Toxicology Research

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/toxicology

Protective effects of lithium against lead-induced toxicities in multiple systems of adult mouse

Jiutao Wang, Lingzhen Song, Kaikai Li, Runchuan Yan, Xinde Hu, Wei Zhang, Yupeng Yin*, Shanting Zhao*

College of Veterinary Medicine, Northwest A&F University, Yangling, shaanxi, 712100, People's Republic of China.

Abstract

Occupational and environmental exposures to lead (Pb), one of the toxic metal pollutants, is of global concern. The present study aims to investigate the protective effects of lithium (Li) against Pb-induced damages *in vivo* and *in vitro*. For this purpose, 3-month-old mice received Li (250 mg/kg body weight, i.p.) and 2 hours later water containing Pb (20 mg/kg body weight, i.p.) for 2-weeks. Treatment of mice with Pb induced remarkable morphological damages in multiple organs, such as swelling and necrosis in liver, kidney and spleen. Immunohistochemistry demonstrated that the number of newly generated cells and immature neurons in hippocampus was significantly decreased in mice exposed to Pb when compared with those that received saline for control or Li. Furthermore, in mice exposed to Pb a higher percentage of newly generated cells differentiated to glial cells and fewer to neurons, and less newborn cells survived compared to those in controls and Li-treated mice. In mice exposed to Pb cognitive tests were impaired. Interestingly, pre-administration of Li markedly decreased Pb-induced pathological and neurological lesions in vivo and in vitro. Specifically, the reduction of hippocampal neurogenesis resulting from Pb exposure was prevented by administration of Li. In addition, we found that pretreatment with Li effectively prevented cognitive impairment in mice exposed to Pb. Furthermore, Li pretreatment significantly improved Pb-induced depletion in p-GSK-3β (Ser9) and microRNA-34c levels in hippocampus. Collectively our findings point to a capacity of Li to attenuate Pb-induced damage.

Keywords: Lead·Lithium·Pathology·Hippocampal neurogenesis·Cognition

Introduction

Environmental exposure to Pb in the general population is a major public health issue [1]. Pb is widely distributed in the environment, and the consequences of chronic Pb exposure in childhood [2] and in juveniles [3] have been the subject of extensive research during the past few decades. Acute exposure to Pb results in gastrointestinal disturbances (anorexia, nausea, vomiting, and abdominal pain), neurological diseases (encephalopathy, malaise, and drowsiness), hepatic damage, and hypertension [4]. With increasing environmental pollution, the chance of Pb exposure to the elderly is rising [5]. Recent advances have improved our understanding of how the toxicity of Pb affects the central nervous system (CNS) [6]. Investigations of the neurotoxicity of this metal have shown that the actions of Pb on glutamate release [7], NMDA receptor function [8, 9] or structural plasticity [7] may underlie the mechanism of Pb-induced perturbations in synaptic plasticity and learning impairments.

*Corresponding author. shantingzhao@hotmail.com and coolyyp@163.com

Several lines of evidence indicate that Pb induces a broad range of physiological, biochemical, and behavioral dysfunctions in experimental Pb-poisoned rats as well as in humans [10]. Blood Pb levels as low as 10 μg/dl inflict serious damage to the CNS of children, leading to developmental delays, lower intelligence quotient (IQ), hyperactivity, learning disabilities, behavioral problems, and school failure [11]. Additional evidence suggests that the proliferation of neural stem cell (NSC) in the adult hippocampus is modulated by Pb exposure [12, 13]. Li is one of the primary drugs used for treating bipolar mood disorder, although its therapeutic mechanism remains unclear. It has been reported that Li induces a significant increase of neural progenitor cells in the dentate gyrus (DG) and enhances synaptic plasticity [14-16]. Downstream activation of the β-catenin pathway using a pharmacological inhibitor of GSK-3β ameliorates the Pb inhibition of Wnt signaling activity in mice [17]. Some distinct molecular and cellular actions of Li have been identified, including its ability to inhibit glycogen synthase kinase-3 (GSK-3β) [18]. Previous studies showed that both Pb and Li act on adult neurogenesis and memory, but have opposite effects. However, no experiment with co-exposure to Li and Pb *in vivo* or *in vitro* has been reported.

Therefore, the present study was designed to examine whether Li is able to antagonize the toxicities induced by Pb *in vivo* and *in vitro*. Li was administered with or without Pb by two separate doses. The protective effects of Li on Pb-induced toxicities and impairment were investigated in liver, spleen, kidney, and brain (adult neurogenesis, learning and memory).

Materials and methods

Chemicals and antibodies

Lead acetate (CAS: 546-67-8, purity > 99.8%), lithium chloride (CAS: 7447-41-8, purity > 99.8%), 5'-Bromo-2'-deoxyuridine (BrdU, CAS: 59-14-3, purity > 99.8%), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, CAS:298-93-1) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sources of the antibodies are as follows: GSK-3β (#9315) and p-GSK-3β (Ser9, #5558) were from Cell Signaling Technology; BrdU (#sc-56258), DCX (#sc-271390), GAPDH (#sc-25778), secondary antibody donkey anti-rabbit IgG-HRP (#sc-2313), goat anti-Rat IgG-CFL 488 (#sc-362263), goat anti-rabbit IgG-CFL 647 (#sc-362292), goat anti-mouse IgG-PerCP-cy3 (#sc-45101), were from Santa Cruz Biotechnology (Oregon, USA), NeuN (#MAB377) was from Chemicon (USA), goat anti-rat-biotinyl, donkey anti-goat-biotinyl, diaminobenzidine (DAB) solution were from Boster (Wuhan, China).

Preparation of primary cultures of mouse hippocampus

Primary cell cultures of hippocampus were prepared from embryonic brains of Kunming mice. On gestation day 20, 3 mice were sacrificed. Their uteri were dissected and the hippocampi of embryos (n=6) were isolated and dissociated. Dissociated cells were resuspended and plated on 48-well multiplates. Cultures were grown at 37 °C in an atmosphere of 5% CO_2, on the 6th day in vitro (DIV 6), culture medium was changed to a serum-free medium. To investigate the effect of Li on cell viability in culture, lithium (5,10 and 20 μM) was added into the medium on DIV 10 and kept for 48 h. Then the cultures were exposed to Pb (10 μM) for 12 h.

Cell viability

Effect of Li on Pb-induced cytotoxicity was studied by MTT assay, a colorimetric assay for assessing cell metabolic activity. NAD(P)H-dependent cellular oxidoreductase enzymes may reflect the number of viable cells under defined conditions. The cultures were incubated with 300 μl of MTT solution (2.5 mg in 5 ml D-PBS) for 4 h. After removing the MTT solution, 400 μl of lysis buffer (99.4 ml of DMSO in 10 g of SDS and 600 μl of HCl) were used to solve the

formazan, before ELISA measurements at 570 were performed.

Animals and experimental design

Sixty 3-month-old male Kunming mice, weighing 25 ± 2 g, were purchased from the Laboratory Animals Center of Xi'an Jiaotong University (Xi'an, China). Mice were maintained under temperature- and light-controlled conditions (22-25 °C, 12 h light/dark cycle) and received commercial chow diet and water ad libitum. The mice were randomly divided into four groups (1-4) containing 15 animals each that were acclimatized for 1 week prior to drug administration. Group 1, 2 and 3 were intraperitoneally (i.p.) injected with a single dose of 0.9% saline (control), Pb (20 mg/kg, 10 μl/g body weight) and Li (25 mg/kg, 10 μl/g body weight) once per day respectively, for 2 weeks. Group 4 (Pb+Li) was i.p. injected with Li (250 mg/kg, 10 μl/g body weight) 2 h prior to Pb (20 mg/kg, 10 μl/g body weight) injection, for 2 weeks. Food intakes and daily body weights were recorded throughout the experimental period of 2 weeks. For evaluation of the adult neurogenesis, 10 mice from each group were given six i.p. injections of BrdU (50 mg/kg bodyweight, twice daily for 3 days) at two time points. Five mice from each group were injected with BrdU on days 12-14 of treatment to examine potential effects of on the proliferation of neural stem cells. Another 5 mice were injected with BrdU on three consecutive days prior to chemical administration to examine the effect of chemical administration on fate decision and survival of newly generated cells. Mice were euthanized one day after the last chemical administration (day 15) or after the memory test (day 20). The last 5 mice were used for western blot. The institutional animal care committee of Northwest A&F University approved the experimental protocol.

Tissue preparation

For the histological study, the mice were deeply anesthetized with an overdose of sodium pentobarbitone (100 mg/g) and then perfused intracardially with saline followed by 4% paraformaldehyde (PFA) for 15 minutes. After fixative perfusion, the brains were removed from the cranium, placed in 4% PFA at 4 °C overnight and then washed with 0.1 M phosphate buffer (PB). The brains were cut serially at 50 μm in the coronal plane using a vibratome (VT 1000S, Leica, Germany). The sections were collected in PB. Only the sections with intact hippocampus (V-shaped dentate gyrus and C-shaped hippocampus proper) were used for immunohistochemistry.

Hematoxylin and Eosin (H&E) staining for pathological examination

After the mice were perfused, the lateral lobe of the liver, entire spleen and kidney were cut, and post-fixed in neutral buffered formalin (10%) for one day. The samples (n=5) were then dehydrated with grades of ethanol (70%, 80%, 90%, 95% and 100%), followed by clearing the samples in two changes of dimethylbenzene. After that the samples were impregnated with two changes of molten paraffin wax, they were embedded and blocked. Sections (5 μm) were stained for hematoxylin and eosin. Stained sections of control and treated mice were examined for alterations in the architecture, portal triads, hepatocytes, sinusoids and for the presence of necrosis, hemosiderosis, pigmentation, fat changes, portal fibrosis and cirrhosis.

Quantification of newly generated cells in dentate gyrus

For this purpose 6 hippocampal coronal sections with typical V-shaped dentate gyrus from each animal injected with BrdU on day 12-14 of chemical administration were used. This procedure was performed as described in a previous study [19]. Briefly, the sections were treated with 0.6% H_2O_2 in PB to block endogenous peroxidase. DNA was denatured by exposing the

sections in 2 N HCl at 37 °C for 30 min. After rinsing in 0.1 M borate buffer (pH=8.5) at RT (room temperature) for 1h, the sections were incubated with rat anti-BrdU antibody diluted 1:1000 in blocking solution (4% BSA, 1% NGS, 0.3% Triton X-100, in phosphate buffer) at 4 °C overnight. After rinsing 3 times in PB, the sections were incubated in biotinylated secondary antibody goat anti-rat IgG diluted (1:300) in 0.1M PB at RT for 3 h, followed by incubation with ABC solution at RT for 1h. Then the sections were incubated in diaminobenzidine (DAB) solution for 2 min. After intensive rinsing, the sections were then mounted on polylysine-coated slides. After dehydration, the sections were mounted in Permount (Fisher # SP15-100). BrdU-labeled cells were counted in 6 sections per brain (every sixth section). All analyses were performed with the experimenter blind to each brain section. The counts were conducted at a magnification of 20x with a Zeiss microscope. The density of BrdU-positive cells ($BrdU^+$) was calculated by dividing the number of $BrdU^+$ cells by SGZ/GCL sectional area (DGZ: subgranular zone; GCL:granule cell layer).

Quantification of newborn neurons

For this purpose 6 hippocampal coronal sections with a typical V-shaped dentate gyrus from each animal injected with BrdU prior to chemical administration were used. To evaluate the phenotype of the newborn cells, immunohistochemistry with an antibody against doublecortin (DCX) was performed. This procedure was performed as described in a previous study [13]. Sections (6 per mouse spaced at 50 μm) were treated with 0.6% H_2O_2 in PB to block endogenous peroxidase and then rinsed in 0.1 M borate buffer. The sections were incubated with the goat anti-DCX antibody diluted 1:1000 in blocking solution (4% BSA, 1% NGS, 0.3% Triton X-100, in phosphate buffer) at 4 °C overnight. After rinsing 3 times in PB, the sections were incubated in biotinylated secondary antibody donkey anti-goat IgG diluted (1:300) in 0.1M PB at RT for 3 h, followed by incubation with ABC solution at RT for 1h. After incubation in diaminobenzidine (DAB) solution for 2 min Nissl staining (Thionine) was performed. After intensive rinsing, the sections were mounted on polylysine-coated slides, and finally embedded in Permount (Fisher # SP15-100) after dehydration. Quantification of newborn immature neurons was performed as the number of DCX^+ cells expressing DCX in SGZ/GCL.

Cell survival and differentiation

For this purpose 6 hippocampal coronal sections with typical V-shaped dentate gyrus from each animal injected with BrdU prior to treatment were used. To evaluate the fate decision (differentiation) of newborn cells, triple-immunofluorescent staining for BrdU, NeuN (marker of mature neurons) and GFAP (marker of astrocytes) was performed as described in our previous study [20]. Sections (6 per mouse spaced at 50 mm) were incubated with mixed primary antibodies (rat anti-BrdU, mouse anti-NeuN and rabbit anti-GFAP diluted 1:1000 in blocking solution) overnight at 4 °C. After rinsing (3×10 min) with PB at RT the sections were incubated in mixed secondary antibodies (goat anti-rat-488, goat anti-mouse-568 and goat anti-rabbit-647 diluted 1:300 in PB) for 3 h in the dark. After intensive washes in PB, sections were mounted with DAKO mounting medium and photographed with an epifluorescent microscope (Axio Observer, Zeiss, Germany). Survival and differentiation of $BrdU^+$ cells expressing NeuN or GFAP were calculated in these five different groups. In every study the analysis of negative controls (omission of primary antibody) was simultaneously performed in order to exclude the presence of non-specific immunofluorescent staining and cross-immunostaining..

Morris Water Maze (MWM) Behavior Test

The MWM procedure was performed as described [21]. Briefly, this spatial reference memory test is commonly used to examine impairments of spatial learning and memory, and requires mice to find a hidden platform (10 cm in diameter) just below the surface (0.5 cm) of a circular pool of opaque water (120 cm in diameter×35 cm high, maintained at 23-25 ℃). Accurate navigation to the platform is rewarded by allowing the animal to escape from the water. The maze was placed in a room with dimmed lights, and visible cues were placed on walls adjacent to the pool to aid the mice in determining the location of the platform. Mice were given 4 consecutive days of training after vehicle or compound treatment for 14 days, with 4 trials per day, with the four starting locations varied between trials. At the beginning of each trial, mice were immersed in the water, facing the wall, at one of three randomly assigned start positions (located in the center of each quadrant not containing the platform). If the platform was not located within the maximum time of 60 s, the mouse was guided to the location. Once a mouse reached the platform, it was allowed to remain there for 15 s. Extra-maze visual cues around the room remained in fixed positions throughout the experiment. For each trial, latency to find the platform (maximum 60 s) was recorded by a video tracking/computer-digitizing system (Imaris). On day 5, the platform was removed from the pool and each mouse received one 30 s swim probe trial. The starting point was set in the south-east quadrant. Percentage of time spent in the target sector was recorded.

Real-time quantitative RT-PCR

Total RNA including miRNAs was extracted from hippcumpas using TRIzol (Invitrogen, USA). cDNA synthesis for miRNAs was performed with 500 ng of total RNA according to the manufacture's instructions (Thermo, USA), and 5 S rRNA was used as an internal control. Primer sequences for RT-PCR of target miRNA and 5 S rRNA are as follows: mircroRNA-34c-5p: RT-5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCAATC-3'; Forward-5'-GCAGCCAGGCAGTGTAGTTAGC-3'; Reverse-5'-GTGCAGGGTCCGAGGT-3'; rRNA-5S:RT-5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAGC G-3';Forward-5'-CTGGTTAGTACTTGGATGGGAGAC-3';Reverse-5'-GTGCAGGGTCCGAG GT-3' (RT is reverse transcription using hairpin primer.) All primers were synthesized by the Sangon technical services, Shanghai Co., Ltd. According to SYBR Premix Ex TaqTM Kit manufacturer (Takara, Japan), all reactions were performed on CFX 96 System (Bio-Rad, USA) with 20 μl reaction system. We used a two-step method: 95 ℃ initial denaturation for 3 min followed by 39 cycles at 95 ℃ for 15s and 62.5 ℃ for 30s, run in triplicate. For melting curve analysis, the temperature was increased by 0.5 ℃ from 65 ℃ to 95 ℃. To ensure accuracy, this progress should avoid contamination and strong light. The expression of miR-34c was determined according to the method of 2-ΔΔCT.

. Western blot analysis

The hippocampi from animals without BrdU-injection was homogenized with a manual pestle in ice-cold RIPA lysis buffer containing 50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% Sodium Deoxycholate, 0.1% SDS, Sodium Orthovanadate, Sodium Fluoride, EDTA and 1mM PMSF. The homogenized tissue was centrifuged at 13000 rpm at 4 ℃ by Centrifuge 5804 R (Eppendorf, Hamburg, Germany) and the supernatant was collected. 5 to 10 μg tissue extracts were applied to 15% sodium dodecyl sulsate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, MA, USA). The blots were blocked with 5% skimmed milk powder for 2 h at RT and then probed with

primary antibodies, rabbit anti-phospho-GSK-3β (Ser9), rabbit anti-GSK-3β and mouse anti-GAPDH, respectively. After three washes, the blots were subsequently incubated with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:2000) or goat anti-mouse IgG (1:2000) for 3 h at RT. Immunoreactive proteins were visualized via chemiluminescent detection and scanned with a chemiluminescent imaging system (Beijing sage creation company, Beijing, China).

Statistical analysis

All data are presented as mean ± SEM. Student's two-tailed t test was used for statistical comparisons between two groups, and one-way ANOVA followed by post hoc Newman-Keuls test was used for comparisons between more than two groups by using GraphPad Prism 5.0 software.

Results

Li-pretreatment significantly prevented Pb-induced reduction of body weight and increase of liver, spleen and kidney weight

The changes in body weight and spleen, liver and kidney weight are shown in Table 1. The body weight gain of Pb-exposed mice was lower compared with that of control mice, while the weight of liver (1.43±0.08), spleen (0.19±0.04) and kidney (0.53±0.05) were increased because the Pb accumulated and induced hyperemia and swelling of these organs. The coefficients of liver (5.01±0.21), spleen (0.63±0.04) and kidney (1.70±0.04) in Pb-exposed mice remarkably increased compared with those of control mice. Interestingly, Li pretreatment significantly offset Pb-induced reduction of body weight or increase of liver, spleen and kidney weight. Collectively the results strongly suggested the protective effect of Li on Pb-induced damages in rodent models.

Table 1: Effects of Pb with or without Li injection on body weight and spleen, liver and kidney weight.

Groups	*n*	IBW(g)	FBW(g)	LW(g)	LC(%)	SW(g)	SC(%)	KW	KC（%）
Control	10	25.28±2.26	34.29±2.43	1.35±0.06	4.19±0.27	0.13±0.02	0.39±0.01	0.45±0.04	1.33±0.03
Pb	10	25.43±1.72	29.06±3.08*	1.43±0.08	5.01±0.21*	0.19±0.04	0.63±0.04*	0.53±0.05	1.70±0.04*
Pb+ Li	10	25.68±2.23	33.75±2.91$^{\#}$	1.39±0.09	4.37±0.19$^{\#}$	0.15±0.03	0.46±0.02$^{\#}$	0.47±0.02	1.41±0.04$^{\#}$
Li	10	24.99±2.14	34.68±2.57$^{\#}$	1.35±0.05	4.21±0.15$^{\#}$	0.14±0.02	0.41±0.02$^{\#}$	0.45±0.02	1.32±0.02$^{\#}$

IBW: initial body weight; FBW: final body weight; LW: liver weight; LC: liver coefficient; SW: spleen weight; SC: spleen coefficient; KW: kidney weight; KC: kidney coefficient. Data are expressed as mean ± SEM. *, $p<0.05$, vs. control mice; #, $p<0.05$, vs. Pb mice.

Li-pretreatment protected the spleen, liver and kidney from Pb-induced toxicities

The spleen, liver and kidney in mice were examined grossly and microscopically with H&E staining (Fig.1). Obvious morphological changes like swelling and necrosis were observed in spleen, liver and kidney of Pb exposed mice. As for the liver, the margin became pale??? compared with that of control mice due to the serious swelling. Histopathological alterations were also observed in sections of spleen exposed to Pb, such as hemosiderosis and necrosis, decreased lymphocytes in white pulp, fibrosis and abundant connective tissue in the red pulp. The architecture of the spleen in co-treated mice did not show obvious changes compared with that of control mice. Histopathological alterations were found in the Pb-exposed liver, such as nuclear vesiculation, cytoplasmic inclusions and swelling. However the architecture of the liver in Pb-exposed mice pretreated with Li did not show obvious alterations compared with that in control mice. As for the kidney in Pb-exposed mice, the tubes were all filled with secretion (asterisks) and the necrotic renal epithelial cells were found in the tubes (right fourth panel). The

structure of the renal epithelial cells looked normal except some swelling in mice treated with both P band Li. (right fifth panel). The results indicated that excessive Pb exposure induced pathological changes of the liver, spleen and kidney. However, Li injection could ameliorate the lesions in these organs.

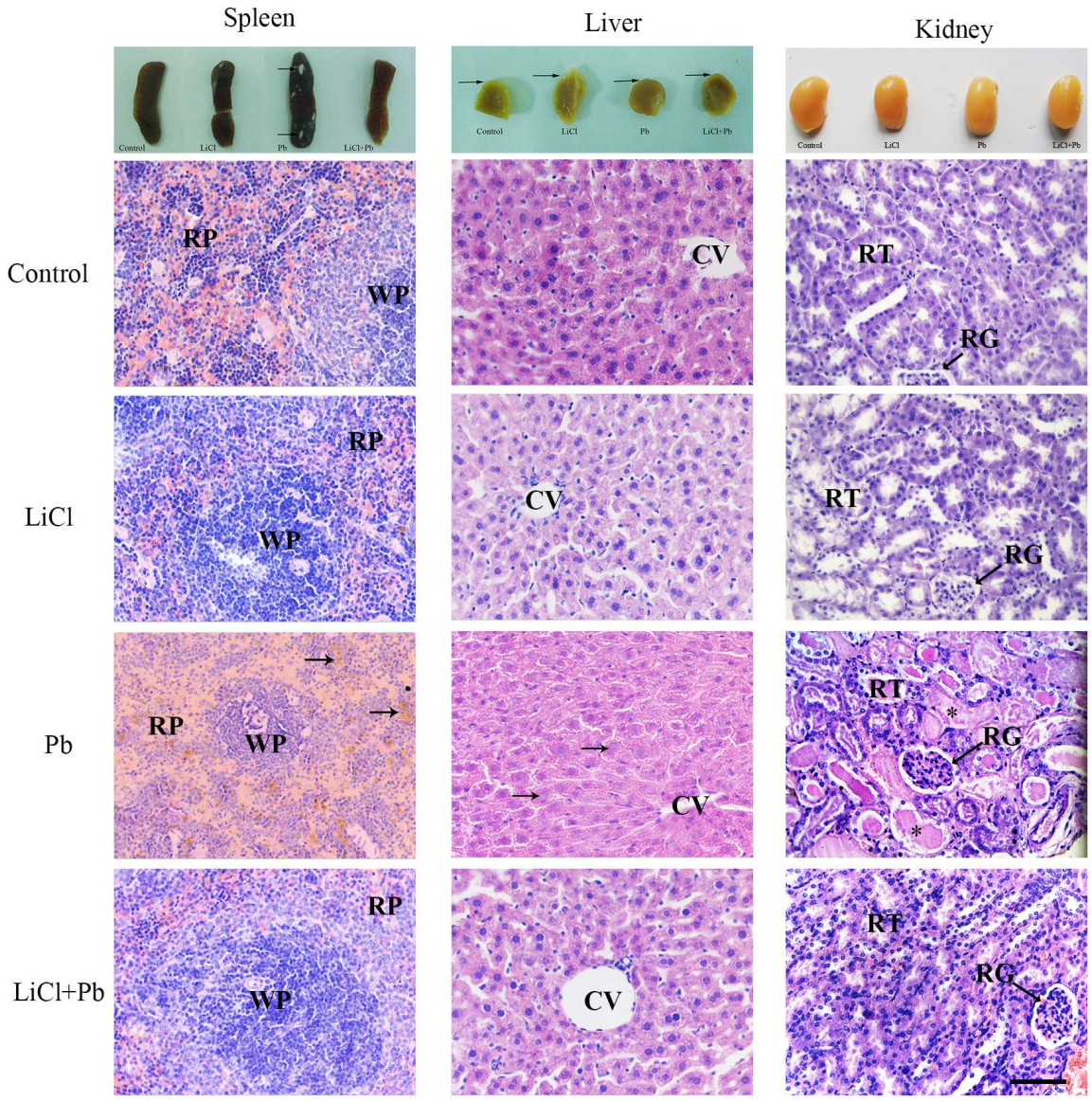


Fig.1. Pb-exposure induced pathological changes of spleen, liver and kidney. Top three panels show photographs of spleen, liver and kidney. Other panels show representative micrographs of H&E staining of spleen, liver and kidney in the different groups (n=5), scale bar =100 μm. The spleen of Pb-exposed mice was remarkably enlarged due to swelling and necrosis (arrows)

compared with that of the other groups (top left panel). The margin of the liver in Pb-exposed mice became blunt (arrows) due to serious swelling compared with that of the other groups (top middle panel). Weight and size of the kidney were larger in Pb-exposed mice (top right panel). No obvious changes were found in spleen, liver and kidney of Li-exposed mice and Pb-Li co-treated mice compared with those of control mice. Serious histopathological alterations were found in the spleen of Pb-exposed mice (left fourth panel), such as hemosiderosis (arrows) and necrosis, decreased lymphocytes in the white pulp (WP), fibrosis and abundant connective tissue in the red pulp (RP) (left fourth panel). No obvious histopathological changes were found in the spleen of the other groups. Serious histopathological alterations were also observed in the liver of Pb-exposed mice (middle fourth panel), such as nuclear vesiculation (arrows), cytoplasmic inclusions and swelling. No obvious histopathological change was found in livers of the other groups. As for the kidney in Pb-exposed mice, the tubes were all filled with secretion (asterisks), necrotic renal epithelial cells were found in the tubes (right fourth panel). In contrast, the structure of renal epithelial cells looked normal except some swelling in co-treatment mice (right fifth panel). RP: red pulp; WP: white pulp; CV: central vein; RT: renal tube; RG: renal glomerulus.

Co-treatment with Li restored Pb-induced Cell death

In order to test the protective effects of Li against Pb induced toxicities in vitro, primary hippocampal cell cultures were prepared. After DIV 12, normal cell bodies and dendrites could be found in control cells (Fig. 2A). However, most of the cells were dead and destroyed after Pb was administrated (Fig. 2B). Interestingly, the cells recovered and were protected to some extent, respectively, when they were pretreated with lithium (Fig. 2C-E). In order to investigate cell viability, MTT assay was used in this study. The results showed that 73.35% of the cells died of Pb compared with the cells in the control group. In accordance with the pictures shown, most cells pretreated with Li survived. Compared with the Pb group, cell viability remarkably increased to 101.27%, 197.28% and 192.28% in three co-treated groups respectively (Fig. 2F). Li itself did not change cell viability. Our findings indicated that the toxicity induced by Pb was significantly reduced when the cells were pretreated with Li.

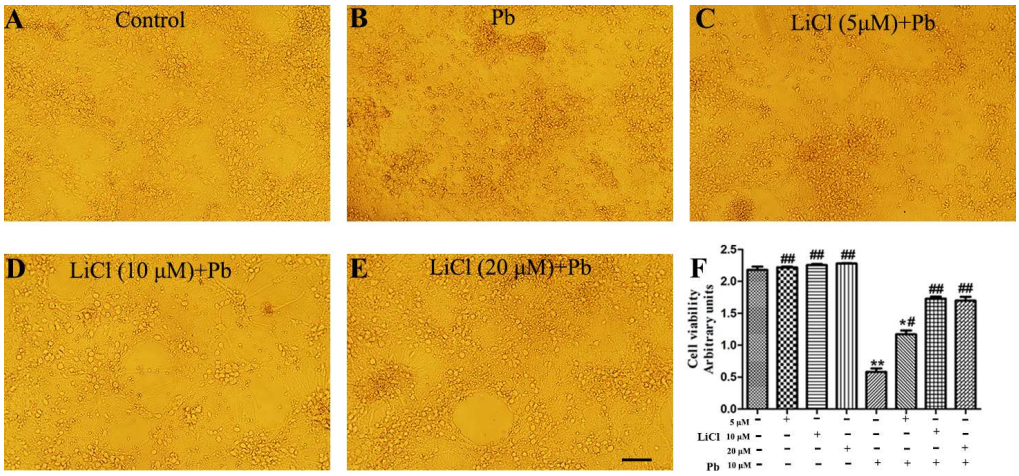


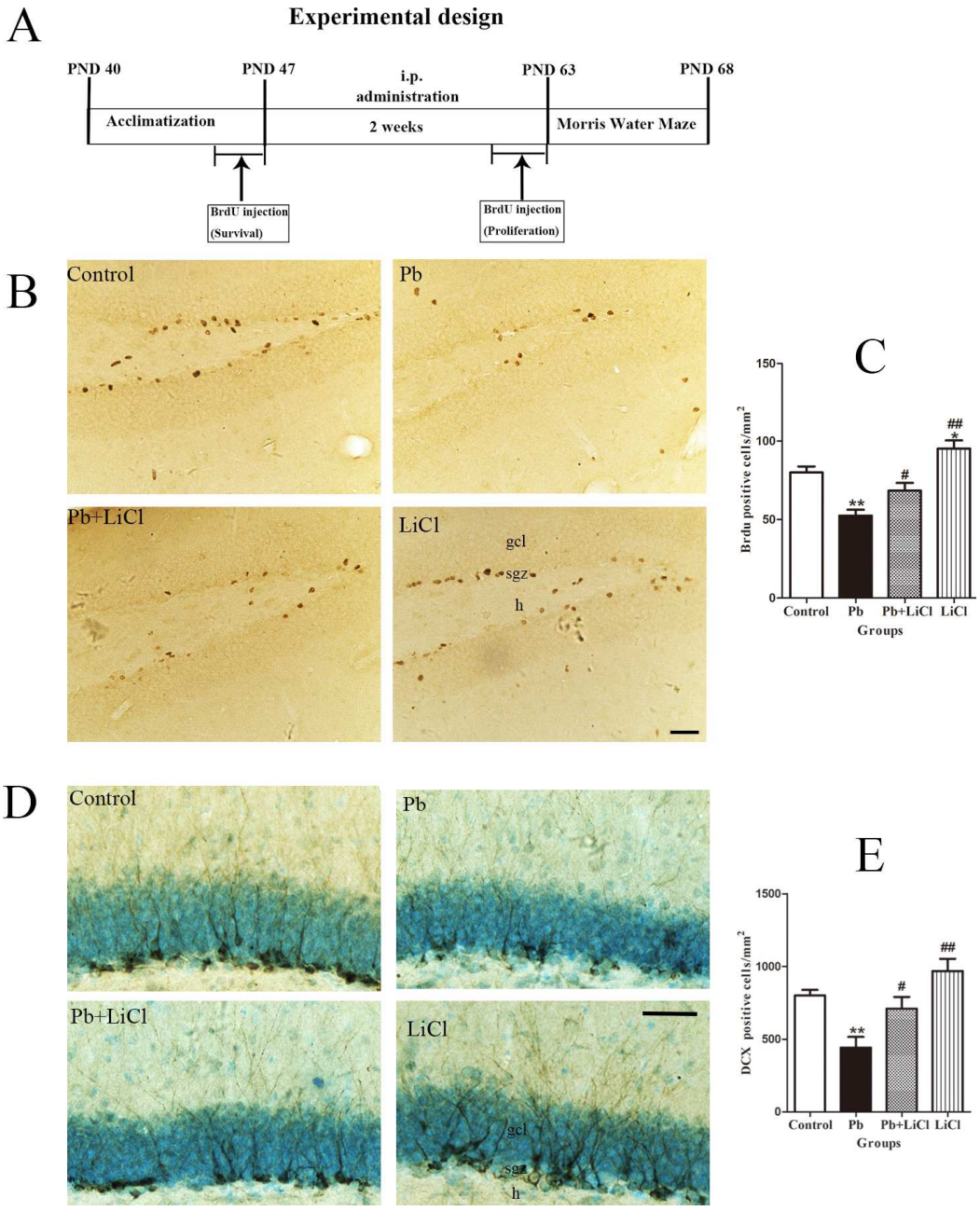
Fig.2. Effects of Li on Pb toxicity, Total cell survival in primary cell cultures. (A-E) Representative photographs of primary cell cultures after Li with or without Pb treatment. Scale bar = 50 μm. Regular cell bodies and dendrites could be detected in control cells. Most of the cells were dead and destroyed after Pb was administrated, but the cells could be rescued and/or

protected to some extent when pretreated with Li. (F) Analysis of cell viability in the different groups (n=3). Li itself did not change cell viability but significantly promoted cell viability when co-treated with Pb. *, $p<0.05$, **, $p<0.01$ vs. control group; #, $p<0.05$, ##, $p<0.01$, vs. Pb group.

Pretreatment with Li restored reduced proliferation of progenitor cells and the generation of neurons in the mouse hippocampus exposed to Pb

In order to investigate the effects on adult neurogenesis, we next examined the proliferation of neural progenitors in the hippocampus of the animals. Mice were injected with BrdU during the last 3 days of treatment and then sacrificed 24 h after the final BrdU injection. Newly generated BrdU-positve ($BrdU^+$) cells were found in the dentate gyrus (DG) of all groups (Fig.3A). The majority of $BrdU^+$ cells were located in the subgranular zone (SGZ) of DG (Fig. 3B). After Pb exposure, the number of $BrdU^+$ cells was significantly decreased in the DG compared to that in controls. However, Li pretreatment significantly attenuated the inhibitory effect of Pb on proliferation of neural progenitors. Administration of Li promoted the proliferation of progenitors and increased the number of $BrdU^+$ cells compared to that of controls (Fig. 3C). We next investigated the morphology of newly generated neurons using immunohistochemistry using an antibody against doublecortin (DCX), a microtubule-binding protein that is expressed only in neuroblasts in an early stage of differentiation. The somata of DCX-positive (DCX^+) cells were located in the SGZ, while their dendrites extended through the granular layer to the molecular layer (Fig.3D). Pb exposure significantly decreased the number of DCX^+ cells in DG. However, Li pretreatment attenuated the Pb-induced decrease in DCX^+ cells. The number of DCX^+ cells in the Li group was similar to that of control mice (Fig. 3E). In sections counterstained with thionine (Nissl staining) no obvious pathological signs were detected. No significant differences were found in neuronal density and the morphology of DCX^+ cells in the hippocampus of all groups (Fig. 3D). Our results indicate that Pb-exposure impaired hippocampal neurogenesis, which could be rescued by pretreatment with Li.

Fig.3. Pb-induced impairment of adult neurogenesis in the hippocampus was rescued by Li pretreatment. (A) Timeline of experimental design. (B) Representative images showing $BrdU^+$ cells (brown dots) in each group. Scale bar =100 μm. (C) Quantitative analysis of the number of $BrdU^+$ cells in the DG. Compared with control mice, Pb-exporure significantly decreased the number of $BrdU^+$ cells. Li remarkably attenuated the Pb-induced decrease in the number of $BrdU^+$ cells in the DG. (D) Representative images showing DCX^+ cells counterstained with thionine. The somata of DCX^+ cells (black) were located in SGZ. They gave rise to dendrites (brown) that extended through the granule cell layer to the molecular layer, scale bar =50 μm. (E) Quantitative analysis of the number of DCX^+ cells in the DG. Compared with the control group, Pb-exposure significantly decreased the number of DCX^+ cells in the DG, while Li-treatment led to an increase in DCX^+ cells. Moreover, Li-pretreatment significantly attenuated Pb-induced decrease in the number of DCX^+ cells in the DG. Data are presented as mean ± SEM. gcl-granular cell layer, sgz-subgranular zone, h-hilus.*, $p<0.05$, **, $p<0.01$ vs. control mice; #, $p<0.05$, ##, $p<0.01$, vs. Pb mice.



Li prevented the toxicity of Pb on the survival and differentiation of newly generated cells

To further analyze the effect of Pb and Li on neurogenesis, the survival and differentiation of the newly generated cells in SGZ were examined 2 weeks after the last BrdU administration prior to Pb and/or Li administration. To identify the cell types of the survived cells, triple immunohistochemical analysis of BrdU with NeuN and GFAP was carried out (Fig. 4A). The number of $BrdU^+$ cells was reduced in all groups compared to that of the respective groups at 24 h after BrdU injection, indicating that many newly generated cells in SGZ did not survive to

maturity. Specifically, the survival rate of $BrdU^+$ cells was much lower in Pb-administrated mice (28.26%) than that in Li-treated mice (61.09%) and in control mice (52.95%). Interestingly, the survival rate of $BrdU^+$ cells was also enhanced in mice that received both Pb and Li (44.23%) compared to that in Pb-treated mice (Fig. 4B). This result suggested that Li treatment significantly restored the survival of progenitors in the Pb-administered mice. Two weeks after BrdU injection, the majority of $BrdU^+$ cells differentiated into neurons in all groups. Quantitative analysis of the NeuN-immunoreactive cells within the SGZ-GCL demonstrated that about 90% of the $BrdU^+$ newborn cells were NeuN-positive, indicating mature neurons. No significant difference was detected among the five groups. However, the percentage of $BrdU^+/GFAP^+$ cells in Pb mice was increased compared with that in control mice, indicating that more newborn cells differentiated to glial cells when administering Pb (Fig. 4C). The results indicated an protective effect of Li on cell survival and differentiation.

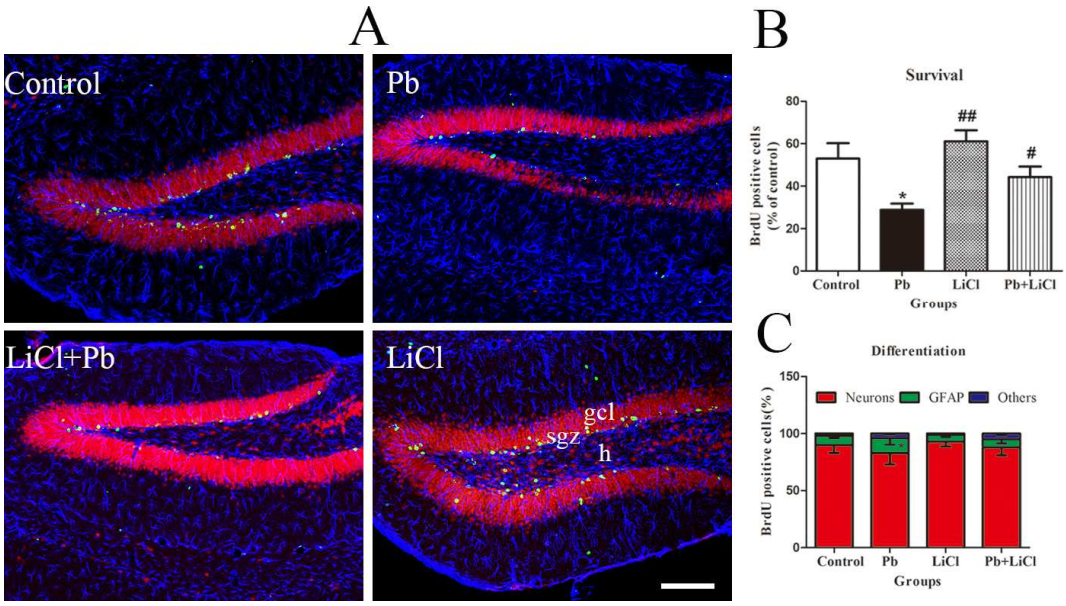


Fig.4. Effects of Pb and/or Li on the survival and differentiation of newly generated cells in the dentate gyrus. To determine the fate of newly generated cells in the DG after Pb-treatment with or without Li, triple immunohistochemistry was performed in the hippocampus labeled with BrdU in prior to compound administration. (A) Representative photographs of the dentate gyrus immunostained with antibodies against BrdU(green)/NeuN(red)/GFAP(blue). The cell types of newly generated cells were determined by co-labeling of BrdU with either marker for mature neurons (NeuN) or astrocytes (GFAP). (B) Quantification of the survival of $BrdU^+$ cells in the GCL of the DG. The analysis showed that the survival rate of $BrdU^+$ cells decreased in Pb administered mice, while it was promoted by administrating Li. (C) Quantification of the differentiation of $BrdU^+$ cells in the GCL of the DG. The results showed that about 90% of the newborn cells differentiated into neurons. No significant difference was detected between these four groups. However, percentage of $BrdU^+/GFAP^+$ cells in Pb mice was increased compared with that in control mice, indicating more newly generated cells differentiated into astrocytes. Data are presented as mean ± SEM. gcl-granular cell layer, sgz-subgranular zone, h-hilus.*, $p<0.05$ vs. control mice; #, $p<0.05$, ##, $p<0.01$, vs. Pb mice. Scale bar = 100 μm.

Li restored Pb-induced impairment on hippocampus-dependent learning and memory

Adult neurogenesis in the hippocampus is closely associated with learning and memory performance. Therefore, we used the Morris Water Maze (MWM) test to investigate alterations in spatial learning and memory caused by Pb and Li treatment. The test was performed for 5 consecutive days after treatment with Pb and Li for 2 weeks. The animals were naive to the water maze and showed no deficiencies in swimming abilities, directional swimming toward the platform or climbing onto the hidden platform during training trials. Notably, Pb-exposed mice displayed significantly decreased escape latencies in the hidden platform paradigm. However, the escape latencies in Pb-exposure mice pretreated with Li were almost restored compared with that in control mice (Fig. 5A). Furthermore, when the platform was removed during the probe test performed after 4 days of training, Pb-exposed mice spent less time in the target sector in a probe trial, whereas mice treated with both Li and Pb spent more time (Fig. 5B). Interestingly, we found that the microRNA-34c, which has been confirmed to be closely and negatively correlated to learning and memory, was highly expressed in hippocampus of Pb-exposed mice. Exposure to Li alone did not affect the expression level of microRNA-34c compared with that in control mice (Fig. 5C). The expression level of microRNA-34c returned to control level in Li-pretreated, Pb-exposed mice. These findings indicated protective effects of Li against Pb-induced spatial memory impairment. Interestingly, this process might be related to microRNA-34c in the hippocampus.

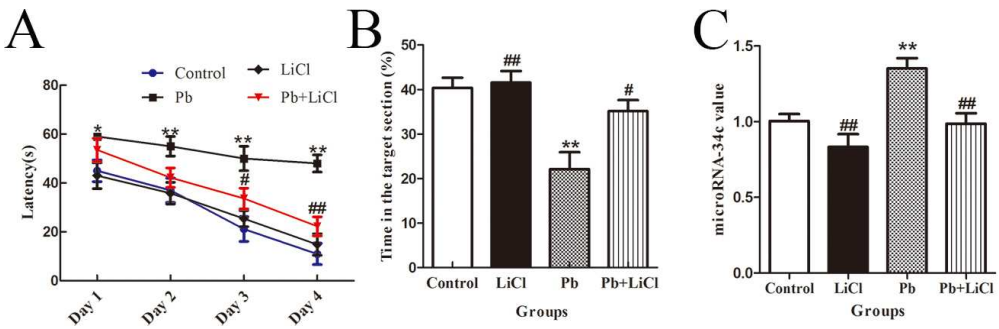


Fig.5. Pb-induced impairment of learning and memory was restored by pretreatment with Li. Escape latencies of mice in all groups were examined with the Morris Water Maze hidden platform test. (A) Training trials. Latencies to find the platform during the training session are reported as mean ± S.E.M. Each point represents the mean daily values of four trials per day. In Pb-exposed mice the recorded latencies were significantly longer than those of control mice, while the escape latency decreased in Pb-exposed mice treated with Li compared with that in Pb-exposed mice without Li. (B) Bar graphs show the percentage of time spent in the target sector during the probe trial within 30 s on day 5. Pb-exposure mice spent less time in the target quadrant compared with that in control mice and that co-treated mice. (C) MircroRNA-34c level in mice of all groups examined by using Real-time PCR. Less mircroRNA-34c was detected in Li mice than in control mice, while more was found in Pb-exposed mice *, $p<0.05$, **, $p<0.01$ vs. control mice; #, $p<0.05$, ##, $p<0.01$, vs. Pb mice.

GSK-3β was activated by Pb-exposure and inhibited by Li

Glycogen synthase kinase 3β (GSK-3β) protein is a key regulator of neurogenesis, neuronal differentiation and polarization during neurodevelopment. Phosphorylation of the N-terminal serine 9 residue of GSK-3β results in inactivation of the kinase. To examine whether Pb impairs neurogenesis and memory through GSK-3β, immunoblot analysis was carried out using antibodies specifically against phosphorylated (p)- and total proteins of GSK-3β (Fig. 6A). The total protein level of GSK-3β was not affected in all experimental groups compared with that in control mice. However, Pb-exposure significantly reduced p-GSK-3β (Ser9) level, while Li-treatment remarkably facilitated p-GSK-3β (Ser9) (Fig. 6B and C). Higher p-GSK-3β (Ser9) protein level was detected in Li-pretreated Pb-exposed mice compared with that in Pb-exposed mice. The variation trend of GSK-3β suggested that Li tended to ameliorate the reduction of p-GSK-3β in Pb mice. These results indicated that Pb induced impairment of adult hippocampal neurogenesis and spatial memory by activating the GSK-3β signaling pathway.

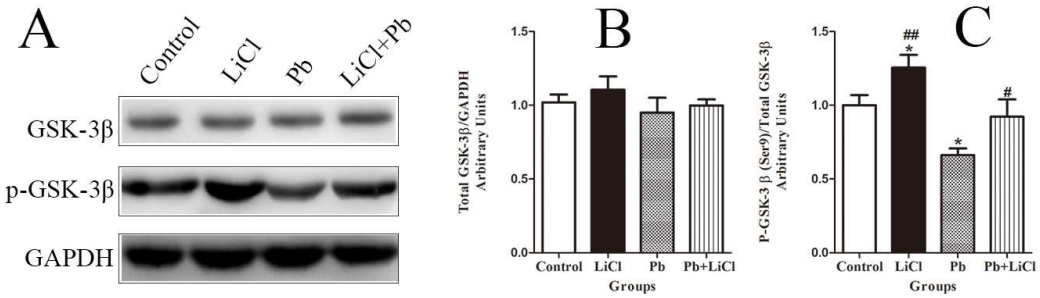


Fig.6. Pb and lithium contrarily regulate the activity of GSK-3β, through inhibition and promotion of phosphorylation of GSK-3β, respectively. Qualitative Western blotting and densitometric analysis of total GSK-3β and p-GSK-3β (Ser9). GAPDH served as a protein loading control. (A) Western blotting analysis of total and phosphorylated GSK-3β. (B and C) Densitometric quantification of total GSK-3β and p-GSK-3β. No difference was evident in the total GSK-3β levels in all groups. Pb-exposure remarkably reduced phosphorylation of GSK-3β. In contrast, lithium-exposure significantly increased the level of p-GSK-3β. Further, p-GSK-3β (Ser9) was significantly increased in co-treated mice compared with that in Pb-exposed mice. *, $p<0.05$, **, $p<0.01$ vs. control mice; #, $p<0.05$, ##, $p<0.01$, vs. Pb mice.

Discussion

In spite of the strict regulatory measures that have been taken by most countries to decrease environmental Pb burden, human Pb-exposure continues to remain an important public health issue particularly in developing countries with a lack of public control [22]. In the present study, we adopted both *in vivo* and *in vitro* experiments to investigate the protective effects of Li against Pb-induced damages based on pathological and neurological criteria.

Pb is a widespread environmental pollutant, which has been implicated in toxic processes that affect several organs in human and animals [23]. The histopathological alterations of organs including liver, spleen and kidney that have been described after chronic Pb exposure are anisokaryosis, nuclear vesiculation, binucleation, cytoplasmic inclusions and swelling, hydropic

degeneration, cell necrosis and vacuolization. Reduction in glycogen content, occasional fatty change and hemosiderosis were also found to be induced by Pb toxicity [24, 25]. In accordance with previous studies, our present work found similar histopathological alterations of Pb exposure in different organs of mice, such as hemosiderosis, fibrosis in spleen; nuclear vesiculation, cytoplasmic inclusions and swelling in liver; hydropic degeneration, cell necrosis and abnormal secretion in renal tubes. Interestingly, pretreatment with Li protected these organs from Pb-induced histopathological alterations to some extent. Moreover, the protective effects of Li against Pb-induced toxicities were also observed in primary cell culture. More cells survived after Pb-exposure when they were pretreated with Li.

Compared to other organs, the nervous system appears to be the most sensitive and main target for Pb-induced toxicity [26]. Fetuses and young children are especially vulnerable to the neurological effects of Pb as the developing nervous system absorbs a higher fraction of Pb. Children with higher Pb level may be affected with delayed growth, decreased intelligence, short-term memory and lower intelligence quotient (IQ) along with learning disabilities, behavioral problems, and school failure [11, 27, 28]. The incorporation of newly generated neurons into hippocampal circuits supports spatial learning and memory. Cognitive stimuli promote the survival of newborn cells [29, 30]. Our previous work also showed that an interplay seems to exist in the hippocampus between neurogenesis and learning and memory [20].

In order to quantify proliferation of the precursor cells, we used the synthetic thymidine analogue BrdU which incorporates into the DNA of dividing cells. Based upon BrdU uptake and labeling, we assessed proliferation of precursor cells in the DG. DCX is often used as a marker for migrating neuroblasts and immature neurons [31]. In consistence with the study of Gilbert et al.,(2005) [19], we observed a significant decrease of the proliferation of neural precursor cells in the hippocampus of Pb-exposed mice. In order to investigate the survival and differentiation of the newly generated cells, triple immunofluorescence staining with antibodies against BrdU, NeuN and GFAP was performed. Several studies in Pb-exposed rats have shown that the expression, synthesis, and concentration of GFAP, as well as the number of astrocytes, were increased in specific brain regions. This increase is associated with the formation of reactive gliosis in the rat brain, suggesting a primary response of astrocytes to Pb [32, 33]. Our present work also detected less BrdU-positive cells 28 days later and a higher percentage of GFAP positive cells in Pb-exposed mice. However, the survival and differentiation of newly generated cells was restored in animals pretreated with Li. In the present study, cognition was significantly impaired in Pb-exposure mice, which was also observed by other investigators [34]. As in previous studies [35], we also observed that spatial learning was significantly decreased when the mice were exposed to Pb. Moreover, neurocognitive functions in the MWM were preserved by litium treatment, suggesting that Li could effectively prevent Pb-induced impairment in memory and neurogenesis.

It is believed that Pb targets learning and memory processes in the brain by inhibiting the N-methyl-D-aspartate receptor (NMDAR), which is essential for hippocampus-mediated learning and memory [36]. Pb is a better activator of PKC than Ca^{2+}, and the activation of PKC induces expression of genes that regulate the formation of the AP-1 transcriptional regulatory complex. This activation disturbs signaling mechanisms in the hippocampus, causing impairment in long-term potentiation and memory in adult rats [37-39]. It has also been reported that microRNAs are key regulators of transcriptome plasticity and have been implicated in the pathogenesis of brain diseases. Recent studies suggest that microRNA-34c could be a marker for the onset of

cognitive disturbances linked to Alzheimer's disease and indicate that targeting microRNA-34c could be a suitable therapy [40, 41]. MicroRNA-34c is implicated in hippocampal function suggesting a potential role for microRNA-34c in the regulation of memory function. High microRNA-34c level is linked to the pathogenesis of learning impairment [42, 43]. Interestingly, our present data showed that memory was seriously impaired by Pb-exposure, accompanied by an increase in mircroRNA-34c in the hippocampus. Li itself did not change the level of microRNA expression. However, pretreatment with Li significantly decreased the expression of mircroRNA-34c in Pb-exposed mice. Our findings further confirmed that microRNA-34c is closely and negatively correlated with hippocampal-dependent cognition.

Pb is probably the most extensively studied heavy metal. Studies carried out in this field have reported the presence of various cellular, intracellular and molecular mechanisms behind the toxicological manifestations caused by lead. Chronic developmental Pb^{2+} exposure results in altered MAPK signaling, calcium/calmodulin kinase II (CaMKII) activity, and altered phosphorylation and binding affinity of cyclic AMP response element binding protein (CREB) [44, 45]. Ionic mechanisms of action for lead mainly arises from its ability to substitute other bivalent cations like Ca^{2+}, Mg^{2+}, Fe^{2+} and monovalent cations like Na^{+}, affecting various fundamental biological processes [46]. The ionic mechanism contributes principally to neurological deficits, as lead, after replacing calcium ions, becomes competent to cross the blood-brain barrier (BBB) at an appreciable rate. Lead, even in picomolar concentration, can replace calcium, thereby affecting key neurotransmitters like glutamate, which is involved in synaptic excitation and memory storage. It also affects the sodium ion concentration, which is responsible for numerous vital biological activities like uptake of neurotransmitters (choline, dopamine and GABA) and regulation of uptake and retention of calcium at the synapse [38]. On the other hand, Li-induced activation of Akt and/or CaMKII and reduced CREB phosphorylation may also contribute to the therapeutic effect of lithium in ischemia-induced brain injury and bipolar disorders [47, 48]. At therapeutic concentrations, Li competes with the cofactor magnesium (mg^{2+}), thereby inhibiting a broad range of enzymatic second messengers [49]. As previous studies, our work found that Li promoted the level of p-GSK-3β (Ser9) in mouse hippocampus. Interestingly, our work also found that p-GSK-3β (Ser9) level was significantly decreased in Pb-exposed mice. Moreover, Li could correct the depletion of p-GSK-3β (Ser9) induced by Pb. GSK-3β plays multiple roles, including modulation of correct growth and patterning of the embryonic dentate gyrus [50] as well as maintaining embryonic stem cells [51], hematopoietic stem cells [52] and adult neurogenesis [53]. It has been proved that Li regulates adult hippocampal progenitor development through canonical Wnt pathway activation [54]. In the present study, Li was administered prior to Pb-exposure and successfully inhibited or decreased the toxicities induced by Pb-exposure in mice in vivo and cells in vitro. Based on our findings, Li possibly acts as a much more potent competitor than Pb to combine the same target proteins like CaMKII, CREB, GSK-3β. Once Li combines with these target proteins, the tight interaction cannot be broken by Pb. This is probably the mechanism how Li protected the mice from Pb intoxication.

In conclusion, pretreatment with Li could protect the toxic effects in Pb-exposed mice, particularly with respect to hippocampal neurogenesis and memory retention. Both, Li and Pb directly or indirectly interact with the same target proteins like p-GSK3β (Ser9) or other molecules like microRNA-34c but cause opposite effects. Additional data are required before Li may become a potential prophylactic treatment of pb intoxication.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (No. 31071873 to S.Z.) and (No. 31301970 to Y.Y.). Foundation for Special Talent of NWSUAF (No.Z111021101 to S.Z.). The authors wish to thank Professor Michael Frotscher for helpful comments on the manuscript.

References

1. E. Brodkin, R. Copes, A. Mattman, J. Kennedy, R. Kling and A. Yassi, *CMAJ : Canadian Medical Association journal = journal de l'Association medicale canadienne*, 2007, **176**, 59-63.
2. R. L. Canfield, C. R. Henderson, Jr., D. A. Cory-Slechta, C. Cox, T. A. Jusko and B. P. Lanphear, *The New England journal of medicine*, 2003, **348**, 1517-1526.
3. Y. M. Jiang, L. L. Long, X. Y. Zhu, H. Zheng, X. Fu, S. Y. Ou, D. L. Wei, H. L. Zhou and W. Zheng, *Toxicology letters*, 2008, **181**, 118-125.
4. L. Seddik, T. M. Bah, A. Aoues, M. Slimani and M. Benderdour, *The Journal of toxicological sciences*, 2011, **36**, 797-809.
5. B. S. Schwartz, W. F. Stewart, K. I. Bolla, P. D. Simon, K. Bandeen-Roche, P. B. Gordon, J. M. Links and A. C. Todd, *Neurology*, 2000, **55**, 1144-1150.
6. L. D. White, D. A. Cory-Slechta, M. E. Gilbert, E. Tiffany-Castiglioni, N. H. Zawia, M. Virgolini, A. Rossi-George, S. M. Lasley, Y. C. Qian and M. R. Basha, *Toxicology and applied pharmacology*, 2007, **225**, 1-27.
7. L. Struzynska, *Neurochemistry international*, 2009, **55**, 151-156.
8. A. P. Neal, P. F. Worley and T. R. Guilarte, *Neurotoxicology*, 2011, **32**, 281-289.
9. C. D. Toscano, H. Hashemzadeh-Gargari, J. L. McGlothan and T. R. Guilarte, *Brain research. Developmental brain research*, 2002, **139**, 217-226.
10. D. T. Wigle, T. E. Arbuckle, M. C. Turner, A. Berube, Q. Yang, S. Liu and D. Krewski, *Journal of toxicology and environmental health. Part B, Critical reviews*, 2008, **11**, 373-517.
11. D. Sen, H. Wolfson and M. Dilworth, *Occupational Medicine (Lond)*, 2002, **52**, 49-54.
12. K. Jaako-Movits, T. Zharkovsky, O. Romantchik, M. Jurgenson, E. Merisalu, L. T. Heidmets and A. Zharkovsky, *International journal of developmental neuroscience : the official journal of the International Society for Developmental Neuroscience*, 2005, **23**, 627-635.
13. T. Verina, C. A. Rohde and T. R. Guilarte, *Neuroscience*, 2007, **145**, 1037-1047.
14. G. Chen, G. Rajkowska, F. Du, N. Seraji-Bozorgzad and H. K. Manji, *Journal of neurochemistry*, 2000, **75**, 1729-1734.
15. S. S. Shim, M. D. Hammonds, S. J. Ganocy and J. R. Calabrese, *Progress in neuro-psychopharmacology & biological psychiatry*, 2007, **31**, 343-347.
16. A. Fiorentini, M. C. Rosi, C. Grossi, I. Luccarini and F. Casamenti, *PloS one*, 2010, **5**, e14382.
17. E. E. Beier, T. J. Sheu, D. Dang, J. D. Holz, R. Ubayawardena, P. Babij and J. E. Puzas, *The Journal of biological chemistry*, 2015, DOI: 10.1074/jbc.M114.629204.
18. W. Noble, E. Planel, C. Zehr, V. Olm, J. Meyerson, F. Suleman, K. Gaynor, L. Wang, J. LaFrancois, B. Feinstein, M. Burns, P. Krishnamurthy, Y. Wen, R. Bhat, J. Lewis, D. Dickson and K. Duff, *Proceedings of the National Academy of Sciences of the United States of*

America, 2005, **102**, 6990-6995.

19. M. E. Gilbert, M. E. Kelly, T. E. Samsam and J. H. Goodman, *Toxicological sciences : an official journal of the Society of Toxicology*, 2005, **86**, 365-374.
20. J. Wang, L. Song, Q. Zhang, W. Zhang, L. An, Y. Zhang, D. Tong, B. Zhao, S. Chen and S. Zhao, *Toxicology letters*, 2014, **232**, 263-270.
21. B. Shukitt-Hale, A. N. Carey, D. Jenkins, B. M. Rabin and J. A. Joseph, *Neurobiology of aging*, 2007, **28**, 1187-1194.
22. K. Radad, K. Hassanein, M. Al-Shraim, R. Moldzio and W. D. Rausch, *Experimental and toxicologic pathology : official journal of the Gesellschaft fur Toxikologische Pathologie*, 2014, **66**, 13-17.
23. M. Correa, A. F. Roig-Navarro and C. M. Aragon, *Life sciences*, 2004, **74**, 2009-2021.
24. C. A. Dooyema, A. Neri, Y. C. Lo, J. Durant, P. I. Dargan, T. Swarthout, O. Biya, S. O. Gidado, S. Haladu, N. Sani-Gwarzo, P. M. Nguku, H. Akpan, S. Idris, A. M. Bashir and M. J. Brown, *Environmental health perspectives*, 2012, **120**, 601-607.
25. C. Nava-Ruiz, M. Mendez-Armenta and C. Rios, *Journal of molecular histology*, 2012, **43**, 553-563.
26. D. A. Cory-Slechta, *Otolaryngology--head and neck surgery : official journal of American Academy of Otolaryngology-Head and Neck Surgery*, 1996, **114**, 224-226.
27. L. M. Cleveland, M. L. Minter, K. A. Cobb, A. A. Scott and V. F. German, *The American journal of nursing*, 2008, **108**, 40-49; quiz 50.
28. L. M. Cleveland, M. L. Minter, K. A. Cobb, A. A. Scott and V. F. German, *The American journal of nursing*, 2008, **108**, 40-47; quiz 47-48.
29. J. J. Rodriguez, V. C. Jones, M. Tabuchi, S. M. Allan, E. M. Knight, F. M. LaFerla, S. Oddo and A. Verkhratsky, *PloS one*, 2008, **3**, e2935.
30. T. T. Chuang, *Biochimica et biophysica acta*, 2010, **1802**, 872-880.
31. J. G. Gleeson, P. T. Lin, L. A. Flanagan and C. A. Walsh, *Neuron*, 1999, **23**, 257-271.
32. K. L. Kumawat, D. K. Kaushik, P. Goswami and A. Basu, *Neurotoxicology*, 2014, **41**, 143-153.
33. L. Struzynska, I. Bubko, M. Walski and U. Rafalowska, *Toxicology*, 2001, **165**, 121-131.
34. R. G. Morris, E. Anderson, G. S. Lynch and M. Baudry, *Nature*, 1986, **319**, 774-776.
35. M. K. Nihei, N. L. Desmond, J. L. McGlothan, A. C. Kuhlmann and T. R. Guilarte, *Neuroscience*, 2000, **99**, 233-242.
36. J. Z. Tsien, P. T. Huerta and S. Tonegawa, *Cell*, 1996, **87**, 1327-1338.
37. R. A. Goyer, *Annual review of nutrition*, 1997, **17**, 37-50.
38. J. Bressler, K. A. Kim, T. Chakraborti and G. Goldstein, *Neurochemical research*, 1999, **24**, 595-600.
39. Y. Gu, L. Wang, C. Xiao, F. Guo and D. Y. Ruan, *Neuroscience*, 2005, **133**, 679-690.
40. A. Zovoilis, H. Y. Agbemenyah, R. C. Agis-Balboa, R. M. Stilling, D. Edbauer, P. Rao, L. Farinelli, I. Delalle, A. Schmitt, P. Falkai, S. Bahari-Javan, S. Burkhardt, F. Sananbenesi and A. Fischer, *The EMBO journal*, 2011, **30**, 4299-4308.
41. N. Liu, M. Landreh, K. Cao, M. Abe, G. J. Hendriks, J. R. Kennerdell, Y. Zhu, L. S. Wang and N. M. Bonini, *Nature*, 2012, **482**, 519-523.
42. W. Konopka, A. Kiryk, M. Novak, M. Herwerth, J. R. Parkitna, M. Wawrzyniak, A. Kowarsch, P. Michaluk, J. Dzwonek, T. Arnsperger, G. Wilczynski, M. Merkenschlager, F. J.

Theis, G. Kohr, L. Kaczmarek and G. Schutz, *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 2010, **30**, 14835-14842.

43. T. W. Bredy, Q. Lin, W. Wei, D. Baker-Andresen and J. S. Mattick, *Neurobiology of learning and memory*, 2011, **96**, 89-94.
44. F. M. Cordova, A. L. Rodrigues, M. B. Giacomelli, C. S. Oliveira, T. Posser, P. R. Dunkley and R. B. Leal, *Brain research*, 2004, **998**, 65-72.
45. C. D. Toscano, J. P. O'Callaghan and T. R. Guilarte, *Brain research*, 2005, **1044**, 51-58.
46. T. I. Lidsky and J. S. Schneider, *Brain : a journal of neurology*, 2003, **126**, 5-19.
47. T. Sasaki, F. Han, N. Shioda, S. Moriguchi, J. Kasahara, K. Ishiguro and K. Fukunaga, *Brain research*, 2006, **1108**, 98-106.
48. D. Tardito, E. Tiraboschi, J. Kasahara, G. Racagni and M. Popoli, *International Journal of Neuropsychopharmacology*, 2007, **10**, 491-496.
49. R. S. Williams, L. Cheng, A. W. Mudge and A. J. Harwood, *Nature*, 2002, **417**, 292-295.
50. J. Galceran, E. M. Miyashita-Lin, E. Devaney, J. L. Rubenstein and R. Grosschedl, *Development*, 2000, **127**, 469-482.
51. N. Sato, L. Meijer, L. Skaltsounis, P. Greengard and A. H. Brivanlou, *Nature medicine*, 2004, **10**, 55-63.
52. T. Reya, A. W. Duncan, L. Ailles, J. Domen, D. C. Scherer, K. Willert, L. Hintz, R. Nusse and I. L. Weissman, *Nature*, 2003, **423**, 409-414.
53. D. Sinha, Z. Wang, K. L. Ruchalski, J. S. Levine, S. Krishnan, W. Lieberthal, J. H. Schwartz and S. C. Borkan, *American journal of physiology. Renal physiology*, 2005, **288**, F703-713.
54. E. M. Wexler, D. H. Geschwind and T. D. Palmer, *Molecular psychiatry*, 2008, **13**, 285-292.