Deciphering the possible role of H$_2$O$_2$ in methylmercury-induced neurotoxicity in *Xenopus laevis*

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Abstract

**Backgrounds** Methylmercury (MeHg) is regarded as a developmental neurotoxicant but the detailed mechanism remains not completely clear.

**Methods** The *Xenopus laevis* embryos were exposed to methylmercury chloride and the expression of neurodevelopment and oxidative stress genes was detected by qRT-PCR or Western blotting. PC12 cells were exposed to various levels of H$_2$O$_2$, and then cell cycle, neurite length, neurodevelopment-related genes, protein expression of apoptosis and autophagy were detected.

**Results** The genes of neurodevelopment and oxidative stress were disrupted by methylmercury chloride and H$_2$O$_2$ were increased interestingly in *X. laevis* embryos. Then, PC12 cells were exposed to H$_2$O$_2$ and the results showed the cell cycle, neurite length, and neurodevelopment-related genes, the proteins apoptosis and autophagy were changed.

**Conclusion** These results supported the idea that neurodevelopment-related gene expression was regulated by oxidative stress and that apoptosis and autophagy pathways were activated by H$_2$O$_2$ and involved in methylmercury neurotoxicity.

Keywords Methylmercury · Neurotoxicity · H$_2$O$_2$ · Oxidative stress · Apoptosis · Autophagy

Introduction

Mercury circulates in the ecosystem, dissolving in fresh water and sea water, and it is transformed to methylmercury by anaerobic bacteria and aquatic organisms, methylmercury has a long half-life in aquatic organisms resulting bioaccumulation and then travels through the food chain and accumulates in humans (Hsu-Kim et al. 2013). Methylmercury (MeHg) is studied mostly as a developmental neurotoxicant because low concentrations of MeHg have been detected in cord blood and placental tissue; this contaminant can pass through the placental and blood–brain barriers, which makes the developing brain vulnerable to MeHg exposure (Hu et al. 2010). Sensory deficits, motor impairment, and an overall neurocognitive decline were found in aquatic animals after MeHg exposure (Cordier et al. 2002).

The effects of methylmercury exposure on development have been evaluated in rodent models, and low-level MeHg exposure has been found to impair motor coordination (Fujimura et al. 2012; Cheng et al. 2013). Additionally, MeHg exposure has been reported to induce apoptosis in cerebellar cells (Daré et al. 2001, 2015); for cerebellar granule cells in particular, oxidative stress has been linked to heavy-metal-induced neurotoxicity and several neurodegenerative disorders (Marcelo et al. 2013). However, the detailed mechanism of MeHg neurotoxicity remains to be elucidated.

In previous study, we used methylmercury chloride (MeHgCl) to investigate the effects of MeHg on the
embryonic development of X. laevis, and the results showed that X. laevis embryos were exposed to 300 and 400 nmol/L MeHgCl for 96 h presented an abnormal motor behavior (Fu et al. 2014). To further know the mechanism, the related genes of neurodevelopment and oxidative were investigated in this study, the results showed that neurodevelopment and oxidative stress were markedly altered and that high levels of hydrogen peroxide (H_{2}O_{2}) were generated. To validate the important role of H_{2}O_{2} in neurodevelopmental toxicity, we further investigated its toxic mechanism in a cell-culture model consisting of rat pheochromocytoma cells (PC12 cells) with nerve growth factor (NGF) (Yang et al. 2018; Fu et al. 2016). In the current study, the cell cycle, neurite length, and neurodevelopment-related gene expression were affected and the markers of apoptosis and autophagy were detected. Therefore, this study could provide new insights into the mechanisms of MeHg-induced developmental toxicity and neurotoxicity.

Materials and methods

Animal husbandry and breeding

X. laevis frogs (Nasco, Fort Atkins, WI, USA) were fed in glass tanks containing charcoal-filtered tap water at 22–24 °C under a photoperiod of 12 h light:12 h dark. A pair of adult X. laevis were injected with human chorionic gonadotropin (250 IU for males and 500 IU for females, Sigma, St. Louis, MO) to stimulate breeding. All eggs were collected after 12 h and stripped of the jelly coat with 2% cysteine solution. Normally developing embryos at the NF8 (middle blastocyst) to NF11 (early gastrula) stages were chosen for exposure experiments.

Exposure of X. laevis to methylmercury chloride

Methylmercury chloride (MeHgCl, CAS number 115-09-3, Dr. Ehrenstorfer GmbH, Germany) was dissolved in dimethyl sulfoxide (DMSO) to prepare the stock solution (10 mol/L). FETAX (frog embryo teratogenesis assay-Xenopus test) is a method to test the teratogenic toxicity of chemicals during the development of frog embryos. This solution consists of 0.01 mol/L-NaCl, 1 mmol/L-NaHCO_{3}, 0.625 mmol/L-MgSO_{4}, 0.35 mmol/L-CaSO_{4}·2H_{2}O, 0.4 mmol/L-KCl and 0.15 mmol/L-CaCl_{2} in ddH_{2}O, pH 7.2–7.4. Twenty five embryos per exposure level were placed in a Petri dish and in 37 °C for 96 h.

Cell culture and H_{2}O_{2} treatment

The PC12 rat pheochromocytoma cell line was purchased from the Shanghai cell bank of the Chinese Academy of Sciences; when cultured in cell medium with 50 ng/mL NGF, the cells developed neuron-like characteristics. The cells were cultured with Dulbecco’s modified Eagle’s medium (DMEM) (HyClone, Logan, USA, SH30022.01B) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, USA, SH30084.03) and 1% penicillin/streptomycin (HyClone, Logan, USA, SV30010) at 37 °C in 5% CO_{2}. PC12 cells were treated with 50, 100, 200 or 300 µmol/L of hydrogen peroxide for 35 h (the period determined by an optimization process). The effects of the treatment on gene expression, the cell cycle, neurite length and intracellular ROS were tested.

RNA extraction and gene expression analysis

Total RNA was isolated from the heads of the embryos by vigorously agitating them in TRIzol reagent according to the manufacturer’s instructions. The concentration and quality of total RNA were measured using a NanoDrop 2000 (Thermo Scientific, USA). RNA (1 µg) was reverse transcribed into cDNA using a Quantscript RT Kit (Tiangen, Beijing, China). The cDNA was stored at −80 °C for further expression analysis. The expression levels of select neuron-related genes were analyzed by qPCR. Ribosomal protein L8 (rpl8) as reference gene in X. laevis embryos and β-actin as reference gene in cellular experiment, and primer information was showed in Table 1.

Western blot

After being exposed to various concentrations of MeHgCl for 96 h, ten the heads of X. laevis embryos were collected and mixed with a homogenizer. PC12 cells were collected and washed two times with PBS, and centrifuged at 10,000g for 15 min at 4 °C. The supernatant of samples was collected for a quantitative test with a BCA kit (Dingguo, Beijing, China). Each sample was separated by SDS-PAGE and transferred to a PVDF membrane. The membrane was then blocked and incubated with primary antibody (at 1:1,000 dilution). After being washed three times with PBST, the membrane was incubated with a secondary antibody (at 1:2,000 dilution) at room temperature for 2 h, and protein was detected with an enhanced chemiluminescence kit (Thermo Scientific, Boston, USA). Antibodies against GAP43, βIII-tubulin, acetylated α-tubulin, and Bax were purchased from Santa Cruz Technology Co. (Santa Cruz, California, USA); antibodies against caspase-3, Beclin-1, α-tubulin, GAPDH and LC3 were purchased from Abcam Ltd. Co. (Headquarters, Cambridge, UK). The Western blots were analyzed using ImageJ software to quantify relative protein expression.
Hydrogen peroxide assay

Levels of H$_2$O$_2$ were measured using a commercial hydrogen peroxide assay kit (Beyotime Biotech, China) according to the manufacturer’s instructions. Briefly, embryos were weighed and immersed in hydrogen peroxide lysis solution, and tissues were mixed with a homogenizer; subsequently, the supernatant and testing solution were added to each well of a 96-well plate, and measured immediately with a spectrophotometer at a wavelength of 560 nm, and the content of H$_2$O$_2$ (μg) per gram of embryo was calculated.

ROS measurement

Cells were collected and resuspended in PBS at 1 × 10$^5$ cells/mL. MitoSOX Red dye (mitochondrial ROS specific, Invitrogen-Life Technologies, New York, USA) was added to the cells for a concentration of 5 μmol/L, after which the cells were incubated at 37 °C for 30 min. Untreated cells served as negative controls. Red fluorescence was detected using a BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA). Cell cycle profiles were calculated by C6 software (Instrument’s own software from BD Biosciences.).

Immunofluorescence and neurite length measurement

PC12 cells were seeded into 12 wells plate, the plate had put the corresponding size glass piece before, adding DMEM medium with final concentration of H$_2$O$_2$ at 0, 50, 100, 200 and 300 μmol/L, which cultured at 37 °C in 5% CO$_2$. After 35 h, medium was removed and cells were fixed with 4% methanol for 10 min and washed two times with PBS, βIII-tubulin antibodies (at 1:100 dilution) were incubated for 2 h at 37 °C, and second antibodies (FITC labeled at 1:200 dilution) were incubated for 1 h. After washing two times with PBS, the cell climbing piece were got out and pasted in a glass slide. Immunofluorescence images were taken with confocal microscopy (Leica, Berlin, Germany) and neurite length was measured with ImageJ software.

Cell cycle analysis by flow cytometry

PC12 cells were harvested and washed in cold PBS, then incubated in 50 μg/mL propidium iodide (PI) solution (containing 0.03% Triton X-100) at room temperature for 20 min. For each sample, at least 2 × 10$^5$ cells/mL were analyzed by an BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA). Cell cycle profiles were calculated by C6 software (Instrument’s own software from BD Biosciences.).

Data analysis

All of the data were expressed as the means ± SD. The statistical differences were analyzed using Student’s t test, and a $P$ value less than 0.05 was considered significantly different. Histograms were drawn with GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA).
Results

The expression of neurodevelopment-related genes was affected by MeHgCl

The expression levels of neurodevelopment-related genes including growth associated protein-43 (gap-43), neurogenic differentiation (neurod), class III β-tubulin isotype (βIII-tubulin) and neural cell adhesion molecule (ncam) were measured by qRT-PCR, and the result showed that gap-43 (Fig. 1a) was increased after MeHgCl exposure at 400 nM, while neurod (Fig. 1b), βIII-tubulin (Fig. 1c) and ncam (Fig. 1d) were decreased after MeHgCl exposure at 300 nmol/L and 400 nmol/L. Then, the protein expression levels of neurodevelopment genes were measured by Western blot; the result showed that the protein expression of GAP-43 (Fig. 1e, f) was increased at 300 nmol/L, while other neurodevelopment proteins such as βIII-tubulin (Fig. 1e, g) and acetylated α-tubulin (Fig. 1e, h) were decreased at 200 nmol/L and 300 nmol/L (Fig. 1e–h). These results indicated that MeHgCl affected the neurodevelopment of X. laevis embryos in terms of mRNA as well as protein levels.

Oxidative stress was activated by MeHgCl

To investigate the mechanisms whereby MeHgCl affected X. laevis embryos, we used qRT-PCR to measure the expression of genes related to oxidative stress, including matrix metalloproteinase (mmp1), thioredoxin 1 (txn1), glutathione reductase (gsr), glutathione S-transferase 6 (gstf6), selenoprotein P (selenop) and glutathione peroxidase 1 (gpx1). Increasing concentrations of MeHgCl were associated with up-regulation of mmp1 (Fig. 2a), txn1 (Fig. 2b), gsr (Fig. 2c) and gstf6 (Fig. 2d) expression, as well as down-regulation of selenop (Fig. 2e) and gpx1 expression (Fig. 2f). These results indicated that the contents of the key oxido-reductases associated with ROS production were altered at high concentrations of MeHgCl. Thus, the oxidation–reduction system of X. laevis embryos undergoing neurodevelopment was damaged by high MeHgCl levels. Then, H2O2 was extracted from the embryonic bodies and measured after 96 h of MeHgCl exposure; the level of H2O2 was obviously increased after MeHgCl exposure at 200–400 nmol/L (Fig. 2g). This result suggests that H2O2 generation damages the antioxidant defense system in X. laevis embryos at high MeHgCl levels.

Exogenous H2O2 induces neurotoxicity in PC12 cells

To investigate the effect of H2O2 in MeHgCl-exposed X. laevis embryos, we generated a cell culture model of neurons by adding NGF to the culture medium of PC12 cells, creating an ideal model to further study the developmental neurotoxicity of H2O2 exposure. After 35 h of H2O2 exposure, the morphology of the PC12 cells was photographed, and neurite length was analyzed; the results showed that the neurite length of PC12 cells was obviously shortened after 200 and 300 μmol/L·H2O2 exposure (Fig. 3a, b). Then, intracellular ROS levels were measured by flow cytometry, revealing high ROS at 200 and 300 μmol/L·H2O2 exposure, the
same concentrations at which neurite length was shortened (Fig. 3c). Subsequently, the mRNA levels of neurodevelopment genes were tested by qRT-PCR; the results showed that the expression of \textit{gap-43} (Fig. 3d) was increased while the expression levels of \textit{neurod} (Fig. 3e), \textit{βIII-tubulin} (Fig. 3f) and \textit{ncam} (Fig. 3g) were decreased after high-level H$_2$O$_2$ exposure. To test for effects on proliferation, we also analyzed the cell cycle in PC12 cells by flow cytometry after 35 h of H$_2$O$_2$ exposure, and the results showed that the cell cycle was blocked at the S-G2/M checkpoint by high levels of H$_2$O$_2$, i.e., 200 and 300 μmol/L (Fig. 4a, b). These results indicate that H$_2$O$_2$ can induce neurotoxicity and inhibit neurite growth by increasing intracellular ROS levels in PC12 cells.

**Discussion**

Previous studies had reported that exposure of children to MeHg could cause severe neurotoxicity affecting brain development (Grandjean et al. 1997, 1998). To further investigate the mechanisms of MeHg toxicity, \textit{X. laevis} embryos were exposed to a series of MeHgCl concentration for 96 h after fertilization. We have previously reported that motor behavior of the embryos were abnormal after exposure to MeHgCl (Grandjean et al. 1997). In this study, we found that MeHgCl exposure can influence the expression of neurodevelopment genes in \textit{X. laevis} embryos in terms of transcription (\textit{gap-43, neurod, βIII-tubulin} and \textit{ncam}) and translational levels (GAP-43, βIII-tubulin and acetylated α-tubulin), and genes related to oxidative stress, including \textit{mmp1}, \textit{txn1}, \textit{gsr} and \textit{gstf6}, were regulated. GAP-43 is widely distributed in neurons and axons and is associated with neural development, axonal regeneration and synaptic plasticity (Benowitz et al. 1987). In addition, NeuroD, NCAM and βIII-tubulin play important role in neural development (Cho 2004; Kastens et al. 2001; Paratcha et al. 2003).

Selenoproteins are involved in protection of macromolecules and cells against oxidative stress. The well-known redox-active selenoenzymes such as glutathione peroxidases and thioredoxin reductases. The antioxidant and ROS scavenging capacity of thioredoxin can be damaged by mercury...
compounds, which can target thioredoxin and activate apoptosis pathway in neuronal cells (Branco et al. 2017). In this present study, the expression of selenop and gpx1 were decreased but mmp1, txnl, gsr and gstf6 were increased after MeHg exposure, which is a possible compensatory mechanism to restore cellular antioxidant capacity.

Some studies have reported mercury can induce cytotoxicity as well as nerve cell damage through inducing oxidative stress (Mailloux et al. 2015; Wehe 2014; Aschner et al. 2004; Syversen et al. 2005). This present study indicated that MeHg can elevate oxidative stress and induce neurotoxicity by increasing H₂O₂. To further validate the underlying mechanism, we exposed PC12 cells to various levels of H₂O₂. H₂O₂, a non-radical derivative of oxygen, is a reactive oxygen species and can be readily converted into oxygen radicals in the cell. In our study, the expression of neurodevelopment-related genes was reduced except in the case of GAP-43, whose expression increased after MeHgCl as well as H₂O₂ exposure. Our results showed gap43 mRNA levels increased at 400 nmol/L of MeHgCl but GAP 43 protein expression increased at 300 nmol/L, it indicated that microRNAs, protein modification and biphasic dose–response pattern played important roles in this phenomenon (Masoud et al. 2018).

In the present study, the expression levels of enzymes related to the antioxidant defense system were regulated in an MeHgCl dose-dependent manner. Some studies have reported that methylmercury-induced neurotoxicity by activating oxidative stress (Carneiro et al. 2014; Beatriz et al. 2016). One of the principal endogenous antioxidant systems is glutathione regulation; reduced glutathione is very important to ROS balance and the oxidative defense process.

![Figure 3](Image)

**Fig. 3** H₂O₂ induces neurotoxicity by increasing intracellular ROS in PC12 cells. **a** The morphology of PC12 cells after 35 h of exposure to H₂O₂ (0, 50, 100, 200 and 300 μM). **b** Semi-quantitative analysis of neurite length in (a). **c** A semi-quantitative analysis of ROS levels in PC12 cells was conducted by flow cytometry after 35 h of H₂O₂ exposure. **d–g** The expression of neurodevelopment genes including gap-43 (d), neurod (e), βIII-tubulin (f) and ncam (g) was analyzed by qRT-PCR after 35 h of H₂O₂ exposure.
based on glutathione reductase. In this study, the antioxidant balance was damaged by MeHgCl, which increased H$_2$O$_2$ levels and altered the expression of antioxidase and oxidase enzymes. Additionally, cell cycle progression, neurite length, neurodevelopment-related gene expression, apoptosis and autophagy markers were assessed after H$_2$O$_2$ exposing. The results showed that cell cycle of exposed PC12 cells was blocked at the S-G2/M checkpoint; it is possible that intracellular H$_2$O$_2$ impaired DNA replication, resulting in the stagnation of cellular growth and proliferation. In addition, oxidative stress might induce the cell cycle arrest and DNA damage at S and G2/M checkpoints (Bijur et al. 2015).

Fig. 4 The cell cycle was blocked in PC12 cells after 35 h of high-level H$_2$O$_2$ exposure. a Profiles of cell cycle stages after 35 h of H$_2$O$_2$ exposure at concentrations of 0, 50, 100, 200 and 300 μM were determined by flow cytometry. b Semi-quantitative analysis of cell cycle phases in (a). *denotes p < 0.05 (n = 3)

Fig. 5 Apoptosis and autophagy were activated by H$_2$O$_2$. a Western blot profile of caspase-3 and LC3 expression in PC12 cells exposed to 200 μM H$_2$O$_2$ for various lengths of time. b Semiquantitative analysis of activated caspase-3 (17 kDa) in (a). c Semiquantitative analysis of LC3-II/LC3-I in (a). d Western blot profile of Bax and Beclin-1 expression in PC12 cells exposed to 200 μM H$_2$O$_2$ for 35 h. e Semiquantitative analysis of Bax in (d). f Semiquantitative analysis of Beclin-1 in (d)
After \( H_2O_2 \) exposure, neurite length and mRNA expression of neurodevelopment-related genes were also measured; the results showed that neurite length was shortened and neurodevelopment-related genes were influenced. Similar to these results, Tusi et al. (2014), in studying the functional effects of \( Salvia macilenta \), found that the neurite length of PC12 cells was affected by \( H_2O_2 \) exposure (Tusi et al. 2014).

Then, to further investigate the mechanisms of \( H_2O_2 \)-induced neurotoxicity, we assessed apoptosis and autophagy through Western blots measuring the expression of caspase-3, Bax, LC3-II/I and Beclin-1. The results demonstrated that caspase-3, Bax, LC3-II/I and Beclin-1 were up-regulated after \( H_2O_2 \) exposure, suggesting that \( H_2O_2 \) can cause neurotoxicity in PC12 by through activating the apoptosis and autophagy pathways. In addition, ROS-induced cell apoptosis through the previously identified mitochondrial pathway; thus, our results showed that Bax and caspase-3 were up-regulated and suggested the \( H_2O_2 \)-induced PC12 apoptosis through the mitochondrial pathway. It is reported that \( H_2O_2 \)-induced autophagy depends on the induction of intracellular production of ROS and activation of the p38 mitogen-activated protein kinase \( \alpha \) (p38 MAPK\( \alpha \)) pathway (Cantarella et al. 2003). In this study, LC3-II/I and Beclin-1, examined as markers of autophagy, were up-regulated after \( H_2O_2 \) treatment, indicating that \( H_2O_2 \) can induce autophagy in PC12 cells. Therefore, \( H_2O_2 \) caused neurotoxicity in PC12 cells through apoptosis as well as autophagy.

\section*{Conclusion}

Our studies provide an evidence that oxidative stress contributed to MeHg-induced developmental neurotoxicity in \( X. laevis \) embryos; our work also verified, through in vitro exposure of the PC12 nerve cell model to endogenous ROS, that such toxicity occurred through apoptosis and autophagy. Both apoptosis and autophagy are important cellular responses to oxidative stress, and their dysregulation has been implicated in impaired brain development in infants and children. Therefore, our results suggest that MeHg-induced neurodevelopment toxicity is mediated by an increase in endogenous ROS that induces apoptosis and autophagy, and MeHg may induce apoptosis and autophagy in \( X. laevis \) embryos.

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\section*{Compliance with ethical standards}

\section*{Conflict of interest}

Xufeng Fu, Xiuyu Yang, Xing Du and Qinghua Cui declare that they have no conflict of interest.

\section*{Human and animal rights}

All animal procedures were conducted according to Regulations for the Administration of Affairs Concerning Experimental Animals (The Ministry of Science and Technology of the People’s Republic of China, 1988).

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