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INCREASED TAU PHOSPHORYLATION AND BETA AMYLOID IN THE HIPPOCAMPUS OF MOUSE PUPS BY EARLY LIFE LEAD EXPOSURE

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The aim of this study was to investigate the effects of maternal lead exposure on the learning and memory ability and expression of tau protein phosphorylation (P-tau) and beta amyloid protein (Aβ) in hippocampus of mice offspring. Pb exposure initiated from beginning of gestation to weaning. Pb acetate administered in drinking solutions was dissolved in distilled deionized water at the concentrations of 0.1%, 0.5% and 1% groups. On the 21th of postnatal day, the learning and memory ability of the mouse pups was tested by Water Maze test and the Pb levels in blood and hippocampus of the offspring were also determined. The expression of P-tau and Aβ in hippocampus was measured by immunohistochemistry and Western blotting. The Pb levels in blood and hippocampus of all exposure groups were significantly higher than that of the control group ($P < 0.05$). In Water Maze test, the performances of 0.5% and 1% groups were worse than that of the control group ($P < 0.05$). The expression of P-tau and Aβ was increased in Pb exposed groups than that of the control group ($P < 0.05$). Tau hyper-phosphorylation and Aβ increase in the hippocampus of pups may contribute to the impairment of learning and memory associated with maternal Pb exposure.

Keywords: Lead (Pb) – tau phosphorylation – beta amyloid – neurotoxicity – learning and memory

INTRODUCTION

Lead (Pb) produces neurotoxic effects, which result into an impairment of learning and memory and other neurological dysfunctions [32]. Neurotoxic effects from exposure to low levels of Pb in the environment are a problem of significant magnitude in the whole world, especially in children and infants [21, 30]. Results from prospective cohort studies provided evidence that low-level in utero Pb exposure could impair infant growth, development and cognitive function [6, 10]. The extensive studies revealed that prenatal and early postnatal Pb exposure even at low doses is extremely dangerous and can cause varied neurological disturbances [6, 17, 31]. Though numerous studies have shown that Pb could accumulate in the brain when its concentration in the blood is elevated [5], the mechanisms of Pb deposition in brain are not yet fully known.

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The research on Pb neurotoxicity has been tested for a long time, but its Alzheimer-like effects in brain have scarcely been investigated. Alzheimer's Disease (AD) is a progressive, irreversible neurodegenerative disease [29]. The neuropathology of AD is characterized by senile plaques (SPs) and neurofibrillary tangles (NFTs). SPs are the extracellular deposits mainly composed of aggregated β-amyloid (Aβ) protein, whereas NFTs are formed due to the intracellular accumulation of hyperphosphorylated tau protein [24]. Furthermore, several studies have previously suggested a link between early-life disturbances (risk factors) and the development of AD [1]. Despite several genetic mutations [11, 18] found in AD patients, more than 90% of AD cases are sporadic [4]. Therefore, it is plausible that environmental exposure may be an etiologic factor in the pathogenesis of AD.

In the present study, the effects on learning and memory of lead exposure during gestation and lactating period were evaluated in mouse pups, and the expression of P-tau and Aβ proteins in hippocampus was investigated. The developing brain shows intensive cellular proliferation, differentiation, and synaptogenesis. It is very sensitive to environmental hazard at this stage. It is known that early life Pb exposure could cause severe consequences for brain development. However, the underlying mechanism of Pb exposure on brain development is still unclear. In order to learn about the potential mechanism, this study will test the expression of hippocampal P-tau and Aβ in Pb exposed mouse pups to know whether these proteins are the media between Pb exposure and brain damage.

MATERIALS AND METHODS

Animals and treatment

All procedures involving animals were carried out in strict accordance with the international standards of animal care guidelines and were approved by the local Care of Experimental Animals Committee. Forty pregnant Kunming female mice were ordered from Henan laboratory animal center. The animals were maintained on a 12–12 h light/dark cycle with food and water available *ad libitum*. All experiments were carried out in accordance with the regulations of the Zhengzhou University Committee on Ethics in the Care and Use of Laboratory Animals. Every effort was made to minimize animal usage and discomfort. The pregnant mice were randomly divided into four groups and caged individually. The groups were named as control (no Pb exposure), low, medium and high dosage of Pb exposure. The animals were fed with lab chow pellet (supplied by Henan laboratory animal center). Pb exposure was conducted by drinking Pb containing water. Pb acetate (luoyang chemical agent factory, China) was dissolved into distilled at three different concentration levels, 0.1%, 0.5% and 1% for low, moderate and high concentration, respectively. Lead exposure started from beginning of gestation and lasted until weaning. The pups were weaned at 21 days of age. At birth, all litters were culled to eight pups. In the process

of experiment, littermates were not used within a group, but they were used across groups. Maternal body weight, the consumption of water and food were record on a weekly basis during the experimental period. The body weight of pups at birth, the end of first, second and third week after birth was also measured.

Determination of Pb concentrations in blood and hippocampal samples

Sample collection

The blood samples were collected from the tail of the pups on the 21st postnatal day after wiping the skin to remove the contaminated Pb. Then, the pups were anesthetized under ether inhalation and the brain was dissected. Both sides of the hippocampus were isolated.

Sample preparing for assay

Total of 100 μl of blood was mixed well with 3.9 ml of 0.5 N nitric acid (Luoyang chemical agent factory, China) containing 0.01% Triton X-100 (Shanghai chemical agent company, China) by vortexing for 10 seconds and followed by centrifugation for 10 minutes at 7500 r/min at room temperature. The supernatant was collected for further analysis of Pb content. The isolated hippocampus from each sample was homogenized in a mixture of 0.5 N nitric acid, 0.5 N perchloric acid (Luoyang chemical agent factory, China) and 0.01% Triton X-100 to make 1:10 (w/v) of diluted homogenate.

Pb levels in blood and hippocampus tissue were analyzed by using graphite furnace atomic absorption spectrometry (HITACHI, Japan).

Morris Water Maze

Beginning on PND 21, learning and memory function was assessed using a Morris Water Maze (MWM) task with a standard reversal procedure introduced. In brief, animals received 4 trials per day, for a total of 8 test sessions. Data from 4 trials were averaged to represent a performance block. The pool consisted of a white circular fiberglass tank measuring 1.5 m in diameter. Water temperature was maintained at approximately 22 °C and was made opaque by the addition of Funstuff© Liquid Tempera black paint (Reeves & Poole Group; Toronto, ON, Canada). Four points around the edge of the pool were arbitrarily designated as north, south, east and west. A clear plexiglass escape platform (15 × 15 cm) was submerged approximately 2 cm below the water surface, and placed initially, in the centre of the "eastern" quadrant

of the maze (approximately 32 cm from the wall of the pool). A reversal task was then introduced where the platform was moved to the centre of the diagonally opposite "western" quadrant for the three days, and finally the platform was returned to its original location.

The dependent measures included escape latency(s) and the number of crosses from initiation point to the platform locations.

For each trial, the mouse pups were placed in the pool facing the perimeter, and allotted 60 s to locate the hidden platform. If the animal failed to locate the platform within the given time, it was placed on the platform by the experimenter. The animal was permitted to remain on the escape platform for 10 s and was then placed in a holding cage for a 60 s inter-trial interval before being returned to the maze for the next trial. Mouse pups were given 4 trials/day, with start locations pseudo-randomly selected (i.e. selected without replacement). Order of start location was kept consistent between all mouse pups on a given test day, but was changed for the next time.

Sample preparing and immunohistochemistry

Three pups from each group were selected for histological study. The selected pups were anesthetised by an overdose of sodium nembutal (35 mg/kg, i.p.; Solarbio, China). Once the pedal reflex was absent, perfusion was performed via the heart with ice cold PBS, pH 7.0–7.4 for 1 min followed by 4% paraformaldehyde (Solarbio, China) in 0.1 M PBS for 10 min. One hour later, the whole brain was dissected and placed in a freshly made post-fixative solution of 4% paraformaldehyde in 0.1 M PBS for 2 h at 4 °C and then in 30% sucrose (Amresco, USA) for cryoprotection in 0.1 M PBS at 4 °C overnight. Cryostat (LEICA, Germany) sectioning was then performed. The fixed brain samples were cut at 5-μm thickness starting at 3 mm posterior to the anterior pole. Sections were collected onto poly-L-lysine-coated slides and allowed to air dry.

Two proteins, named P-tau and Aβ, were detected in the brain slides. The tissue slides were treated by microwave in 10 mM citrate buffer (pH = 6) for 3 min and followed by blockade of non-specific binding by incubation in 0.1 M PBS containing 3% normal goat serum. Sections were then subsequently incubated with primary antibodies overnight at room temperature. The primary antibodies were rabbit anti-phospho-tau (pSer 404) (P-tau) (Santa cruz, USA) and rabbit monoclonal anti-Aβ (Santa Cruz, USA). After extensive rinsing steps in 0.1 M PBS, the sections were incubated in biotinylated goat anti-rabbit antibody (Santa Cruz, USA) for 1 h at room temperature and followed by using the Vector ABC system (Vector, USA). Subsequent incubation in diaminobenzidine (Wuhan yuancheng technology limited company, China) was performed for visualization of the reaction product. For negative controls, the primary antibody was omitted.

Protein isolation and Western blotting

The dissected hippocampus samples were homogenized in lysis buffer containing 2% SDS (Solarbio, China), 10% glycerol (Solarbio, China), 2% 2-mercaptoethanol (Solarbio, China), 0.002% bromphenol blue (Solarbio, China) in 75 mM Tris-HCl (Solarbio, China). The samples were heated at 95 °C for 10 min before separating on 10% Tris/Glycine/SDS acrylamide gels (Bio-Rad, USA). The proteins were subsequently transblotted to polyvinylidene difluoride (Solarbio, China) membranes and blocked in 5% dry milk for 2 h at room temperature. The membrane was incubated with rabbit anti-phospho-tau (pSer 404) (P-tau) (Santa Cruz, USA) and anti-Aβ antibody (Santa Cruz, USA) for 2 h at 37 °C. After three washes with TBS/0.05% Tween-20, the membrane was incubated with a horseradish peroxidase-conjugated goat anti-rabbit antibody (Santa Cruz, USA) for 1 h at 37 °C. Protein signal was visualized using the SuperSignal West Pico Chemiluminescent Substrate (PIERCE, USA) and detected with Imaging System (Syngene, USA). β-actin protein was visualized and detected as above.

Statistical analysis

All data were expressed as mean ± SEM. One-way ANOVA and a *post hoc* Bonferroni's test in SPSS12.0 soft ware was used to analyze the differences of P-tau, Aβ and lead content in blood and hippocampus tissue between groups. *P* values less than 0.05 were considered to be significant.

RESULTS

Pb content in blood and hippocampal of pups

Figures 1 and 2 illustrate the profile of Pb levels in blood and hippocampus of pups at PND21 in different groups with maternal lead exposure during pregnancy and lactation period. A significant increase of lead level in blood and hippocampus content was found in lead exposure groups as compared with control group ($P < 0.05$) and the lead levels were positively related to the exposure dosage. The body weight of the pups were not significantly different between groups (data not shown).

Effect of lead exposure on learning and memory

The escape latency and the number of crossing the platform area were used to evaluate the performance of learning and memory. Data were shown in Table 1. The low lead exposure group showed no significant difference when comparing it with control group ($P > 0.05$). However, the performance of control group was significantly different from the other two exposure groups ($P < 0.05$, $P < 0.01$) (see Table 1).

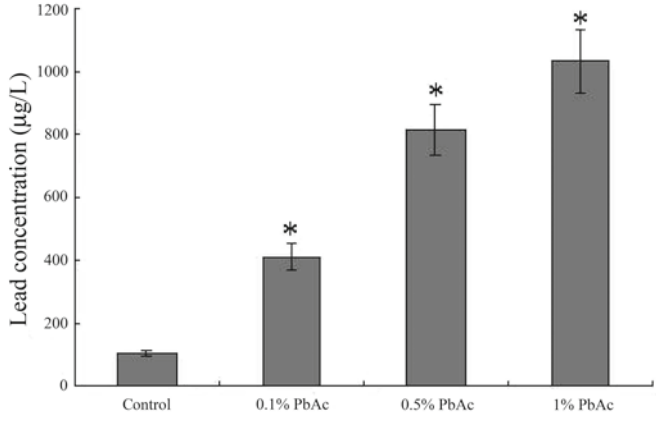

Fig. 1. Blood Pb levels in control and Pb-exposed animals at PND21. Each value represents the mean ± S.E.M. of ten different litters at same group. Only one animal per litter at each group was used. Blood Pb levels from the control animals were significant lower than in the Pb-exposed animals (*$P < 0.05$)

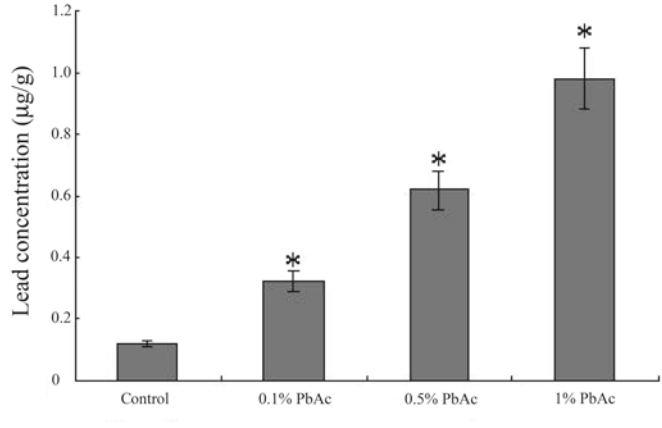

Fig. 2. Hippocampal Pb levels in control and Pb-exposed animals at PND21. Each value represents the mean ± S.E.M. of ten different litters at each group. Only one animal per litter at each age group was used. Hippocampal Pb level in control group was significant lower than in the Pb-exposed groups (*$P < 0.05$)

Effect of lead exposure on the expression of P-tau in hippocampus

Figure 3 illustrates the results of immunoreaction for P-tau. Figure 3a shows the expression of P-tau in hippocampus at control group. Figure 3b shows the influence of lead exposure on P-tau expression in 1% PbAc group. For an overview of the results, P-tau was expressed in a large body of cells. Figure 4 illustrates the effects of

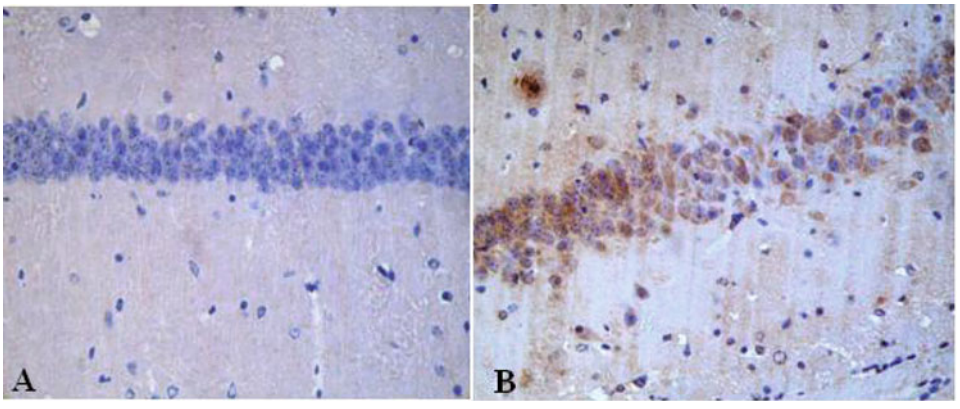

Fig. 3. P-tau immunoreactivity in CA1 region of hippocampus. **A** control group, **B** 1% PbAc group (40 × 10 magnification) (n = 3)

Table 1
The results of Morris Water Maze task x (n = 10, $\bar{x} \pm s$)

Groups	Number of crossed (times)	Escape latency(s)
Control	1.73±0.07	39.13±6.12
0.1% PbAc	2.31±0.24	43.22±9.86
0.5% PbAc	5.89±0.54*	50.85±12.35*
1% PbAc	9.53±1.03**	62.23±15.36**

* P<0.05; **P<0.01 when compared with the control group

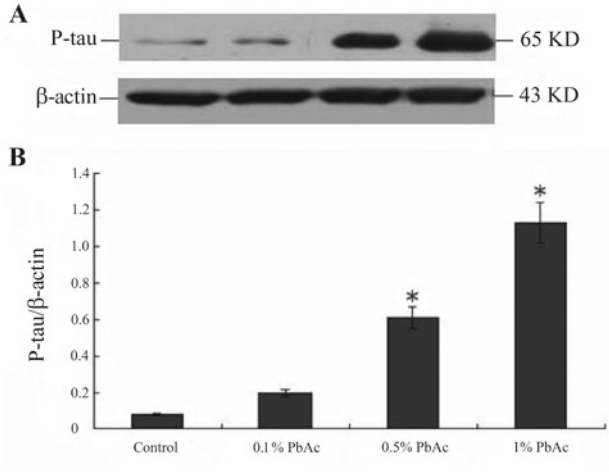

Fig. 4. Western blot analyses of P-tau protein in hippocampus tissue. **A** P-tau protein was analyzed by western blot in hippocampus of pups in different groups. β-actin is shown as a loading control. **B** P-tau protein expression levels analyzed by western blot in each group. The values represent the ratio of P-tau/β-actin intensity (n = 8) (*P < 0.05)

lead exposure on the expression of P-tau quantitatively. Figure 4a is a representative result of western blotting. Figure 4b shows the expression of P-tau in hippocampus of pups in different groups. The quantitative results showed that P-tau protein level was very low in the control and 0.1% PbAc group. P-tau protein level had no significant difference between 0.1% PbAc group and control group ($P > 0.05$). But, Its expression levels were higher in 0.5% and 1% PbAc groups than the control group and the differences are statistically significant ($P < 0.05$).

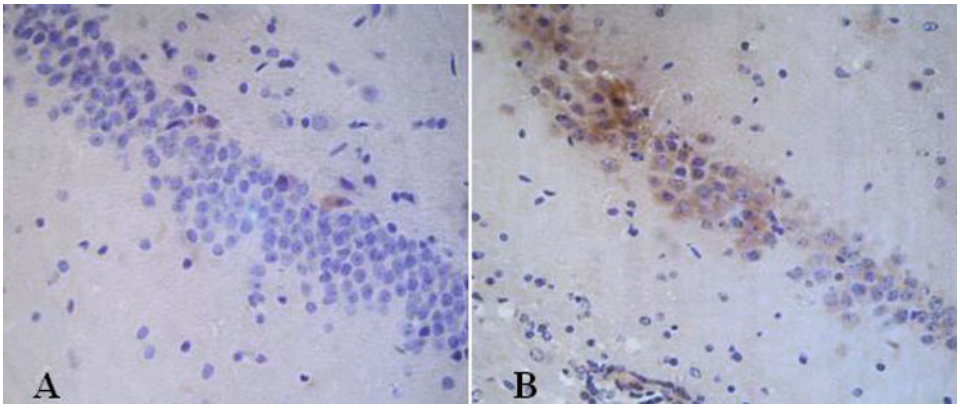

Fig. 5. Aβ immunoreactivity in CA1 region of hippocampus. **A** control group, **B** 1% PbAc group (40 × 10 magnification) (n = 3)

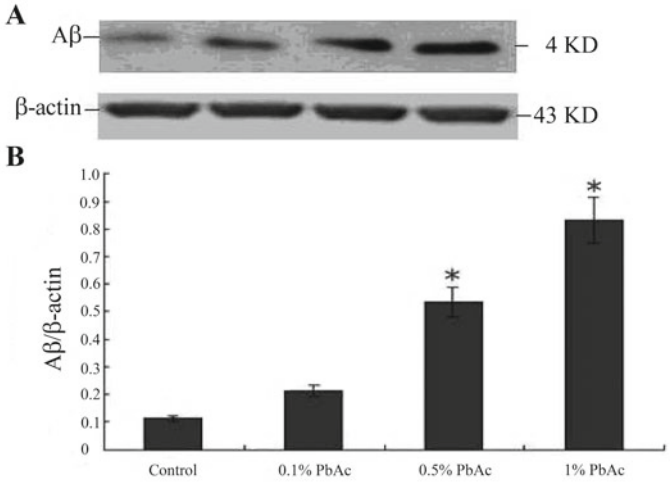

Fig. 6. Western blotting analyses of Aβ protein in brain tissue. **A** Aβ-protein was analyzed by western blotting in hippocampus of pups in different groups. **B** Aβ-protein expression levels analyzed by western blot in each group. The values represent the ratio of Aβ/β-actin intensity (n = 8) (*$P < 0.05$)

Effect of lead exposure on the expression of Aβ in the hippocampus

Figure 5 illustrates the results of immunoreaction for Aβ. Figures 5a and 5b show the expression of hippocampal Aβ in the control and 1% PbAc groups, respectively. The effects of lead exposure on the expression of Aβ are shown quantitatively in Fig. 6. Figure 6a is one of the results of Western blotting. Figure 6b shows the expression of Aβ in each group. The expression of Aβ in the hippocampus of pups was significantly increased in 0.5% and 1% PbAc groups when compared with the control group ($P < 0.05$), while in the 0.1% PbAc group showed no significant difference when comparing with control group ($P > 0.05$).

DISCUSSION

Chronic exposure to low levels of Pb has been a matter of public health concern worldwide. Epidemiological investigations have revealed the relationship between chronic developmental lead (Pb) exposure and cognitive impairments in young children. Lead neurotoxicity has been defined as a significant pediatric health problem. The fetal stage is a very susceptible period to Pb insult even exposured at the levels under 10 μg/dL, the lower bound threshold for Pb neurotoxicity in children [20]. The nervous system is the primary target for the Pb-exposure and the developing brain appears to be especially vulnerable [3, 22]. However, the mechanisms of lead exposure on brain deficits remain unclear [9].

Previous studies indicated that in 1999, a population-based case-control study found that chronic occupational exposure to Pb as well as other metals were associated with Parkinson's disease [13]. In 2002, Kamel et al., evaluated the relationship between Pb exposure and Amyotrophic Lateral Sclerosis (ALS) [16]. They found that the risk for ALS was associated with elevations in both blood and bone Pb levels suggesting that Pb exposure played a role in the etiology of ALS. While these studies provided hints as to the possible connection between Pb exposure and neurodegenerative disease, more convincing evidence was provided by Stewart et al., in 2002. They analyzed occupational exposure to Pb and risk factors for AD. In this seminal work, Stewart et al., examined tibia Pb levels in 529 former organo-lead workers and its relationship to the ApoE genotype, a known risk factor for AD. They concluded that the persistent CNS effects of Pb are more toxic in individuals with at least one ApoE (4 allele) [28]. The link between past adult Pb exposure and neurodegeneration was further established by this group using brain MRI imaging [27] and was consistent with their previous work which showed an association between Pb exposure and longitudinal cognitive decline. The possibility that toxic levels of Pb in any form could result in the formation of Alzheimer's neurofibrillary tangles was studied from the findings on a patient who suffered from severe Pb encephalopathy at 2 years of age, but died of severe mental deterioration at the age of 42 [19]. Atomic absorption spectrophotometry disclosed a tenfold increase of Pb in frontal and temporal cortices as compared to the control. A high concentration of Pb has also been reported in

patients with diffuse neurofibrillary tangles with calcification (DNTC) [14]. Pb is thus suspected to play a role in the pathogenesis of DNTC, a form of presenile dementia.

A candidate cellular process that may be implicated in memory loss is the remodeling of spine synapses. Evidence has been accumulated suggesting that rapid remodeling and stabilization of small spines and their associated synaptic contacts might represent a mechanism by which new memories are made and stored [25]. Spines of hippocampal pyramidal neurons are unique cellular compartments with the critical function of receiving the vast majority of glutamatergic, excitatory input via their asymmetric synaptic specializations [23]. During LTP formation (long-term potentiation, the widely accepted synaptic plasticity model of learning and memory) the number of spine synapses increases [26]. LTP is decreased as a consequence of lead exposure in parallel with poor memory function [7, 12]. In voltage clamp experiments, lead (Pb) increased glutamate-activated chloride currents in *Helix pomatia* L. neurons [23]. The cognitive effects of lead neurotoxicity may result from effects on LTP [8]. These above findings suggested that environmental influences occurring during brain development may regulate the expression of tau hyper-phosphorylation and Aβ (beta amyloid protein) later in life, potentially influencing the course of learning and memory.

The present study investigates the effects of lead exposure on the learning and memory function and the expression of tau protein phosphorylation (P-tau) and beta amyloid protein (Aβ) in hippocampus during pregnancy in mice offspring. The results demonstrate that the immunoactivities of Ser^{404} (P-tau) and Aβ were significantly inceased in hippocampus of lead exposure groups. In addition, transgenic mice expressing a phosphorylation-prone truncated tau show a deficit in the Morris Water Maze test, with no changes in spontaneous locomotor activity and no anxiety [15]. Hyperphosphorylated tau causes instability of microtubules, leading to neuronal dysfunction, and induction of tau phosphorylation by aggregation of Aβ, leads to an impairment of spatial memory. In Alzheimer's disease and other dementia-related disorders, there is a significant increase of Ser^{404} (P-tau) and Aβ expression in the brain due in part to neuron impairment [2]. As hypothesized, increase of immunoactivity for Ser^{404} (P-tau) and Aβ may contribute to the progression of memory decline in mouse pups with maternal Pb exposure.

It is concluded that the increased Ser 404 (P-tau) and Aβ expression with coexisting neurodegenerative features as a consequence of Pb exposure during pregnancy and lactating period are excessively expressed in hippocampus of the offspring. The results imply a potential mechanism for early life Pb exposure on poor performance of learning and memory function and neuropathological processes under conditions of maternal Pb exposure.

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