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Effect of lead sulfide nanoparticles exposure on calcium homeostasis in rat hippocampus neurons

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ABSTRACT

PbS nanoparticles (NPs) is an important nanomaterial for biomedical imaging in living tissues. However, concerning the high toxicity, especially neurotoxicity, of Pb element, it is crucial that the toxicity assessment of "naked" PbS NPs should be adequately studied. In the current study, we systematically explored the neurotoxicity of PbS NPs in rats by measuring the body weight and brain coefficient changes, testing memory behaviors in Y-electric maze, and studying the neuronal ultrastructure and pathology in hippocampus. Furthermore, in order to study the toxic mechanism, we performed Pb and Ca content measurements in various organs, and investigated Ca^{2+}-ATPase activity and L-type calcium channel subunit expression. Our results confirmed that PbS NPs showed high neurotoxicity, while a possible mechanism was suggested to be due to the PbS NPs-induced calcium homeostasis disorder which was caused by the abnormal calcium transportation.

1. Introduction

Currently, the potential toxicity of nanoparticles (NPs) is highly concerned due to the rapid growth of NP applications in living beings [1,2]. With the advantages of small size and unique properties, nanoparticles have been widely used in consumer products, food, drugs, and medical devices. Typically, lead sulfide (PbS) nanoparticles have shown great promising for biomedical imaging in living tissues [3–6], since they have the unusual and important properties of large exciton Bohr radii, large optical dielectric constant, narrow fundamental bandgaps, negative pressure coefficients and large static dielectric constants [7–10]. However, lead is highly toxic and as an important nerve poison in the environment, it can cause nervous system damage, especially in learning and memory [11]. In order to block its direct contact with living tissues and lower its potential toxicity, surface coating method has been reported for preparing shell or ligands coated PbS nano-composites [4,5]. These coated-PbS NPs, however, still have the risks of incomplete coating and possible degradation under certain in vivo conditions. Therefore, the toxicity assessment of "naked" PbS NPs is an important issue and till now, it has not been adequately studied.

The hippocampus is a very important area of the limbic system [12], and highly correlated with learning and memory, emotion behavior and neuroendocrine activity. In hippocampus neurons, a major intracellular messenger that mediates many physiological responses of neurons to chemical and electrical stimulation is calcium ion (Ca^{2+}) [13,14]. As an important second messenger, Ca^{2+} signaling connects membrane excitability and cell biological functions of neurons. From studies of cultured neurons, excessive Ca^{2+} loading has become central to most hypotheses of excitotoxic neuronal injury [15,16]. In addition, evidence indicated that neuronal Ca^{2+} signaling was abnormal in many of neurodegenerative disorders [17]. Intracellular calcium homeostasis disorder may lead to neuronal damage, leading to the learning, memory dysfunction [18]. Previous study has suggested that lead may interfere the cytoplasmic calcium level ($[Ca^{2+}]i$) homeostasis and the long-term potentiation (LTP), showing neurotoxicity [19].

Herein, in order to examine the possible PbS NPs neurotoxicity in rats, we systematically monitored the body weight and brain coefficient variations, memory behavior, Pb content and Ca content. It is found that PbS could influence calcium homeostasis significantly. Based on the

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Ca^{2+}-ATPases activities assay and L-type calcium channel subunits expression studies, it is clear that the calcium homeostasis is caused by the abnormal transmembrane transport of Ca^{2+}.

2. Experimental

2.1. Materials and animals

Male Sprague Dawley (SD) rats (180–240 g body weight) were purchased from Beijing (License number SCXK jing 209-00047). Male, age-matched rats were bred and maintained in-house and used at the age of 12–14 weeks. They were housed under controlled temperature and light conditions with free access to standard rat chow and water. Care and use of the laboratory animals were in accordance with NIH guidelines and the local Ethics Committee. Chemicals and bio-reagents were bought from Sinopharm and Sigma.

2.2. Synthesis of PbS nanoparticles

PbS NPs were synthesized by the reported colloidal chemistry method [20,21]. Briefly, a solution of 0.1 M Na_2S was added dropwise to an equal-volume of 0.2 M $PbCl_2$ solution under vigorous agitation. The as-prepared products were washed with water and ethanol for several times. X-Ray diffraction analysis was carried out using an X-ray diffractometer (XRD, Model D/MAX2500, Rigaka) with Cu Kα radiation (λ = 1.54056 Å). The morphology of the as-prepared products was characterized by transmission electron microscope (TEM, JEOL-2010).

2.3. Experimental groups and treatments

The SD rats were randomly divided into 3 groups (20 rats per group) based on their body weight after adapting to the new environment. Control group was exposed to physiological saline; low exposure group (PbS NPs: 15 mg/kg) and high exposure group (PbS NPs: 30 mg/kg) were exposed to PbS NPs by trachea once every 7 days for 3 consecutive months (13 perfusion times in total).

Rats' weight changes were monitored every week and general indexes variation was calculated. Electricity maze experiment was used to detect the activity of learning and memory. The whole brain samples were weighted and the brain coefficient was calculated according to the formula (brain coefficient (%) = brain quality (g) / body quality (g) × 100%). Fluo-3/Am calcium ion fluorescence indicator was used to measure Ca^{2+} level in hippocampal neurons. Ca^{2+}-ATPase activities and hippocampal pathology were performed.

Hippocampus was conventionally fixed in 2.5% glutaraldehyde solution, dehydrated, penetrated, ultra-thin sliced and electron stained. Ultrastructure changes were observed with a transmission electron microscope (TEM, Hitachi H-7650).

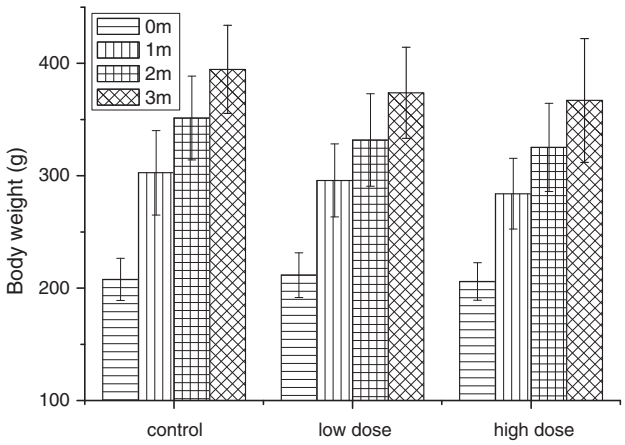

Fig. 2. Body weight values.

3. Results

3.1. Characterization of PbS nanoparticles

The morphology of the PbS sample was examined by transmission electron microscopy. Fig. 1a shows a typical TEM image from which uniform nanoparticles with average diameter of 36.8 nm (calculated with ImageJ software) could be seen obviously. Powder X-ray diffraction was used to characterize the crystal structure of the obtained products. As shown in Fig. 1b, it can be seen that the XRD pattern is in conformity with cubic PbS (JCPDS: 05-0592). The observed peaks could be assigned to diffraction from the (111), (200), (220), (311), and (222) faces and there is no characteristic peak for other impurities.

3.2. Body weight

After exposure to PbS NPs for 0–3 months, body weight of each rat was measured. The data (Fig. 2 and Table S1) shows that there was no significant difference between the three groups ($P > 0.05$).

3.3. Brain coefficient

As the exposure dose increased, the brain coefficient also increased (Fig. 3 and Table S2). The coefficient of the brain increased in the low and high dose group compared with the control group respectively ($P < 0.05$).

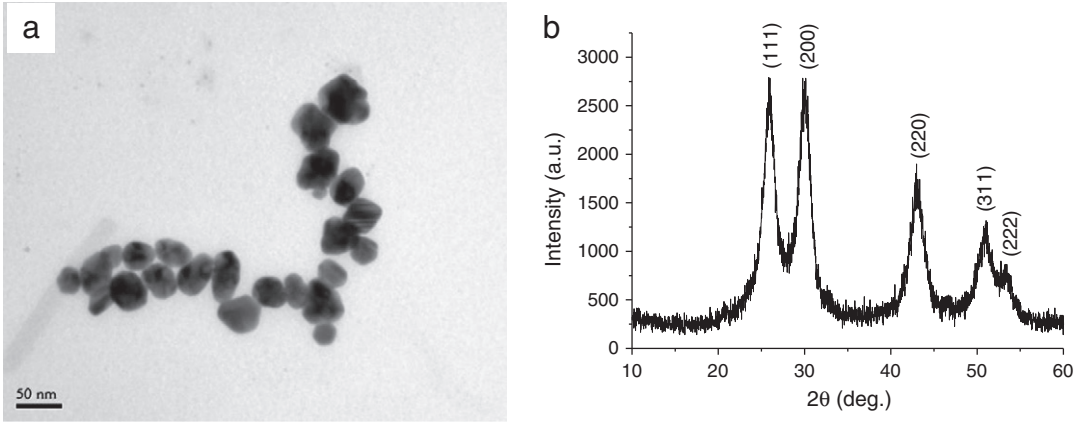

Fig. 1. Characterizations of PbS nanoparticles: (a) TEM image, and (b) XRD pattern.

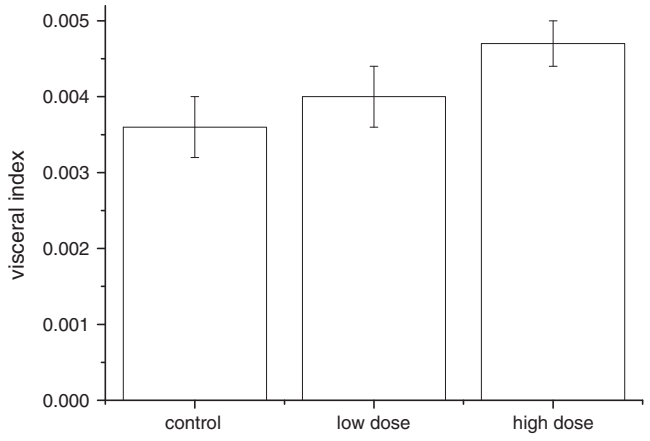

Fig. 3. Brain coefficient values.

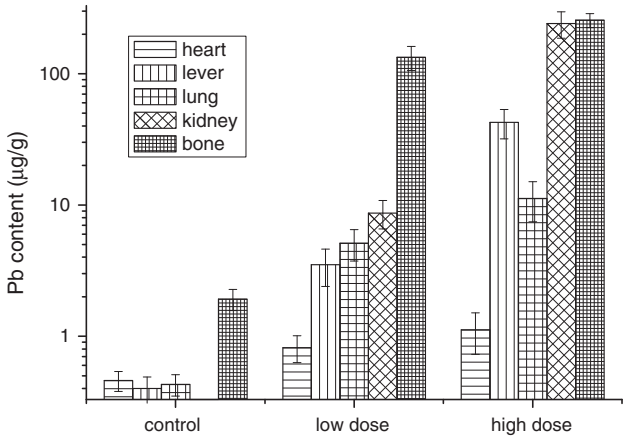

Fig. 5. Pb levels in organs.

3.4. Memory behavior

To detect the ability of learning and memory of rats that were treated with PbS NPs for three months, we used Y electric maze test in dark and quiet environment. Results of Y-electric maze test were showed in Fig. 4 and Table S3. With the increase of PbS concentration, average number of errors and escape latency were increased ($P < 0.05$).

3.5. Pb levels in organs

The lead levels in organs, blood and brain structures including hippocampus and cortex have been determined. We observed that in all measured tissues lead levels increased significantly as the treatment dose increased ($P < 0.05$). Compared with heart and lung, high lead levels were found in liver and kidney after high dose PbS NP treatments. The lead levels were particularly high in bone regardless of the dose of PbS NPs treatment, indicating the accumulation of lead (Fig. 5 and Table S4). In blood and brain structures, lead levels increased approximately 3.58 (low dose treatment) and 6.45 (high dose treatment) times in blood, 5.29 and 8.24 times in cortex, 10.33 and 13.40 times in hippocampus (Fig. 6 and Table S5).

3.6. Calcium concentration in hippocampal neurons

In order to examine whether PbS NPs affect $[Ca^{2+}]i$, the change of $[Ca^{2+}]i$ was measured using the Ca^{2+} indicator Fluo-3/AM. $[Ca^{2+}]i$ in neurons were showed in Fig. 7 and Table S6. With the increase of PbS NPs concentrations, $[Ca^{2+}]i$ increased. Calcium fluorescence index was 63.88 ± 8.16 in control group. However, in high dose PbS NPs-treated group, $[Ca^{2+}]i$ increased approximately 1.42 fold ($P < 0.05$).

3.7. Ca^{2+}-ATPase activity assay

After neurons were treated with PbS NPs for three months, we observed a significant increase of Ca^{2+}-ATPase activity in a concentration-dependent manner (Fig. 8 and Table S7). In low dose and high dose PbS NP-treated groups, Ca^{2+}-ATPase activities significantly increased approximately 13.89 and 33.55% ($P < 0.05$).

3.8. Effect of PbS NPs on neuronal ultrastructure in hippocampus

As shown in Fig. 10, there were clear neuronal caryotheca, large nucleolus and well-distributed chromatin in hippocampus for control group. PbS NPs caused neuronal ultrastructure changes including nuclear membrane shrinkage and lysosomal inclusion bodies in hippocampus.

3.9. Expression of L-type calcium channel α1 and β subunits on rat hippocampal neurons

The expression of L-type calcium channel α1 and β subunits are all in CA1 area of neurons in hippocampus. Immunohistochemical staining was employed for characterizing L-type calcium channel α1 and β subunits on rat hippocampal neurons. With PbS NP dose increasing, strong

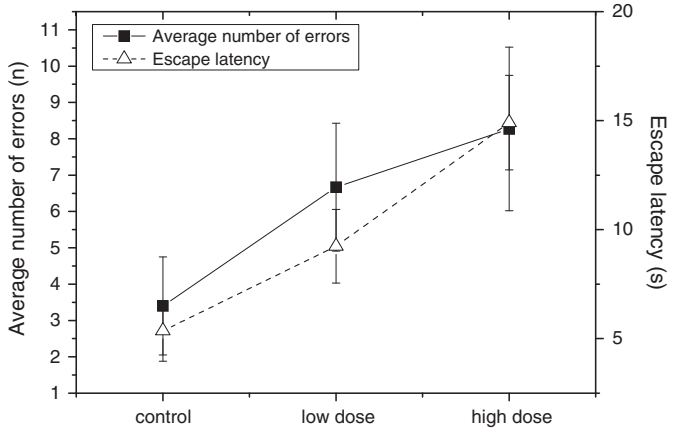

Fig. 4. Memory behavior.

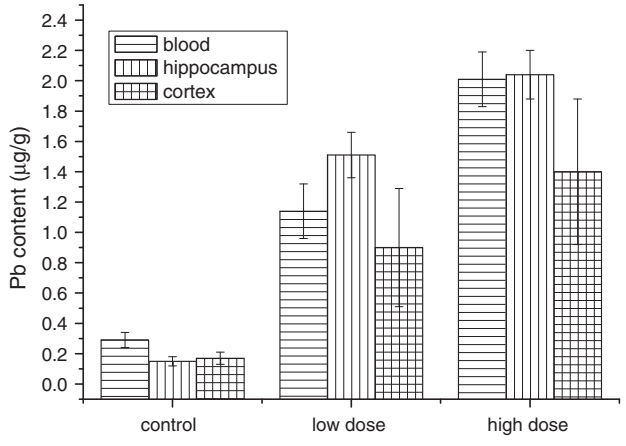

Fig. 6. Pb levels in blood, hippocampus and cortex.

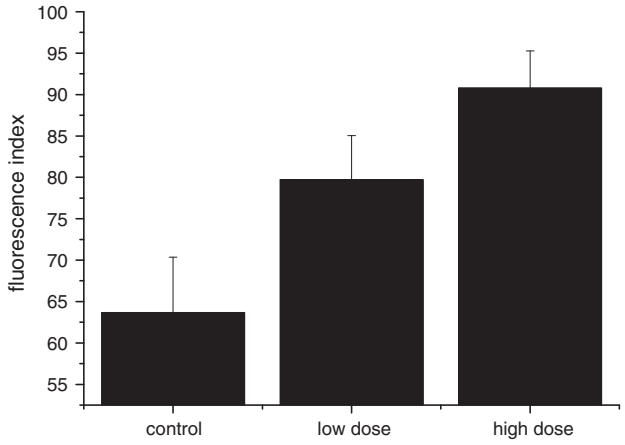

Fig. 7. Ca levels.

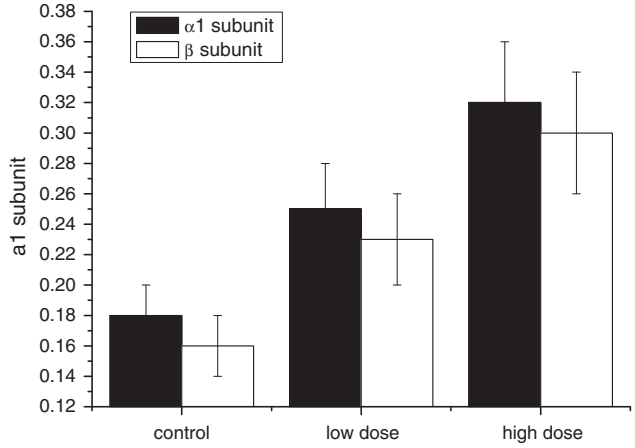

Fig. 9. Protein-expression of L-type calcium channel α_1 and β subunits in hippocampus.

immunohistochemical staining in the neuron's membrane could be observed, indicating that the expression of L-type calcium channel α1 and β subunits increased significantly (Figs. 9 and 11 and Table S8).

3.10. Hippocampal pathological findings

Hippocampal pathology pictures were shown in Fig. 12. For control group without PbS NP treatment, hippocampal cells with similar size arranged in neat rows could be observed. For low dose-treated group, the arrangement of hippocampal cells is not well anymore, while the cell contour is unclear and vacuolization could be seen. Furthermore, slight increases in the inter-cell distance and cell number, as well as a decrease in the cell volume are found. Compared with the control and low dose-treated groups, the above changes could be observed more obviously from the high dose-treated group. The cell contour is unclear and the number of cells increases significantly. The distance between cells is large and the arrangement is messy. Nucleus becomes pyknotic and strong vacuolization is found.

4. Discussion

In animal toxicology studies, bogy weight change and more importantly, organ coefficient change, are two widely used, objective and simple indicators for poisoning assessment. Specifically, brain coefficient, which reflects the ratio of brain weight to body weight, is very important in assessing the brain and nerve injury [22]. In the present study, we found that the body weights of PbS NPs-treated rats were slightly lower than that of the saline-treated rats, while no statistically significant difference was observed between these groups. However, significant difference in the brain coefficient could be found between PbS NP-treated groups and the control group. Given the evidence that the brain coefficients have increased after PbS NP treatments, there may have some abnormities such as congestion, edema, or hypertrophy in the brain tissue [22].

On the other hand, the neurobehavioral changes were assessed by a Y-electric maze test which was used for the detection of learning and memory in rats. The results showed that the memory behavior of average wrong choice of frequency increased and escape latency extended. In order to further study the PbS NPs caused brain damage, we carried out the lead contents measurements and neuronal ultrastructure characterization. It is not surprising that the lead contents in all measured

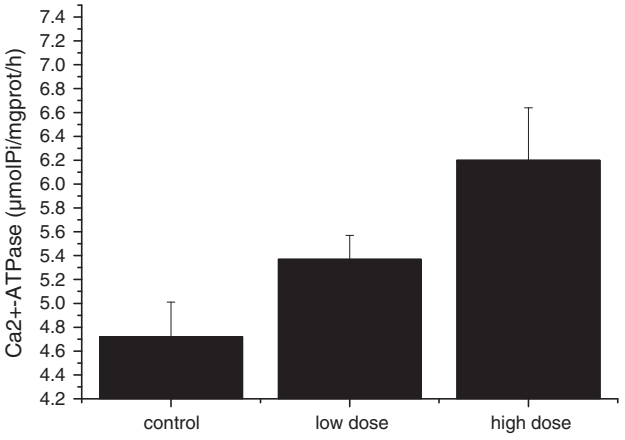

Fig. 8. Ca^{2+}-ATPase activity assay.

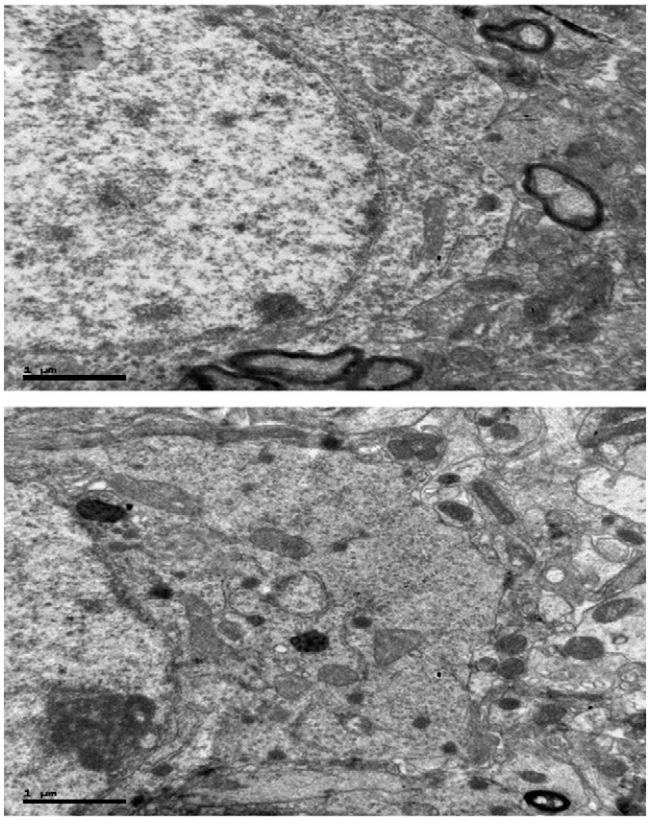

Fig. 10. Effect of PbS NPs on neuronal ultrastructure in the hippocampus (top: control group, bottom: high dose group).

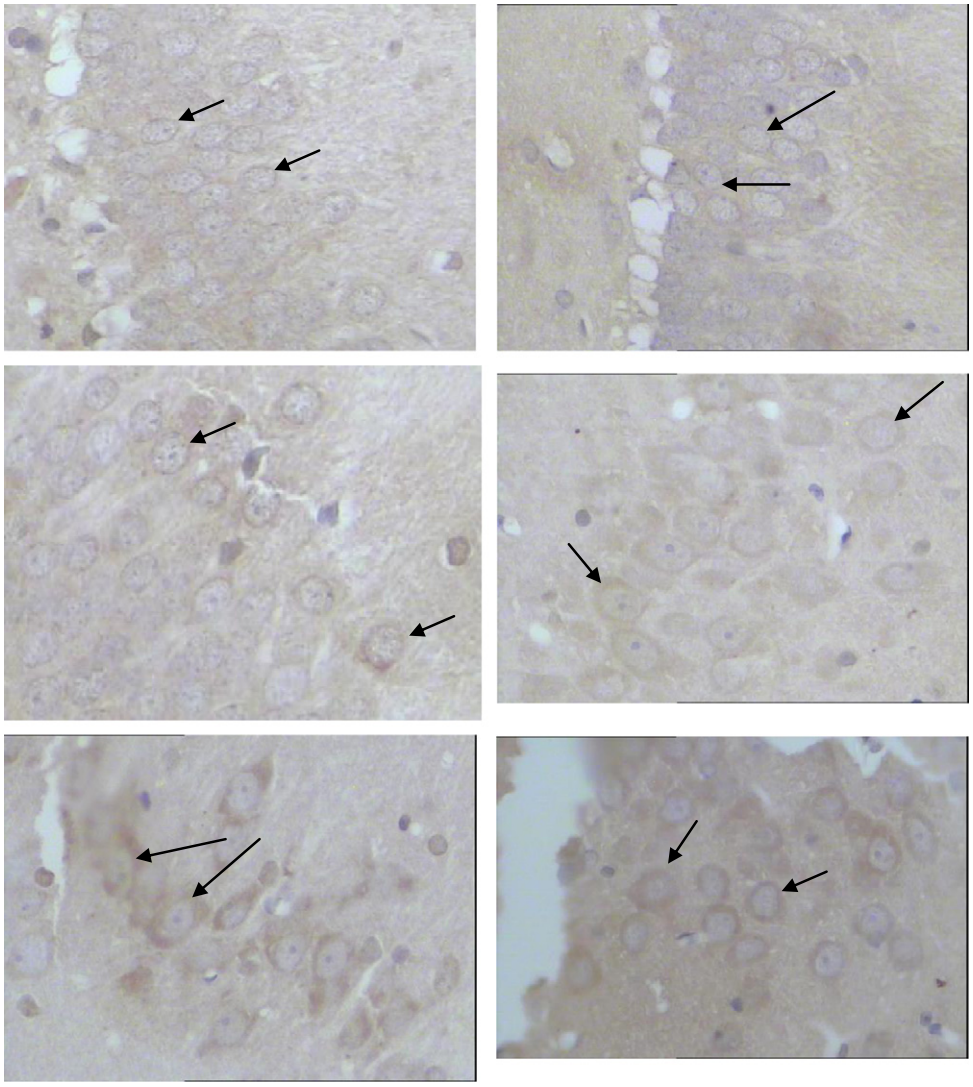

Fig. 11. Expression of L-type calcium channel α1 subunit (top left: control, middle left: low dose, bottom left: high dose) and β subunit (top left: control, middle left: low dose, bottom left: high dose) in CA1 area of neurons in hippocampal (×400). The black arrows indicate the locations of α1 and β subunits on rat hippocampal neurons.

organs, especially in hippocampus, increased significantly after exposure to PbS NPs, implying that PbS NPs can enter the brain tissue via the respiratory tract and therefore the lead accumulation in the hippocampus can damage learning and memory. This phenomenon is consistent with reported works [23–25] that lead exposure can damage rats' LTP. This hypothesis further supported the neuronal ultrastructure characterization with TEM method. As shown in Fig. 10, neuronal ultrastructure changes including nuclear membrane shrinkage and lysosomal inclusion bodies in hippocampus were observed in PbS NPs-treated groups.

In order to study the mechanism of PbS NPs induced neurotoxicity, we carried out the $[Ca^{2+}]i$ measurement in hippocampal neurons. Previous studies [19,26] have indicated that lead can lead to imbalances of intracellular calcium. Alshuaib et al. [27] showed that the $[Ca^{2+}]i$ was significantly higher in nerve cells of chronic lead exposed rats compared with controls, and the $[Ca^{2+}]i$ in nerve cells was also significantly correlated with the lead content in blood. Fox et al. [28] find that with the increase of lead dose and time of incubation, the $[Ca^{2+}]i$ increased significantly in hippocampal neurons of rats. Our data also showed that compared with the control group, hippocampal cellar $[Ca^{2+}]i$ level of PbS NP exposed rats was significantly higher, indicating that PbS NPs will also influence the intracellular calcium content, and therefore influence the rat's LTP.

Concerning the increasing of cytoplasmic Ca^{2+} concentration, there may have two reasons. One could be caused by the increasing of Ca^{2+} flow into the cell; the other one is the possible decreased outflow of intracellular Ca^{2+}. Regarding the Ca^{2+} inflow, a possible reason is the increased expression of membrane calcium channels, leading to endoplasmic reticulum and mitochondrial Ca^{2+} release and increased cytoplasmic Ca^{2+}. L-type voltage-dependent calcium channels (L-VDCCs) is a long process Ca^{2+} channel, consisting of α1, α2, β, γ and δ subunits. Two α1 subunits are the key parts of the ion channel, while other subunits are auxiliary subunits. In the present study, we found that in the PbS NP exposed rats, both α1 and β subunits of the L-type calcium channel over-expressed in the cell membrane in the CA1 region of the hippocampal. This result suggested that the over-expression of α1 and β subunits may lead to the increased opening of calcium channels in hippocampus cell membrane, causing increased Ca^{2+} influx and elevated $[Ca^{2+}]i$.

On the other hand, ATP-driven plasma membrane calcium pump (Ca^{2+}-ATPase) and Na^{+}/K^{+} exchanger are of crucial importance in maintaining a low intracellular Ca^{2+} concentration by controlling the outflow of Ca^{2+}. Ca^{2+}-ATPases exist in cell membrane and endoplasmic reticulum, muscle plasma, and is an important carrier that can transport cytoplasm Ca^{2+} to extracellular environment or endoplasmic reticulum. However, in this study, it was found that both the cytoplasmic

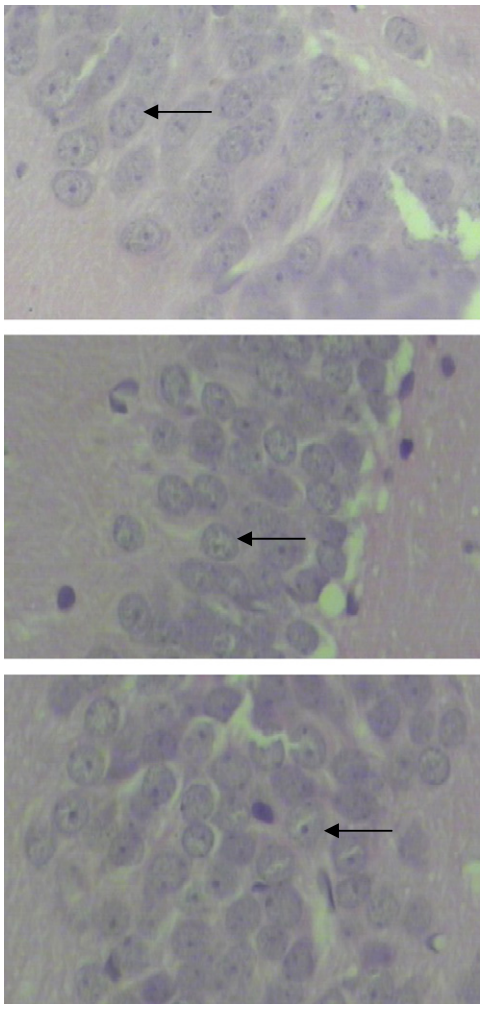

Fig. 12. Hippocampal pathology pictures (top: control, middle: low dose, bottom: high dose, ×400). The black arrows indicate the hippocampal cells.

Ca^{2+} concentration and the Ca^{2+}-ATPase activity increased in rats with PbS NP exposure. We suggest that the disorder of cytoplasmic Ca^{2+} induced the increased activity of Ca^{2+}-ATPase, which is a compensatory mechanism.

Hippocampus is the key to learning and memory. LTP is one of the possible mechanisms of hippocampal memory formation [29], in which calcium plays a key role. Lead may affect the intracellular free calcium levels through a variety of ways, leading to interfering with cell signaling, and damaging learning and memory abilities. In this study PbS NP exposure can cause inflammation of central nervous system, which could be the result of lung inflammation factors induced systemic inflammation through circulation system.

5. Conclusions

In conclusion, the present results showed that PbS NPs could induce neuronal damage by increasing $[Ca^{2+}]i$. Due to the complexity of the central nervous system, more studies need to focus on clarifying the exact neuronal damage mechanism. However, these results could give insight into the neurochemical alternations taking place in NPs-treated neurons.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jinorgbio.2013.05.008.

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