EXPERIMENTAL STUDY

Tanshinone IIA ameliorates lead (Pb)-induced cognitive deficits and oxidative stress in a rat pup model

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ABSTRACT

OBJECTIVE: Chronic lead (Pb) exposure affects the developing central nervous system, whereas Tanshinone IIA (TSA) improves cognitive deficits.

METHODS: In this study, we investigated the effects of TSA against lead-induced neurotoxicity in a rat pup model. A total of thirty two healthy male Wistar rats were randomly divided into four groups: lead-treated group, lead plus TSA-treated 1 group, lead plus TSA-treated 2 group, and controls. After a 4-week lead exposure, memory function was determined using Morris water maze and the concentration of lead was measured in blood. Total superoxide dismutase (T-SOD), glutathione (GSH), malonaldehyde (MDA) and brain-derived neurotrophic factor (BDNF) activities were determined in hippocampus samples.

RESULTS: Lead exposure causes decrease of body weight; increase of the blood lead concentration; decrease of antioxidant activities and BDNF content. However, co-administration of TSA with lead ameliorated the weight loss. Furthermore, TSA inhibited neurotoxicity as evidenced by decreased latency period and increase in percentage of time spent in the target quadrant. Administration of TSA also improved antioxidant activities by increased T-SOD, GSH, and decreased MDA activities compared to lead-treated group.

CONCLUSION: This study provides evidence of that TSA has a neuroprotective effect against lead-induced cognitive deficit by enhancing antioxidant activities in the brain *(Tab. 2, Fig. 3, Ref. 27).* Text in PDF *www.elis.sk.*

KEY WORDS: lead exposure, Tanshinone IIA, memory, oxidative stress, neurotoxicity.

Introduction

Metals are an important gamut of pollutants because they are bio-accumulative, immutable, and non-degradable (1). Lead (Pb) is a heavy metal and common environmental contaminant that has debilitating effects in the body (2). Studies have proven a definite relationship between low-level lead exposure and development of neuronal dysfunction, especially in developing brain (3, 4). However, reversal of the neurological deficits does not take place by means of chelation therapy, and lead deposited in the brain cannot be removed by chemical chelating agents (5).

Tanshinone IIA (TSA) is a phenanthrenequinone derivative of the traditional Chinese medicine "Danshen", which is used to promote the circulation of blood and eliminate stasis (6). TSA has been shown to possess anti-inflammatory and anti-oxidative properties, and proposed as a potential therapeutic agent in heart disease, liver disease, and cancer treatment (7, 8). However, the neuroprotective mechanism of TSA on lead-induced deficits has never been investigated.

The research data suggested that oxidative stress might contribute to learning and memory deficits following oxidative brain damage (9). Oxidative stress has been identified as a common mechanism for the toxic effects of lead. Antioxidants counteract the detrimental effects of oxidative stress in biological systems. TSA can increase neural progenitor cell line viability and protect cells against mitochondrial damage owing to its anti-oxidative activities (10). In particular, TSA alleviates H_2O_2-induced hippocampal long-term potentiation impairment against the neurotoxic effects of oxidative damage (11). Therefore, TSA may ameliorate cognitive deficits and oxidative stress against lead intoxication.

Therefore this study is of great interest to investigate if TSA can be effective to improve learning and memory ability in the experimental lead-induced rat model. Moreover, the study will also attempt to delineate the association between the cognitive impairment, oxidative stress, and BDNF expression in lead-induced rat brain tissue, and the findings will enhance our understanding of the neuroprotective effect of TSA in Wistar rats exposed to lead.

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Materials and methods

Animals and TSA administration

The experiments were carried out on the male rats of Wistar strain (45–60 g, aged three weeks) obtained from Shanghai Slac laboratory Animal Co., Ltd. (China). All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Wenzhou Medical University, followed the Ethical guidelines for Investigations of Experimental Pain in Animals (12).

After a 7-day acclaim, the animals were randomly assigned into 4 groups ($n = 8$ per group). Group I received distilled water via oral gavage once daily (control group). Group II was administrated 0.2 % lead acetate as the only drinking fluid (lead exposure group). Group III was exposed to lead through drinking water and received oral administration of 4 mg/kg of TSA concomitantly (lead plus TSA-treated 1 group, LPT1). Group IV was exposed to lead through drinking water and received oral administration of 8 mg/kg of TSA concomitantly (lead plus TSA-treated 2 group, LPT2).

The selected doses of TSA (4 mg/kg and 8 mg/kg) used in this study have been reported previously in studies on its neuroprotective effects in rat brain (13). At the end of experiment, 24 h after the last TSA treatment, all rats were weighed and then euthanized by CO_2 inhalation to examine the neuroprotective effects of TSA.

Behavioral testing

The Morris water maze has become one of the most frequently used research tools for testing the learning and memory ability of the rodents (14). Rats were evaluated using Morris Water Maze (MWM) test, as described in a previous report (15). Briefly, the apparatus consists of large circular pool (150 cm in diameter, 50 cm in height) filled to a depth of 30 cm with water at 25 ± 1 °C. The water was made opaque with milk. A submerged platform (10×10 cm), painted white was placed in the middle of the target quadrant (2 cm below surface of water). The position of platform was kept unaltered during the training period. The tank was located in a room where there were numerous extra-maze cues external to the maze. The position of the cues remained constant throughout the experiment. The procedure included training and test sessions. The behaviors of the rats (latency period, path length, swim speed, and navigation path) were monitored by a video camera mounted on the ceiling above the center of the pool. Each animal was trained for four trials in one day and continued 5 days. Twenty-four hours after the last training session, a space exploration trial was performed.

Blood and tissue sampling

Twenty-four hours after the last behavioral test, 2 mL of blood were drawn by cardiac puncture, transferred into heparinized tubes, and centrifuged at 4000 rpm for 15 min at 4 °C. The samples were stored at –80 °C until analysis. The brain tissue samples were immediately obtained and hippocampus region was isolated on ice. Then the hippocampi were washed immediately with ice-cold saline, frozen in liquid nitrogen, and stored at –80 °C till further processing for carrying out biochemical studies. T-SOD, GSH, MDA and BDNF activity were analyzed in hippocampus samples. Pb levels were assessed in plasma samples.

Blood lead determination

The concentration of lead in blood was measured after HNO_3-H_2O_2 wet digestion using a microwave digestion system (model MARs-5; CEM, Matthews, NC). Then determination of blood Pb was performed using a coupled plasma-mass spectrometry (ICP-MS, model 7500ce; Agilent, Waldbronn, Germany). The assay wavelength was set at 283.3 nm.

Assessment of oxidative stress in hippocampus

The hippocampus samples were homogenized in 10 volumes of ice-cold sodium phosphate buffer (50 mM, pH 7.4) at 4 °C using a motor driven Teflon Potter homogenizer. The homogenate was centrifuged at 10,000 rpm for 15 min at 4 °C and its supernatant was separated for the assessment of oxidative stress level. The activity of T-SOD, the levels of GSH and MDA were measured using ELISA assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

T-SOD levels were estimated by using the method as described previously (16). MDA content was measured by the method of

Tab. 1. Effect of lead and TSA on body weight of the control, lead, LPT1, and LPT2 groups.

Groups	Body weight (g)				
	0st week	1st week	2nd weeks	3rd weeks	4th weeks
Control	92.6±8.7	121.4±11.5	161.3±16.9	218.9±19.5	272.4±24.6
Lead-treated	97.1±9.6	114.7±10.8	136.2±13.2	162.9±14.4[#]	201.7±19.2[##]
LPT1	95.8±9.2	116.4±12.9	142.2±15.7	174.5±24.3[#]	219.3±21.6[#]
LPT2	94.2±8.3	119.4±11.7	147.5±18.7	177.6±15.2[#]	221.8±21.7[#]

Data are expressed as mean ± SEM, n = 8 rats per group. Superscript letters represent the statistically significant findings by ANOVA followed by Tukey's multiple comparison tests. [#] p < 0.05, [##] p < 0.01 when compared with control group

Tab. 2. Lead concentrations in blood of the control, lead, LPT1, and LPT2 groups.

Groups	Control	Lead-treated	LPT1	LPT2
Blood(μg/dl)	2.18±0.11	245.27±3.53[##]	241.36±3.16[##]	256.69±3.84[##,*]

Data are expressed as mean ± SEM, n = 8 rats per group. Superscript letters represent the statistically significant findings by ANOVA followed by Tukey's multiple comparison tests. [##] p < 0.01 when compared with control group; * p < 0.05, when compared with lead exposure group
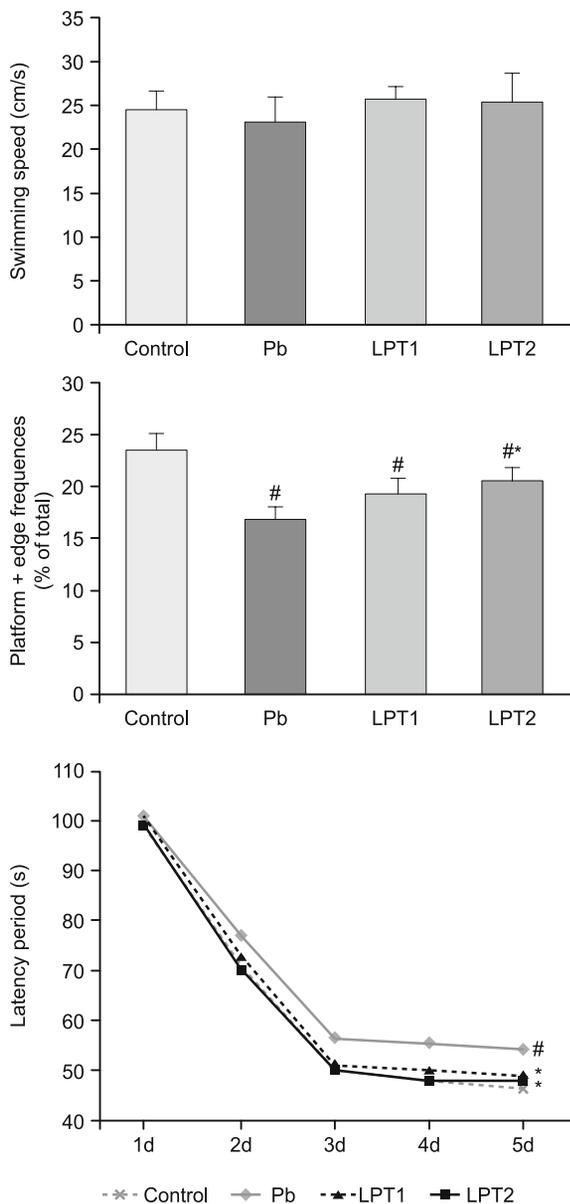
Fig. 1. TSA attenuates Pb-induced injuries in spatial reference memory. **(A)** Swimming speed in the water maze on the first day of the training; **(B)** The percentage of time spent in the target quadrant, as indicated by the percentage of time swimming across the area or the edge of the target quadrant to the four quarters. **(C)** Latency to find the hidden platform in the water maze. The data are expressed as means ± SEM, $^{\#}p < 0.05$ when compared with control group, $*p < 0.05$, when compared with lead exposure group.
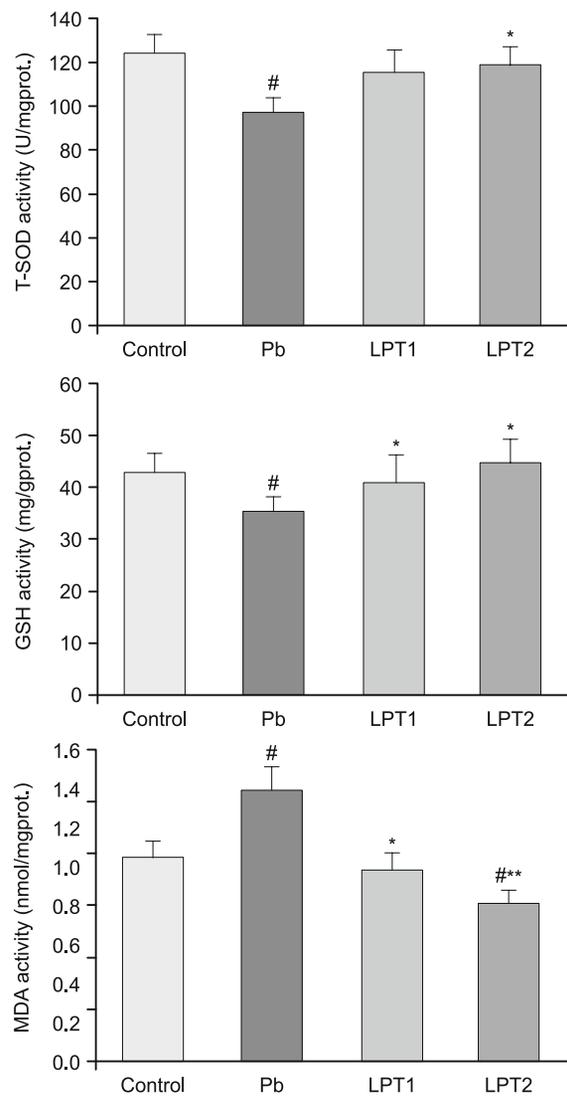

Fig. 2. Histogram showing the selected parameters which indicate the extent of oxidative stress in hippocampus in all four groups. **(A)** Effects of lead and TSA on T-SOD activity in the hippocampus. **(B)** Effects of lead and TSA on GSH content in the hippocampus. **(C)** Effects of lead and TSA on MDA content in the hippocampus. The data are expressed as means ± SEM, $^{\#}p < 0.05$ when compared with control group, $*p < 0.05$ and $**p < 0.01$, when compared with lead exposure group.

Buege and Aust (17). GSH levels in hippocampi were estimated by employing the method as described previously (18).

BDNF level determination

The BDNF levels were measured using sandwich enzyme-linked immunosorbent assay kit according to the manufacturer's protocol. The concentration of BDNF in the sample was determined using a microplate reader (Biotech ELx800) at 450 nm wavelength. The standard curve of BDNF that shows a direct relationship between BDNF concentration and corresponding ODs (absorbance) was established to calculate the concentration in the sample.

Statistical analysis

All Data are expressed as mean±SEM. Data were analyzed by a one-way analysis of variance (ANOVA) followed by Tukey's multiple range test. p<0.05 was considered as statistically significant.
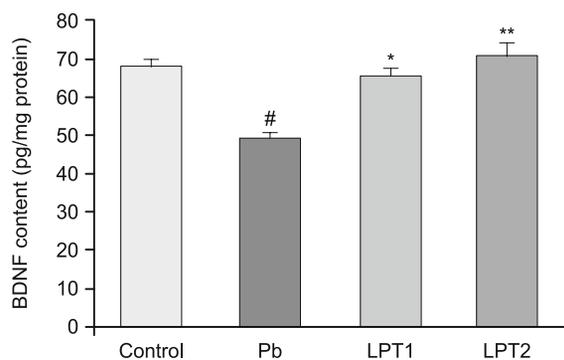
Fig. 3. Histogram showing the effects of lead and TSA on BDNF level in hippocampus of four groups. The data are expressed as means ± SEM, #p < 0.05 when compared with control group, *p < 0.05 and **p < 0.01, when compared with lead exposure group.

Results

Effect of lead and lead+TSA on body weight

The change in body weight of the tested rats after the treatment with TSA for 4 weeks is summarized in Table 1. Body weights at the beginning of the treatment period were not significantly different among the four groups. In contrast to control group, there was a weight loss among the lead exposure groups after 3 weeks of induction. However, treatment with TSA had no significant effect on body weight of lead exposed rats.

Effects of TSA on lead-Induced injuries to rats' spatial reference memory

The possible protective effect of TSA against Pb-induced neurotoxicity was evaluated in an in vivo Pb exposure rat pup model. The blood lead concentrations (BLCs) showed that the BLCs in the lead-treated group and lead + TSA groups were much higher compared with the control group after Pb exposure for 4 weeks (Tab. 2). However, high-dose TSA induced a slight increase in BLCs compared with that of lead exposure group (lead-treated: 245.27 ± 3.53 μg/dl, LPT2: 256.69±3.84 μg/dl, p < 0.05), indicating that TSA could not reduce the BLCs, at least during developmental lead exposure. Next, MWM task test was used to examine the effects of TSA on Pb exposure rats' spatial reference memory. As shown in Figure 1A, B, there were no obvious differences in swimming speed among the four groups, which suggested that lead treatment or lead plus TSA treatment had no significant influence on rats' motor ability. However, Pb exposure dramatically affected animals' spatial reference memory ability, and lower percentage of time spent in the target quadrant (platform + platform edge) was observed in the lead-treated group as compared with the control group (Control: 23.63 ± 1.52 %, lead exposure: 16.85 ± 1.24 %, p < 0.05). Furthermore, in rats treated with Pb it resulted in a significant increase in escape latency compared with the control (Control: 46.29 ± 0.73 sec, lead exposure: 54.11 ± 1.06 sec, p < 0.05), however, shorter latency period was observed in LPT1 and

LPT2 groups as compared with lead exposure group (lead-treated: 54.11 ± 1.06 sec, LPT1: 49.16 ± 0.88 sec, LPT2: 47.96 ± 0.91 sec, p < 0.05) (Fig. 1C). To some extent, TSA treatment alleviated lead-induced impairment in the spatial reference memory, but it cannot entirely reverse the lead-induced depression.

Effects of TSA on lead-induced alterations in hippocampus antioxidant activities

The selected parameters that indicate the extent of oxidative stress in Pb exposed rats' hippocampi are summarized in Figure 2. The mean concentration of T-SOD in the hippocampus of the lead-treated group (96.38 ± 5.72 U/mgprot.) was significantly lower than in the control (123.64 ± 8.57 U/mgprot., p < 0.05) and LPT2 groups (118.29 ± 8.36 U/mgprot., p < 0.05). Moreover, GSH levels in the hippocampus of the lead-treated group (35.77 ± 2.63 mg/gprot.) were significantly lower than in the control group (43.17 ± 3.95 mg/gprot., p < 0.05), LPT1 group (41.43 ± 5.31 mg/gprot., p < 0.05) and LPT2 group (45.22 ± 4.31 mg/gprot., p < 0.01). In addition, the MDA hippocampus concentration was significantly higher in the lead-treated group (1.38 ± 0.12 nmol/mgprot.) when compared to the control group (1.04 ± 0.08 nmol/mgprot., p < 0.05), LPT1 group (0.97 ± 0.06 nmol/mgprot., p < 0.05) and LPT2 group (0.81 ± 0.09 nmol/mgprot., p < 0.01). In summary, we found lower levels of T-SOD and GSH, as well as higher level of MDA, in the lead-intoxicated group, and lower MDA level, but higher T-SOD and GSH levels, in the TSA-protected groups, when compared with the control group.

Effects of TSA on hippocampal BDNF content in lead-induced rat

To determine the underlying mechanism of TSA's neuroprotective effects, BDNF protein contents were detected in the hippocampus using ELISA assays. The BDNF contents were found to be significantly decreased in lead-treated group (49.37 ± 1.36 pg/mg protein) as compared to control group (68.18 ± 1.62 pg/mg protein, p < 0.05) (Fig. 3). Notably, administration of TSA was able to significantly ameliorate the lead-induced decrease in the hippocampal BDNF contents.

Discussion

The results of this study suggest that administration of lead acetate increased the uptake of Pb by the blood. TSA possesses neuroprotective effects against lead-induced neurobehavioral deficits by improving spatial reference memory, ameliorating cognitive impairment and increasing antioxidants status.

In the present study, Rats' body weights in lead group, LPT1 group and LPT2 group were significantly different compared to the control group owing to Pb exposure. Similar decrease in the body weight was observed in growing or adult rats exposure to lead acetate (19) On the other hand, it has been reported that Pb has no effect on body weight (20). In contrast, according to the results of other studies it has been demonstrated that exposure to Pb increased body weight (21).

The mechanisms underlying the toxicity of Pb are supposed to impair spatial reference memory. Our data demonstrated that the

BLCs in rat pups co-treated with Pb and TSA were much higher than that in the control group, however, a slight increase was induced in LPT2 group compared with that of Pb exposure group. These results suggest that neuroprotection of TSA against lead-exposed neurotoxicity may not affect the absorption or elimination of Pb. In the MWM test, the behavioral experimental data suggested that treatment with TSA effectively diminished lead-induced cognitive function impairment. These data indicated that lead exposed rats showed significantly longer latency period and lower percentage of time spent in the target quadrant than the control animals. Hence, lead exposure affected animals' spatial reference memory ability. In agreement with our findings, memory loss and impaired spatial learning has been associated with Pb exposure in experimental animal model (22). Also high dosage of TSA significantly increased percentage of time spent in the target quadrant and decreased the latency period. Performances of rats during the MWM test in the present study showed that although there was not a significant influence on rats' motor ability, TSA treatment alleviated lead-induced impairment in the spatial reference memory.

As one of the important complex mechanisms of Pb toxicity, oxidative stress has been reported in different animal models (23). In the present study, we found increased level of lipid peroxidation as shown by the higher MDA level and lower antioxidant enzymes in Pb exposure rats' hippocampi compared to the control group as shown by the T-SOD and GSH levels. In agreement with this, oxidative stress associated with Pb exposure has been noted in experimental animals and humans (24). Wang et al (11) also reported that lower T-SOD activity and higher MDA content were found in Pb exposure 30-day-old mice. These findings suggest that TSA acts by increasing the antioxidant capacity to mitigate the effect of the oxidative stress induced by Pb.

One of the best indicators for the impact of pollutants, like heavy metals, is the level of antioxidant enzyme. The T-SOD level of tissues is a good biochemical parameter of those toxicological effects. Meantime, GSH is also an important compound for the detoxication and excretion of heavy metals. Increased MDA content, as well as decreases in reduced GSH were noted in lipid peroxidation from lead-treated rats. TSA showed protective effect against oxidative damage in the liver (25) and kidney (26). In this study, Pb decreased T-SOD and GSH activities, and increased MDA level in the hippocampus of animals. Against the lead-treated rats, higher T-SOD and GSH activities and lower MDA content were observed in LPT and LPT2 groups. As an antioxidant, TSA is capable of reducing the activity of T-SOD, increasing the inhibition of the serum MDA activity via the HO-1, Akt and p38 MAPK signaling pathway.

BDNF is a neurotrophin that has an important function in the development, survival, and function of neurons. Stansfield et al (27) revealed that Pb exposure decreases BDNF gene and protein expression during the period of synaptogenesis of hippocampal neurons in culture. Consistent with these findings we have shown in our study a decreased concentration of BDNF in the lead-treated rat pups compared to control animals. And treatment of TSA (LPT1 and LPT2) was able to significantly ameliorate lead-induced decrease in the hippocampal BDNF contents.

In conclusion, the present results indicated that developmental Pb exposure during critical periods of brain development caused significant oxidative stress in the hippocampus of rat pups. Moreover, the administration of antioxidant agent such as TSA can ameliorate these effects of Pb toxicity.

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