm of MeHg for 4 h, the morphological changes of apoptosis were effectively inhibited (Figure 4C–E).

Annexin V-FITC and PI double staining was performed to further identify the apoptosis progression. Exposure to MeHg resulted in the percentage of both early apoptotic cells (Annexin V-FITC$^+$/ PI$^-$) and late apoptotic cells (Annexin V-FITC$^+$/ PI$^+$) increased to $60.7 \pm 3.89\%$ and $31.4 \pm 1.13\%$ as compared to $3.9 \pm 0.28\%$ and $5.0 \pm 0.35\%$ in the normal control population (Figure 5A and B). After pre-treatment with PQQ, then exposed to MeHg, the percentage of both decreased to $40.8 \pm 0.49\%$ and $26.4 \pm 1.27\%$ of 3 nM, $26.1 \pm 0.92\%$ and $22.9 \pm$

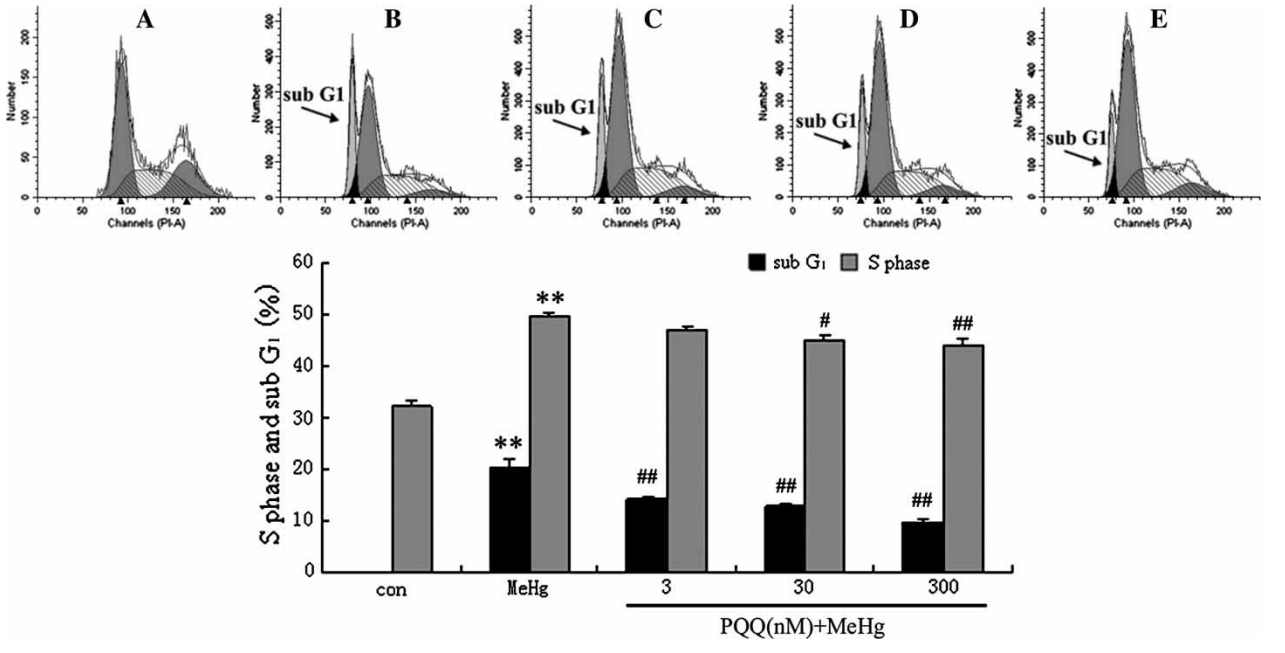

Figure 3. Effect of PQQ on MeHg-induced changes in cell cycle. (A) control. (B) PC12 cells were treated with MeHg for 4 h. (C–E) Cells were pre-treated with PQQ for 30 min and then were exposed to MeHg for 4 h. $^*p < 0.05$ or $^{**}p < 0.01$, compared with control, $^{\#}p < 0.05$ or $^{\#\#}p < 0.01$, compared with MeHg treatment group.

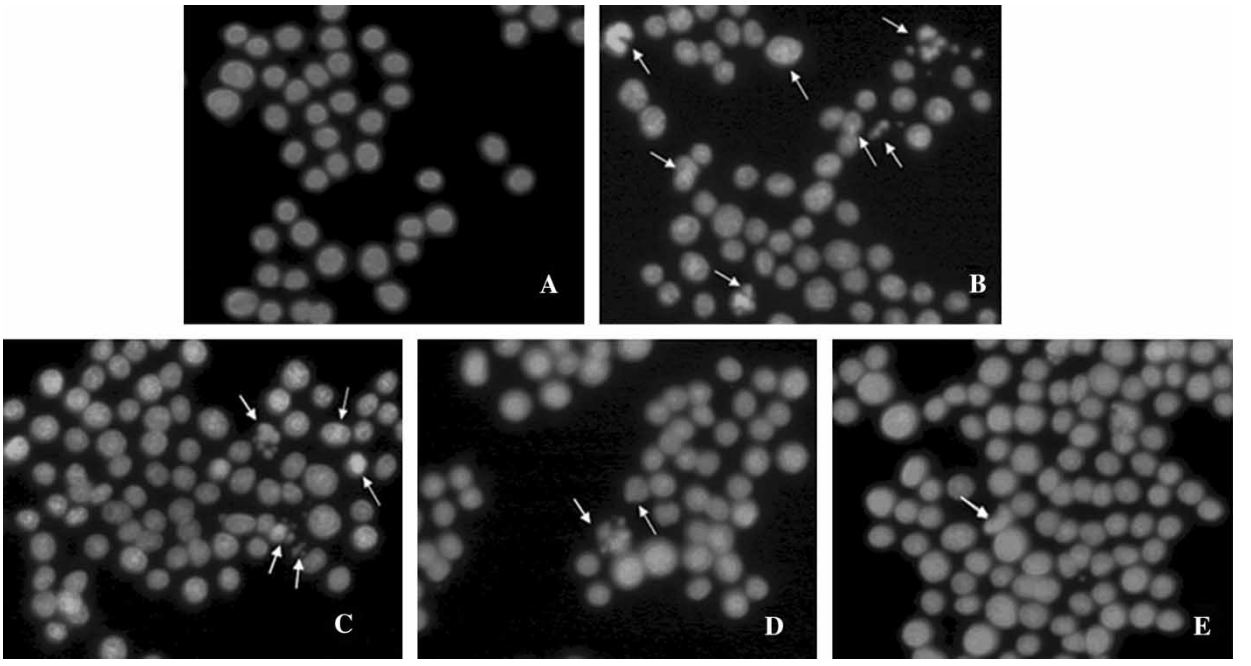

Figure 4. Effect of PQQ on morphological changes of MeHg-induced apoptosis by Hoechst 33258 staining (400 ×). (A) control. (B) PC12 cells were treated with MeHg for 4 h. (C–E) Cells were pre-treated with PQQ for 30 min and then were exposed to MeHg for 4 h.

1.77% of 30 nM and 22.9 ± 1.98% and 12 ± 2.55% of 300 nM, respectively (Figure 5C–E).

Effect of PQQ on ROS production and lipid peroxidation in cells exposed to MeHg

As shown in Table I, compared with the control group, there was a significant increase in $O_2\cdot^-$, H_2O_2 and ·OH production in PC12 cells treated with MeHg. When PC12 cells were pre-treated with PQQ prior to MeHg treatment, the above ROS production was evidently reduced.

MDA is a marker of lipid peroxidation. Compared with the control group, MDA production in PC12 cells treated with MeHg was found to be statistically significant, whereas PQQ could evidently reduce the MDA production.

Effect of PQQ on antioxidant enzyme activities in cells exposed to MeHg

Information on SOD, CAT and GSH-Px activities is shown in Table II. When PC12 cells were treated with MeHg for 4 h, SOD, CAT and GSH-Px activities significantly decreased in comparison with the control group ($p < 0.01$). After pre-treatment with PQQ (30 and 300 nM), the SOD activity was evidently increased from 17.04 ± 1.21 U/mg prot to 20.81 ± 0.69 U/mg prot ($p < 0.05$) and 24.12 ± 1.38 U/mg prot ($p < 0.01$), respectively. This is consistent with the tendency of GSH-Px (160.92 ± 13.49 vs 124.59 ± 9.68 U/mg prot, $p < 0.05$; 189.59 ± 8.58 vs 124.59 ± 9.68 U/mg prot, $p < 0.01$); 300 nM PQQ also elevated the CAT activity in comparison with the MeHg treatment group ($p < 0.01$).

Discussion

Through a series of experiments from many laboratories, it has been previously demonstrated that MeHg has toxic effects on animals, as well as on humans [36–38]. So far medical treatment is limited to the rapid decontamination of MeHg by means of chelating agents, dialysis and the oral administration of thiol resin. However, these treatments are not sufficient to protect against the intracellular behaviour of MeHg and cellular response to it. The present study indicated that exposure to MeHg resulted in the PC12 cells injury. The nanomolar levels of PQQ significantly increased PC12 cells viability in a dose-dependent 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. He et al. [39] found that PQQ could interact with RPMI 1640 medium to generate H_2O_2 and the extracellular generation of this oxidant may contribute to the cell death. The present results agree with the above observations. Various concentrations of PQQ have no toxicity to PC12 cells in a short time incubation. As the incubation time increased, the micromolar levels of PQQ clearly inhibited the cell viability. We suspected that the relative high dosage of PQQ could generate some oxidative species, such as $O_2\cdot^-$, H_2O_2 and ·OH, and thereby result in the cell death. Moreover, the fact that the micromolar levels of PQQ promoted the toxicity of MeHg to PC12 cells may be also due to the combination effect of MeHg and these oxidative species.

It is very important to note that compounds that engage in redox cycling can also be effective free radical

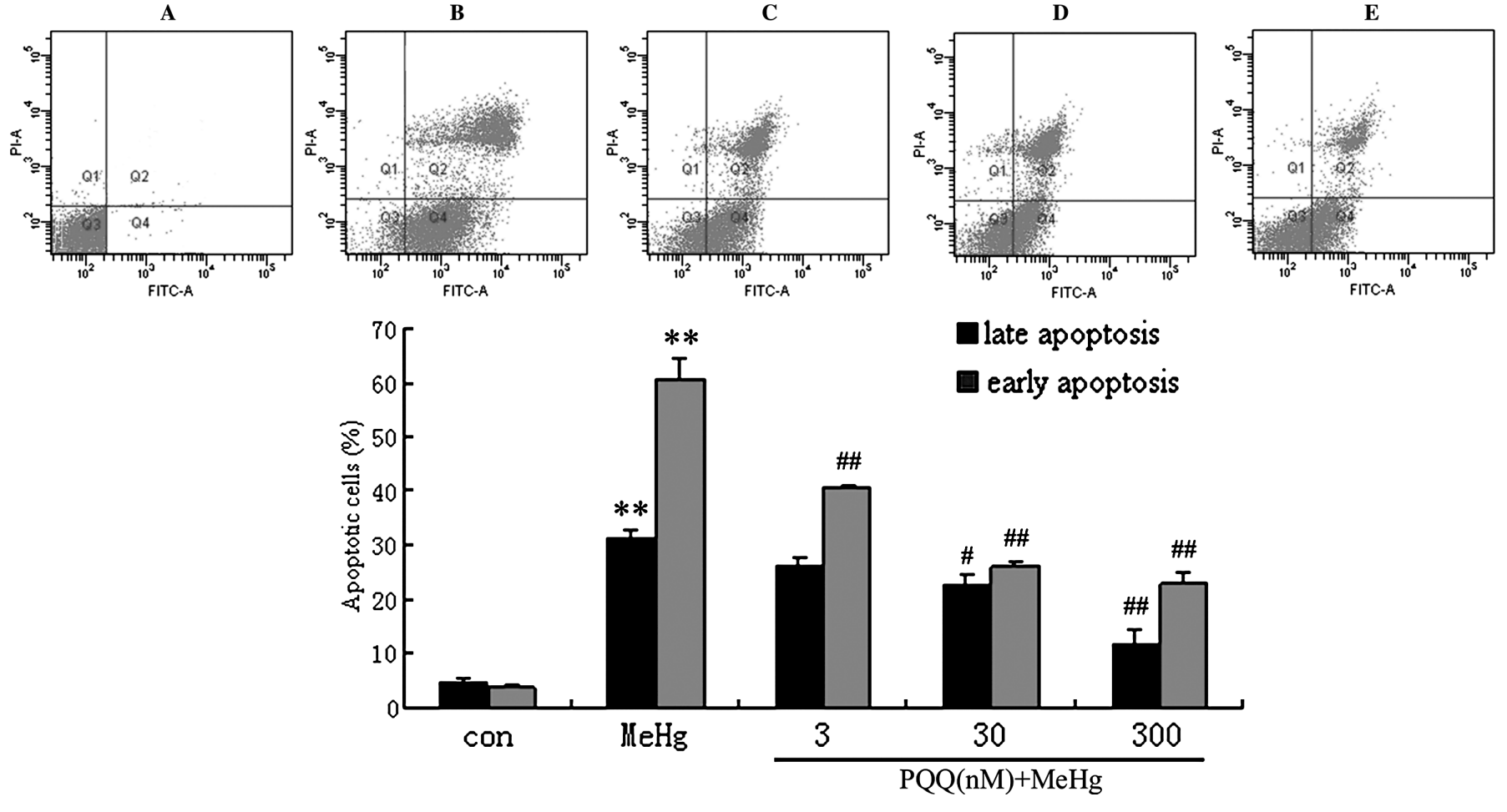

Figure 5. Effect of PQQ on MeHg-induced distributive change of early and late apoptotic cells. (A) control. (B) PC12 cells were treated with MeHg for 4 h. (C–E) Cells were pre-treated with PQQ for 30 min and then were exposed to MeHg for 4 h. *$p<0.05$ or **$p<0.01$, compared with control, #$p<0.05$ or ##$p<0.01$, compared with MeHg treatment group.

Table I. The levels of $O_2^{\cdot -}$, H_2O_2, $^{\cdot}OH$ and MDA

	$O_2^{\cdot -}$ (% of control)	H_2O_2 (% of control)	$^{\cdot}OH$ (U/ml)	MDA (nmol/ml)
control	100	100	91.5±0.5	2.9±0.04
MeHg	112.5±1.5**	240.1±0.9**	242.4±11.5**	8.7±0.1**
PQQ (3 nM)	111.1±1.9	216.3±12.0#	202.9±7.2##	7.1±0.8
PQQ (30 nM)	108.4±1.3#	178.6±6.4##	182.6±1.4##	5.9±0.8#
PQQ (300 nM)	105.5±0.7##	162.5±0.7##	163.7±7.2##	4.2±0.9##

*$p<0.05$ or **$p<0.01$, compared with control, #$p<0.05$ or ##$p<0.01$, compared with MeHg treatment group.

initiators. Due to its antioxidant and pro-oxidant properties, PQQ may be therefore regarded as a janus-faced molecule. Hiraku and Kawanishi [40] found that only 2 μM PQQ initiated DNA damage in the presence of NADH and Cu^{2+}. He et al. [39] reported that high concentrations of PQQ (>50 μM) caused Jurkat cell death after 24 h incubation by the measurement of the loss of intracellular LDH activity, whereas 25 μM PQQ induced apoptotic cell death after 5 h incubation by increasing the caspase-3 activity. When pyrroloquinoline quinone (PQQ) was intraperitoneally injected into rats daily for 4 days at a dose of 11.5 mg/kg body weight/injection, nephrotoxicity were clearly observed [41]. The current investigations demonstrated that PQQ (>3 μM) resulted in the decrease of PC12 cell viability. These results indicated that the ultimate effect of PQQ is strongly dependent on its concentrations, redox system and microenvironment. However, it is worth noting that nanomolar levels of PQQ could protect PC12 cells against MeHg-induced neurotoxicity and may be worth further research to determine its therapeutic potential.

Apoptosis is a mode of cell death characterized by distinct morphological and biochemical features. Morphologically, apoptosis is often characterized by cytoplasmic shrinkage, active membrane blebbing and chromatin condensation with or without nuclear fragmentation into membrane-enclosed vesicles. This visible morphological transformation is accompanied by several important biochemical changes including externalization of phosphatidylserine to the outer leaflet of the plasma membrane, degradation of the chromosomal DNA into high molecular weight and oligosomal-sized fragments, as well as the cleavage and proteolysis of a variety of specific cellular proteins called death substrates. In this study, when PC12 cells were treated with MeHg, morphological changes typical of apoptosis were observed. Flow cytometric analysis demonstrated that an increase in the proportion of cells in S-phase was evident after MeHg treatment. Moreover, accompanied by the increase of S-phase arrest, the proportion of cells in the sub-G_1 area also correspondingly increased. Through annexin V-FITC and PI staining simultaneously, we further identified the percentage of both early apoptotic cells and late apoptotic cells increased due to MeHg exposure. The addition of PQQ evidently reduced the number of apoptotic cells, ameliorated S-phase arrest and decreased the peak of sub-G_1 area. Further, both of the early apoptosis and late apoptosis populations were reduced concentration-dependently by PQQ. These results suggested that PQQ can protect PC12 cells against MeHg-induced apoptotic cell death.

Table II. The activities of SOD, CAT and GSH-Px (U/mg prot).

	SOD	CAT	GSH-Px
control	29.3±1.5	7.1±0.1	277.3±6.0
MeHg	17.1±1.2**	3.8±0.1**	124.6±9.7**
PQQ (3 nM)	18.5±0.5	4.1±0.2	143.3±5.3
PQQ (30 nM)	20.8±0.7#	4.8±0.4	160.9±13.5#
PQQ (300 nM)	24.1±1.4##	5.7±0.5##	189.6±8.6##

*$p<0.05$ or **$p<0.01$, compared with control, #$p<0.05$ or ##$p<0.01$, compared with MeHg treatment group.

Oxidative stress is the result of an imbalance in the pro-oxidant/antioxidant homeostasis and has been pointed to as an important molecular mechanism in MeHg neurotoxicity [42,43]. It is widely accepted that ROS, such as $O_2^{\cdot -}$, H_2O_2 and $^{\cdot}OH$, are responsible for various stress-induced damages to macromolecules and ultimately to cellular structures. Some of these stress responses have been attributed to changes in the activities of ROS scavenging enzymes, such as SOD, CAT and GSH-Px. SOD catalyses the dismutation of superoxide radicals, whereas CAT eliminates hydrogen peroxide. GSH-Px provides an efficient protection against oxidative damage and free radicals in the presence of GSH through reduction of both hydrogen peroxide and organic hydroperoxides. A decrease in the activity of these antioxidants can lead to an excess availability of $O_2^{\cdot -}$ and H_2O_2, which in turn generate $^{\cdot}OH$, resulting in initiation and propagation of lipid peroxidation. *In vivo* and *in vitro* biochemical studies employing neuronal cultures and mixed neuronal/glial cultures have shown increased ROS formation with MeHg exposure [44–46]. These studies are consistent with our investigation that the levels of $O_2^{\cdot -}$, H_2O_2 and $^{\cdot}OH$ increased after exposure to MeHg. Moreover, in our study, MeHg caused the oxidative lipid damage, as indicated by increase of MDA. MDA is a marker of lipid peroxidation resulting from the direct interaction of ROS and unsaturated fatty acids [47,48] and it was used as one representative of ROS accumulation. Previous studies have indicated that PQQ can scavenge $O_2^{\cdot -}$ and $^{\cdot}OH$ efficiently [49], inhibit lipid peroxidation [50]

and also protect the isolated heart from reoxygenation injury [51]. Therefore, PQQ is thought to be a potent antioxidant. Our studies indicated that PQQ can significantly decrease the production of intracellular ROS and reduce the level of lipid peroxidation in comparison with the MeHg treatment group.

To further understand the underlying protective mechanism of PQQ, we examined the changes of SOD, CAT and GSH-Px activities. SOD is an essential component of the antioxidative defense system. In the present study, MeHg treated cells showed a serious decrease in the level of antioxidant enzymes SOD, CAT and GSH-Px compared to the control group. The reduction in the activities of antioxidant enzymes like SOD, CAT and GSH-Px could reflect the adverse effect of MeHg on antioxidant system of cells. A reduction in the activity of SOD causes a rise in the level of $O_2{\cdot}^-$, which inactivates CAT activity [52]. CAT or GSH-Px fails to eliminate H_2O_2 from the cell and the accumulated H_2O_2 has been shown to cause inactivation of SOD [53]. Compared with the MeHg treatment group, pretreatment with PQQ mildly elevated the activities of SOD, CAT and GSH-Px. These observations permitted us to propose that PQQ may exert its beneficial effect against MeHg-caused toxicity by scavenging the excess $O_2{\cdot}^-$, H_2O_2 and $\cdot OH$. Moreover, PQQ increases the activities of antioxidant enzymes in MeHg-treated PC12 cells implying that reactivating the antioxidant defense system may be an important mechanism for PQQ-mediated protective effect.

In conclusion, the results summarized herein overwhelmingly documented the ability of PQQ to inhibit apoptosis and reduce oxidative stress induced by MeHg *in vitro*. Data from this study demonstrated that MeHg is highly toxic to neuronal cells by producing the excessive ROS and inhibiting the antioxidant enzymes activities and eventually triggering the apoptotic cell death. Addition of PQQ reduced the damage probably due to its strong antioxidant property. However, our study found that the concentration range that PQQ exerted its protective effect on PC12 cells exposed to MeHg was narrow. Thus, it is likely to limit a possible therapeutic role. This needs extensive *in vitro* and *in vivo* investigation to evaluate its application effect. In any case, the present results obtained highlighted the potential of PQQ in offering protection against oxidative stress and supported the fact that PQQ may provide health benefits. The detailed mechanisms of PQQ against MeHg-induced neurotoxicity need to be further investigated in the future.

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