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Are newborn rat-derived neural stem cells more sensitive to lead neurotoxicity?★

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Abstract

Lead ion (Pb^{2+}) has been proven to be a neurotoxin due to its neurotoxicity on mammalian nervous system, especially for the developing brains of juveniles. However, many reported studies involved the negative effects of Pb^{2+} on adult neural cells of humans or other mammals, only few of which have examined the effects of Pb^{2+} on neural stem cells. The purpose of this study was to reveal the biological effects of Pb^{2+} from lead acetate [Pb $(CH_3COO)_2$] on viability, proliferation and differentiation of neural stem cells derived from the hippocampus of newborn rats aged 7 days and adult rats aged 90 days, respectively. This study was carried out in three parts. In the first part, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT viability assay) was used to detect the effects of Pb^{2+} on the cell viability of passage 2 hippocampal neural stem cells after 48-hour exposure to 0–200 μM Pb^{2+}. In the second part, 10 μM bromodeoxyuridine was added into the culture medium of passage 2 hippocampal neural stem cells after 48-hour exposure to 0–200 μM Pb^{2+}, followed by immunocytochemical staining with anti-bromodeoxyuridine to demonstrate the effects of Pb^{2+} on cell proliferation. In the last part, passage 2 hippocampal neural stem cells were allowed to grow in the differentiation medium with 0–200 μM Pb^{2+}. Immunocytochemical staining with anti-microtubule-associated protein 2 (a neuron marker), anti-glial fibrillary acidic protein (an astrocyte marker), and anti-RIP (an oligodendrocyte marker) was performed to detect the differentiation commitment of affected neural stem cells after 6 days. The data showed that Pb^{2+} inhibited not only the viability and proliferation of rat hippocampal neural stem cells, but also their neuronal and oligodendrocyte differentiation *in vitro*. Moreover, increased activity of astrocyte differentiation of hippocampal neural stem cells from both newborn and adult rats was observed after exposure to high concentration of lead ion *in vitro*. These findings suggest that hippocampal neural stem cells of newborn rats were more sensitive than those from adult rats to Pb^{2+} cytotoxicity.

Key Words

neural regeneration; stem cells; neural stem cells; adult; neonate; mammals; Pb^{2+}; neurotoxicity; viability; proliferation; hippocampus; photographs-containing paper; neuroregeneration

Research Highlights

(1) Viability, proliferation, neuronal and oligodendrocyte differentiation of rat neural stem cells were significantly decreased after lead exposure.
(2) Lead exposure promoted astrocyte differentiation from rat neural stem cells.
(3) Newborn rat-derived neural stem cells are more sensitive to lead neurotoxicity than their adult counterparts.

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INTRODUCTION

Lead ion (Pb^{2+}) is a common element that has been widely used in modern industries like building industry, battery production, radiation shield, or munitions industry. Neurotoxicity of lead on nervous system has been extensively studied since 1943, in which, the first report about influences of lead neurotoxicity on mental development was published[1]. Over years, a host of studies have demonstrated the negative effects of Pb^{2+} on adult neural cells of humans and mammals[2-6].

Pb^{2+} can seriously impair the development and functions of mammalian nervous system. A study in 2003 showed that 12–14 week old rats, after being treated with 15 mg/kg daily dose of lead acetate for 7 days, had a significant increase in both blood lead level (17–19 folds) and Bax (an apoptotic protein)/Bcl-2 (an anti-apoptotic protein) expression ratio in the hippocampus, showing a significant lead-induced apoptosis[5]. The number of viable newborn cells in the dentate gyrus of rats greatly decreases after lead exposure[7]. Lead exposure can also reduce the weight and size of the hippocampus[8]. Lead ions at low level impaired the sialylation pattern of neural cell adhesion molecules chronically and thus affected the formation of synapse[9]. Lead acetate, at the level of 30 μg/mL, was toxic to neurons differentiated from cerebral cortical precursor cells; however, it raised the number of the cortical precursors at low concentrations[10]. Glial cell is also a target for lead cytotoxicity[11]. Lead exposure results in loss of viability and enzymatic activity of oligodendrocytes[11]. Lead might inhibit brain development by delaying the differentiation of oligodendrocyte progenitors *in vitro* [12]. Myelination in adults is permanently altered by childhood lead exposure[13]. Exposure of oligodendrocyte progenitor cells from newborn rat pups to 1 μM lead acetate for 24 hours before the culture in differentiation medium leads to a significant reduction in expression of galactolipid metabolic enzymes, affecting the level of galactolipids in myelin[14]. The expression of astrocytes was enhanced by acute lead exposure, demonstrating the neuroprotective role of astrocytes against lead toxicity[15-17].

Although many reported studies have extensively examined the negative effects of lead on human neural cells, only a few addressed the influences of Pb^{2+} on human neural stem cells. Neural stem cells are defined as the cell populations which are capable of self-renewal and with multipotency that can generate the main phenotypes of the nervous system including neurons, astrocytes and oligodendrocytes[18]. Neurons are the basic units of the nervous system, which are excitable and inhibitory cells transmitting and processing nervous information electrically and chemically[19]. Astrocytes take up glucose from the blood circulation for metabolism and neurotransmitters from the axon terminals for recycling[20]. They also maintain a suitable ionic environment for neurons by removing excess potassium ions from the extracellular fluid[20]. Oligodendrocytes form myelin sheath in the central nervous system wrapping the axonal membranes to provide mechanical support and insulation for efficient electrical signal propagations along the nerve fibers[21]. Due to the critical role played by neural stem cells in the development, plasticity and regenerative biology of the nervous system, the *in vitro* culture model has been developed as an important platform for the studies involving environmental neurotoxicity, drug screen and development-relevant researches.

There are few studies that used neural stem cells or other stem cells as *in vitro* models to study neurotoxicity of lead[12, 22-23]. Huang and Schneider isolated embryonic neural stem cells from several brain regions of rats to investigate the effects of lead ions on neural stem cells[22]. They found that lead ions inhibited the proliferation, neuronal and oligodendrocyte differentiation of striatum- and ventral mesencephalon-derived neural stem cells but not cortex-derived neural stem cells[22]. Apart from neural stem cells, bone marrow mesenchymal stem cells are also a target for lead neurotoxicity[23]. The neuronal gene expression and the percentage of differentiated neurons of bone marrow mesenchymal stem cells were greatly reduced after lead exposure[23]. The current use of neural stem cells in lead neurotoxicity studies, however, is still limited.

The present study evaluated and compared the neurotoxic effects of lead on both newborn and adult hippocampal neural stem cells. The hippocampus, one of the most abundant sources of neural stem cells in humans, was employed in this study, because of its crucial roles in memory and spatial navigation[24-25]. In order to assess the influences of Pb^{2+} on the viability, proliferation and differentiation of neural stem cells, Pb^{2+} at different concentrations (0, 1, 10, 50, 100, and 200 μM), was added to the proliferation medium of passage 2 neural stem cells for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide viability assay, bromodeoxyuridine proliferation assay and immunocytochemical staining. The sensitivity of newborn

and adult hippocampal neural stem cells to Pb^{2+} was also compared to investigate the influences of age and environmental lead concentration on neural stem cell behavior *in vitro*. A better understanding of the influences of Pb^{2+} on neural stem cells would help establish regulations for the discharge standard of Pb^{2+} for the sake of public health.

RESULTS

Free-floating neurospheres containing neural stem cells

Single cell suspensions harvested from the hippocampi of both newborn rats (aged 7 days old) and adult rats (aged 90 days old) were able to generate free-floating neurospheres and increased in size thereafter. The sizes of the neurospheres from newborn rats were slightly larger than those from adult rats after 3 and 6 days of culture (Figures 1A–D). The neurospheres formed after primary cultures were dissociated to produce single cell suspension.

The dissociated cells from both newborn and adult rats showed positive staining for nestin (a specific marker for neural stem cells), indicating the presence of neural stem cells of the neurospheres (Figures 1E, F).

Significant inhibitory effects of lead on the viability and proliferation of newborn and adult rat neural stem cells

MTT viability assay showed that there was no significant reduction in cell viability after adding 1μM lead ions to both newborn and adult hippocampal neural stem cells ($P < 0.05$, *vs*. control). However, the number of viable newborn rat hippocampal neural stem cells significantly decreased ($P < 0.05$, *vs*. control) after addition of 10 μM Pb^{2+} but no significant effect on the percentage of viable adult rat hippocampal neural stem cells was found at this concentration compared to the negative control (0 μM) (Figure 2E). Therefore, the newborn rat hippocampal neural stem cells were more vulnerable to the toxicity of Pb^{2+} than adult rat hippocampal neural stem cells in this study. Higher concentrations of Pb^{2+} remarkably reduced the survival of hippocampal neural stem cells from both newborn and adult rats (Figure 2E). After addition of 50, 100 and 200 μM Pb^{2+}, the percentage of viable newborn and adult hippocampal neural stem cells decreased significantly when compared with the negative control (0 μM Pb^{2+} treatment) ($P < 0.01$, *vs*. control; Figure 2E).

Bromodeoxyuridine was used to evaluate the proliferation of neural stem cells after exposure to different concentrations of Pb^{2+}.

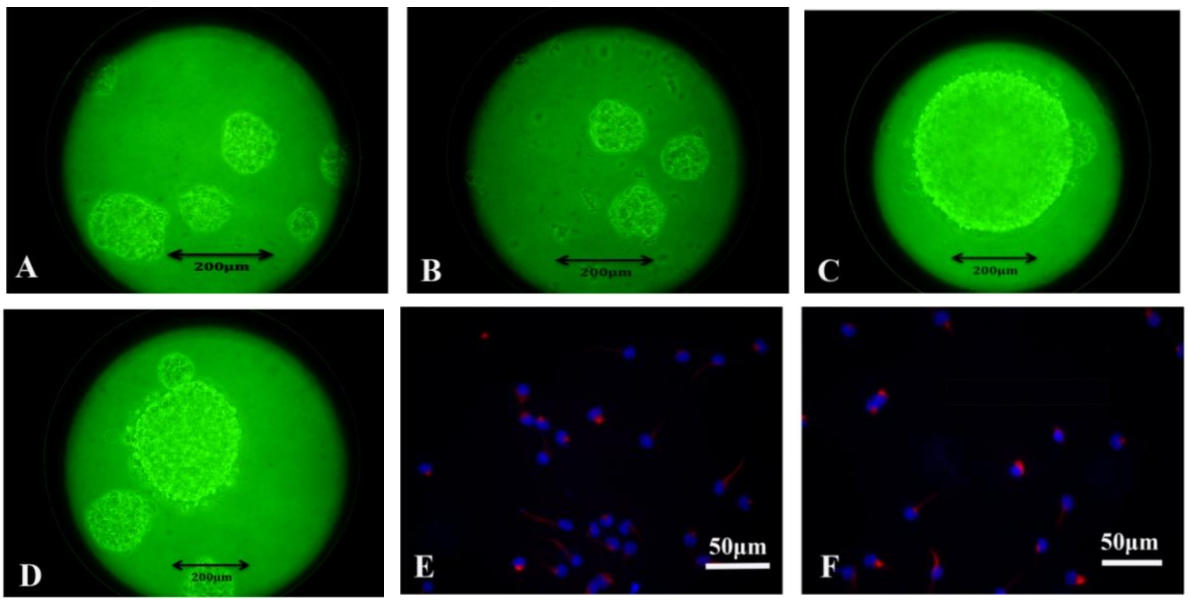


Figure 1 Neural stem cells generated from newborn and adult rat hippocampi.

Neurospheres generated from the neural stem cells of newborn and adult rats after 3 days of culture as shown in (A) and (B) respectively. The individual neurospheres generated from the neural stem cells of newborn and adult rats after 6 days of culture are shown in (C) and (D) separately. These images were captured by light microscopy with a green filter.

The dissociated neural stem cells of newborn and adult rats shown in (E) and (F) were obtained using double labeling immunocytochemistry, respectively (red for nestin-positive signal and blue for 4',6-diamidino-2-phenylindole-stained nuclei).

The scale bars: A–D, 200 μm; E and F, 50 μm.

As shown in Figures 2A–D and F, newborn rat hippocampal neural stem cells were more susceptible to Pb^{2+} in cell proliferation. Even a low concentration of Pb^{2+} (1 or 10 μM) could result in significant reduction in the proliferation of newborn rat hippocamal neural stem cells ($P < 0.01$, *vs*. control). Low concentrations of Pb^{2+} (1 or 10 μM) showed no significant effect on the proliferation of adult rat hippocampal neural stem cells. High concentrations of Pb^{2+} (50, 100 and 200 μM) significantly inhibited the proliferation of both newborn and adult hippocampal neural stem cells compared to the negative control ($P < 0.01$; Figure 2F).

Lead inhibited neuronal and oligodendrocyte differentiation but promoted astrocyte differentiation

Exposure to 50 μM or above Pb^{2+} negatively affected the neuronal differentiation of newborn rat hippocampal neural stem cells ($P < 0.01$, *vs*. control group; Figures 3C, E and G). Low concentrations (1 and 10 μM) of Pb^{2+} did not significantly influence the neuronal differentiation of newborn hippocampal neural stem cells (Figures 3A and G). Adult rat hippocampal neural stem cells were less sensitive to lead toxicity than newborn rat hippocampal neural stem cells in neuronal differentiation (Figures 3B, D and F). Only high concentrations of lead ions (100 or 200 μM) could significantly inhibit the neuronal differentiation of adult rat hippocampal hippocampal neural stem cells ($P < 0.01$, *vs*. control). As shown in Figure 3G, no significant decrease in the percentage of differentiated neurons was observed in the groups with lower concentrations of Pb^{2+} (1–50 μM). In addition to the influences of lead toxicity on the percentage of differentiated neurons, Pb^{2+} could impair the neuronal differentiation by greatly reducing the sizes of differentiated neurons of newborn rats (Figures 3A, C and E). However, this was not observed in the adult hippocampal neural stem cells (Figures 3B, D and F).

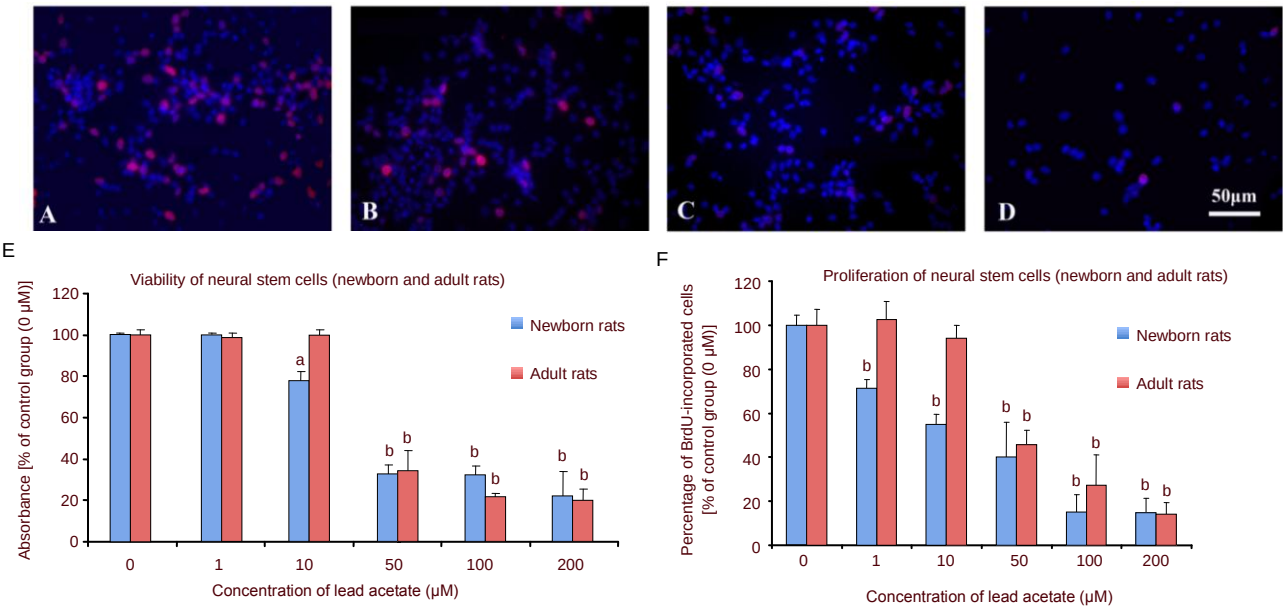


Figure 2 Inhibitory effects of Pb^{2+} on the viability and proliferation of newborn and adult rat hippocampal neural stem cells.

Through the fluorescence microscopy using double labeling of 4',6-diamidino-2-phenylindole (blue) and anti-bromodeoxyuridine (red), the proliferation of newborn rat hippocampal neural stem cells exposed to Pb^{2+} is shown in (A, 0 μM Pb^{2+}) and (C, 100 μM Pb^{2+}) respectively. Additionally, the proliferation of adult rat hippocampal neural stem cells exposed to Pb^{2+} is shown in (B, 0 μM Pb^{2+}) and (D, 100 μM Pb^{2+}) respectively. Scale bars: 50 μm for A–D.

Hippocampal neural stem cells from newborn rats were more vulnerable to the toxicity of Pb^{2+} than those from adult rats. 10 μM Pb^{2+} initially led to significant death of neural stem cells from the newborn rats compared to the control group with 0 μM Pb^{2+} treatment ($^{a}P < 0.05$), but there was no significant difference in cell death between adult rat hippocampal neural stem cells and the control with the treatment of 0 μM Pb^{2+} (E). 50–200 μM lead acetate treatments resulted in a significant decrease in the number of viable newborn and adult rat neural stem cells compared to the control group with 0 μM Pb^{2+} treatment ($^{b}P < 0.01$). Regarding the proliferation assay, all concentrations of lead acetate (1, 10, 50, 100 and 200 μM) in this study caused a significant inhibitory effect on the proliferation of newborn rat neural stem cells when compared to the control without Pb^{2+} treatment ($^{b}P < 0.01$, *vs*. control group). However, only higher concentrations of Pb^{2+} (50, 100 and 200 μM) could result in a significant inhibitory effect on the proliferation of adult rat neural stem cells compared to the control group with 0 μM Pb^{2+} treatment ($^{a}P < 0.05$, $^{b}P < 0.01$) (F). One-way analysis of variance was used to analyze the difference and the data were expressed as mean ± SEM.

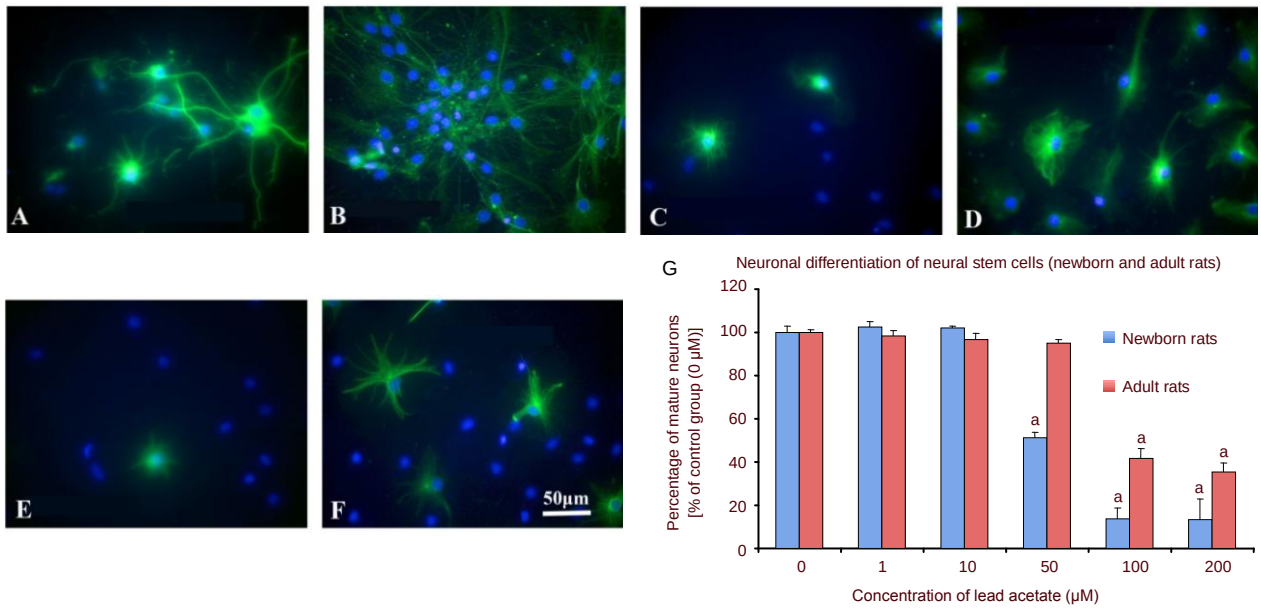


Figure 3 Inhibitory effects of Pb^{2+} on neuronal differentiation of hippocampal neural stem cells from the newborn and adult rats.

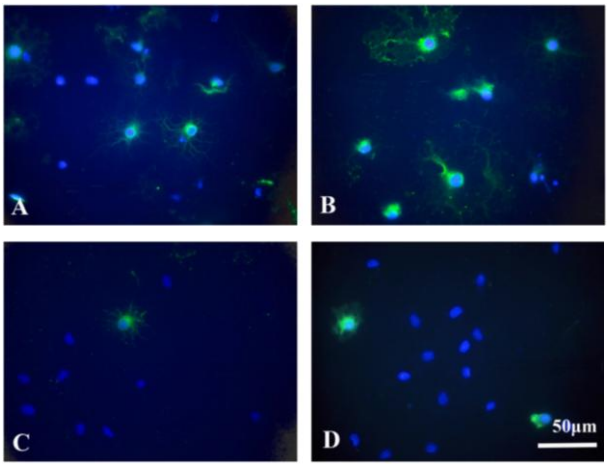
Through the fluorescence microscopy using double labeling of 4',6-diamidino-2-phenylindole (blue) and microtubule-associated protein 2 (green), the neuronal commitment of newborn rat hippocampal neural stem cells after exposure to Pb^{2+} is shown in (A, 0 μM Pb^{2+}), (C, 50 μM Pb^{2+}) and (E, 200 μM Pb^{2+}) respectively. Moreover, the neuronal commitment of adult rat hippocampal neural stem cells after exposure to Pb^{2+} treatment is shown in (B, 0 μM Pb^{2+}), (D, 50 μM Pb^{2+}) and (F, 200 μM Pb^{2+}) respectively. Scale bars: 50 μm for A–F.

The percentage of mature neurons differentiated from newborn rat neural stem cells decrease significantly ($^aP < 0.01$) when exposed to 50 μM or above lead acetate; however, the percentage of neuronal differentiation of adult rat hippocampal neural stem cells did not change significantly until the concentration of Pb^{2+} reached 100 and 200 μM (G). One-way analysis of variance was used to analyze the difference between groups and the data were expressed as mean ± SEM. The present results indicated that newborn rat hippocampal neural stem cells were more sensitive to lead toxicity in neuronal differentiation compared to adult rat hippocampal neural stem cells.

Lead exposure at the concentrations of 10–200 μM significantly impaired the oligodendrocyte differentiation ability of newborn and adult rat hippocampal neural stem cells ($P < 0.01$, *vs*. control; 50–200 μM Pb^{2+} groups of newborn rat hippocampal neural stem cells shown in Figures 4A and C, E and 10–200 μM Pb^{2+} groups of adult rat hippocampal neural stem cells shown in Figures 4B, D, E). These results demonstrated that newborn and adult rat hippocampal neural stem cells were relatively more sensitive to lead toxicity in oligodendrocyte differentiation than neuronal differentiation, in which, a significant neuronal loss was only found in the groups with higher Pb^{2+} concentrations (> 50 μM for newborn rat hippocampal neural stem cells and > 100 μM for adult rat hippocampal neural stem cells; $P < 0.05$, *vs*. 10 μM Pb^{2+} control group for newborn rats).

Moreover, results of astrocyte differentiation were quite different from those of neuronal and oligodendrocyte differentiation. Generally, Pb^{2+} significantly inhibited the neuronal and oligodendrocyte differentiation of newborn and adult rat hippocampal neural stem cells. However, an increase in the percentage of differentiated astrocytes was found after exposure to certain levels of Pb^{2+}. The percentage of astrocytes generated from newborn rat hippocampal neural stem cells increased significantly after addition of 10–200 μM Pb^{2+} ($P < 0.01$, *vs*. control group) (Figures 5 A, C and E).

There was also an increase in the percentage of differentiated astrocytes of adult rat hippocampal neural stem cells after addition of 50–200 μM Pb^{2+} (Figures 5B, D, E). The newborn rat hippocampal neural stem cells were more sensitive to Pb^{2+} stimulation for astrocyte differentiation than adult rat hippocampal neural stem cells as a significant increase in the percentage of differentiated astrocytes was observed at a relatively low concentration (10 μM) of Pb^{2+} ($P < 0.01$, *vs*. control group). Higher concentration of Pb^{2+} (200 μM) could further increase the percentage of mature astrocytes significantly ($P < 0.05$, *vs*. the group with 100 μM Pb^{2+}) compared to newborn rat neural stem cells (Figure 5E)



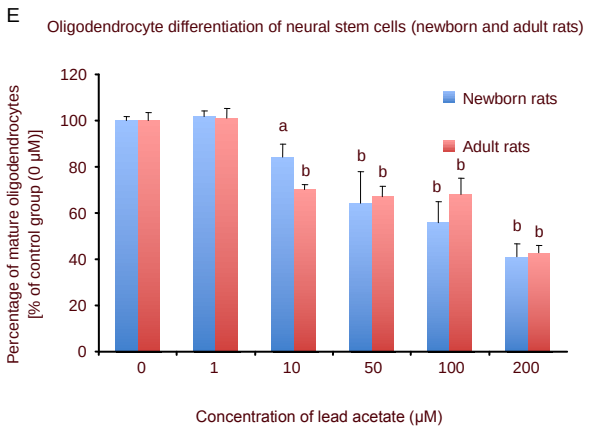
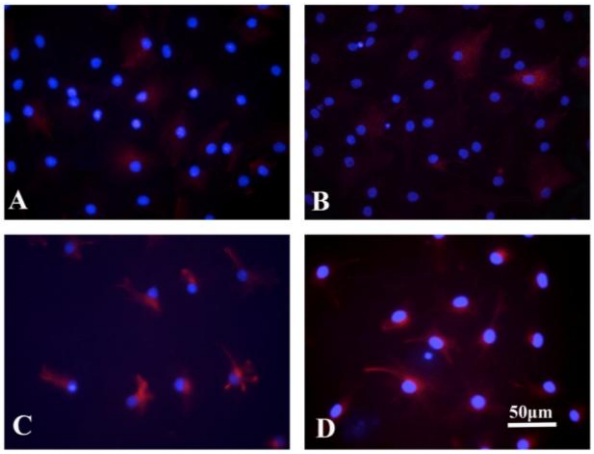


Figure 4 Negative effects of Pb^{2+} on oligodendrocyte differentiation of hippocampal neural stem cells from the newborn and adult rats.

Through the fluorescence microscopy using double labeling of 4',6-diamidino-2-phenylindole (blue) and RIP (green), the oligodendrocyte commitment of newborn rat hippocampal neural stem cells after exposure to Pb^{2+} treatment is shown in (A, 0 μM Pb^{2+}) and (C, 100 μM Pb^{2+}) respectively. Furthermore, the oligodendrocyte commitment of adult rat hippocampal neural stem cells after exposure to Pb^{2+} treatment is shown in (B, 0 μM Pb^{2+}) and (D, 100 μM Pb^{2+}) respectively. Scale bars: 50 μm for A–D.

(E) Pb^{2+} exposure at the concentration of 10–200 μM significantly impaired oligodendrocyte differentiation of newborn and adult rat hippocampal neural stem cells compared to the control group with 0 μM lead acetate treatment ($^{a}P < 0.05$, $^{b}P < 0.01$, analysis of variance). One-way analysis of variance was used to analyze the difference and the data were expressed as mean ± SEM.



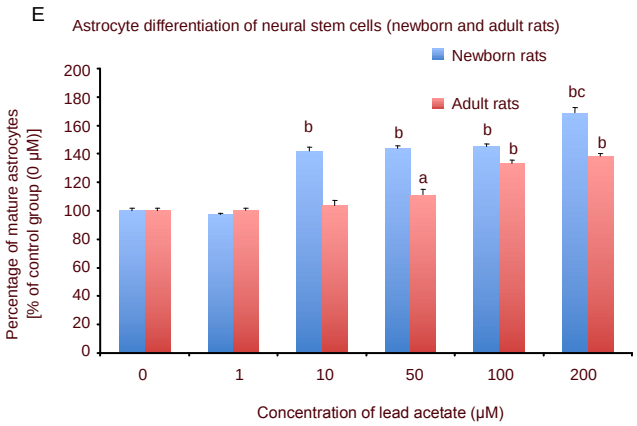


Figure 5 Up-regulation of astrocyte differentiation of hippocampal neural stem cells derived from both newborn and adult rats after exposure to Pb^{2+}.

Through the fluorescence microscopy using double labeling of 4',6-diamidino-2-phenylindole (blue) and glial fibrillary acidic protein (red), the astrocyte differentiation of newborn rat hippocampal neural stem cells after exposure to Pb^{2+} is shown in (A, 0 μM Pb^{2+}) and (C, 100 μM Pb^{2+}) respectively. Furthermore, the astrocyte commitment of adult rat hippocampal neural stem cells after exposure to Pb^{2+} is shown in (B, 0 μM Pb^{2+}) and (D, 100 μM Pb^{2+}) respectively. Scale bars: 50 μm for A–D.

The newborn rat hippocampal neural stem cells were more sensitive to Pb^{2+} exposure in astrocyte differentiation indicated by a significant increase in the percentage of mature astrocytes even at a relatively low concentration (10 μM) of lead acetate ($^{b}P < 0.01$, *vs*. control group). In contrast, higher concentrations of Pb^{2+} exposure (50–200 μM) led to a significant increase in the percentage of mature astrocytes differentiated from adult rat hippocampal neural stem cells compared to the negative control (E, $^{a}P < 0.05$, $^{b}P < 0.01$, analysis of variance). Notably, 200 μM Pb^{2+} exposure could further increase the percentage of mature astrocytes (E, $^{c}P < 0.05$ for comparison between newborn and adult groups with 200 μM lead acetate treatment) in the study. One-way analysis of variance was used to analyze the difference and the data were expressed as mean ± SEM.

DISCUSSION

Effects of Pb^{2+} exposure on viability of neural stem cells

Neural cell death induced by lead toxicity has been shown in various studies[1, 5, 7, 11, 26-27]. One of the mechanisms to explain lead neurotoxicity is that Pb^{2+} might compete with zinc ion for the zinc-binding site of some DNA-binding protein complexes[28-29]. Zinc finger proteins, acting as DNA-binding factors, regulate multiple gene expression[28-29]. Substitution of zinc ions by Pb^{2+} undoubtedly dampened the gene expression for normal cellular activities including cell survival, differentiation and signaling pathways[28-29].

It is generally believed that children neural cells are more vulnerable to the toxicity of Pb^{2+}[30-31]. Low-level lead exposure could impair the cognitive ability of children[30]. In the present study, a significant neural stem cell death was found in the newborn rat hippocampal neural stem cell samples cultured with equal or over 10 μM Pb^{2+}. However, in the adult counterparts, a significant cell death was only observed after addition of 50 μM or above Pb^{2+}. These results indicate that children neural stem cells are more vulnerable to lead toxicity. Even a low concentration (10 μM) of Pb^{2+} could initiate significant neural stem cell death *in vitro*.

Effects of Pb^{2+} exposure on proliferation of neural stem cells

In this study, neural stem cells were cultured in proliferation medium with or without Pb^{2+} for 48 hours, followed by incorporation of bromodeoxyuridine into the DNA of dividing cells to measure the rate of proliferation. It is not surprising that newborn rat hippocampal neural stem cells were more susceptible to lead toxicity than adult rat hippocampal neural stem cells in cell proliferation. Nevertheless, it is important to note that 1 μM Pb^{2+} significantly inhibits the proliferation of children's neural stem cells, which is regarded as a relatively low but environmentally significant concentration[22]. Suppression of neural stem cell proliferation can occur without obvious cell death, indicating that lead-induced inhibition on cell proliferation may be stronger than that on viability of neural stem cells in children.

Again, adult rat hippocampal neural stem cells were more resistant to lead toxicity. The cell proliferation was only inhibited significantly when 50 μM Pb^{2+} was added into the proliferation medium for adult neural stem cell culture. Our findings could be explained by previous findings that Pb^{2+} inhibited the N-methyl-D-aspartic acid receptor-ion channel in an age-dependent manner[30]. Since N-methyl-D-aspartic acid receptor-ion channel regulates the changes in synaptic plasticity as well as the influx of calcium ions, inhibitions by Pb^{2+} regulate the cell proliferation and survival negatively[30, 32]. Collectively, significant inhibition of proliferation was observed at a relatively lower concentration (1 μM) of Pb^{2+} in newborn rat hippocampal neural stem cells compared to the counterparts of adult rats[30, 32].

Effects of Pb^{2+} on differentiation of neural stem cells into neurons, oligodendrocytes and astrocytes

Previous studies have reported the influences of lead ions on mature neurons or their progenitors. Lead exposure at the level higher than 60 μM led to significant reduction in percentage of mature neurons and expression of neuronal genes of bone marrow mesenchymal stem cells[23]. Postnatal exposure to lead could decrease the number of spontaneously active midbrain dopamine neurons in rats[33]. Lead also induced P53-mediated apoptosis of hypothalamic GT1-7 neurons in rats[34]. Consistent with these findings, results of the present study suggested that introduction of moderate to high levels (50–200 μM) of Pb^{2+} could significantly suppress the neuronal differentiation of neural stem cells *in vitro*. Although the percentage of differentiated neurons found in the samples of newborn rats with lower lead concentrations (1–10 μM) did not change significantly, the number of stained nuclei decreased obviously after addition of Pb^{2+}, indicating a decrease in the total number of neurons due to cell death.

The influences of lead on the functions and development of oligodendrocytes and their progenitors have been well studied which showed that lead exposure could reduce the viability and intracellular enzymatic activity of oligodendrocytes and delay the oligodendrocyte differentiation[11-12]. Pb^{2+} not only inhibits myelin formation in developing nervous system but also causes demyelination of axons[35-36]. Parallel to these previous studies, exposure to moderate to high levels (10–200 μM) of Pb^{2+} significantly decreased the percentage of differentiated oligodendrocytes from both newborn and adult rat neural stem cells in the present study. The number of mature oligodendrocytes decreased with the increasing concentrations of Pb^{2+} which was similar to the results of inhibition of neuronal differentiation with designed treatments. One of the important functions of oligodendrocytes is to support the survival of neurons and neuronal differentiation[36-37]. Fewer oligodendrocytes were

present to support the survival of neurons as well as the neuronal differentiation from neural stem cells, which indicated that lower percentages of mature neurons were found in the groups with higher concentrations of Pb^{2+}.

It is known that astrocytes play a protective role to maintain the homoeostasis in normal central nervous system[15-16, 33, 38-40]. Astrocytes sequester lead ions, maintaining ionic balance in the nervous system thus protecting neurons from lead toxicity[15-16, 33]. The present study showed that the percentage of astrocytes increased significantly after addition of moderate to high level of Pb^{2+} (>10 μM for newborn rats and > 50 μM for adult rats), indicating that Pb^{2+} might enhance astrocyte differentiation to generate more astrocytes to exert protection of the neurons involved. Despite of the neuroprotection from astrocytes, an increase in percentage of differentiated astrocytes after lead exposure might indicate an alternative decrease in percentage of differentiated neurons and oligodendrocytes, which would significantly affect the normal functions of developing nervous systems.

To summarize, the present study demonstrates the neurotoxic effects of Pb^{2+} on the viability, proliferation and neural differentiation of neural stem cells derived from the hippocampi of newborn and adult rats. The results suggest that Pb^{2+} have negative effects on viability, proliferation, neuronal and oligodendrocyte differentiation of both newborn and adult neural stem cells *in vitro*. In contrast, certain levels of Pb^{2+} activate astrocyte differentiation in both age groups. Of note, newborn rat neural stem cells are more sensitive to lead neurotoxicity than their adult counterparts. The underlying molecular and cellular mechanisms warrant further studies with *in vitro* and *in vivo* models.

MATERIALS AND METHODS

Design

An *in vitro* cell experiment.

Time and setting

The experiment was performed at the Department of Anatomy, Li Ka Shing Faculty of Medicine, the University of Hong Kong between January and August 2012.

Materials

Ten newborn female Sprague-Dawley rats aged 7 days old and two adult female Sprague-Dawley rats aged 90 days old were used in this study. The rats were provided by Laboratory Animal Unit, the University of Hong Kong, China.

Methods

Neural stem cell harvest and primary culture

After anesthesia by intramuscular injection of 20% Dorminal (200 mg/mL, 0.5 mL/kg), newborn and adult Sprague-Dawley rats were sacrificed and their brains were placed into cold, sterile Hank's Buffered Salt Solution (HBSS; Life Technologies™ Inc., Hong Kong, China) immediately. The hippocampi, identified as a seahorse-shaped structure in medial temporal lobes, were washed with HBSS once and dissociated into single cell suspensions by 2.5% trypsin (Life Technologies™ Inc.) for 15 minutes. The dissociated cells were re-suspended in HBSS and filtered through a sterile cell strainer (70 μm pore size) to obtain single-cell suspensions. An aliquot of dissociated cells was diluted with HBSS and counted using a hemocytometer to estimate the number of cells obtained for both rat groups (newborn and adult). Single-cell suspensions were plated onto a 25cm^2 cell culture flask at a density of 1 × 10^5/mL in proliferation medium for neural stem cells [dulbecco's modified eagle's medium (DMEM)/F12 (1:1), B27 supplement, N2 supplement, 20 ng/mL basic fibroblast growth factor and 20 ng/mL epidermal growth factor; Life Technologies™ Inc.]. The cells were incubated in an incubator at 37°C with 5% CO_2 for several days.

Formation of free-floating neurospheres and secondary culture of neural stem cells (passage 1)

In order to obtain sufficient number of neural stem cells, passage 1 neural stem cells were further cultured for one more generation. After primary culture, free-floating neurospheres formed. Neurospheres were collected into 15 mL centrifuge tubes and centrifuged at 800 r/min for 5 minutes. The cell pellets at the bottom of the centrifuge tube were washed once with HBSS and 0.5% trypsin (Life Technologies™ Inc.) was added to dissociate the cell pellets at 37°C for 3 minutes. DMEM/F12 with 10% fetal bovine serum (Life Technologies™ Inc.) was added to stop the enzymatic digestion. Digested neurospheres were centrifuged at 800 r/min for 5 minutes. The dissociated cells were re-suspended in DMEM/F12, and an aliquot of dissociated cells was further diluted with HBSS and the cell number was counted using a hemocytometer. Dissociated cells at the density of 1 × 10^5/mL were plated onto a 25 cm^2 cell culture flask containing

proliferation medium for neural stem cells [DMEM/F12 (1:1), B27 supplement, N2 supplement, 20 ng/mL basic fibroblast growth factor and 20 ng/mL epidermal growth factor]. The cells were incubated at 37°C with 5% CO_2 for several days.

Dissociation of neurospheres

After 5–8 days, a plenty of passage 2 free-floating neurospheres formed. The neurospheres were collected and centrifuged at 800 r/min for 5 minutes. The cell pellets were washed once with HBBS and trypanized by 0.5% trypsin at 37°C. DMEM/F12 with 10% fetal bovine serum was added to stop the enzymatic digestion. Digested neurospheres were centrifuged at 800 r/min for 5 minutes. The dissociated cells were re-suspended in DMEM/F12, and an aliquot of dissociated cells was diluted with HBSS and counted using a hemocytometer to estimate the number of cells.

Characterization of neurospheres

Single cell suspensions obtained from neurospheres were plated onto the poly-L-lysine-coated coverslips at a density of 2.5×10^4 cells/cm^2 per well in 500 μL proliferation medium [DMEM/F12 (1:1), B27 supplement without vitamin A, N2 supplement, 20 ng/mL basic fibroblast growth factor and 20 ng/mL epidermal growth factor]. The cells were incubated at 37°C with 5% CO_2 for 12 hours to allow the cells to attach to the coverslips.

After 12 hours, the cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature and permeabilized by 0.3% triton X-100 solution in PBS. Thereafter, the cells were incubated with 5% bovine serum albumin (Sigma-Aldrich China Inc., Hong Kong, China) in PBS to avoid non-specific binding of antibody. After three rinses with PBS, cells were incubated with mouse anti-nestin (a neural stem marker; Life Technologies™ Inc.) at 1:200 dilution in 1% bovine serum albumin/PBS for 24 hours at 4°C, washed three times with PBS and then incubated with goat anti-mouse IgG secondary antibody (Alexa Fluor®647; Life Technologies™ Inc.) at 1:500 dilution in 1% bovine serum albumin/PBS for 2 hours at room temperature. The stained cells were washed three times with PBS and counterstained with 4',6-diamidino-2-phenylindole (1:1 000 dilution in 1% bovine serum albumin/PBS; Life Technologies™ Inc.) for 20 minutes. After being washed with PBS once, coverslips were mounted onto slides with mounting medium and allowed to dry overnight. The stained cells were viewed under light and fluorescence microscopes (Zeiss, Oberkochen, Germany) (100 ×).

Preparation of culture media with lead

In order to study the effect of Pb^{2+} on the survival of neural stem cells, lead acetate powder [$Pb(CH_3COO)_2 \cdot 3H_2O$; Sigma-Aldrich China Inc.] was used. Lead acetate was first dissolved in warm water (37°C) and filtered through a 0.22 μm membrane filter to remove bacteria. Lead acetate solution was added into the proliferation or differentiation medium to prepare media with different concentrations of Pb^{2+} (0, 1, 10, 50, 100 and 200 μM).

Cell viability assay

Passage 2 cells obtained from neurospheres were seeded onto the poly-L-lysine-coated wells of a 96-well plate at a density of 2.5×10^4 cells/cm^2 per well in 100 μL culture medium without Pb^{2+}. The cells were incubated at 37°C with 5% CO_2 for 24 hours. Different concentrations (0, 1, 10, 50, 100 and 200 μM) of lead acetate were prepared with proliferation medium of neural stem cells. After 24-hour incubation, the wells were washed with growth medium to remove non-adherent cells. 100 μL of culture medium with different concentrations of lead acetate was added into each well of the 96-well plate, followed by further incubation at 37°C with 5% CO_2 for 48 hours. After the incubation, 20 μL 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich China Inc.) solution was added into each well and the cells were further incubated for 4 hours. Then, the culture media with MTT solution were discarded and replaced with 200 μL dimethyl sulfoxide (DMSO, 1.1g/mL; Sigma-Aldrich China Inc.), followed by incubation at room temperature with shaking for 5 minutes. The absorbance of each well was measured at 570nm by enzyme-linked immunosorbent assay (ELISA) reader (Biotmed LLC, Northborogh, MA, USA).

Cell proliferation assay

Dissociated passage 2 cells were added onto the poly-L-lysine-coated coverslips in 24-well plate at a density of 2.5×10^4cells/ cm^2 per well in 500 μL culture medium without lead acetate and incubated at 37°C with 5% CO_2 for 24 hours. The coverslips were washed with growth medium to remove non-adherent cells. The wells were filled with the culture media with different concentrations of lead acetate (0, 1, 10, 50, 100 and 200 μM) and the cells were further incubated at 37°C with 5% CO_2 for 48 hours. Then, bromodeoxyuridine solution was added into each well to a final concentration of 10 μM, followed by incubation at 37°C with 5% CO_2 for additional 24 hours to allow the incorporation of bromodeoxyuridine into the DNA of the cells.

After that, the cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature and permeabilized by 0.3% triton X-100 solution in PBS for 5 minutes. The DNA of the cells was denatured by 2 M hydrochloric acid at room temperature for 1 hour, followed by neutralization by 0.1 M borate buffer. The cells were then incubated with 5% bovine serum albumin/PBS for 30 minutes to prevent non-specific binding of antibodies and then rinsed by PBS three times. Afterwards, the coverslips were incubated with mouse anti-bromodeoxyuridine (1:200; Life Technologies™ Inc., Hong Kong, China) in 1% bovine serum albumin/PBS for 24 hours at 4°C, rinsed three times with PBS/Tween20, and stained with goat anti-mouse IgG secondary antibody (Alexa Fluor®647; Life Technologies™ Inc.) at 1:500 dilution in 1% bovine serum albumin/PBS for 2 hours at room temperature. The cells stained by the secondary antibody were washed with PBS three times, counterstained with 4',6-diamidino-2-phenylindole (Life Technologies™ Inc.) (1:1 000 dilution in 1% bovine serum albumin/PBS) for 20 minutes and then washed once with PBS. Finally, the coverslips were mounted onto slides with mounting medium and allowed to dry overnight. The stained cells were visualized under a fluorescent microscope (100 ×). Ten random fields of view were taken for each coverslip, and the number of nuclei and bromodeoxyuridine-incorporated cells were recorded.

Immunochemistry for differentiated neural cells from neural stem cells

Different concentrations (0, 1, 10, 50, 100 and 200 μM) of lead acetate were prepared in differentiation medium of neural stem cells (neuronal differentiation: neurobasal® medium, B27supplement, 1% fetal bovine serum and GlutaMAX™-I; astrocyte differentiation: neurobasal® medium, N2 supplement, 1% fetal bovine serum and GlutaMAX™-I; oligodendrocyte differentiation: neurobasal® medium, B27supplement, and GlutaMAX™-I; Life Technologies™ Inc.). Single cell suspensions were first seeded onto the poly-L-lysine-coated coverslips in 24-well plate at a density of 2.5×10^4cells/cm^2 per well in 500 μL differentiation medium free of Pb^{2+}, and incubated at 37°C with 5% CO_2 for 24 hours to allow the cells to attach to the coverslips. After 24 hours, coverslips were rinsed with neurobasal® medium to get rid of non-adherent cells, and the wells were re-filled with lead acetate-added differentiation medium for subsequent 6-day culture. The medium was renewed every 3 days.

After 6-day culture, 4% paraformaldehyde was added to fix the cells for 15 minutes at room temperature, followed by permeabilization of the cells by 0.3% Triton X-100 in PBS for 5 minutes. The blocking agent 5% bovine serum albumin/PBS was added to block the cells thereafter. Next, the cells were incubated with primary antibodies [rabbit anti-microtubule-associated protein 2 (Life Technologies™ Inc.) (1:200 dilution in 1% bovine serum albumin/PBS); rabbit anti-glial fibrillary acidic protein (Life Technologies™ Inc.) (1:200 dilution in 1% bovine serum albumin/PBS); mouse anti-RIP (Life Technologies™ Inc.) (1:50 dilution in 1% bovine serum albumin/PBS)] at 4°C for 24 hours.

At the end of the incubation, the cells were rinsed three times with PBS and incubated with secondary antibodies [goat anti-rabbit Alexa Fluor®488 for rabbit anti-microtubule-associated protein 2 (1:500 dilution in 1% bovine serum albumin/PBS), goat anti-rabbit Alexa Fluor®647 for rabbit anti-glial fibrillary acidic protein (1:500 dilution in 1% bovine serum albumin/PBS) and goat anti-mouse Alexa Fluor®488 for mouse anti-RIP (1:500 dilution in 1% bovine serum albumin/PBS); Life Technologies™ Inc.) at room temperature for 2 hours. Following three rinses in PBS after the incubation, the cells were counterstained with 4',6-diamidino-2-phenylindole at 1:1 000 dilution in 1% bovine serum albumin/PBS for 20 minutes at room temperature. Finally, the coverslips were washed with PBS once, mounted onto the slides with mounting medium, and allowed to air-dry overnight. The stained nuclei and differentiated cells were counted in 10 random fields of view per group under a fluorescent microscope (100 ×).

Statistical analysis

Five independent samples for each concentration of lead acetate were analyzed. Data were collected in a group identity-blinded way and differences ($P < 0.05$ and $P < 0.01$) were set for intergroup comparisons. The data were expressed as mean ± SEM and analyzed by SPSS 17.0 software (SPSS, Chicago, IL, USA). One-way analysis of variance was used to analyze differences among multiple groups and $P < 0.05$ and $P < 0.01$ were regarded as statistically significant differences.

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Ethical approval: The use of all experimental animals in this study was carried out in accordance with the regulations of the Committee on the Use of Live Animals for Teaching and Research of the University of Hong Kong, China.

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