*Original Research*

# The expression of MMP2 and MMP9 in the hippocampus and cerebral cortex of newborn mice under maternal lead exposure

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## Abstract

The current study focused on the MMP2 and MMP9 expression in cerebral cortex and hippocampus of newborn mice under maternal lead exposure. Lead exposure was initiated from gestation to weaning. Lead acetate was dissolved in deionized water with concentration of 0.1, 0.2, and 0.5% and was absorbed through daily drinking. On day 21 after birth, lead in blood and tissue levels was examined by Graphite Furnace Atomic Absorption Spectrum (GFAAS). The protein expressions of MMP2 and MMP9 in hippocampus and cerebral cortex tissues were tested by western blotting and immunohistochemistry. Compared to the control group, blood, cerebral cortex, and hippocampus lead levels of newborn mice in 0.1, 0.2, and 0.5% lead exposure groups were markedly high ($P < 0.05$), and mice within the 0.2 and 0.5% lead exposure groups performed much worse than that of the control group in Water Maze test ($P < 0.05$). Compared with the control group, MMP2 and MMP9 expressions in hippocampus were up-regulated in the lead exposure groups ($P < 0.05$), and the MMP2 and MMP9 expressions in cerebral cortex were also higher ($P < 0.05$). The increased expression of MMP2 and MMP9 in the hippocampus and cerebral cortex may lead to the neurotoxicity in the context of maternal lead exposure.

**Keywords:** Lead, hippocampus, cerebral cortex, MMP2, MMP9, neurotoxicity

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## Introduction

Lead (Pb) neurotoxicity remains an important health problem worldwide. Although the occurrence of childhood $Pb^{2+}$ poisoning nationally has fallen sharply in the past 10 years, more than 250,000 children aged from one to five years old have been found to have blood lead levels higher than 10 μg/dL, which recently prompted the United States Centers for Disease Control and Prevention to take action on lead levels.[1] One of the heavy metals, lead has threatened environment sustainability and our future generations. Although research on lead neurotoxicity has been of interest for many years,[2–7] the effects of lead on the expression of MMP2 and MMP9 in the cerebral cortex and hippocampus still need to be elucidated.

The matrix metalloproteinase (MMPs) family includes five members, including the membrane-type MMP, gelatinases, matrilysin, collagenases, and stromelysin.[8–10] All extracellular MMPs secrete proenzymes which are needed for activation.[11,12] Recent results have shown that the MMPs participate in various pathological and physiological processes, such as wound repair, reproduction, angiogenesis, inflammation, the placenta morphogenesis and development, cancer cell invasion/metastasis, and nervous system diseases.[12]

Some studies have focused on the potential role of MMP on neural plasticity and neuropathology in the brain.[13,14] Two gelatin enzyme subgroups of matrix metalloproteinase, MMP2 and MMP9, are activated in cerebral vascular diseases.[15] However, the function of MMP2 and MMP9 in neural plasticity is little known.[16] The mouse hypothalamus has been found to express MMP2 and MMP9.[17] Studies have proved the fundamental importance of MMP9 function regulating hippocampus synaptic plasticity in learning and memory. Additionally, microarray experiments have detected an increase in MMP9 protein transcription in the magnocellular supraoptic neurons of the rat after dehydration.[18]

Matrix metallopeptidase-2, named gelatinase A (72 kD type II collagenase) has vital functions in immunity and inflammation, not to mention its physiological functions of remodeling and degrading extracellular matrix. MMP-2 expressions increased in animal models of inflammation and immune diseases, and in human diseases. After the analysis of MMPs of white blood cells, monocytes seemed to be

mainly involved in inflammatory cells in multiple sclerosis (MS). Comparing with normal subjects, a high level of monocyte-expressed MMP2 and MMP14 was found in MS patients.[19] In addition, another study showed that MMP2 could be expressed not only in monocytes but also in a microglial cells, strocytes, and macrophages.[11] Although in the acute phases, the relationship between the frequency of reactive glial cells and macrophages to axon injuries has been demonstrated, the precise role of MMP-2 in the immune pathogenesis of MS is little known.[20]

MMP-9, also named the gelatine enzyme and collagenase, is secreted from macrophages, neutrophilic granulocytes, and certain forms of fermentation bacteria cellules. After activation, MMP-9 performs in many inflammatory processes, and in the progression of rheumatoid arthritis, cardiovascular disease, MS, and chronic obstructive pulmonary disease.[21] MMP-9 plays a pivotal role in protease and modulation; it reduces the alpha 1-antitrypsin serine protease inhibitors, which may cause the destruction of the lungs.[22] Various studies have shown an increased expression of MMP-9 in MS. For example, a study indicated that the MMP-9 level increased in the cerebrospinal fluid of MS and central nervous system infection and other inflammatory diseases. A study showed that young mice with MMP-9 knockout were partly opposed to experimental autoimmune encephalomyelitis (EAE) development.[21] However, as both gelatinases (MMP2 andMMP9) were genetically knocked out, we were able to witness a whole oppose against myelin oligodendrocyte glycoprotein induced by EAE.[22] The latter research was important because it showed many proteases in the cascade or network. The research described above has shown that MMP2 and MMP9 are related to the development of many brain diseases.

The developing brain undergoes intensive differentiation, cellular proliferation, and synaptogenesis and is therefore particularly sensitive to environmental risks. Research has indicated that early life Pb exposure causes harm to brain development.[5,6] Both MMP2 and MMP9 play a vital role in the central nervous system. However, the potential effect of lead neurotoxicity remains unclear. With the purpose of clarifying the underlying mechanisms, our study aims to detect MMP2 and MMP9 expression in the hippocampus and cerebral cortex, key points in the central nervous system of mouse offspring with different maternal lead exposure to ascertain whether these two proteins are involved in lead neurotoxicity.

## Materials and methods

### Animals and treatment

All procedures on animals were approved by the local experimental animals care committee, and experimental animals were covered by strict regulations according to international standards of animal care. Forty Kunming pregnant mice (SCXK 2010-0002) were obtained from the experimental animal center of Henan province. They were divided into four different groups randomly and different groups were housed in different cages. The control group was provided with distilled deionized water without lead pollution. PbAc was administered to the water at three different levels, 0.5% (5000 ppm), 0.2% (2000 ppm), and 0.1% (1000 ppm), representing high, moderate, and low level, respectively. The lead exposure time, by drinking water with lead, started from the beginning of pregnancy to weaning (day 21).[23] The animals were kept in a 12–12 h dark/light cycle and ate food and drank water freely. All the tests were conducted according to guidelines of the Ethics and Laboratory Animals Use Committee of Zhengzhou University. After birth, eight healthy pups per litter (both male and female) were used. Just one mouse per nest mouse in each group was used in the experiment.[24]

### Measurement of lead concentrations in samples

*Sample preparation.* After cleaning and eliminating lead pollution in the skin, blood samples were collected from the pup tail on postnatal day 21. Then, the pups were put into a coma by ether narcosis and the whole brains were separated to obtain hippocampus tissues and cerebral cortex tissues.

*Sample preparing for assay.* Fifty microliters blood, 1.95 mL 0.5 N nitric acid (included 0.01% Triton X-100) were blended by eddy current for 10 s and then centrifuged for 10 min at 7500 r/min at room temperature. The liquid supernatants were collected for lead level analysis. The isolated hippocampus tissue samples were homogenized in 0.5 N perchloric acid, 0.01% Triton X-100, and 0.5 N nitric acid mixture to make a diluted homogenate with 1:10 (w/v).

The measurement of lead concentrations in samples was described.[19,25,26] A 20 μL sample was put into the autosampler of a graphite furnace atomic absorption spectrum (GFAAS) spectrometer (Hitachi Construction Machinery). 0.2% magnesium nitrate 20 μL was used as a modifier. Lead concentration determination of blood and the brain was conducted at 283.3 nm. Pure argon was used as a clean and protective gas. Analysis of the replication and high average peak values were calculated. 1000 ppm lead chloride in dilute nitric acid 0.3–5, 10, and 20 μL containing 0.01% Triton X-100 0.2% nitric acid was used to make the standard curve. To examine the recovery rate, standard Pb solution was added (final concentration: 10 ppm) to blood and brain samples as internal standard. The brain and blood recovery rate were 98.6 and 97.5%, respectively.

### Morris Water Maze

A Morris Water Maze was used to evaluate the animals' learning and memory ability.[27–29] Beginning on day 21, the animals were subjected to two tests per day for about five days. The test duration was 30 min every day. The animals were put in warm water and faced to the sidewall of the tank to look for the platform to escape in 60 s. If the animals found the platform within 60 s, the times of escape latency were recorded, and the animals received 10 s rest before the next training.

The average of four test data represented a grade. The test tank of the Water Maze was an off-white round fiberglass pool with a diameter of 1.5 m with black paint (Reeves and Poole Group, Canada). The temperature of the water was kept at about 23°C. Four test points were named as north, south, east, and west at the edge of the pool. About

2 cm below the surface of the water was an escape platform (0.15 m × 0.15 m) that was placed initially in the middle of the ''eastern'' quadrant of the Water Maze (about 0.32 m to the pool wall). Then, a reversal task was introduced, from the escape platform to the center of the opposite ''western'' quadrant, for about three days, and finally the escape platform was put back to the original place. The hidden platform stayed in the same place for each part of the learning and memory experiment. The error data and the latency(s) of escape of the pups from the start point to the escape platform were measured to analyze the ability of learning and memory.

### Sample preparation and immunohistochemistry

The mice in four different groups were selected for the immunohistochemistry study.[30,31] Selected pups were anesthetized by ether inhalation. The pup brain was isolated. The fixed brain samples were cut at 5 μm thickness starting at 3 mm posterior to the anterior pole. The tissue slides were subjected to microwave treatment with citric acid buffer (pH = 6) for 3 min and then blockaded by nonspecific binding by 0.1 mol/L PBS with 3% serum, and incubated by the first antibodies overnight at room temperature. The first antibodies were rabbit anti-MMP2 (1:200, Santa Cruz Biotechnology) and anti-MMP9 (1:200, Santa Cruz Biotechnology). Slides were rinsed five times in 0.1 mol/L PBS cleaning step and then incubated in second antibody IgG (1:300, Santa Cruz) for 1 h at room temperature. The Vector ABC system was used for visualization of the reaction results. The primary antibody was omitted for negative controls.

### Western blotting analysis

The brains were separated into hippocampus tissues and cerebral cortex tissues. The tissue samples were homogenized in tissue lysis buffer, composed of 10% glycerol, 2% SDS, 0.002% bromphenol blue, and 2% 2-mercaptoethanol added in 75 mmol/L Tris–HCl.[19] All the tissues were heated for about 10 min at 95°C and then separated on 10% Tris–acetate/SDS/glycine acrylamide gelatins.

Protein was transported to polyvinylidene fluoride (PVDF) membranes and blocked by 5% milk power at room temperature for about 2 h. The film was treated with rabbit anti-MMP2 (1:200, Santa Cruz Biotechnology) and anti-MMP9 (1:200, Santa Cruz Biotechnology) at 37°C for about 2 h.[32] After washing using thermomorphic biphasic solvent (TBS) with 0.05% Tween-20 for three times, films were incubated with the second antibody IgG (1:300, Santa Cruz Biotechnology) at 37°C for about 1 h. The Super Signal West Pico Chemiluminescent Substrate (Pierce Chemical Company) and Imaging Detection System (Syngene Gene Company) were used to visualize the protein signal. Beta actin was found and also visualized according to the previous description.

### Statistical analysis

All data appear as mean ± *s*. A *post hoc* Bonferroni's test and One-way ANOVA in SPSS12.0 software were chosen to analyze the ability of learning and memory, the differences of blood lead content and brain tissue, and the expression differences of MMP2 and MMP9 in hippocampus and cerebral cortex tissue among these four different groups. $P < 0.05$ was considered to be indicative of significance.

## Results

### Lead levels in hippocampus, cerebral cortex, and blood of mouse pups

Figure 1 shows the $Pb^{2+}$ content in the blood (a), hippocampus (b), and cerebral cortex (c) of mouse pups (on day 21) in four different groups under maternal lead exposure. Comparing to the control groups, lead levels in the blood, hippocampus, and cerebral cortex were significantly increased under lead exposure ($F = 55.14$, $P < 0.05$; $F = 9.49$, $P < 0.05$; $F = 11.38$, $P < 0.05$).

### Pb exposure effect on learning and memory

The crossing numbers and escape latency to the flat pallet were chosen to determine learning and memory ability (see Table 1).

Mice in the 0.2 and 0.5% lead exposure group showed a higher escape latency in platform retrieval ($F = 38.61$, $P < 0.05$; $F = 58.04$, $P < 0.05$ versus control group) and also higher numbers of entering non-exit errors ($F = 29.17$, $P < 0.05$; $F = 35.27$, $P < 0.05$ versus control group) than the control group in the Water Maze task. However, there was no significant difference between the 0.1% $Pb^{2+}$ exposure group and the controls on the memory ability of the mice ($F = 15.32$, $P > 0.05$; $F = 19.75$, $P > 0.05$).

### Effects of lead exposure on MMP2 expression in cerebral cortex and hippocampus

Figure 2(a) and (b) depicts the results of the immune response of MMP2. Figure 2(a) illustrates the expression of MMP2 in the hippocampus of the control without lead group. Figure 2(b) indicates the maternal lead exposure effect on MMP2 expression in the 0.5% PbAc group. Figure 2(e) and (f) quantitatively show maternal lead exposure influences on MMP2 expression. Figure 2(e) is a typical western blot result. Figure 2(f) describes MMP2 expression in the hippocampus of the three different exposure groups. Quantitative results showed that the expression of MMP2 in the control group was relatively low, and the PbAc-treated groups showed higher levels of MMP2 expression, and the difference was statistically significant ($F = 42.19$, $P < 0.05$).

Figure 2(c) and (d) shows the results of MMP2's immune response in the cerebral cortex. Figure 2(c) illustrates the MMP2 expression in the cerebral cortex of the control group without lead. Figure 2(d) indicates the maternal lead exposure effect on MMP2 expression in the 0.5% PbAc group. Figure 2(g) and (h) quantitatively show maternal lead exposure influences on MMP2 expression in the cerebral cortex. Figure 2(g) is a typical western blot result. Figure 2(h) describes MMP2 expression in the cerebral cortex of the three different lead exposure groups. Results showed that the MMP2 expression level in the cerebral cortex of the control was very low, but higher levels of

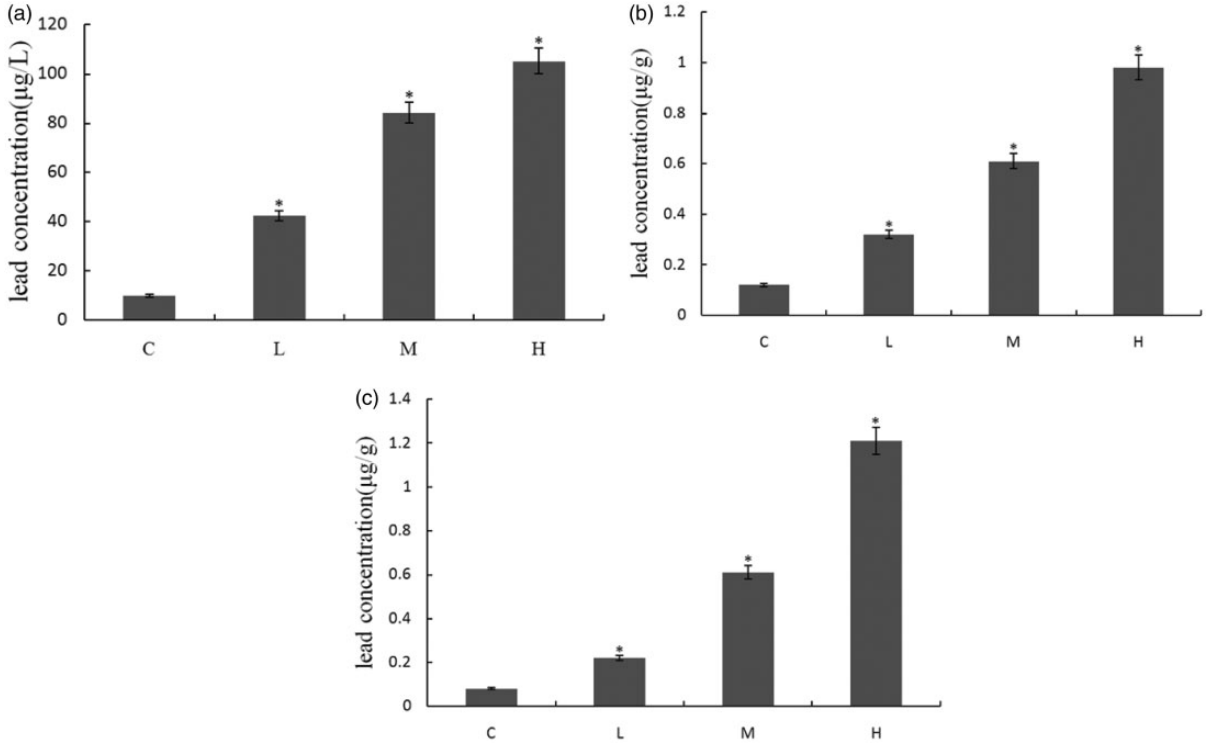


**Figure 1** (a)–(c) Pb levels in blood (a), hippocampus (b), and cerebral cortex (c) of different Pb-exposed animals at PND21. Each value represents the mean ± SEM of 10 different litters at same group. Pb levels from the control animals were significantly lower than the Pb-exposed animals. (n = 10 per group) (*$P < 0.05$). C, L, M, H group refers to control, 0.1, 0.2, and 0.5%, respectively

**Table 1** The results of Water Maze task ($\bar{x} \pm s$)

| Groups | n | Number of errors (times) | Escape latency(s) |
|---|---|---|---|
| Control | 10 | 1.73 ± 0.07 | 39.13 ± 6.12 |
| 0.1% PbAc | 10 | 2.31 ± 0.24 | 43.22 ± 9.86 |
| 0.2% PbAc | 10 | 4.43 ± 0.31* | 48.97 ± 10.02* |
| 0.5% PbAc | 10 | 5.89 ± 0.54* | 50.85 ± 12.35* |

Note: * $P < 0.05$ when compared with the control group.

MMP2 expression were found within the PbAc-treated groups other than the control, and the difference was statistically significant ($F = 76.18$, $P < 0.05$).

### Effects of lead exposure on MMP9 expression in the mouse pups' hippocampus and cerebral cortex

Figure 3(a) and (b) shows the results of MMP9's immune response in the hippocampus. Figure 3(a) illustrates the MMP9 expression in the hippocampus of control group without lead. Figure 3(b) indicates the lead exposure effect on the expression of MMP9 in the 0.5% PbAc group. Figure 3(e) and (f) quantitatively describe the lead exposure influences on MMP9 expression in the hippocampus. Figure 3(e) is a typical western blot result. Figure 3(f) indicates the MMP9 expression in the hippocampus of the three different lead exposure groups. The results showed that the MMP9 expression in the hippocampus of the control group was lower than that of the PbAc-exposed groups, and the difference was statistically significant ($F = 68.24$, $P < 0.05$).

Figure 3(c) and (d) shows the results of MMP9's immune response in the cerebral cortex. Figure 3(c) illustrates the MMP9 expression in the cerebral cortex of control group without lead. Figure 3(d) indicates the lead exposure effect on the expression of MMP9 in the 0.5% PbAc group. Figure 3(g) and (h) quantitatively describe the lead exposure influences on MMP9 expression in the cerebral cortex. Figure 3(g) is a typical western blot result. Figure 3(h) indicates the MMP9 expression in the cerebral cortex of three different lead exposure groups. The results showed that the MMP9 expression in cerebral cortex of control was also very low, and the PbAc groups display significantly higher levels of MMP9 expression than the control group without lead ($F = 765.19$, $P < 0.05$).

## Discussion

Even low levels of lead exposure during development adversely affects many behavioral functions, and neurochemical and cognitive systems, leading to defects in attention, administrative, and learning/memory abilities in childhood that continue to later adulthood.[33–37] The primary target of lead exposure is the nervous system and the growing nervous system seems to be particularly vulnerable.[38,39] However, the functional mechanisms of maternal lead exposure on the offspring's brain deficits still remain unclear.[38] Adult disability or disease symptoms are consistent with persistent effects occurring in early life Pb exposure that arise from physiological reprogramming.[40]

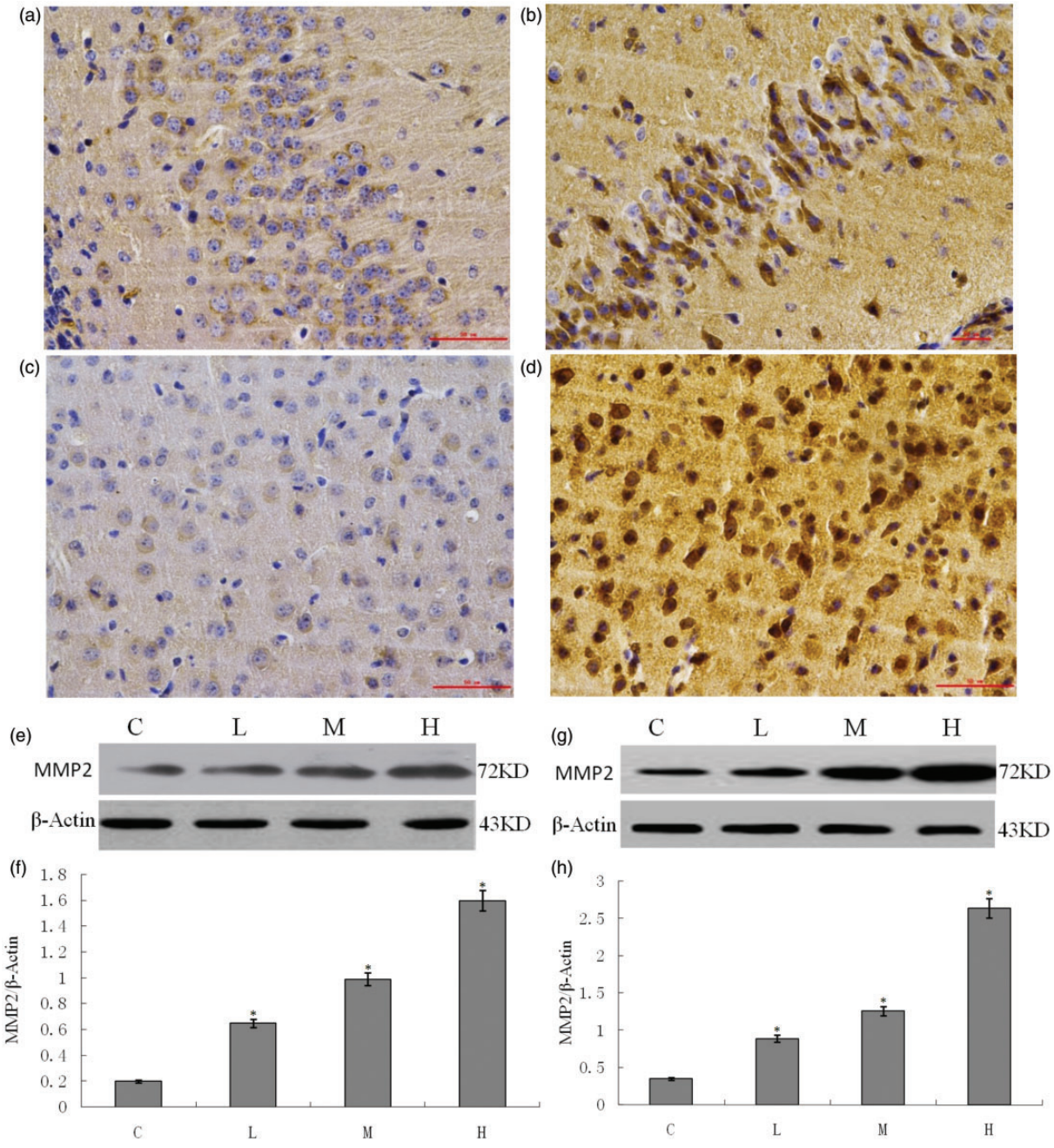


**Figure 2** MMP2 expression in hippocampus and cerebral cortex of offspring with maternal lead exposure. (a)–(d) represent MMP2 immunoreactivity in CA1 region of hippocampus (a, b) and cerebral cortex (c, d). (a) and (c) control group, (b) and (d) 0.5 % PbAc group, respectively. (e)–(h) Western blot analyses of MMP2 protein in hippocampus and cerebral cortex tissue. MMP2 protein was analyzed by western blot in hippocampus (e, f) and cerebral cortex (g, h) tissue of pups in different groups. β-actin is shown as a loading control. The values represent the ratio of MMP2/β-actin intensity (n = 10 per group) (*$P < 0.05$). C, L, M, H group refers to control, 0.1, 0.2, and 0.5%, respectively. (A color version of this figure is available in the online journal.)

Lead has effects on various behavioral and cognitive functions, and its influences on the brain developmental cortical–subcortical system, which programs and promotes vital fields of cognition, are not well known.[41] In a normal physiological situation, MMPs are expressed at a low level in the central nervous system. However, in various situations of neurological damage or disorders, gelatinolytic MMPs (MMP2 and MMP9) are notably increased.[13] In the rodent brain, MMP9 has been reported to increase in a bilateral manner. After ischemia, a peak of MMP9 appeared first in the acute period (within 24–48 h), and subsequently in the delayed period (on days 7–14). In the acute-outbreak period of ischemia, MMP9 played a detrimental function by causing brain edema, hemorrhage, and cell death and damaging the brain–blood barrier.[42] However, in the delay period, MMP9 was helpful in repairing activity by advancing functional recovery, facilitating re-myelination and neurovascular remodeling.[43–45]

Studies have indicated that MMP-2 was up-regulated in brain plaques, but also seemingly up-regulated in the normal brain white matter near the severe plaques in EAE disease.[46,47] In these acute plaques, the reactive glial cells

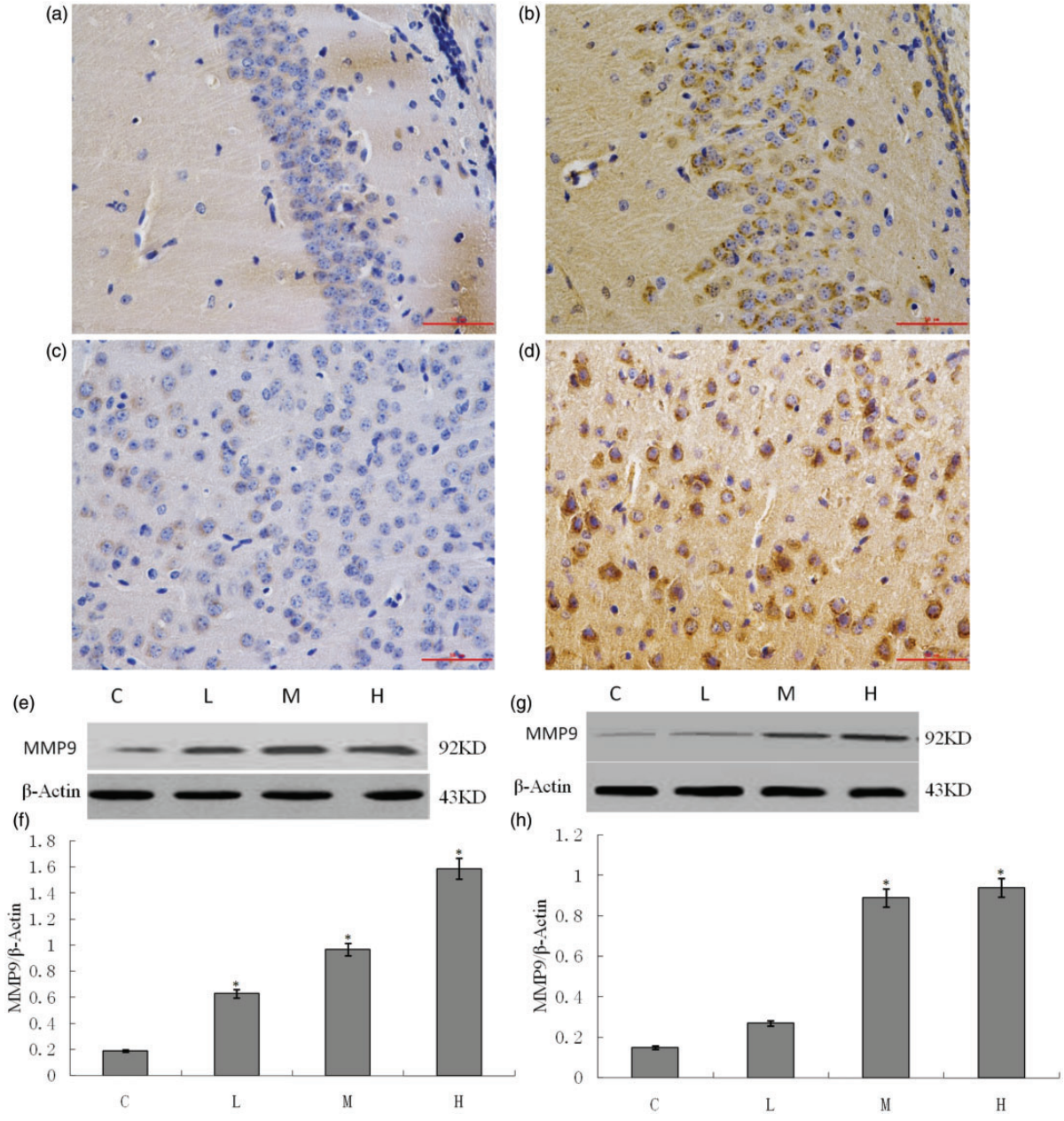


**Figure 3** MMP9 expression in hippocampus and cerebral cortex of offspring with maternal lead exposure. (a)–(d) represent MMP9 immunoreactivity in CA1 region of hippocampus (a, b) and cerebral cortex (c, d). (a) and (c) control group, (b) and (d) 0.5 % PbAc group, respectively. (e)–(h) Western blot analyses of MMP9 protein in hippocampus and cerebral cortex tissue. MMP9 protein was analyzed by western blot in hippocampus (e, f) and cerebral cortex (g, h) tissue of pups in different groups. β-actin is shown as a loading control. The values represent the ratio of MMP9/β-actin intensity (n = 10 per group) (*$P < 0.05$). C, L, M, H group refers to control, 0.1, 0.2, and 0.5%, respectively. (A color version of this figure is available in the online journal.)

and macrophages degraded and engulfed the myelin, resulting in demyelination. Another study showed that rat subcortical white matter microinjected by MMP-2 led to serious axonal injury.[48] As compared with controls, this study indicated that MMP9 and MMP2 co-labeled with newborn neurons, endothelial cells, and astrocyte markers in DG, and the percentage of co-expression also markedly up-regulated in the following postischemic disfunction.[49,50] With maternal hypoxia exposure, the brain and body size were markedly decreased, and MMP-9 was significantly increased at day 0 and day 4, and MMP-2 was significantly increased at day 0.[51] Similarly, in the present study, the MMP-2 expression was also up-regulated in the hippocampus and cerebral cortex of offspring with maternal lead exposure, as compared with the control group.

In the central nervous system, the meninges, vascular endothelial cells, astrocytes, microglia, accumulated inflammatory cells, and inflamed MS plaques as well as cerebral-infarction tissue or in the seemingly normal white matter can express MMP-9.[52] As an unspecific laboratory marker of inflammation, MMP-9's high levels are related to the IgG index in patients with MS. For example, MMP-9 expression and other MMPs (such as MMP-2, MMP-7, and MMP-12) were up-regulated in brain slices of MS patients when detected through immunohistochemical assay.[47] Moreover, MMP-9 levels in leucocytes or serums were

also high. In addition, experiments showed that MMP-9 steady-state mRNA levels were enhanced in MS by MRI.[53]A study has shown that in patients up to one week after an infarction, neutrophils strongly expressed MMP-9, and that at that time, MMP-9 was expressed by many macrophages. These studies have shown that some patients, such as those with MS or infarction, the MMP9 expression is up-regulated in the brain. Similarly, in the present study, maternal lead exposure increased the MMP-9 expression in the cerebral cortex and hippocampus of offspring compared with the control group.

The present study showed that, compared with the control group, the lead levels in mice blood, hippocampus, and cerebral cortex all increased significantly and that MMP-2 and MMP-9 expression also increased significantly in the hippocampus and cerebral cortex of offspring with maternal lead exposure groups. In the Water Maze test, the learning and memory abilities of mice in medium and high lead exposure groups were worse than those of the control group. Increased MMP2 and MMP9 expression in the hippocampus and cerebral cortex of the offspring and poor learning and memory ability were consequently the result of the mice experiencing a lead exposure effect during pregnancy and the lactating period. These results indicate another potential mechanism for neuropathological processes with maternal lead exposure.

**Author's contribution:** NL: The writer of the paper and the person in charge of all the project; XL: Sample preparing and immunohistochemistry; LL: Protein isolation and western blotting; PZ: Statistical analysis; MQ: Animal feeding experiment; QZ: Animal rising; LS: Sample collecting; ZY: Papers amending and reviewing.

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#### DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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