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Prenatal and Lactational Lead Exposure Enhanced Oxidative Stress and Altered Apoptosis Status in Offspring Rats' Hippocampus

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Abstract Oxidative stress and apoptosis facilitation in the developing central nervous system (CNS) have been inferred as two mechanisms related to lead's neurotoxicity, and excessive reactive oxygen species (ROS) can promote oxidative stress and apoptosis facilitation. Few studies systematically investigated the potential relationship among oxidative stress, ROS generation, and apoptosis facilitation after lead exposure in earlier life as a whole. To better understand the adverse effect on the developing central nervous system (CNS) after lead exposure during pregnancy and lactation, the indexes of oxidative stress, apoptosis status, and Bax and Bcl-2 expression of offspring rats' hippocampus were determined. Pregnant rats were randomly divided into four groups and given free access to drinking water which contained 0 %, 0.05 %, 0.1 %, and 0.2 % $Pb(AC)_2$ respectively from gestation day 0 to postnatal day 21 (PND21). Results showed that ROS and malondialdehyde level of either PND7 or PND21 pups' hippocampus were significantly raised; reduced glutathione level and superoxide dismutase activity were obviously decreased following the increase of blood and brain lead level. Similar to apoptotic indexes, Bax/Bcl-2 ratio increased after 0.1 % and 0.2 % $Pb(AC)_2$ exposure, especially for the pups on PND7. Comparing with cortex, the hippocampus seemed much more sensitive to damage induced by lead. We concluded that the disruption of pro-oxidant and antioxidant balance and apoptosis facilitation could be associated with the mechanisms of neurotoxicity after lead exposure in earlier life.

Keywords Lead · Developing neurotoxicity · Oxidative stress · Apoptosis · Bax/Bcl-2

Introduction

Lead (Pb) is a toxic heavy metal which is widely distributed in the environment. As a neurotoxic agent, lead has been known to cause damage in the nervous system. The developing central nervous system (CNS) is far more vulnerable to lead's toxic effects than the mature brain [1]. Epidemiology evidence suggested that the adverse effects caused by lead included intellectual and behavioral deficits in children [2, 3]; furthermore, the deficits in CNS functioning will persist into young adulthood [4]. Similarly, learning and memory impairments in experimental animals exposed to lead during embryonic and gestational stages have also shown continuous brain damage till adult stage [5–12]. Therefore, lead exposure during CNS development could induce serious effects and remain a potentially large public health problem in the world [13, 14].

One possible molecular mechanism involved in the lead neurotoxicity is the disruption of the pro- and antioxidant balance, which can lead to brain injury via oxidative damage to critical biomolecules, such as lipids, proteins, and DNA. Jin reported that lipid peroxidation in blood increased in the children with blood lead levels (BLLs) ≥100 μg/L [15]. Some studies reported that Pb exposure can increase the level of reactive oxygen species (ROS) and oxidative stress in the CNS [16–19]. Since the developing brain is rich in unsaturated fatty acids, it is more vulnerable to ROS which

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can cause lipid peroxidation. In addition, excessive ROS generation is considered a trigger of the programmed cell death by apoptosis [20]. It was shown that the low micromolar doses of Pb^{++} that promote apoptosis are well within the blood level range reported to impair CNS function in children and alter synaptogenesis in the neonatal rat brain [21]. Apoptosis is a gene regulated programmed cell death [22], among which Bax and Bcl-2 are the major proteins acting as apoptotic inducer and inhibitor, respectively [23, 24]. The expression of Bax and Bcl-2 proteins during the development of CNS varies according to the developmental period, and is also involved in discriminative cell death [25, 26]. Several reports found that lead exposure can induce a higher ratio of Bax/Bcl-2 protein expression, which reminded that lead's neurotoxicity may be due to a facilitation of apoptosis in the brain development.

The hippocampus, which is functionally related to vital behaviors and intellectual activities such as memory and learning, has been shown to be affected by lead, particularly in young children with an unknown mechanism [27]. For this reason, we selected the hippocampus as the target location in the present study. To date, few studies systematically investigated the inter-relationship among oxidative stress, ROS generation, and apoptosis as a whole. We hypothesized that those factors could impact each other and induce adverse damage in the developing brain due to lead exposure. Therefore, in order to clarify the possible association among those factors and investigate the dynamic change in brain development after birth, we examined the effects of Pb on the hippocampus in the weaned pups on postnatal days (PND) 7 and 21, respectively, whose dams were exposed to lead during pregnancy and lactation.

Materials and Methods

Reagents and Laboratory Wares

Lead acetate [$Pb(AC)_2$, analytical grade] was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in sterile distilled drinking water to the desired concentrations. 7′-dichlorofluorescin-diacetate (DCFH-DA) was purchased from Sigma Chemical Corporation (MO, USA). Analysis kits for MDA, GSH, and SOD were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). TUNEL DNA Fragmentation Detection Kits were purchased from Huamei Biotech Corporation (Beijing, China). Immunohistochemistry (IHC) staining kits of Bcl-2 and Bax were purchased from Zhongshan Biotech Corporation (Beijing, China). All glasses and plastic wares were washed with detergent and acid, and then rinsed with redistilled water to eliminate metal leaching.

Experiment Design

Female Wistar rats (weighing 220–250 g) were obtained from the Experimental Animal Centre of China Medical University. All the animals were acclimated for a week before experiments and were housed in an air-conditioned room with controlled temperature (25±2 °C). They were fed basal pellet diet and water ad libitum. After a week, animals were mated (one female and one male per cage) in the evening. Pregnancy was determined by the presence of sperm in vaginal smears the next morning. The day when sperm plug was confirmed was designated as day 0 of gestation (GD 0). The pregnant rats were randomly divided into four groups of seven to nine animals each. Each group was given free access to drinking water (sterile distilled water) that contained 0 %, 0.05 %, 0.1 %, and 0.2 % $Pb(AC)_2$ from GD 0 to postnatal day 21 (PND21). All dams were allowed to deliver naturally and rear their offspring. The litters of each group were culled on PND4 to keep litter size at 8–10 pups per litter with an equal distribution of males and females. The rats were observed daily, and their body weight and water consumption were recorded. Pups were sacrificed to collect samples of brain on PND7 and 21, respectively, stripping the hippocampus rapidly. A single cell suspension of hippocampus or cortex was prepared to determine the levels of ROS. At least five pups' brains were removed and fixed in the freshly prepared paraformaldehyde for 72 h, and subsequently embedded into paraffin. At the same time, 100 μL blood was collected by cardiac puncture into heparinized tubes for lead determination.

Lead Determination in Blood and Brain

The blood samples were collected into heparinized centrifuge tubes and digested in digestive acid comprised of perchloric acid and nitric acid (1:1). The brain tissue samples were weighed and digested in 1 mL digestive acid and incubated for 2 h at 120 °C. Atomic absorption spectrophotometry graphite furnace (Varian spectra-AA 40P; USA) was used for blood and brain lead determination following a standardized analytical method with an accuracy of ±5 μg/L. Instrumental parameters used for sample analysis were drying for 65 s between 85 and 120 °C, charring for 30 s between 300 and 480 °C, atomization for 3 s at 1,850 °C, and cleaning for 4 s at 2,700 °C. Photometry was performed at a wavelength of 283.3 nm and using a lead hollow cathode lamp with a current supply of 7.5 mA, taking advantage of Zeeman background correction. Duplicate determinations were carried out for each sample and the average was taken as a measure. Quality control was performed by determination of the reference samples from the US CDC as a participation in

the CDC Proficiency Testing Program. The test results were in good agreement with the reference values.

Measurement of Oxidative Stress Indexes

Malondialdehyde (MDA) The measurement of malondialdehyde (MDA) using the thiobarbituric acid method was described by Satoh [28]. Briefly, samples of the hippocampus were homogenized in cold saline solution. Five hundred microliters of homogenate was incubated with the TBA reagent (0.5 % thiobarbituric acid, 16 % trichloroacetic acid, and 2.5 % hydrochloric acid in deionized water) for 30 min at 95 °C. After cooling, the samples were centrifuged (3,000 rpm for 10 min) and absorbance of the supernatant was measured at 530 nm. The protein content for all the assays was determined according to Lowry assay. The content of MDA product was expressed as nanomoles per milligram of protein.

Reduced Glutathione (GSH) The intracellular glutathione (GSH) level in the hippocampus was assayed. The fresh tissues were homogenized in saline solution on ice, and then 10 % trichloroacetic acid was added and the homogenate was centrifuged. One milliliter of the supernatant was treated with 0.5 mL of Ellman's reagent [19.8 mg of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) in 100 mL of 0.1 % sodium nitrite] and 3.0 mL of 0.2 M phosphate buffer (pH 8.0). The absorbance was read at 412 nm.

Superoxide Dismutase (SOD) Superoxide dismutase (SOD) assay was performed as follows. A 10 % homogenate was prepared using a homogenizing buffer (100 mM phosphate buffer, pH 7.5) and was then centrifuged at 4,000 rpm for 10 min at 4 °C. The supernatant containing the enzyme was used for assay. A solution of hydroxylamine, xanthine oxidase, hypoxanthine, and sample was incubated at pH8.2, 37 °C for 30 min. Diazo dye forming reagent was added and the absorption was measured at 550 nm. One unit of SOD was defined as the amount of enzyme required to cause 50 % inhibition of nitrite production per milliliter of assay solution. Enzyme activity was reported in nitrite units per gram of protein.

Reactive Oxygen Species (ROS) The levels of ROS were detected by fluorescent probe DCFH-DA (from Sigma) using a fluorescence-activated cell sorter [29]. Briefly, the hippocampus and cortex of rats on PND7 and PND21 were stripped and sheared into pieces in PBS, respectively, and digested with trypsin to obtain single cell suspension. After incubated with 10 μmol/L fluorescent probe DCFH-DA for 30 min, the samples were detected by flow cytometry (BD Company, USA), while the ROS levels were expressed as the average fluorescence intensity.

Quantification Assay of Apoptosis

Terminal deoxynucleotidyl-transferase mediated biotin–deoxyuridine triphosphate nick-end labeling (TUNEL) assay was performed according to the manufacturer's instructions of TUNEL detection kit. The paraffin block was cut into sections of 5 μm in thickness, and was dewaxed and dehydrated using xylene and graded alcohol series. The sections were stained using the terminal deoxynucleotidyl transferees (TdT) method. Endogenous peroxidase was first quenched with 2 % hydrogen peroxide and the sections were permeabilized using the supplied equilibration buffer. The 3′ OH ends of DNAs were reacted with TdT and digoxigenin-labeled ATP for 30 min. After washing with PBS, the cells on slides were incubated with an anti-digoxigenin antibody conjugated to peroxidase, washed, and developed with diaminobenzidine tetrachloride (DAB). Apoptotic cells (TUNEL-positive) were identified by the presence of dark brown staining in nucleus. At least 1,000 cells were counted under ×20 magnification. Then, counting was continued in the successive ten defined areas (10 mm^2).

Immunohistochemistry (IHC) of Bax and Bcl-2 Expression

Paraffin sections were deparaffinized and dried at 37 °C. Following antigen retrieval, endogenous peroxidase activity was blocked by incubation in 1 % hydrogen peroxide in methanol for 20 min. Slides were blocked with 1.5 % normal goat serum. Negative control slides were prepared without primary antibody. Sections were incubated in polyclonal rabbit-derived primary antibodies to Bax and Bcl-2 overnight, respectively (1:500 dilutions in PBS). After washing

Table 1 Comparison of lead content in blood and brain on PND7 and 21 in different Pb $(AC)_2$-exposed groups ($\bar{x} \pm s$, n=20)

Group	PND7		PND21	
	Blood lead (μmol/L)	Brain lead (μg/g)	Blood lead (μmol/L)	Brain lead (μg/g)
Control	0.26±0.09	0.06±0.02	0.29±0.04	0.13±0.07
0.05 % $Pb(AC)_2$	1.27±0.34*	0 .23±0.07**	1.65±0.06*	0.37±0.07*
0.1 % $Pb(AC)_2$	2.02±0.79**	0.39±0.26**	2.71±0.32**	0.64±0.18**
0.2 % $Pb(AC)_2$	3.54±0.96**	0.59±0.15**	3.59±0.64**	0.99±0.14**

*Comparing with the control, P <0.05; **comparing with the control, P<0.01

and incubating with a biotinylated goat anti-rabbit secondary antibody for 30 min at room temperature, the ABC kit was used to bind antibodies according to the manufacturer's instructions. Slides were developed with DAB, counterstained with hematoxylin, and mounted. The slides were observed under a bright field microscope and photographed. Bax or Bcl-2 positive cells were identified by the presence of dark brown staining in cytoplasm and mitochondrial membrane. The evaluation of Bax or Bcl-2 expression was done by counting the grade of dark

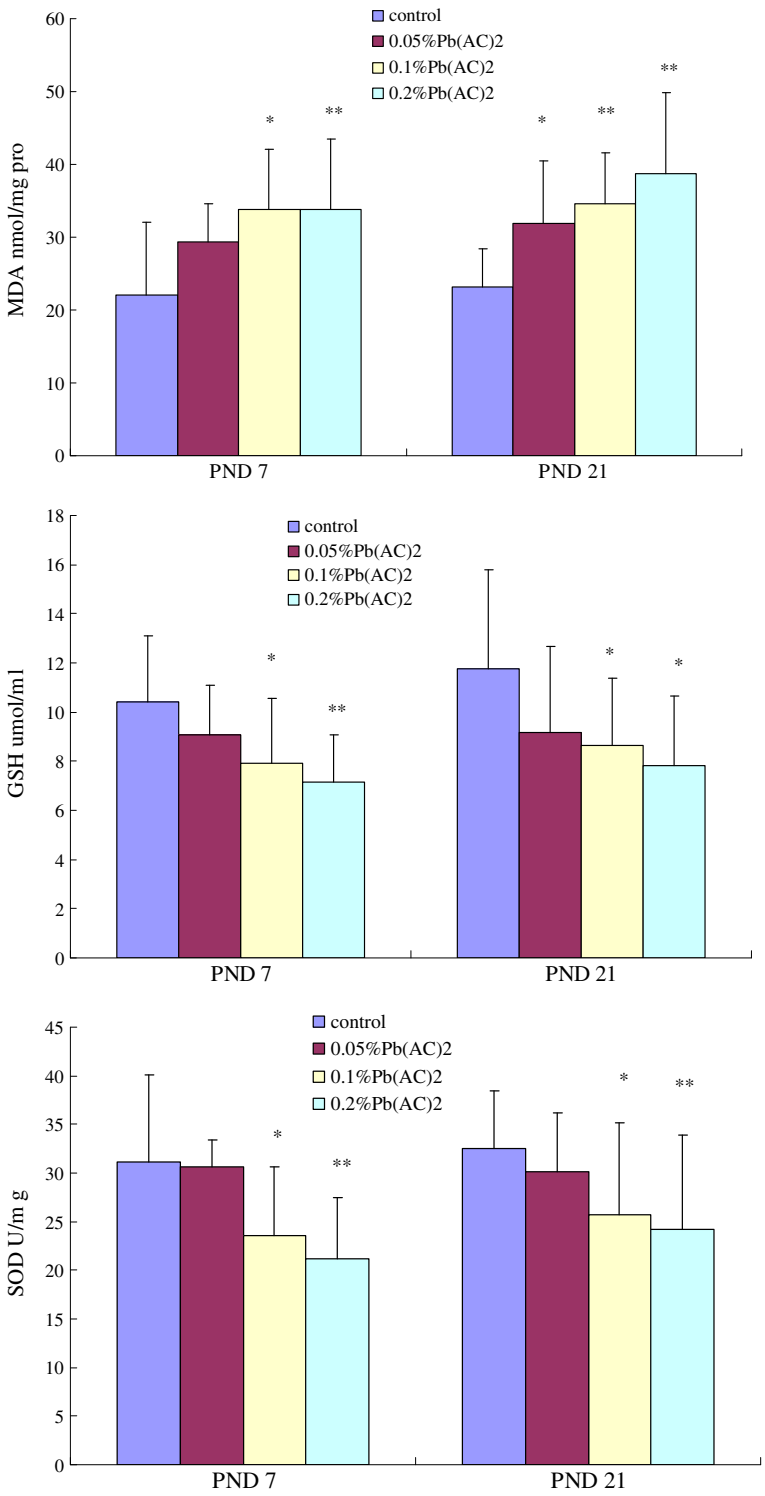

Fig. 1 Comparison of oxidative stress indexes in pup's hippocampus between lead-exposed groups and control on PND7 and 21, respectively (n=20). *Comparing with the control, P<0.05, **comparing with the control, P<0.01

Table 2 Comparison of the average fluorescence intensity in pups' hippocampus and cortex in lead-exposed groups and control on PND7 and 21, respectively ($\bar{x} \pm s$, n=3)

Group	PND7		PND21	
	Hippocampus	Cortex	Hippocampus	Cortex
Control	494.26±96.81	247.39±55.71	275.13±17.35	191.60±23.29
0.05 % $Pb(AC)_2$	484.21±44.37	295.09±34.61	395.00±102.13	238.71±4.898
0.1 % $Pb(AC)_2$	564.74±78.24*	375.16±19.13*	467.15±63.91	289.94±54.88
0.2 % $Pb(AC)_2$	690.11±50.03**	473.60±91.57**	656.20±228.25**	459.26±90.79*

*Comparing with the control, P <0.05; **comparing with the control, P<0.01

brown staining. No dark brown staining denoted negative, visible dark brown staining denoted positive, and then the grade evaluation of each section was given. Counting was continued in the successive ten defined areas (10 mm^2). At least 1,000 cells were counted under ×40 magnification per section.

Statistical Analysis

All analyses were performed with SPSS 15.0 for Windows. Data presented as mean±SD. Statistical analysis of the data was performed with one-way ANOVA or non-parametric Kruskal–Wallis test according to the normality and variance of homogeneity of data, and followed by Student–Newman–Keuls test to figure out the differences between the two groups. All tests were two-sided and P <0.05 was considered significant.

Results

Total Lead Content in Blood and Brain

The total Pb content in blood and brain of pups on both PND7 and 21 is displayed in Table 1. The total Pb content in blood and brain of 0.05 %, 0.1 %, and 0.2 % $Pb(AC)_2$-exposed groups were higher than the control on both PND7 and PND 21(P<0.05, P<0.01). Dose-dependant trend was found between blood lead level and Pb concentration in drinking water. Similarly, there is a close relationship between brain lead and blood lead level.

Oxidative Stress Indexes of Hippocampus

Oxidative stress indexes including malondialdehyde (MDA), reduced glutathione (GSH) level, and superoxide

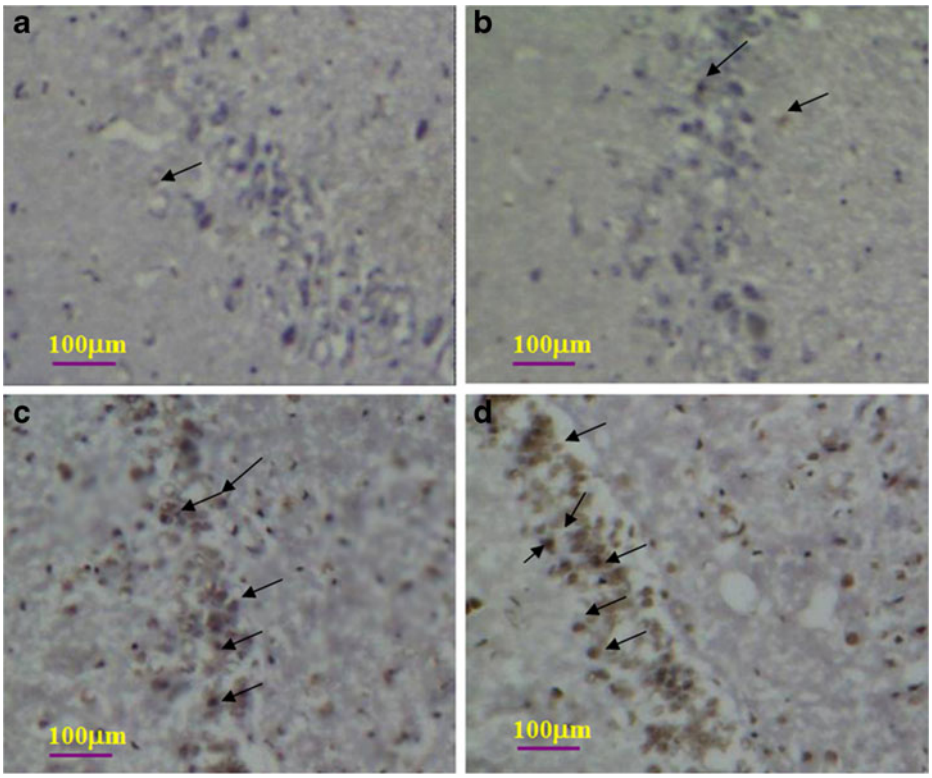

Fig. 2 Representative results of TUNEL staining in pups' hippocampus between lead-exposed groups and control on PND7. An *arrow* in the picture indicates some positive cells of tunnel staining. **a** Control group, **b** 0.05 % $Pb(AC)_2$-exposed group, **c** 0.1 % $Pb(AC)_2$-exposed group, **d** 0.2 % $Pb(AC)_2$-exposed group

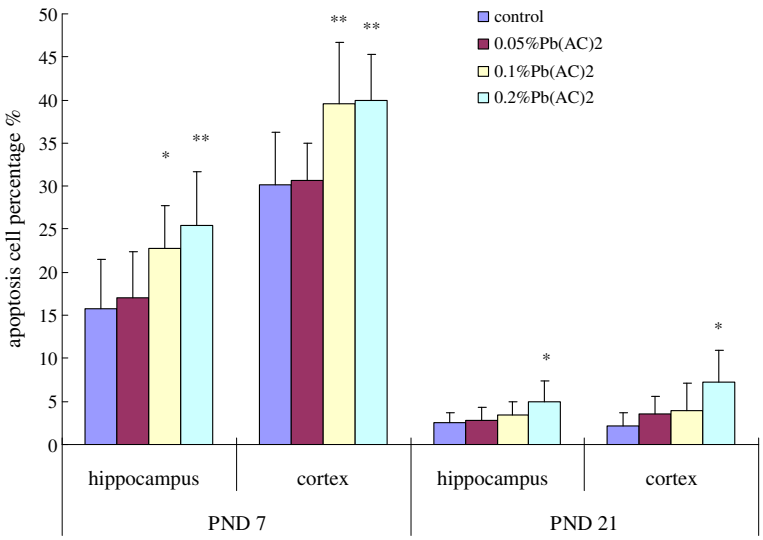

Fig. 3 Comparison of the apoptosis index in pups' hippocampus and its nearby cortex between lead-exposed groups and control on PND7 and 21, respectively (n=5). *Comparing with the control, P <0.05, **comparing with the control, P<0.01

dismutase (SOD) activity in hippocampus of $Pb(AC)_2$-exposed pups on PND7 and 21 are shown in Fig. 1. The levels of MDA in 0.1 % and 0.2 % $Pb(AC)_2$-exposed pups' brain were significantly higher than the control (P<0.05, P< 0.01), and an increase in MDA level after lead exposure on both PND7 and 21 was observed.

We examined GSH level in pups' hippocampus on PND7 and 21. Comparing with the control group, the average concentration of GSH in 0.1 % and 0.2 % $Pb(AC)_2$-exposed groups showed a clear decreasing trend on both PND7 and 21. In addition, we also determined SOD activity in pup's hippocampus on PND7 and PND21. Our results indicate that lead exposure decreased SOD activity in 0.1 % and 0.2 % $Pb(AC)_2$-exposed groups on either PND7 or PND21 (P<0.05, P<0.01).

The levels of ROS in hippocampus and cortex were expressed as the average fluorescence intensity, which are shown in Table 2. Compared with the control, the average ROS levels increased significantly in both 0.1 % and 0.2 % $Pb(AC)_2$-exposed groups on PND7 and also raised in 0.2 % $Pb(AC)_2$-exposed groups on PND21 (P<0.05, P<0.01).

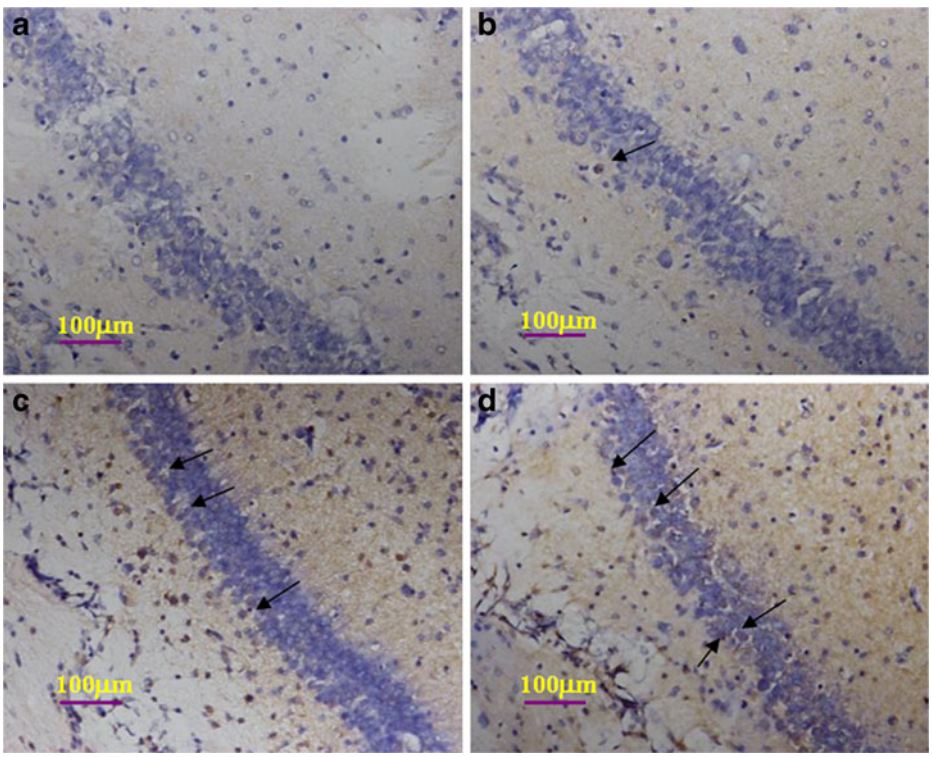

Fig. 4 Representative results of Bax immunohistochemical staining in pups' hippocampus between lead-exposed groups and control on PND7. An *arrow* in the picture indicates some positive cells. **a** Control: Bax protein expression is the lowest. The percentage of positive cell is approximately 2.6 % after counting. **b** 0.05 % $Pb(AC)_2$-exposed group: Bax protein expression has an increased trend compared with the control. The percentage of positive cells is 4.6 % after counting. **c** 0.1 % $Pb(AC)_2$-exposed group: Bax protein expression significantly increased compared with the control. The percentage of positive cell is approximately 11.9 % after counting. **d** 0.2 % $Pb(AC)_2$-exposed group: Bax protein expression is the highest. The percentage of positive cell is approximately 13.3 % after counting

Table 3 Comparison of the average percentage of Bax-positive cells in pups' hippocampus and cortex after lead exposure on PND7 and 21, respectively ($\bar{x} \pm s$, %, $n=6$)

Group	PND7		PND21	
	Hippocampus	Cortex	Hippocampus	Cortex
Control	2.549±1.429	2.208±1.709	1.658±0.310	4.007±1.871
0.05 % $Pb(AC)_2$	4.553±1.747	1.839±0.638	2.601±1.317	6.771±4.657
0.1 % $Pb(AC)_2$	11.887±2.147**	4.289±1.986*	6.256±5.516**	12.448±3.416**
0.2 % $Pb(AC)_2$	13.293±2.858**	9.657±3.820**	11.479±1.459**	20.176±2.757**

*Comparing with the control, $P<0.05$; **comparing with the control, $P<0.01$

Apoptosis Indexes of Hippocampus and Cortex

Apoptosis status in hippocampus and its nearby cortex after lead exposure are shown in Fig. 3. We found that the apoptosis indexes (the ratio of the number of apoptotic cells to the total cells) showed an increasing trend in 0.1 % and 0.2 % $Pb(AC)_2$-exposed groups in either hippocampus or cortex on PND7 ($P<0.05$, $P<0.01$) (see Figs. 2 and 3). Compared with PND7, the amount of apoptotic cells showed a physiological reduction on PND 21, but a clear increase in apoptotic cells caused by lead exposure only occurred in the 0.2 % $Pb(AC)_2$-exposed group ($P<0.05$) (the pictures are not shown and the data are shown in Fig. 3).

Expression of Bax and Bcl-2 Proteins in Hippocampus and Cortex

In order to compare the effect of lead exposure on the different developing stage of brain, the expression Bax and Bcl-2 protein of hippocampus and cortex on PND7 and PND21 were detected by immunohistochemical method. An intense localization of Bax protein was found in both hippocampus and cortex after lead exposure. For PND7

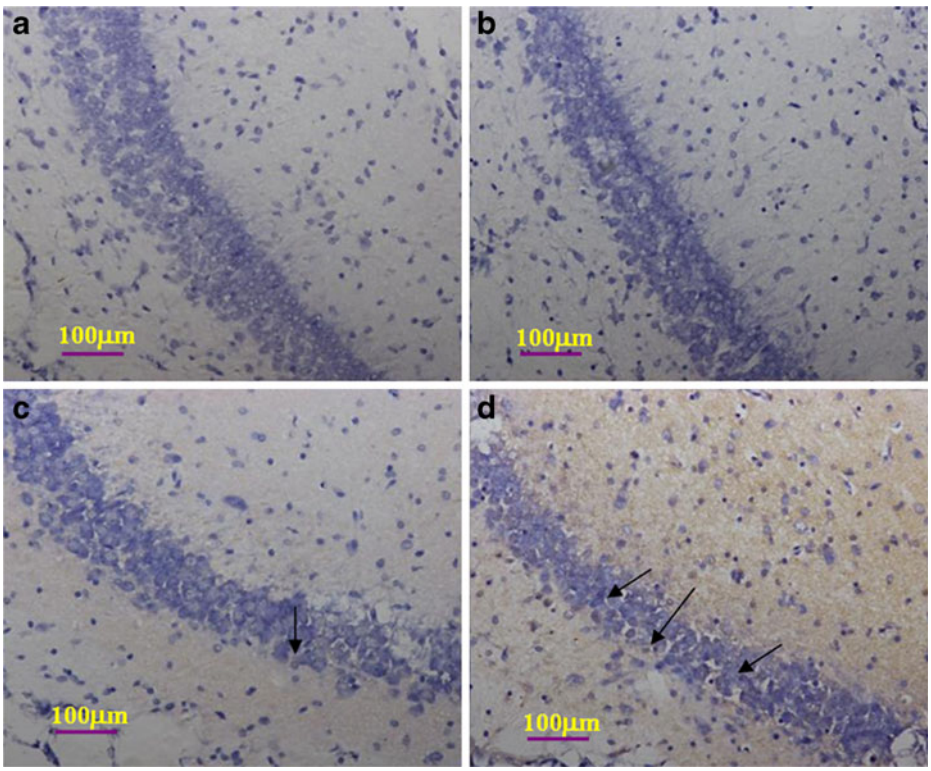

Fig. 5 Representative results of Bcl-2 immunohistochemical staining in pups' hippocampus between different lead-exposed groups and control on PND7 after lead exposure during pregnancy and lactation. An *arrow* in the picture indicates some positive cells. **a** Control: the percentage of positive cell in Bcl-2 protein expression is approximately 6.5 % after counting. **b** 0.05 % $Pb(AC)_2$-exposed group: no significant alteration has been found in Bcl-2 protein expression comparing with the control. The percentage of positive cell is approximately 5.7 % after counting. **c** 0.1 % $Pb(AC)_2$-exposed group: similar to 0.05 % $Pb(AC)_2$-exposed group, Bcl-2 has not shown the difference comparing with the control by statistical analysis. The percentage of positive cell is approximately 8.3 % after counting. **d** 0.2 % $Pb(AC)_2$-exposed group: Bcl-2 protein expression is the highest among all groups. The percentage of positive cell is approximately 15.4 % after counting

Table 4 Comparison of the average percentage of Bcl-2-positive cells in pups' hippocampus and cortex after lead exposure on PND7 and 21, respectively ($\bar{x} \pm s$, %, n=6)

*Comparing with the control, P <0.05; **comparing with the control, P<0.01

Group	PND7		PND21	
	Hippocampus	Cortex	Hippocampus	Cortex
Control	6.512±1.302	8.466±3.493	4.349±1.736	11.294±2.487
0.05 % $Pb(AC)_2$	5.637±1.812	6.409±0.756	7.487±2.588	11.803±6.629
0.1 % $Pb(AC)_2$	8.319±1.895	6.925±2.980	13.139±3.824**	17.727±1.413*
0.2 % $Pb(AC_)_2$	15.480±3.940**	15.497±4.444**	12.551±2.278**	16.110±2.764*

pups, Bax expression in 0.1 % and 0.2 % $Pb(AC)_2$-exposed groups increased (see Fig. 4 and Table 3). Meanwhile comparing with the control, a distinct enhancement in Bcl-2 expression was only shown in 0.2 % $Pb(AC)_2$-exposed group on PND7 (see Fig. 5 and Table 4). Bax protein expression on PND21 had an increasing trend from the middle to high lead exposure in both hippocampus and cortex. We also found an obvious increase in Bcl-2 expression in both 0.1 % and 0.2 % $Pb(AC)_2$-exposed groups on PND21 (the pictures are not shown and the data is shown in Table 4).

A higher level of Bax/Bcl-2 ratio is an important index of apoptotic cell death. We found that although Bcl-2 expression increased following the increase in Bax expression in both hippocampus and its nearby cortex, Bax/Bcl-2 ratio increased in 0.1 % and 0.2 % $Pb(AC)_2$-exposed groups on PND7, while it kept at high level only in 0.2 % $Pb(AC)_2$-exposed groups on PND21. Furthermore, it seemed that hippocampus was much more sensitive to lead exposure because even the low $Pb(AC)_2$ exposure could induce a significant increase in the brain region on PND7 (see Fig. 6).

Discussion

As we have known, the developing organism presents a fivefold greater absorption of lead than the adult organism [30]. So the adverse effects of lead on the developing organism attract much more attention. The prenatal and lactational period is more sensitive and critical than other periods in human development. Large evidences from epidemiology and animal experiments have proved that as a known neurotoxicant in environment, lead is able to transverse the placental barrier and reaches to the conceptus in the case of maternal exposure and also crosses the blood–brain barrier easily due to its lack of function in the developing organism [31]. The current study investigated the adverse effect of lead exposure during pregnancy and lactation on developing organism. The close relationship between brain lead level and blood lead level has been shown and further confirmed the above conclusions. Furthermore, we manifested that the measurement of BLLs could be a wonderful indicator of lead exposure in brain especially for children.

A kind of mechanism related to lead's neurotoxicity is that lead can induce oxidative stress in brain which can be well extrapolated from the increase in lipid peroxidation products such as TBARS [16, 31]. It is widely accepted that oxidative stress causes the lipid peroxidation of PUFA side chains, which in turn produces the toxic substance malondialdehyde that interferes with normal cell function. We also found that 0.1 % and 0.2 % $Pb(AC)_2$ exposure could cause oxidative stress in a dose-dependent way on either PND7 or

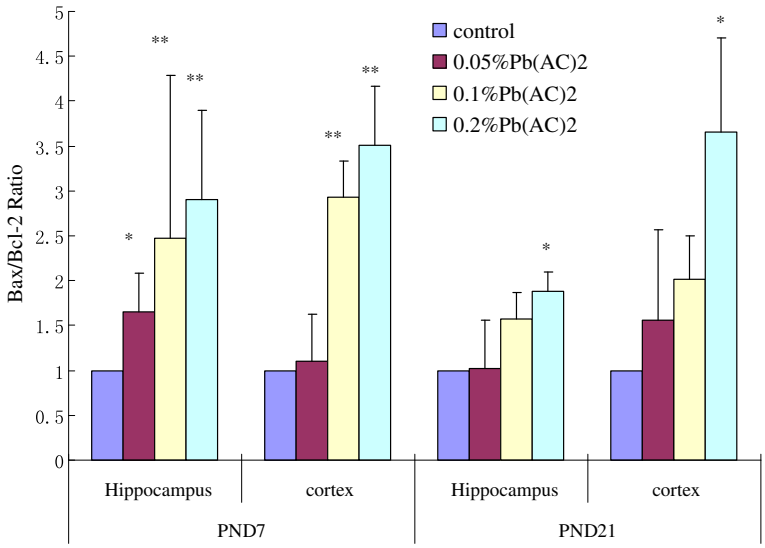

Fig. 6 Alteration of Bax/Bcl-2 ratio in pups' hippocampus and its nearby cortex between lead-exposed groups and control on PND7 and 21, respectively (n=5). *Comparing with the control, P<0.05; **comparing with the control, P<0.01

PND21; meanwhile, the blood lead level reached at 2.02± 0.79 μmol/L. Moreover, GSH level showed a significant reduction in parallel with an increase in MDA. SOD is a free-radical scavenger and metalloenzyme; if its activity is kept down, the amount of free radical expresses an increasing trend and damages the developing nerve system [32, 33]. Our results showed a significant decrease in SOD activity in hippocampus after lead exposure.

Apoptosis is very important in both physiological and pathological conditions. Recent studies indicated that lead-induced cytotoxicity in hippocampus can be mediated via apoptosis facilitation [34–36]. It has also been interesting to investigate which period in pup's development after birth is more vulnerable to be affected by prenatal and early life lead exposure. We attempted to pick two time points on PND7 and PND21 to reflect the dynamic change. Our findings indicated that the hippocampus on PND7 had more apoptotic cells than PND21, so a higher sensitivity in younger animal has been observed. Apoptosis is a phenomenon of naturally occurring cell death that plays a fundamental role during CNS development. Some reports analyzed the distribution of apoptotic cells in rat's different brain regions during the different developmental stages, and found that the number of apoptotic cells in hippocampus progressively increased from birth to the first postnatal week, with a peak at PND7, and subsequently decreased, and stops at PND28 [37, 38]. We found that the younger animals on PND7 showed more apoptotic cells following the peak of physiological apoptosis and a clear increase due to 0.1 % and 0.2 % lead exposure, while on PND21 the obvious increase only occurred in high lead exposure.

As we have known, apoptosis or programmed cell death is a gene-regulated and complicated process. One of the gene families closely related to apoptosis regulatory pathways is the Bcl-2 family, which comprises several genes promoting apoptosis (Bax and Bad) and some others inhibiting apoptosis (Bcl-2, Bcl-Xl, Bcl-w, NR-13, and Mcl-1) [39–41]. As an apoptosis promoter in the family, Bax protein is known to be located in the mitochondrial membrane and apoptosis accompanies the over-expression of Bax [42, 43]. Usually, Bax is stimulated by the signal of apoptosis and causes cytochrome *c* to be released from the mitochondria and promote apoptosis. In contrast, Bcl-2 can inhibit the action of Bax and blocks the release of cytochrome *c* [25, 44]. Therefore, a higher Bax/Bcl-2 ratio can become an important index of apoptotic cell death. We observed that Bax/Bcl-2 ratio in 0.1 % and 0.2 % lead exposure increased on PND7, but it kept high only in 0.2 % $Pb(AC)_2$ group on PND21. Consistent with apoptotic index, PND7 but not PND21 was shown to be more sensitive to lead. In addition, it seemed that the hippocampus was much more sensitive to the damage induced by lead because even 0.05 % $Pb(AC)_2$ exposure could induce a significant increase in Bax/Bcl-2, which suggested that lead exposure converted programmed cell death from a natural physiological phenomenon into a pathological process.

Studies suggested that in vivo generation of high ROS in the aftermath of lead exposure might result in the depletion of intrinsic antioxidant defenses in cells [45]. Furthermore, following ROS increase, lead can affect the mitochondrial permeability, thereby stimulating the release of mitochondrial proteins, like Bax, and disturbing the action of survival factors, like Bcl-2 [46]. In the current study, we found that ROS levels in 0.1 % and 0.2 % lead exposure showed an increase; meanwhile, oxidative stress and apoptosis facilitation were also found. Although excessive ROS and oxidative stress could be found during the whole earlier life, the period sensitive to lead exposure seemed much earlier. Our findings would contribute to further understanding of the mechanisms of lead's developing neurotoxicity and cognition impairment in children after lead exposure during the critical and sensitive stage of CNS development.

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