Journal of Hazardous Materials 194 (2011) 277–282

Contents lists available at ScienceDirect

Journal of Hazardous Materials

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

Effects of lead on neurogenesis during zebrafish embryonic brain development

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ARTICLE INFO

Article history:
Received 17 February 2011
Received in revised form 10 July 2011
Accepted 26 July 2011
Available online 10 August 2011

Keywords:
Lead
Neurogenesis
Zebrafish
Apoptosis

ABSTRACT

Lead neurotoxicity has caused wide public concern in recent decades, yet little is known about its effects on cellular and molecular mechanisms during the sensitive early life stages of animals. This study examines neurological deficits caused by lead acetate (Pb) during early embryonic stages in the zebrafish (*Danio rerio*) and further explores its potential molecular mechanism. Zebrafish embryos showed varying levels of toxicity, which was proportional to the concentration of Pb to which the embryos were exposed. Following Pb exposure (0.2 mM), embryos showed obvious neurotoxic symptoms with "sluggish" action, slow swimming movements and slow escape action. Whole mount *in situ* hybridization showed that *gfap* and *huC* gene expression patterns decreased significantly throughout the brains of the Pb-treated embryos, particularly in the diencephalon region. RT-PCR further proved the downregulation of the two genes. However, *ngn1* and *crestin* gene expression patterns were similar in both the Pb-treated embryos and the control embryos. The TUNEL assay demonstrated that the reduction of nerve cells was due to increased apoptosis of neuron and glia cells. In conclusion, these findings identify that Pb-induced neurotoxicity can be caused by impaired neurogenesis, resulting in markedly increased apoptosis of special types of neural cells, neuron and glia cells.

1. Introduction

Lead is a neurotoxic heavy metal, which can cause a variety of disorders both in the peripheral and central nervous system, in particular, neuronal damage [1]. Acute lead exposure can result in neurotoxic effects such as, behavioral abnormalities, learning impairment, decreased hearing, and impaired cognitive functions in experimental animals [2,3]. Studies have revealed that high lead body burden in children previously related to impaired motor skills and lower school achievement, even low-level lead exposure in perinatal period can damage the cultured fetal central nervous system [4–8]. However, the specific cellular and molecular mechanisms underlying lead-induced neurotoxicity remain obscure.

Zebrafish (*Danio rerio*) embryos are frequently used as a model organism for toxicological studies due to the wealth of knowledge available on molecular genetics and developmental biology in this species [9,10]. Notable similarities and differences exist between embryonic brain development in human and zebrafish species yet the zebrafish represents a valuable model for understanding development of the vertebrate nervous system and brain disorders, such as, autism in humans. The zebrafish brain has the typical vertebrate embryonic brain morphology and its morphogenesis has been conserved through evolution. Homologous or orthologous genes are involved in patterning the brain regions, induction of neurogenesis, neuronal differentiation and axonogenesis and the positions of axonal tracts in zebrafish are also very similar to other vertebrates [11]. Complex behaviors such as learning, cerebral laterization and memory can also be studied in zebrafish [12]. Similar to other vertebrates, embryonic patterning in the zebrafish is regulated by the highly orchestrated temporal and spatial expression patterns of several transcription factors.

In view of the advantages of the model, the zebrafish was used in this study to investigate lead-induced developmental neurotoxicity, and further explore its potential cellular and molecular mechanism during early zebrafish embryonic development.

2. Materials and methods

2.1. Zebrafish strains and maintenance

Wild-type (AB* strain) zebrafish stocks were obtained from International Zebrafish Research Center. Embryos were obtained

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doi:10.1016/j.jhazmat.2011.07.106

from natural spawning of wild type adults. Zebrafish were raised, maintained, and staged as previously described [13].

2.2. Drug treatments

PbAc (Pb, Sigma, USA), was dissolved in ddH_2O at stock concentrations of 100 mM, and then diluted to final concentrations in embryo media at the stages indicated. Control embryos were wide-type zebrafish. All embryos were incubated at 28.5 °C [13]. Embryos were continuously exposed to Pb (0.05–0.7 mM) in embryo medium from 0 to 6 dpf (days post hatch). Three replicates ($n = 3$) containing 20 embryos in a 60-mm diameter Petri dish were cultured in treatment group (a total of 60 embryos). Similar criteria applied to the control group, which was reared in culture medium only.

2.3. Recording of zebrafish behavior

Spontaneous swimming activity (SSA) was measured as described [14,15]. Larvae at 6 dph were tested in groups of fish in a clear acrylic plate. Fish images were captured by a digital camcorder, and the Embryos' swam distance was recorded on video for 20 min after the habituation period and simultaneously analyzed using the ANY-Maze recording software (Stoelting Co., Wood Dale, IL, USA). Spontaneous activity was quantified by counting the number of moving fish across all frames. The SSA (spontaneous swimming activity) index was calculated by dividing the number of movement episodes seen in PbAc-treated group and control (wide type) group. The SSA index was calculated by dividing the number of movement episodes seen in PB (Pb-treated) group and control group. Escape action was measured by the numbers of escape embryos after tip touch tail in the two groups, and calculate the percentage of observed.

2.4. Determination of Pb accumulation in embryos

Determination of Pb was performed with 3 replicates ($n = 3$) containing 200 embryos in a 1.5 ml Eppendorf tube in both control and Pb-treated group. All samples were digested with HNO_3 and $HClO_4$ mixture, and all the concentrations of Pb in the digests were determined by AAS (Solaar MK II M6, Thermo Elemental, Massachusetts, USA).

2.5. Whole-mount RNA in situ hybridization

Developmental markers and regulatory gene expressions were detected by whole mount *in situ* hybridization as described by previous study [16], with modifications. Digoxigenin-labeled antisense riboprobes were synthesized for *ngn1*, *gfap*, *huC*, and *crestin*, which were generously provided by Dr. Liu Dong (Fudan University, China). *In situ* hybridization was performed with 3 replicates ($n = 3$) containing more than 20 embryos in a 1.5 ml Eppendorf tube in both control and Pb-treated group. Zebrafish embryos were dechorionated manually at 24 hpf (hours post fertilized) and fixed overnight in buffers with 4% paraformaldehyde (PFA) at 4 °C. Embryos were dehydrated in methanol and stored at −20 °C. Embryos were rehydrated and digested with 10 g/ml proteinaseK in PBS with 0.1% Tween 20 before incubation with antisense probes overnight at 55 °C. Following hybridization, probes were removed with high-stringency washes, 2× SSC and 0.2× SSC twice each for 30 min, at 62 °C. Embryos were subsequently incubated with pre-absorbed sheep anti-digoxigenin–alkaline phosphatase Fab fragments (Roche, Basel, Switzerland) for 2 h at room temperature. The blue–purple color reaction was performed by using 5-bromo-4-chloroindolyl phosphate (BCIP) as substrate and nitro blue tetrazolium (NBT) as coupler (Roche, Basel, Switzerland).

2.6. Whole-mount TUNEL (terminal deoxynucleotide transferase mediated dUTP nick-end labeling) staining

For whole-mount TUNEL staining, embryos were fixed in 4% PFA at 4 °C overnight, and then rinsed in PBS before proceeding with TUNEL staining using the *in situ* Cell Death Detection kit (Roche) according to the manufacturer's instructions.

2.7. RT-PCR

Total RNA was extracted from 25 to 30 embryos using TRIzol Reagent (Invitrogen, USA) according to manufacturer's instructions. One microgram of total RNA was used as a template in a 20 ml RT-PCR reaction mixture using a one step RT-PCR kit (Qiagen, Germany). The RT-PCR conditions are as described by Chow et al. [19] except for a change in annealing temperature, which depended on the T_m value of the primers. PCR primers used to detect *huC*, *gfap* and *gapdh*. *huC* (Forward primer: 5′-AGA CAA GAT CAC AGG CCA GAG CTT-3′; reverse primer: 5′-TGG TCT GCA GTT TGA GAC CGT TGA-3′); *gfap* (forward primer: 5′-GGA TGC AGC CAA TCG TAA T-3′; reverse primer: 5′-TTC CAG GTC ACA GGT CAG-3′); *gapdh* (forward primer: 5′-ACC ACA GTC CAT CAC3′; reverse primer: 5′-TCC ACC ACC CTG TTG CTG TA-3′).

2.8. Statistical analysis

Data for morphology and gene expression analysis were presented as percentage, and all data were transformed by square root transformation (SRT) before performing Student's *t*-test (SPSS 16.0) to determine the significance between control and lead-treated groups. The level accepted for statistical significance in all cases was $p < 0.05$.

3. Results

3.1. Pb-induced neurotoxicity to zebrafish in early embryonic stage

To investigate the possible influence of Pb on zebrafish neural system development, the SSA assay was employed to reveal spontaneous swimming activity of embryos. In response to Pb treatment, zebrafish embryos exhibited no obvious swimming abnormalities at 6 dph, which we call sluggish action. A concentration-dependent correlation between the sluggish action phenomenon and Pb concentration exposure was observed ($p < 0.05$). The dose response curve demonstrates that treatment with 0.2 mM Pb induced sluggish action phenomenon in 50.9% of the embryos, which was significantly higher than that in the control group ($p < 0.05$, Fig. 1).

Meanwhile, embryonic morphology was observed after exposure of embryos to varying concentrations of Pb (0.05–0.7 mM). After treatment with a concentration of 0.3 mM Pb, the incidence of "S" body malformation (Fig. 2A) and the mortality rate in the Pb-treated group was 54.8% and 25.8%, At this concentration of the drug, there was a significant difference from the results recorded in the control group ($p < 0.05$, Fig. 2B). Yet, the reason for this malformation was suggested for not only neurotoxic effects, but complex developmental toxicity [16]. Therefore, in all future experiments embryos exposed to 0.2 mM Pb are defined as the Pb-treated group.

Pb accumulation in zebrafish embryos of the control and 0.2 mM Pb-treated groups was detected at 24 hpf, 48 hpf and 72 hpf, respectively. Pb could not be detected in all control groups, which was lower than the detection line. Pb accumulation in the Pb-treated groups was determined to be 3.7×10^{-4} g to 9.4×10^{-4} g per embryo. However, there was no significant difference between different treatment time.

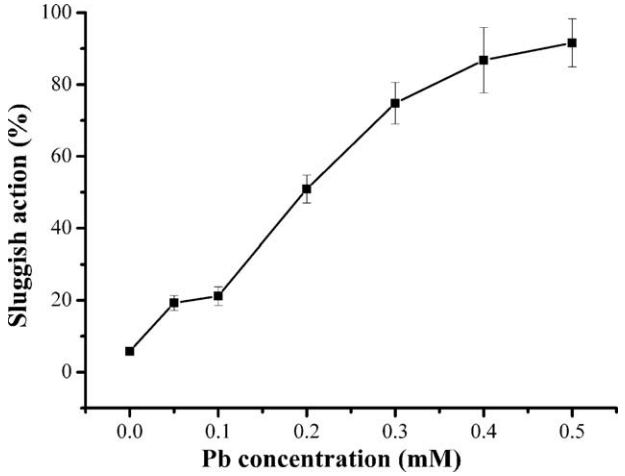

Fig. 1. Dose–response curve for induction of sluggish action phenotype in the Pb-treated zebrafish.

Sensory motor reflexive circuits appeared to be abnormal at early developmental stages, which was determined by spontaneous swimming activity ($p < 0.05$, Fig. 2C). In addition, the escape action was examined by video microscopy, with the control embryos swimming away rapidly upon tactile stimulation (tip touch), while Pb-treated embryos swimming away much less efficiently at 6 dph ($p < 0.05$, Fig. 2D).

3.2. Pb-induced gene expression related to neurogenesis in zebrafish

The sluggish action induced by Pb treatment suggested a specific impairment in the development of the brain. To investigate the possibility of the effect of Pb on neurogenesis, the expression pattern of the neural genes *huC* and *gfap* were examined in Pb-treated embryos and the control embryos. At 24 hpf, expression of the pan-neuronal marker *huC* was strikingly reduced throughout the brain in Pb-treated embryos, particularly in the diencephalon, except in the forebrain region (Fig. 3A–F). These results demonstrate that neurogenesis is significantly compromised by exposure to Pb during embryonic development. However, in other parts of the brain and the neurocoele, *huC* expression was similar to that in the control group. The number of *gfap*-positive cells in the brain region of the Pb-treated group was also significantly decreased, except in the forebrain (Fig. 3C–H). Then, RT-PCR further showed *gfap* and *huC* gene expression downregulated in Pb-treated group (Fig. 3I). These data indicate that the downregulated expression of the two genes examined here may be induced by impaired development of specific types of neurons, neuron cells and glia cells.

The expression patterns of two other genes involved in neurogenesis, *neurogenin1* (*ngn1*) and *crestin*, were also examined. *Ngn1*, which is expressed in the central nervous system (CNS) and otic and epibranchial placodes, is a marker for neuronal precursors at 24 hpf [11]. *Crestin* is expressed in premigratory cranial and trunk neural crest cells and then in actively migrating crest cells [17]. In this study, Pb treatment led to a slight reduction in the expression of *ngn1* and *crestin* (Fig. 4).

3.3. Pb-induced apoptosis of neuron cells in zebrafish

The number of *gfap*-positive and *huC*-positive cells decreased in the brain of Pb-treated embryos suggests a distinct tendency towards cell death (Fig. 2b and d). TUNEL staining was used to examine apoptosis in Pb-treated and control embryos. Extensive apoptosis, indicated by the increased numbers of TUNEL-positive

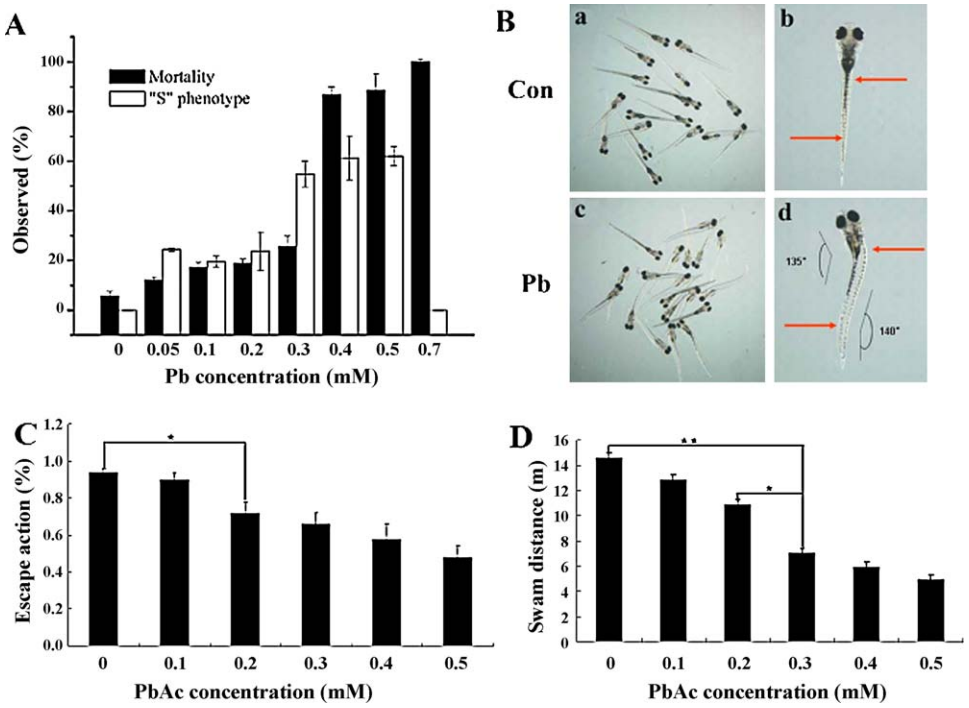

Fig. 2. Combined adverse effects of Pb on zebrafish. (A) Percentage of cumulative mortality at 72 hpf and "S" phenotype observed at 6 dph in zebrafish exposed to Pb with different concentrations. (B) The phenotype of Pb-treated zebrafish at 6 dph. When embryos were exposed to Pb at 0.2 mM, the body exhibit two bend regions, and curvature were 135° and 140°, respectively (red arrow heads). a–d: dorsal views. (C) Swam distances of embryos determined during 20 min of video recording in the plate diving behavioral test. The percentage of sluggish action and swam distance of every fish was scored at 6 dph. Symbols and vertical bars represent the means and SD of three experiments ($p < 0.05$), ★ means $p < 0.05$ and ★★ means $p < 0.01$. (D) The number of zebrafish embryos with escape action (tip touch) induced by PbAc at 6 dpf. Symbols and vertical bars represent the means and SD of three experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

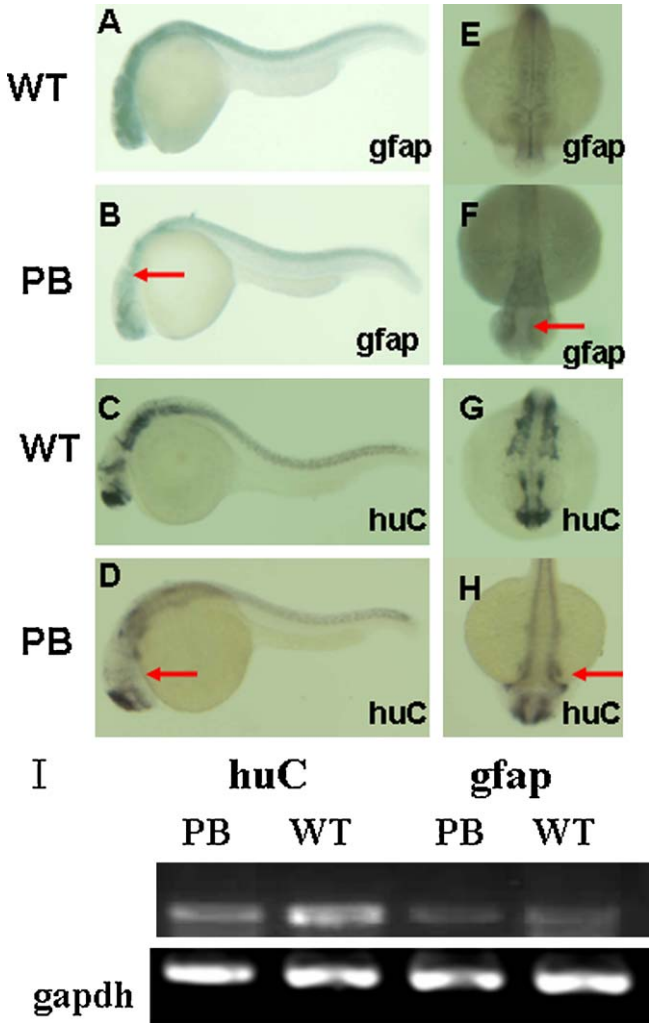

Fig. 3. Effects of Pb exposure on the gene expression of *gfap* and *huC* in zebrafish. Whole mount *in situ* hybridization showed that *gfap* expression cell domain was reduced in embryonic midbrain and hindbrain at 24 hpf (A, B, E and F); Whole mount *in situ* hybridization showing that *huC* expression cell domain was reduced in embryonic midbrain at 24 hpf (C, D, G and H). RT-PCR analysis (I) was performed with RNA samples isolated from embryos at 24 hpf. *gfap* and *huC* specific products were amplified from RNA isolated from whole embryos. The *gapdh* primers amplified a single fragment in all the samples. Expression of marker genes indicates different cells: *gfap* for glia cells and *huC* for neuron cells. Red arrows in B, D, F and H show region *gfap* and *huC* expression reduced. (A)–(D) left lateral view, (E)–(H) dorsal view.

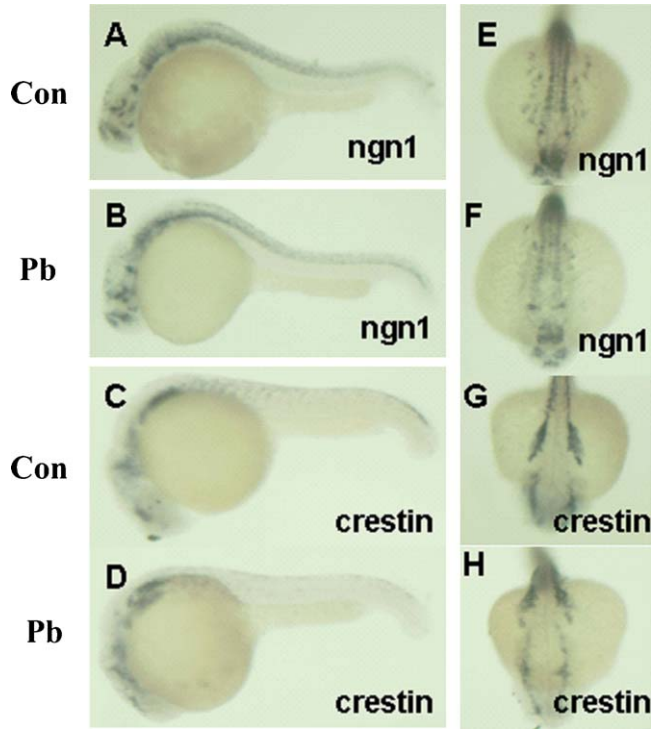

Fig. 4. Effects of Pb exposure on the gene expression of *ngn1* and *crestin* in zebrafish. Whole mount *in situ* hybridization show that Pb-treaded group (B, F, D and H) continues to express *ngn1* and *crestin* strongly as normal as the controls (A, E, C and G). Expression of marker genes indicated different cells: *ngn1* for neural stem cells, and *crestin* for neural crest cells. (A)–(D) left lateral view, (E)–(H) dorsal view.

cells, was observed in the Pb-treated group of embryos at 24 hpf. In contrast, the control embryos displayed only a few scattered apoptotic cells. Moreover, TUNEL-positive cells were concentrated in the head region, which was the area of reduced neural tissue in the CNS. However, the Pb-treated embryos and the control embryos both showed little apoptosis throughout the body of the embryo at 12 hpf (Fig. 5). These data indicate that the downregulated expression patterns of *huC* and *gfap* may be induced by impaired development of neuron and glia cells.

4. Discussion

4.1. Zebrafish as a model for neurotoxicity in human brain development

Zebrafish are used as a model for understanding CNS development due to their similarities with other vertebrates and their ease of use. The organization of the major brain components in zebrafish is highly conserved with that of the human brain [18]. Meanwhile, zebrafish has a relatively simple nervous circuitry that could serve as a screening system for developmental intoxication in the complex mammalian central nervous system. Employing zebrafish as a model species, our results provide insights into lead neurotoxicity in the developing embryonic brain from molecular and regulatory perspectives. In addition, zebrafish embryos are often exposed to chemical contaminants at high concentrations during early life stages [10]. This strategy has been used to screen rapidly for poten-

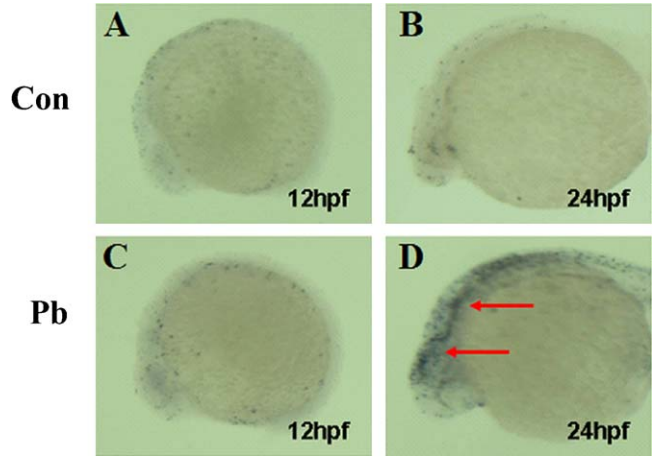

Fig. 5. Pb exposure results in increased neural cell apoptosis. Both Pb-treated embryos and the control embryos show little apoptosis in whole-body at 12 hpf (A and B). The Pb-treated embryos show massive apoptosis concentrated primarily in the CNS region compared to a few scattered positive cells in control embryos at 24 hpf (C and D). Red arrows indicate widespread apoptosis in the midbrain, and hindbrain. Black dots indicate the TUNEL-positive cells, lateral view. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

tial toxicity mechanisms of environmental contaminants such as heavy metals, polycyclic aromatic hydrocarbons and common pesticides [19].

4.2. The relationship between expression of neural genes and neurotoxicity

Pb exposure causes a variety of disorders both in peripheral and central nervous system, especially neuronal damage. Neurogenesis can be subdivided into four processes: selection, specification, determination, and differentiation [20,21]. In zebrafish development, the spatial and temporal patterns of neural induction and axonal tract formation were assessed before 48 hpf. To test whether sluggish action was caused by neural damage at an early stage, we examined the expression of several neuronal markers, including *huC*, *gfap, ngn1* and *crestin*, following PbAc treatment at 24 hpf.

After the specification process, the process of determination establishes the commitment of the neural progenitors to become neurons. This is maintained even in the presence of signals that repress the development of neurons thus allowing expression of early pan-neuron markers, for example, *huC* and its family members [22]. In our study, reduced expression of *huC* in the restricted region (particularly in the diencephalon) correlates with the neuronal defects in the brain of Pb-treated embryos at 24 hpf.

Glial fibrillary acidic protein (GFAP) is a commonly used marker to identify enteric glia in the mammalian gut [23]. Enhanced expression of GFAP is generally accepted as a marker of neurotoxicity. When lead exposure began after the critical period of brain development, an elevation of GFAP expression or mRNA for GFAP was observed in the hippocampus of young rats chronically exposed to Pb [2]. However, prenatal and early postnatal exposures to lead caused a decreased expression of *gfap* mRNA in the rat brain (cerebellum and hippocampus) as a result of altered glia integrity and function [24]. This is similar to the results obtained in this study using the zebrafish as a model species.

Ngn1 is expressed in the CNS and otic and epibranchial placodes, and is a marker for neuronal precursors at 24 hpf [11]. *Crestin* expression is subsequently observed in premigratory cranial and trunk neural crest cells and then in actively migrating crest cells [17]. However, the two biomarkers (*ngn1* and *crestin*) showed little response to lead treatment. It demonstrated that PbAc did not affect the expression of all of these markers of neuronal differentiation, and these cells successfully underwent differentiation in the CNS, as did glial and neural crest cells. These data indicate that downregulated expression of the two genes may be induced by impaired development of the specific types of neurons.

4.3. The apoptosis induced by lead

Apoptosis is a tightly controlled process in which cell death is executed to maintain a steady state under physiological conditions and in response to various stimuli. Recent studies also indicated that apoptosis might be associated with the lead-induced cytotoxicity. Many research groups have revealed that lead could induce apoptosis in a number of experimental systems, including rat brain, testis, fibroblasts, lung, and blood cells [25–29]. Even low to moderate levels of lead exposure produces apoptotic rod and bipolar cell death in developing and adult rats and apoptotic neuronal cell death in primary cultured cells [27]. Regulation of apoptosis is complex, and two critical pathways are known to be involved. In the intrinsic pathway, *p53* shifts the balance in the *Bcl-2* family via both transcription-dependent and transcription-independent mechanisms [30]. A recent study showed that the MAPK pathway may also play an important role in Pb-induced cerebral apoptosis by activating MEK-ERK [27]. Thus, we speculated that lead can induce apoptosis in CNS of zebrafish brain as well as other animal models or cultured cells.

5. Conclusions

This study provides evidence that Pb exposure can lead to neurotoxicity with sluggish action in the zebrafish. Pb exposure in these embryos also resulted in downregulation of *huC* and *gfap* expression in the diencephalon and dorsal hindbrain. The critical event affected by Pb that allows initiation of neural cells excessive apoptosis formation occurs after 24 hpf. Downregulation in the expression of *gfap* and *huC* indicates a decrease in the number of neural cells. *Ngn1* and *crestin* expression were more or less unchanged, which indicates that the determination of neuronal cells and crest cells are not altered as a result of Pb treatment.

Thus, the present findings identified sluggish action induced by lead neurotoxicity and uncovered a possible link between this phenotype and neural cell apoptosis in the midbrain and hindbrain of the zebrafish embryo. However, it still remains unclear why Pb exposure results in damaged cells in particular regions of the brain (mainly in the midbrain and hindbrain) in the zebrafish. Future studies are required to understand the related molecular pathway and how this pathway functions.

Acknowledgements

This work was partially supported by the Open Project Program of Key Laboratory of Non-point Sources Pollution Control, the Ministry of Agriculture of the People's Republic of China (No. KLNSCP2010B01),Science Fundation of Chinese University, and the Environmental Protection Public Welfare Scientific Research Project, Ministry of Environmental Protection of the People's Republic of China (No. 201009041).

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