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Original Article

Effect of Lead (Pb^{2+}) Exposure in Female Pregnant Rats and Their Offspring on Spatial Learning and Memory in Morris Water Maze

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Abstract

Lead (Pb^{2+}) is a well known neurotoxin that was frequently found in the environment and chronic exposure to lead has been matter of public health. In the present study the effect of lead on spatial memory in developmentally exposed rats and their dams in Morris water maze task were investigated. Female rats were divided into three groups and two groups exposed to 250 and 750 part per million (ppm) Pb acetate and one group Na acetate , as a control group, through drinking water, ten days prior to mating and continue through pregnancy, pregnant animals were tested in the swim task at gestation day 14±2. Another group of animals exposed to the same concentration of Pb acetate at different developmental stages including a maternally exposed group (including gestation and lactation period) and continuously exposed group (including gestation, lactation and continue to lead exposure until test time). Rats in these groups were tested for spatial learning and memory in Morris water maze task at post natal day (PND) 56.

Exposure to lead did not affect learning ability of dams in Morris water maze performance indicating by no significant differences in escape latency and traveled distance between groups, but, for both maternally-and continuously-exposed groups treated with 750 ppm lead, average escape latency and traveled distance were increased, indicating significant impairment in spatial learning and memory. These results are more direct evidence that indicate developing brain is more susceptible to Pb^{2+} induced neurotoxicity.

Keywords: Lead (Pb^{2+}); Spatial learning and memory; Morris water maze task; Rat.

Introduction

Lead is a common environmental neurotoxicant that remains as a significant public health problem. It is frequently found in air, drinking water, soil and industrial by products, and also found in lead associated work places such as smelting, battery manufacture, stained glass manufacture, and lead based paint abatement (1). The clinical and animal research have suggested that early exposure to low levels of pb is related to persistent cognitive impairments (2-4).

The central nervous system has been recognized as a primary target site for lead -induced toxicity (5). Epidemiological investigations have repeatedly associated chronic developmental lead exposure with several adverse effects on the neurobehavioral system. These include diminished intelligence, reduced

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learning and memory capacities and deficient IQ scores in children (6, 7). Also animal study indicated similar neurological abnormalities and memory deficit following exposure to Pb^{2+} during gestation or developmental period (8).

These effects have been shown to persist in the adulthood, after exposure has ceased and body burdens have diminished (9).

Neurotoxic effects of lead on different site of brain were reported by several studies but the hippocampus has been the focus of much research on lead effects. Hippocampus is an area, known to undergo morphological changes in rats exposed to lead during development (10). Also it is reported that effect of lead on NMDA (a glutamate receptor) on hippocampal cells is greater than on cortical cells. (11) These finding support the hypothesis that hippocampus is specifically vulnerable to lead. The hippocampus has been shown to be necessary for several types of learning and memory formation in rat and other mammals (12). Also, it is reported that the hippocampus plays a particularly important role in processing and remembering of spatial and contextual information (13) and there is some evidence from animal studies that alternations in the hippocampus disrupt the animal's ability to learn spatial relations (14).

It appears that children are more sensitive to lead intoxication than adults (15) and because of that the effect of chronic lead exposure on developmental individuals have received considerable attention, and therefore, scarce information of lead exposure on the cognitive functions of adult animals is available. In this study, we evaluated performance of female pregnant rats in Morris Water Maze that exposed to lead ten days prior to mating and during pregnancy and their offspring at postnatal day 56 (PND 56). The relationship of learning and memory between dams and offspring has been considered.

Experimental

Animals

Adult female Albino-wistar rats were obtained from the breeding colony of the Pasture Institute of Iran. They were housed in a temperature and light controlled room under a 12/12h light/dark cycle (light on at 07:00) with food and water provided ad libitum.

Experiments

Experiment 1

The aim of this experiment was to assess the effect of lead exposure on female pregnant rats. The animals were divided into three groups and exposed to 250 and 750 ppm Pb as Pb acetate and Na acetate through drinking water, ten days prior to mating. To prevent the formation of Pb precipitate, 0.5 ml of glacial acetic acid was added while stirring to prepare 1000 ml of both solutions (Na and Pb), all solutions prepared freshly (16). Two female and one male rat housed in each cage to mating for five days, then male rat from each cage was removed and female rats was screened for pregnancy, pregnant animals were tested in the swim task at gestation day 14±2.

Experiment 2

The aim of this experiment is to assess the effect of lead exposure on offspring of female rats that were exposed to lead during pregnancy.

Another set of female rats were chosen and received the same concentration of Pb acetate through the same manner mentioned above, for dams exposure to Pb acetate continued throughout gestation and lactation. Litters were culled to eight pups on postnatal day 1 (PND 1). Whenever possible, only male rats were kept within the litter and females were kept just to maintain equal litter sizes. Pups were weaned at 21 days of age and in each lead treated groups divided into two groups, maternally exposed group which weaned on control diet, then exposed to lead only during pregnancy and lactation, continuously exposed group which weaned on dams diet and treated with this diet until experiment time that was 56 days of age. Only male rat was tested and all rats were tested for Morris water maze at 56 days of age. Maternal body weight and fluid consumption were measured on a weekly basis throughout pregnancy and lactation, also, litters body weight from birth time until experiment time (PND 56) were measured on a weekly basis.

Morris water maze task

Behavioral testing of pregnant and PND56

rats in all groups were conducted in Morris water maze. The water maze was a black circular tank (136 cm) in diameter and 60 cm in height. The tank was filled with water (20±2°C) to a depth of 25 cm and was located in a room containing several extra maze cues. The Plexiglas escape platform used for the spatial task was submerged at a depth of 1 cm of water surface. Rats received one training block consisting of four trials in a day and were tested on four consecutive days. A trial was started by placing the rat in the pool facing the wall in one of four quadrants delineated by marks at the four cardinal directions. Rats were allowed to swim to the hidden platform and the escape latency (time to find the hidden platform) and path length (distance traveled to the hidden platform) were recorded. If an animal did not escape within 90 s, it was manually guided to the escape platform by the experimenter. Rats were allowed to rest on the platform for 20 s between each trial. This procedure was repeated with each rat from starting positions in all four quadrants. The submerged platform was located in the same quadrant on every trial. Visual test were performed by extending a flag above the water level from the submerged platform and located it in adjacent quadrant The maximum time allowed was the same as the original training sessions. This test was performed on the last day of training. This procedure is believed to provide information on the possible non-specific effects involving motor, visual, or motivational abilities unrelated to learning and memory. In an effort to further characterize the potential effects of Pb on other factors which may influence the ability of the rat to learn the spatial task, swim speed for each rat was measured during training (17, 18).

Determination of lead in brain

Brain Pb^{2+} levels were determined by graphite furnace atomic absorption spectrometry as described previously (19). For measurement of Pb^{2+} in the brain, animals were anesthetized and then transcardially perfused with 100 ml of normal saline to remove blood from the brain tissue then the whole brain was collected. To prepare 10% (w/v) brain homogenates, the whole brain was homogenized at an appropriate mixture of 0.5 N nitric acid, 0.5 N perchloric acid and 0.01% Triton X-100. To determine Pb^{2+} concentration in samples the same volume of each samples and 0.2% magnesium nitrate (as a modifier) was mixed and 10 µl was injected into graphite furnace of atomic absorption spectrophotometer. Because the homogeneity of regional Pb^{2+} concentration in the rat brain has been reported (20), we elected to use the whole brain.

Statistics

We used repeated measure analysis of variance (RANOVA) to assess daily performance of rats in each group and one-way ANOVA to assess differences between groups. Post hoc analyses were made by Tukey's test. A P value of 0.05 or less was considered statistically significant. All results have been shown as mean±SEM.

Results

Experiment 1

Evaluation of escape latency and traveled distance during training days in the Morris water maze

During four days training in the Morris water maze for all groups in this experiment ,control, 250 ppm and 750 ppm PbOAc treated groups, escape latency and traveled distance measure decrease each day RANOVA analysis shows significant differences between days in control ($F_{3,21}$=49.05, $p<0.0001$ for latency, $F_{3,21}$=47.5, $p<0.0001$ for traveled distance), 250 PbOAc treated ($F_{3,21}$=46.87, $p<0.0001$, for latency, $F_{3,21}$=55.3, $p<0.0001$ for traveled distance) and 750 PbOAc treated ($F_{3,21}$=28.32, $p<0.0001$, for latency, $F_{3,21}$=34.3, $p<0.0001$ for traveled distance) groups, Tukey's post hoc analysis shows significant difference between the first day of training and the other days in all groups for escape latency and traveled distance measure (Figure 1).

Effects of systemic administration of PbOAc on escape latency, traveled distance and swimming speed during training trials

Statistical analysis of average escape latency, traveled distance and swimming speed during training days between control and PbOAc treated groups showed no significant differences for

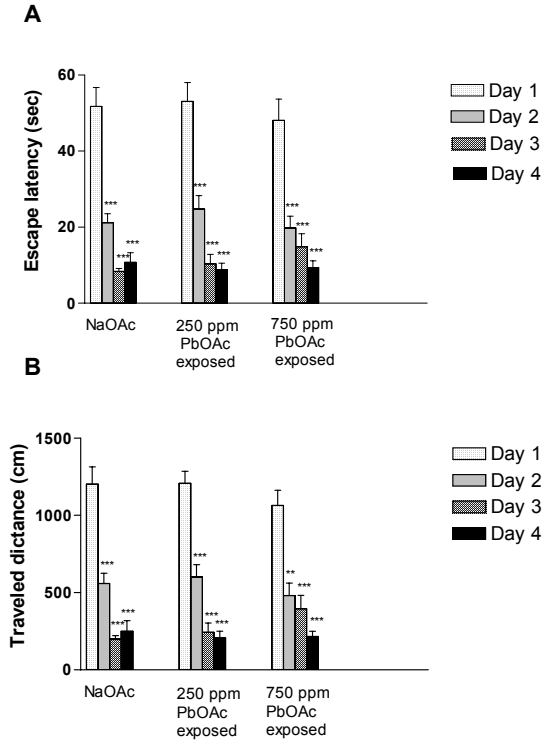

Figure 1. Comparison of learning between pregnant rats exposed to PbOAc. The figure shows that all groups of the animals learned how to find hidden platform. RANOVA analysis shows significant differences in escape latency (A) and traveled distance (B) within all groups, Tukey's post hoc analysis shows significant difference between the first day of training and the other days in all groups for escape latency (***p<0.001) and traveled distance (***p<0.001). Each bar graph shows Mean±SEM for 8 animals.

escape latency ($F_{2,93}$=0.043, p=0.95), traveled distance ($F_{2,93}$=0.028, p=0.97) and speed ($F_{2,93}$=0.15, p=0.85). No significant differences in each day of training between control and Pb^{2+}-exposed groups in latency (Day 1, $F_{3,21}$=0.24, p=0.78; Day 2, $F_{3,21}$=0.71, p=0.5; Day 3, $F_{3,21}$=1.7, p=0.2; Day 4, $F_{3,21}$=0.24, p=0.78) and traveled distance (Day 1, $F_{3,21}$=0.68, p=0.5; Day 2, $F_{3,21}$ =0.64, p=0.53; Day 3, $F_{3,21}$=2.6, p=0.09; Day 4, $F_{3,21}$=0.19, p=0.82) were observed.

Performance of animals in visible platform in all groups were the same and there were no significant difference for escape latency ($F_{2,21}$=1.89, p=0.4) and traveled distance ($F_{2,21}$=2.1, p=0.1) between groups.

Experiment 2

Evaluation of escape latency and traveled distance during training days in the Morris water maze

After four days training in the Morris water maze for all groups in this experiment, control, 250 ppm and 750 ppm PbOAc maternally- and continuously-exposed groups, learned how to find the hidden platform as indicating by decreasing escape latency and traveled distance measure each day, RANOVA analysis shows significant differences between days in control(latency, $F_{3,27}$=45.54, p<0.0001, traveled distance, $F_{3,27}$=63.05, p<0.0001), 250 ppm PbOAc maternally exposed (latency, $F_{3,27}$=24.95, p<0.0001, traveled distance, $F_{3,27}$=17.42 p<0.0001), 250 ppm PbOAc continuously-exposed (latency, $F_{3,27}$=58.63, traveled distance, $F_{3,27}$=63.21 p<0.0001), 750 ppm PbOAc maternally exposed (latency, F3, 27=28.05, p<0.0001, traveled distance, $F_{3,27}$=12.31, p<0.0001) and 750 ppm PbOAc continuously-exposed (latency, $F_{3,27}$=75.29, p<0.0001, traveled distance, $F_{3,27}$=45.34, p<0.0001) groups. Tukey's post hoc analysis shows significant differences between the first day of training and the other days in all groups for escape latency and traveled distance measure. In control group significant differences were not observed between the second, third and forth days but in all PbOAc-exposed groups significant differences were observed between the second and forth days (Figure 2).

Effects of systemic administration of PbOAc on escape latency, traveled distance and swimming speed during training trials

Exposure of rats to Pb^{2+} in both maternally- and continuously-exposed groups increased their average escape latency and traveled distance during four days of training compared to control group that received NaOAc, One-way ANOVA analysis showed significant treatment effect (latency, $F_{4,195}$=4.05, p<0.01, traveled distance, $F_{4,195}$=5.24). Tukey's post hoc analysis showed significant difference between 750 ppm both maternally- and continuously-exposed groups and control group (Figure 3 A-B).Although There was also an increase in escape latency and traveled distance in animals that received PbOAc at 250 ppm concentration, but these changes were not significant (Figure 3 A-B). The swimming speed was not altered during

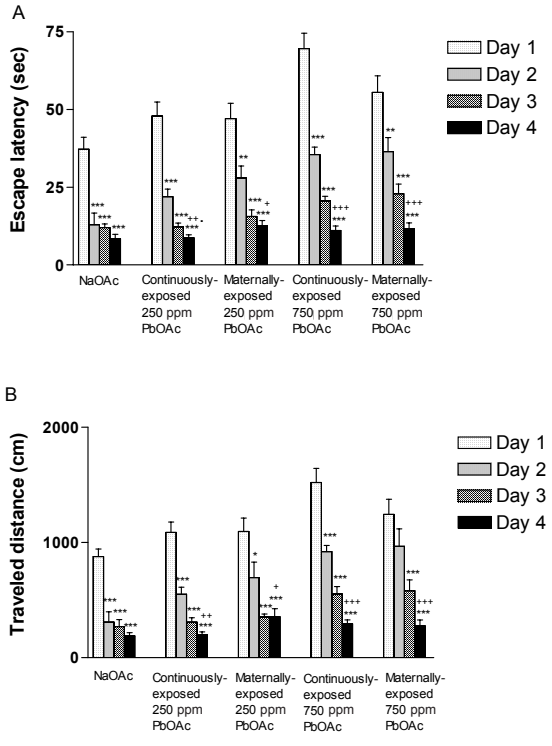

Figure 2. Comparison of learning between developmentally exposed rats to PbOAc in experiment 2. There are significant differences between the first day of training and other days in all groups (***p<0.001), (*) shows significant differences between the first day and the other days, (+) shows significant differences between the second and forth days, in control group significant difference were not observed between the second, third and forth days but in all PbOAc-exposed groups significant differences was observed between the second and forth days. Each bar graph shows Mean±SEM for 10 animals.

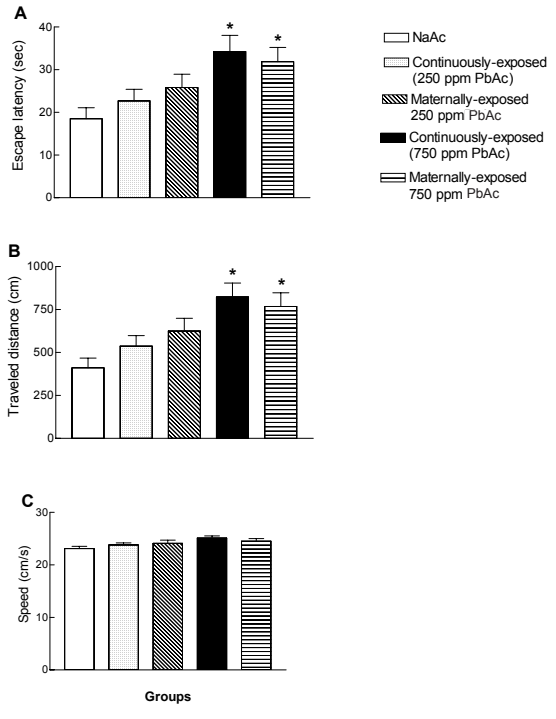

Figure 3. Average escape latency (A), traveled distance (B), and swimming speed (C) across all training days. The figures show both escape latency and traveled distance were significantly increased (*p<0.05) in both maternally-and continuously-exposed groups treated with 750 ppm PbOAc compared to the control group. Similarly, both latency and traveled distance increased in maternally-and continuously-exposed groups treated with 250 ppm PbOAc, but the changes were not statistically significant. The swimming speed in all groups show no significant differences (C). Each bar graph shows Mean±SEM for 10 animals.

training trials in any of the animal groups, suggesting that Pb^{2+} exposure did not cause any motor disturbances involvement in performance tests. (Figure 3 C)

Comparison of animals in each day of training between control and Pb^{2+}-exposed groups indicated a significant difference in latency and traveled distance at the first (latency, $F_{4,36}$=5.3, p<0.01, traveled distance, $F_{4,36}$=4.6, p<0.01), second (latency, $F_{4,36}$=8.01, p<0.001, traveled distance, $F_{4,36}$=6.85, p<0.001) and third (latency, $F_{4,36}$=6.07, p<0.01, traveled distance, $F_{4,36}$=5.1, p<0.01) day of training (Figure 4) and post test analysis showed that the differences between750 ppm maternally-, continuously-exposed groups and control group were significant. On the forth day of training all none of the groups of animals including control, maternally and continuously Pb^{2+}-exposed rats showed no significant differences in escape latency ($F_{4,36}$=2.25, p=0.08) and traveled distance ($F_{4,36}$=1.3, p=0.28) (Figure 4 A-B)

Average escape latencies measured using a visible platform were not significantly different for all groups (latency, $F_{4,36}$=2.1, p=0.1, traveled distance, $F_{4,36}$=1.9, p=0.19,) (Figure 4). These results suggest that developmental Pb^{2+} exposure had no effect on the motivational, visual or motor systems which that also contribute to their water maze performance.

Measuring concentration of Pb^{2+} in the brain

By the end of the training trials, exposure to Pb^{2+} at both 250 and 750 ppm concentrations on continuously-exposed groups and pregnant female rats, resulted in a significant increase in Pb^{2+} levels in the brain of rats compared to the control. Also, an increase in Pb^{2+} levels in the

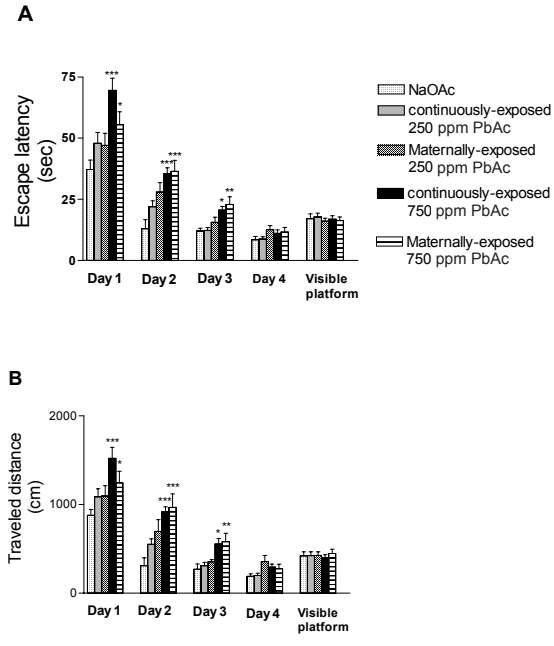

Figure 4. Comparison of escape latency (A) and traveled distance (B) during training days. The figures show the latency and traveled distance significantly increased in both maternally-and continuously-exposed rats treated with 750 ppm PbOAc at the first, second, and third days of training compared to the control group (*p<0.05,**p<0.01,***p<0.001, respectively). There were no significant differences between the treated and the control groups at the forth day of training. In visible platform test there were no significant differences in escape latency and traveled distance. Each bar graph shows Mean±SEM for 10 animals.

brain of rats in PND21 (post natal day 21) was observed in all treated groups. It was also apparent that brain Pb^{2+} levels increased in PND21 in the maternally-exposed groups but they decreased to the control group levels in the test time, Pb^{2+} concentration in maternally exposed-groups in the test time did not significantly differ compared to control group (Table 1).

Effects of PbOAc exposure on body weight and whole-brain wet weight to body weight ratio

A small reduction in body weight was observed in Pb^{2+}-treated groups at birth time and in both 750 ppm Pb^{2+}-treated groups at PND56. Exposure to lead resulted in an increase in whole-brain wet-weight to body weight ratio on PND21 and in both 750 ppm Pb^{2+}-exposed groups at PND56 (Table 2). There were not any differences between control and Pb-treated groups in maternal body weight and fluid intake.

Table 1. Pb^{2+} levels in the rat brain

Exposure groups	Brain Pb^{2+} (ng/g)
NaOAc †	157.75±11.47
Continuously-exposed † (250 ppm PbOAc)	470 ±55.1 ***
Continuously-exposed † (750 ppm PbOAc)	708.25±59.22 ***
Maternally-exposed † (250 ppm PbOAc)	183.5±20.21
Maternally-exposed † (750 ppm PbOAc)	192.5±23.37
NaOAc (PND21)	141±20.6
PbOAc 250 ppm (PND21)	441±67*
PbOAc 750 ppm (PND21)	675.4±69.5***
NaOAc (pregnant female)	157±29.3
PbOAc 250 ppm (pregnant female)	220±32.28*
PbOAc 750 ppm (pregnant female)	395.5±44***

† For all these groups, the test trials were conducted at PND 56. Brain samples were collected at the end of acquisition phase of the memory swim task. Thus, the actual ages of the rats were PND 59.
Each value represents the mean±SEM
Significant differences between control and treated groups (*p<0.05, ***p<0.01, ***p<0.001)
(PND: postnatal day)

Discussion

The present study indicates that the exposure of pregnant female rats to lead had no effect on spatial learning and memory in Morris water maze task as both escape latency and traveled distance had no significant differences between PbOAc exposed groups and control group during training days. Also it is shown that their offspring that continue to be exposed to lead after birth through breast milk and after that through drinking water, impaired in spatial learning and memory in Morris water maze task, indicated by increased average escape latency and traveled distance during training days compared to control group. Exposure of the rats to Pb^{2+} (750 ppm) in both maternally-and continuously-exposed

Table 2. Average body weight in grams and percent brain to body weight ratio.

Exposure groups	age	Body Weight (g)	Brain/Body Weight (%)†
NaOAc	At birth	5.98±0.1	
	PND21	41.52±0.63	2.39±0.07
	PND56	237.1±3.99	0.73±0.0135
Continuously-exposed (250 ppm PbOAc)	At birth	5.44±0.07*	
	PND21	34±0.87*	2.77±0.06 *
	PND56	224.8±4.11	0.76±0.009
Continuously-exposed (750 ppm PbOAc)	At birth	5.31±0.08*	
	PND21	29.95±0.44*	2.86±0.115 *
	PND56	197.78±5.7*	0.857±0.035*
Maternally-exposed (250 ppm PbOAc)	PND56	222.52±4.28	0.75±0.019
Maternally-exposed (750 ppm PbOAc)	PND56	213.22±4.22*	0.84±0.032*

The mean ± SEM are presented for each treatment group

† Brain Weight /Body Weight×100. Body weight used in this ratio were taken when brains were collected, and the actual ages of the rats in PND56 groups was PND59

Significantly different from control rats of the same age ($p<0.05$)

groups increased their average escape latency and traveled distance during four days of training compared to control group. Differences between escape latency and traveled distance between exposure of rats to Pb^{2+} in both maternally- and continuously-exposed groups compared to control group were in the first, second and third day of training days that indicate the acquisition and consolidation phase of learning are done. On the forth day of training no significant differences in escape latency and traveled distance between Pb^{2+} exposed and control groups were observed which indicates that on day four all groups learned how to find hidden platform.

The visual test indicates that the motor, motivational, and visual abilities of animals did not differ significantly among the treated groups. If functional disabilities were present in Pb^{2+}-exposed rats, the animals would have taken significantly longer to locate the visual platform. Swimming speed is considered a good measure of motor and coordination abilities in the context of the swimming task, and the general lack of any differences in swimming speed among the treatment groups indicates that motor skills were not impaired.

A moderate reduction in body weight was indicated at birth time in all Pb^{2+}-exposed rats and in both 750 ppm Pb^{2+}-exposed groups at the time of training trials. One may argue that the effects of Pb^{2+} observed in water maze performance may have been related to the effects on body weight rather than specific effects on learning and memory, but there is experimental evidence that loss in body weight is not correlated with poor performance in the Morris water maze. For instance, it has been reported that chronically malnourished male and female rats with 30-37% reductions in body weight performed similarly to age-matched controls in the Morris swim task (21).

Several lines of evidence have revealed that Pb^{2+} exposure produces neurological damage and behavioral disruptions in human and in experimental animals. It is reported that exposure to Pb^{2+} during brain development results in behavioral alternation and learning and memory deficits (16, 4). Previous studies reported that developmental Pb^{2+} exposure causes an impairment in the acquisition phase in the Morris water maze (2), radial arm maze task (22) and step-down inhibitory avoidance task (23). It has also been demonstrated that gestation-only Pb^{2+} exposure caused significant learning and memory retrieval deficits in young adult offspring (4). This impairment persists into adulthood despite

the removal of Pb^{2+} exposure at weaning (9). Recently, It is reported that developmentally low level lead exposure does not impair spatial learning when they are tested in adulthood but reduces neurogenesis in adult rat hippocampus (24), but in this study the age of rats, training protocol and Pb concentration are different as compared to their study. There is some evidence that the developing relative to the mature brain is more susceptible to neurotoxicity of lead (15) because of that there is a few studies about effect of lead on adult brain. It is reported that post-weaning exposure did not produce learning and memory impairment (9).

Our finding in present study is consistent with the previous study which indicates that developmental exposure cause spatial memory impairment; also we found that dams that were exposed to the same concentration of Pb^{2+} did not show any impairment in memory, 750 ppm PbOAc maternally exposed animals that were exposed to Pb^{2+} during gestation and lactation and their Pb^{2+} level in brain at the test time was low, showed impairment in memory. These findings are consistent with the hypothesis that there is a developmental window of vulnerability to Pb^{2+} neurotoxicity.

The hippocampal formation is widely considered to be the major site of action of lead on the brain (10), and the NMDA receptor has been demonstrated to play a key role in synaptic plasticity underlying learning and memory (25). It was found that learning and memory deficits caused by hippocampal damage are similar to those found in lead induced neurotoxicity (26). Biochemical and electrophysiological studies have shown that lead interacts with the NMDA receptor complex and inhibits receptor activation. It has been suggested that the effects of Pb^{2+} on the NMDA receptor complex may be responsible at least in part, for some of the learning deficits which have been identified in experimental animals exposed to Pb^{2+} during early development (27). There are some evidences that NMDA receptors in hippocampus of young rats are more susceptible to inhibitory affect of Pb^{2+}. In vitro studies from different laboratories have shown that inhibition of NMDA receptor function by Pb is greater in neuronal membrane preparations from young rat brain (28) or in young hippocampal neurons in culture (29) than from adult brain membranes or older hippocampal neurons in culture. Also, marked changes in NMDA receptors were measured in the hippocampus of young but not adult rats chronically exposed to Pb during development (30). Specifically, it has been suggested that the developmental expression of NMDA receptor subunits result in population of receptors expressing differential sensitivity to inhibition by Pb (31).

Some differences in brain concentration of Pb^{2+} were observed between groups. Concentration of Pb^{2+} in brain tissue of both 250and 750ppm continuously-exposed rats and in PND21 of both group were about 50% higher than that of adult rats. These differences in Pb^{2+} concentration may be due to the fact that in developing rats, the gastrointestinal tract can absorb a greater percentage of the ingested Pb^{2+} than in adults (32). Also, the blood-brain barrier, which when fully developed excludes many toxic substances from reaching the brain, is more likely to be compromised during childhood, allowing a greater percentage of the absorbed Pb^{2+} to enter the brain (21).Then, another explanation for memory impairment in developmentally exposed animals but not in adult animals ,observed in this study, is higher level of Pb^{2+} accumulation in brain tissue of developmentally exposed rats.

In conclusion, The current findings could indicate that developmentally exposed rat to Pb^{2+} show impairment in spatial memory in Morris water maze task, but dams that are exposed to the same concentration of Pb^{2+} did not show impairment in swim task performance in Morris water maze. This finding is direct evidence that demonstrate the more susceptibility of developing brain than adult brain to Pb^{2+} neurotoxicity.

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