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Neurotoxicological Evaluation of Long-Term Lanthanum Chloride Exposure in Rats

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With their widespread application in industry, agriculture, medicine, and daily life, rare earth elements (REEs) are widely used in various fields and eventually accumulated in human body. Therefore, understanding the effects of REEs on health has become more and more important. In this work, the neurotoxicity of lanthanum (La) was evaluated. Wistar rats were exposed to lanthanum chloride through oral administration at 0, 0.1, 2, and 40 mg/kg doses from gestation day 0 through 6 months of age. Experiments were carried out to reveal the effects of La exposure on brain functions from four aspects including behavioral performance, $[Ca^{2+}]_i$ level and the activity of Ca^{2+}-ATPase (adenosine triphosphatase) in hippocampal cells, oxidative stress, and Nissl staining. Adverse effects were observed in 2 and 40 mg/kg dose groups and increased with dose. Morris water maze test showed that La exposure at 2 and 40 mg/kg could significantly impair the behavioral performance. (The preference for the target quadrant decreased by 16.6% and 19.4% versus control, respectively.) The neurotoxicological consequences demonstrated that the alteration in homeostasis of $[Ca^{2+}]_i$/Ca^{2+}-ATPase (the ratio of $[Ca^{2+}]_i$ vs. Ca^{2+}-ATPase activity increased by 44% in rats of 40 mg/kg group), the inhibition to activities of antioxidant enzymes, and the subsequent cell damage (18% and 23% cell loss in CA3 subregion of rats in 2 and 40 mg/kg group, respectively) might be involved in the neurological adverse effects of REEs exposure.

Key Words: **intracellular free calcium; lanthanum; Morris water maze; Nissl staining; oxidative stress; rare earth elements.**

Rare earth elements (REEs) constitute a series of 15 transition elements starting with lanthanum (La) (atomic number 57) and ending with lutetium (atomic number 75). Although REEs are not abundant in the earth's crust, cerium (Ce), the most plentiful element of rare earth, is about 100 times more abundant than cadmium (Cd), one of the most well-known heavy metals in toxicology. It was reported that over 900 kg each of cerium, La, and neodymium were released into the environment annually by the coal-fired electric power plants in Europe (Goetz *et al.*, 1982), and these quantities exceeded the environmental releases of mercury and cadmium by 50 times, of selenium, uranium, or tin by 10 times, and of arsenic or molybdenum by two to three times (Sabbioni *et al.*, 1982). The REEs compounds frequently have magnetic, catalytic, and optic properties and therefore they are widely used in industry (Dobrynina *et al.*, 1997). The world consumption of REEs in industry was 84,000 tons rare earth oxides in 2003. In China, they are also used in agriculture (Evans, 1990). REEs-containing fertilizers are applied to over 6 million hectares of farmlands per year. REEs have been used directly in humans for therapy of cancer, inflammation and synovitis and for diagnosis by magnetic resonance imaging (Hirano and Suzuki, 1996). Recently, lanthanum carbonate has been proposed as a calcium- and aluminum-free phosphate binder for the treatment of hyperphosphatemia of chronic renal failure (D'haese *et al.*, 2003). It turns out to be that REEs are in large scale used in various fields and eventually accumulated in human body via inhalation, food chain and clinical treatment.

According to the Hodge-Sterner classification system (Hodge and Sterner, 1949), REEs are generally considered to be of low toxicity. Oral LD_{50} of 2000 mg/kg to more than 10 g/kg body weight has been described for simple rare earth compounds (oxides, chlorides, nitrates, sulfates, acetates, etc.) (Evans, 1990) and no observed adverse effect levels of 250 mg/kg body weight per day have been determined for rats (Tian *et al.*, 1992). However, the results of epidemiological investigations of natural and local populations that reside in the REEs high background regions of south China imply that REEs might be neurotoxic. A study completed the Hiskey-Nebraska Test of Learning Aptitude on children aged from 6 to 9 years and found that the mental age and intelligence quotients (IQ) of the children living at the REEs-high regions were obviously lower than those at normal regions (Zhu et al., 1996). Drawing A Man Test on children aged 7–10 years also showed that the IQ of children living near a REEs ore area was significantly lower than those at normal regions (Fan *et al.*, 2004). A significant negative impact on the signal conduction velocity was found in

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the people lived in rare earth mining area and adverse effects were also found in rat after a long-term intake of small doses of REEs, suggesting REEs exposure could cause subclinic damage in the central nervous system (Zhu *et al.*, 1997a). The neurotoxicity of REEs has been confirmed by several recent animal experiments. Briner *et al.* (2000), studied the neurodevelopmental effects of La exposure, indicating that La was a potential behavioral teratogen with subtle but detectable effects on mouse development. Liu *et al.* (2002) found that long-term intake of REEs in drinking water could disturb metabolism and distribution of some trace elements in rat brain. Yang *et al.* (2006) found that REEs exposure changed the expression of some genes in rat brain and suggested that this might be responsible for the neurotoxicity of rare earths.

In our previous work, the penetrability of REEs across the blood–brain barrier has been proven using radioisotope tracer technique (Xiao *et al.*, 2005a, b). Moreover, We found that chronic exposure to La could impair the learning ability and this deficit may be possibly attributed to the disturbance of the homeostasis of trace elements, enzymes and neurotransmitter systems in brain (Feng *et al.*, 2006a, b). The aim of the present study was to explore the further association between the impaired spatial learning and memory abilities and the altered physical and physiological conditions of brain. The study as a whole was designed to evaluate the neurotoxicity of long-term lanthanum chloride exposure at relative low levels.

MATERIALS AND METHODS

Animals and treatment. $LaCl_3$ solution was prepared from La_2O_3 (99.99%, China Medicine Group Shanghai Chemical Reagent Corporation, Shanghai, China) and hydrochloric acid. Female Wistar rats (age 2 months) were obtained from the Animal Center of Perking University Health Science Center. The animal experiments were carried out in compliance with the ethics guideline for the care and use of animals in research (formulated by Perking University Health Science Center). Each dose group had 10 maternal rats. During the experiment, the maternal rats were housed separately in a polycarbonate cage under a controlled environment of room temperature (19–23°C), humidity (40–60% relative humidity), and lighting (12/12-h light/dark cycle). After 1-week adapting feeding, the female rats mated with males of the same age and strain. Evidence of a copulatory plug was used to indicate successful mating and day 0 of pregnancy. From gestation day 0 through postnatal day 20 (PND20), the pregnant rats were housed singly and orally exposed to $LaCl_3$ by gavage feeding under one of four doses (0, 0.1, 2, 40 mg/kg/day). All the pregnant rats were allowed to litter normally and nurture their offspring. Access to food and water was *ad libitum*. The day of birth was designated PND0. On PND0, female pups were picked out, and all litters were randomly culled to 5 males. On PND21, the male offspring were weaned, separated from their mothers and culled to four per cage randomly and exposed to $LaCl_3$ by gavage feeding under the same dose as their maternal rats until they were killed. The four groups (40 male offspring each) were labeled as C rats (rats of control group), L rats (rats of low-dose group), M rats (rats of middle-dose group), and H rats (rats of high-dose group).

Morris water maze test. At the age of 6 months, 15 rats of each group were randomly chosen to be tested in Morris water maze as described previously (Morris, 1984), with slight modifications. Briefly, a black circular pool (150 cm in diameter and 50 cm in height) filled with water (23–25°C), was placed in the middle of an experimental room with fixed visual clues on walls. Carbon black ink was used to render the water opaque and the pool was divided virtually into four equal quadrants, a black escape platform (9 cm in diameter) was placed in the middle of one quadrant (the target quadrant) and submerged 1.5 cm below the water surface. During one session, a rat was introduced into the pool at different starting points and allowed to swim until the small platform was found. Rats were left on the platform for 15 s. If the platform was not found within 90 s, the animals were directly placed on the platform for 15 s. The trial was recorded by a video tracking system and the escape latency (the time to reach the platform), the general pathway, and the average swimming speed were measured by a PC software (Beijing Lvyiyuan, Ltd., China). Each rat swam a session of four trials per day for 4 consecutive days, with each trial having a trial interval of approximately 2 min.

On the fifth day, a probe trial was performed wherein the extent of memory consolidation was assessed. Rat was placed into the pool as in the training trial, except that the hidden platform was removed from the pool. The time of crossing the target quadrant and the total time of crossing all quadrants were recorded for 1 min. The preference for the target quadrant (percentage of the time spent in the target quadrant) at this test indicated the degree of memory consolidation that had taken place after learning.

To test possible deficits in sensorimotor processes, rats were tested in the water maze with a visible platform on a new location 1 cm above the water line on the final day of training (Kamal *et al.*, 2000).

Determination of La contents by inductively coupled plasma-mass spectrometer. Six rats of each group (not been tested in Morris water maze test [MWM test]) were fasted overnight and then sacrificed under anesthesia. Serum was separated from the blood obtained via heart puncture. Saline perfusion was employed to remove the blood. The brain was collected and the hippocampus and cerebral cortex were dissected and rinsed with ice-cold physiological saline.

The brain samples were dried at −50°C for approximately 5 days in a freeze-drier, and then less than 200 mg of each dried sample was predigested in a pressure Teflon vessel with an acid solution of 4 ml of HNO_3 (70%) and 1 ml of H_2O_2 (30%) at 160°C for 6 h. The serum samples (0.2–0.6 ml) were predigested with an acid solution of 3 ml of HNO_3 (70%) and 0.5 ml H_2O_2 (30%) in the same way. In order to validate the analyses, bovine liver (SRM 1577a, National Institute of Standard and Technology, Gaithersburg, MD) and serum (GBW 09131, Institute of Environmental Health Monitoring, Chinese Academy of Preventive Medicine, Beijing, China) were used for reference samples. All the acid solution and H_2O_2 were MOS-grade reagents (Beijing Institute of Chemical Reagents, Beijing, China). The dilution water was prepared from a Milli-Q ultrapure water purification system and a Thermo Elemental X Series 7 inductively coupled plasma-mass spectrometer was used for trace element analysis.

Intracellular free calcium and Ca^{2+}-adenosine triphosphate activity assay. After finishing the MWM test, the concentration of intracellular free calcium in hippocampal cells was assayed as previously described (Dildy and Leslie, 1989), with some slight modifications. Briefly, seven rats in each group were decapitated, the brain was removed and the hippocampus was dissected on ice, and rinsed with ice-cold Hanks' Balanced Salt Solution (HBSS, containing 137mM NaCl, 5mM KCl, 0.3mM Na_2HPO_4, 6mM glucose, and 10mM N-(2-hydroxyethyl) piperazine-N'-ethanesulfonic acid (HEPES), pH 7.4). Immediately, the left hippocampus was trypsinized with 0.05% trypsin and 0.5mM ethylenediaminetetraacetic acid in HBSS, and cells below 200 mesh were collected. An aliquot of the cell suspension was taken for counting and Trypan blue staining which consistently showed over 90% cellular viability. The hippocampal cells were loaded with 5μM Fura-2/AM in HBSS, and then incubated in assay medium (containing 137mM NaCl, 5mM KCl, 1mM $MgSO_4$, 1mM $CaCl_2$, 0.3mM Na_2HPO_4, 6mM glucose, and 10mM HEPES, pH 7.4) for 10 min at 37°C, for complete hydrolysis of Fura-2/AM to Fura-2.

The fluorescence of Fura-2-loaded cells was monitored at 510 nm using a computer-assisted Spex Fluoromax spectrofluorometer, with dual-wavelength excitation at 340 and 380 nm, using 5-nm slits. The calibration was made in the

presence of 0.2% TritoxX-100 (R_{max}) followed by 25mM Ethylene glycol bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (R_{min}). The fluorescence ratios were converted into $[Ca^{2+}]_i$ values by using the calibration equation for measurements considering the dissociation constant of the Fura-2/Ca^{2+} complex as 224nM (Grynkiewicz *et al*., 1985).

The right hippocampus and cerebral cortex were respectively homogenized in 1: 9 (wt/vol) ice-cold 0.1M phosphate buffer (pH 7.4). The homogenates were kept as individual samples at −80°C for the Ca^{2+}-ATPase activity and oxidative stress assays and total protein concentrations were determined with bicinchoninic acid assay (Beyotime Biotechnology, China). The Ca^{2+}-ATPase activity was measured with kits (obtained from Nanjing Jiancheng Biological Engineering Institute) at 37°C in terms of the release of inorganic phosphate, measured colorimetrically.

Oxidative stress assays. The homogenates of right hippocampus and cerebral cortex from the seven decapitated rats were also used to investigate the degree of LPO and the activity of antioxidant enzymes. The level of LPO was measured by determining thiobarbituric acid–reactive substances following the method of Ohkawa *et al.* (1979). 1,1,3,3-Tetraethoxypropane served as malondialdehyde (MDA) standard. The values were expressed in nmol MDA/mg of total protein.

The homogenates were centrifuged at 10,000 × g for 10 min and the supernatant was used to measure the activity of antioxidant enzymes. Catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) were assayed with kits, following the instruction provided by the producer (Nanjing Jiancheng Biological Engineering Institute), the methods and procedures were described in the previous papers (Aebi, 1984; McCord and Fridowich, 1969; Paglia and Valentine, 1967). Three replicates were used for each sample.

Nissl staining. Four rats (after completing the MWM test) from each group were anesthetized deeply with sodium pentobarbital (50 mg/kg i.p.) and perfused transcardially with phosphate buffer for 10 min followed by 4% paraformaldehyde in 250 ml of 0.1M phosphate buffer. The brains were removed and postfixed by immersion in the phosphate-buffered 4% paraformaldehyde solution for 6 h and then cryoprotected in 30% sucrose for 72 h. Coronal sections (20 μm) were cut at the level of the dorsal hippocampus using a cryostat (Reichert-Jung 2700, Germany) and the sections were stained with 0.05% cresyl violet.

The numbers of surviving neurons in the CA1, CA3, and DG cell layers from three sections per animal at the dorsal hippocampal level were counted by a blinded observer using light microscopy (Olympus IX 71, Japan). Only whole neurons with visible nucleus were counted. The data were expressed as surviving cell number per square mm.

Statistical analysis. The results of each group were calculated and expressed as mean ± SEM. Between-group differences in MWM test were analyzed by the two-way ANOVA followed by *post hoc* Tukey multiple comparison for all groups. For the other data, one-way ANOVA was employed, and least significant difference (LSD) multiple comparison was applied to examine significance where appropriate. Significant difference was defined as that with a p value < 0.05. All the statistical analysis was implemented using SPSS 10.0 (SPSS Inc., Chicago, USA).

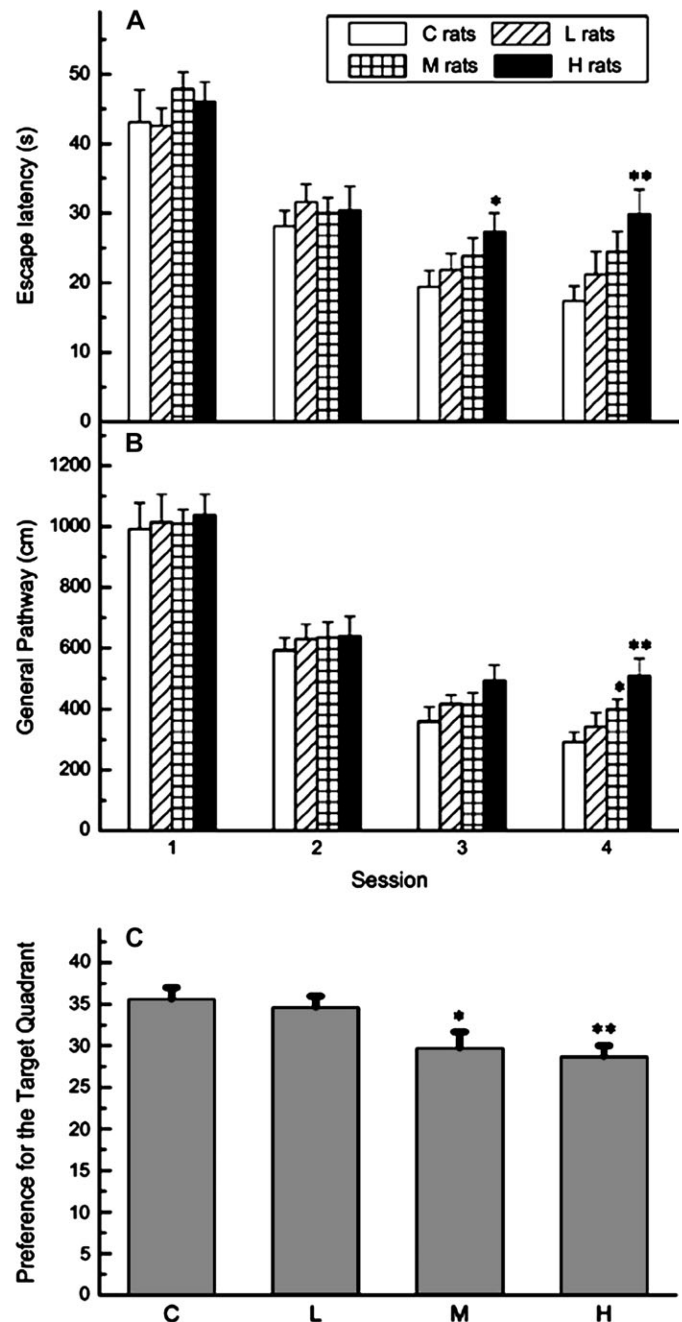

FIG. 1. Results of MWM test. (A) Escape latency in the training sessions (s). (B) General pathway in the training sessions (cm). (C) Preference for the target quadrant in the probe trial session. Data are expressed by mean ± SEM; $n = 15$. *$p < 0.05$, **$p < 0.01$ versus C rats.

RESULTS

Effects of La Exposure on Behavioral Performance

Because it was previously shown that body weight could influence the outcome of behavioral tasks, the animals were weighed before the behavioral tests and the one-way ANOVA analysis showed no significant differences in body weight (C: 479.6 ± 8.6 g, L: 479.8 ± 9.1 g, M: 476.8 ± 7.0 g and H: 472.3 ± 8.2 g, $F_{3,224} = 0.180$, $p = 0.91$).

The overall analysis of the escape latencies revealed the main effects of session ($F_{3,224} = 64.510$, $p < 0.001$; Fig. 1A) and treatment ($F_{3,224} = 4.806$, $p = 0.003$), and no interaction of session versus treatment ($F_{9,224} = 0.842$, $p = 0.578$); similarly, there were main effects of session ($F_{3,224} = 107.331$, $p < 0.001$; Fig. 1B) and treatment ($F_{3,224} = 2.722$, $p = 0.045$) to the general pathway, and no interaction of session versus treatment ($F_{9,224} = 0.437$, $p = 0.914$). The influences of motoric deficits could be excluded as there were no differences

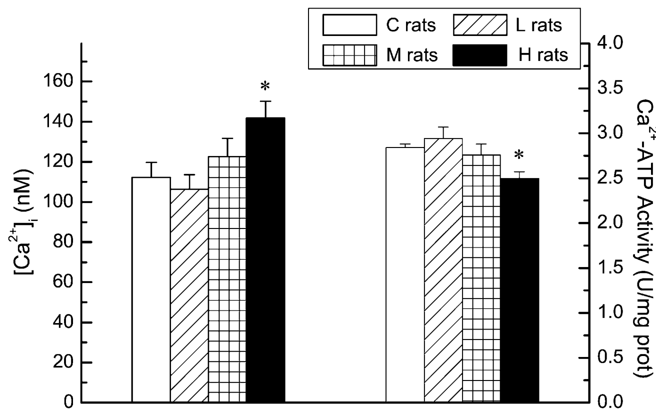

FIG. 2. $[Ca^{2+}]_i$ (nM) and Ca^{2+}-ATPase activity (U/mg protein) in the hippocampus. Data are expressed by mean ± SEM; $n = 7$. *$p < 0.05$ versus C rats.

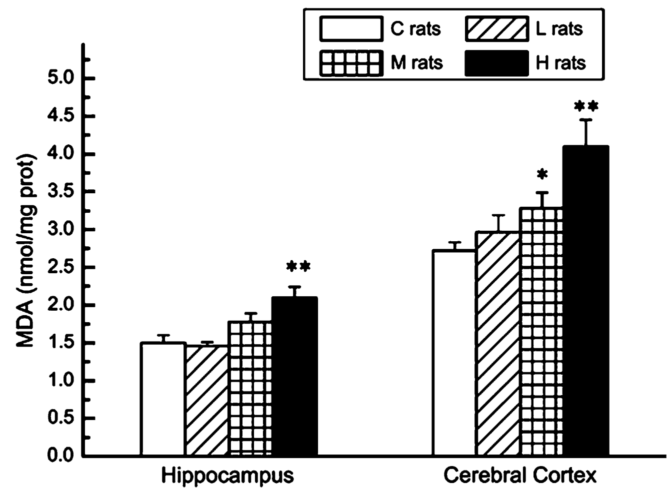

FIG. 3. MDA contents in brain regions (nmol/mg protein). Data are expressed by mean ± SEM; $n = 7$. *$p < 0.05$, **$p < 0.01$ versus C rats.

in swim speed between treatment groups ($F_{3,224} = 0.548$, $p = 0.650$; data not shown). The further analysis of post hoc multiple comparisons showed significant prolongations in the mean escape latency ($p < 0.01$) and the mean general pathway ($p < 0.05$) of the H rats, indicated impaired acquisition of spatial learning and memory after La exposure.

Spatial reference memory was tested on the fifth day in a probe trial, and the results are shown in Fig. 1C, revealing a significant difference between groups ($F_{3,56} = 4.151$, $p = 0.010$). In the probe trial, the M rats ($p < 0.05$) and H rats ($p < 0.01$) spent less time in the target quadrant than did C rats (the preference for the target quadrant decreased by 16.6% and 19.4% vs. control, respectively), indicating memory deficits in these animals.

All groups were submitted to a test of their ability to escape to a visible platform. The performance of all the groups of rats in the trial with the visible platform was not significantly different (the escape latency: $F_{3,56} = 0.246$, $p = 0.864$; the general pathway: $F_{3,56} = 0.659$, $p = 0.581$).

La Contents in Serum and Brain

Significant increases in the La contents (Table 1) were found in the serum ($F_{3,20} = 9.174$, $p = 0.001$), hippocampus ($F_{3,20} = 5.198$, $p = 0.008$) and cerebral cortex ($F_{3,20} = 78.292$, $p < 0.001$).

$[Ca^{2+}]_i$ and Ca^{2+}-ATPase Activity in Hippocampal Cells

There was an increase in the $[Ca^{2+}]_i$ ($F_{3,24} = 3.807$, $p = 0.023$) concomitant with a decrease in the activity of Ca^{2+}-ATPase ($F_{3,24} = 3.675$, $p = 0.026$) in hippocampal cells (Fig. 2). The level of $[Ca^{2+}]_i$ in hippocampal cells of the H rats was significantly elevated, whereas the Ca^{2+}-ATPase activity was decreased compared with those of the C rats, and the ratio of $[Ca^{2+}]_i$ versus Ca^{2+}-ATPase activity increased by 44%.

LPO and Antioxidant Enzymes Activities

The determination of MDA contents (Fig. 3) revealed that long-term La exposure could lead to an aggravation of LPO in hippocampus ($F_{3,24} = 8.310$, $p = 0.001$) and cerebral cortex ($F_{3,24} = 6.332$, $p = 0.003$). Compared with the C rats, the MDA contents in the hippocampus of H rats and in the cerebral cortex of M and H rats were significantly increased. The assays of activities of antioxidant enzymes (Fig. 4) showed that treatment did significantly affect the activities of CAT ($F_{3,24} = 11.684$, $p < 0.001$) and GPx ($F_{3,24} = 3.450$, $p = 0.032$) in hippocampus, SOD ($F_{3,24} = 3.057$, $p = 0.048$) and GPx in cerebral cortex ($F_{3,24} = 3.101$, $p = 0.046$), but no effect on the activities of SOD ($F_{3,24} = 0.576$, $p = 0.637$) in hippocampus and CAT ($F_{3,24} = 0.791$, $p = 0.511$) in cerebral cortex. The CAT contents in hippocampus of M and H rats, the SOD content in the cerebral cortex of H rats, and GPx contents in both hippocampus and cerebral cortex of H rats were decreased when compared with C rats.

Nissl Staining

Obvious loss of pyramidal cells was found in the CA3 subregion of hippocampus (Figs. 5 and 6) after a long-term $LaCl_3$ exposure ($F_{3,12} = 5.697$, $p = 0.012$; 18% and 23% cell loss in M rats and H rats, respectively); no marked histological shift was observed in the other regions.

DISCUSSION

Data from the training sessions and probe trial of the MWM study, which measures how well the animals had learned and consolidated the platform location, indicated significant differences between the four groups. The result of the escape latencies showed that H rats spent significantly longer time to

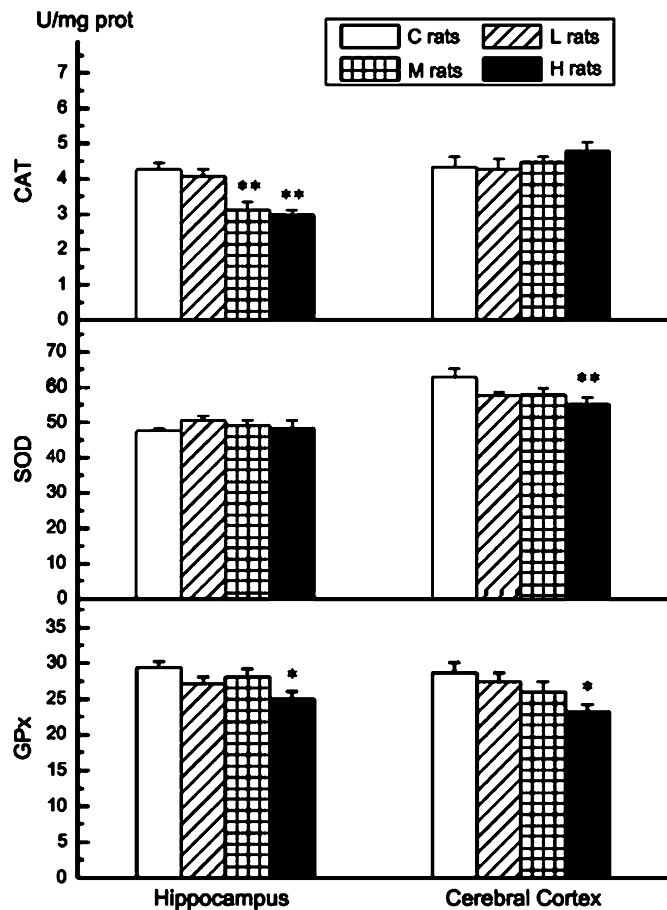

FIG. 4. Antioxidant enzymes activities in brain regions (U/mg protein). Data are expressed by mean ± SEM; $n = 7$. *$p < 0.05$, **$p < 0.01$ versus C rats.

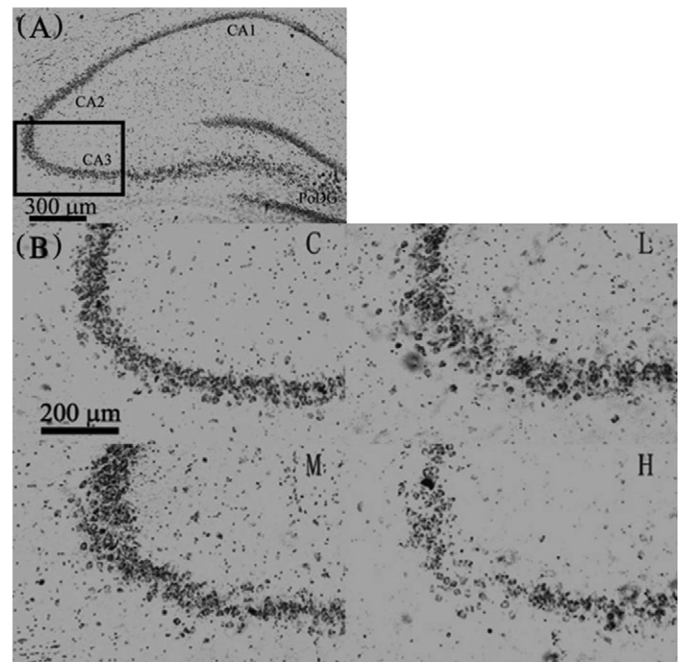

FIG. 5. Nissl-stained sections of hippocampus from the rats of four groups. (A) A low magnification photo of the rat hippocampus, scale bar: 300 µm. (B) A magnified section of CA3 cell layers, scale bar: 200 µm.

find the escape platform than C rats in session 3 and session 4. Similar results were obtained in our previous work. But in the present experiment, the application of video tracking system made it possible to record the general pathway to the escape platform and the preference for the target quadrant, and the results demonstrated that besides H rats, M rats were also affected by $LaCl_3$ exposure. Our findings suggest that the rat models we established are suitable models to research how long-term La exposure on rats results in impaired spatial learning and memory ability. Moreover, our research provides an approach to examine if there is a link between the high REEs background and the reduction of IQ of local children.

The concentrations of La in serum and brain regions are good indicators of La accumulation in rats. La contents in the hippocampus and cerebral cortex of H rats increased significantly, though the concentration of La in the serum was extremely low, which suggested La could penetrate the blood–brain barrier and accumulated in brain, and this conclusion agreed with the views in our previous reports (Xiao *et al.*, 2005a, b).

In the present work, the intracellular free calcium in hippocampal cells increased significantly after exposure to 40 mg/kg $LaCl_3$ for 6 months. Correspondingly, there was a significant decrease in the activity of Ca^{2+}-ATPase in hippocampus of H rats. Intracellular Ca^{2+} ions play an important role as the second messengers that mediate the effects of a variety of extracellular signals. There is hardly any cellular function that is not directly or indirectly affected by the ubiquitous second messenger Ca^{2+} (Cheek, 1991). Homeostatic mechanisms maintain $[Ca^{2+}]_i$ at low concentrations and are a prerequisite for normal neuronal functioning. Cellular Ca overload results in further damage to cell by reducing ATP synthesis with impaired function of the Ca^{2+}-ATPase, which in turn would increase the passage of additional Ca into the cell (Fujita and Palmieri, 2000). In other words, a decline in the activity of Ca^{2+}-ATPase would result in $[Ca^{2+}]_i$ increase,

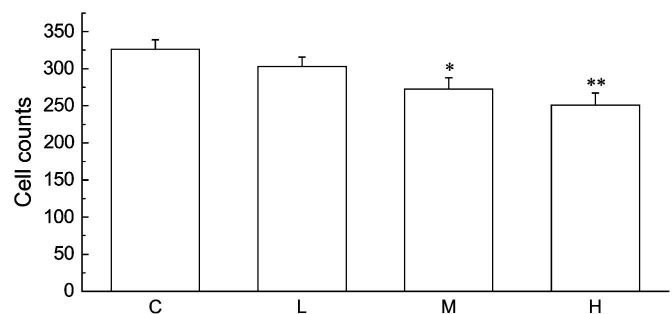

FIG. 6. The cell numbers in the CA3 region of the hippocampus (number/mm^2). Data are expressed by mean ± SEM; $n = 4$. *$p < 0.05$, **$p < 0.01$ versus C rats.

TABLE 1
Contents of La in the Serum, Hippocampus, and Cerebral Cortex of the Filial Rats (♂) at the Age of 6 m

	Dose group			
Samples	C	L	M	H
Serum[a]	< 0.0001	< 0.0001	0.0002 ± 0.0001	0.0006 ± 0.0001**
Hippocampus[b]	0.003 ± 0.003	0.008 ± 0.002	0.007 ± 0.001	0.014 ± 0.003**
Cerebral cortex[b]	0.004 ± 0.001	0.004 ± 0.001	0.007 ± 0.001	0.040 ± 0.004**

Note. Data are expressed by mean ± SEM; $N = 6$. **$p < 0.01$ versus C rats.
[a]The unit is μg/ml.
[b]The unit is μg/g dry weight.

overloaded $[Ca^{2+}]_i$ could also lead to impairment of Ca^{2+}-ATPase function. They influenced each other, and could both be regulated by other signal pathway, so it was difficult to judge which alteration was triggered first and how it took place. But the alteration in $[Ca^{2+}]_i/Ca^{2+}$-ATPase homeostasis itself could be regarded as a signal of cell damage and eventual nervous system dysfunction induced by long-term La exposure.

Activities of antioxidant enzymes, such as SOD, CAT, and GPx, are related to exposure to xenobiotics. One of the important features of these enzymes is their inducibility under conditions of oxidative stress, and such induction can be an important adaptation to pollutant-induced stress (Romeo *et al*., 2000). CAT is responsible for the reduction of hydrogen peroxide, whereas GPx catalyzes the reduction of both hydrogen peroxide and lipid peroxides and is considered an efficient protective enzyme against LPO (Winston and Di Giulio, 1991). In the present findings, the SOD and PGx activities in the cerebral cortex of H rats were inhibited. Meanwhile, the CAT and GPx activities in the hippocampus of H rats decreased. Possible mechanisms may include direct metal-mediated structural alteration of the enzymes and the depression of enzymes synthesis. A reduction in GPx activity paralleled LPO increase in both hippocampus and cerebral cortex, demonstrating that the disruption of oxidant/antioxidant balance induced by $LaCl_3$ exposure was conductive to oxidative damage. Neuropathological investigation showed that La neurotoxicity caused pyramidal cell breakdown and loss in the subfield of CA3; no marked histological shift was observed in the other regions, suggesting the CA3 region of the hippocampus was susceptible to La exposure.

The neurotoxicological consequences found in the present work demonstrated that the rise in intracellular free calcium in hippocampal cells, the inhibition to activities of antioxidant enzymes, the aggravation in LPO and the subsequent cell damage might contribute to the impairment in spatial learning and memory ability caused by long-term La exposure. However, these results are not enough to reveal the dose–response relation of oral REEs exposure. It is partly because the biological effect spectrum of REEs is wide and the dose–response relationship is complicated (Zhao *et al*., 2004)—both the positive and negative effects of REEs exposure have been reported (Wang *et al*., 2003). Another reason is that the deposition of REEs in body takes place at an extremely low speed after oral administration, and the consequences are subtle (Albaaj and Hutchison, 2005; Persy *et al*., 2006).

Considering of the low La contents in brain regions of H rats (Table 1), the impairment in spatial learning and memory of H rats may be not a direct consequence of the toxicity of La deposited in brain. $[Ca^{2+}]_i/Ca^{2+}$-ATPase homeostasis, antioxidant enzymes, and LPO might be influenced via an indirect, multistep mechanism under a long-term exposure to La.

Calcium overload followed by neuron damage or dysfunction might be one of the possible indirect-mechanisms. Although the cause of $[Ca^{2+}]_i$ overload in hippocampal cell is not addressed in this experiment, some facts reported by the papers listed below imply that the disturbance of calcium balance is one of the consequences of long-term exposure to La. According to the theory of calcium paradox disease, calcium deficiency would lead to a decrease in serum calcium and an increase in the occurrence of arteriosclerosis, and it also might result in Ca^{2+} overload in cells (Fujita and Palmieri, 2000). Acute La exposure had a dose-dependent inhibition on calcium absorption (He *et al*., 2007a). In our previous research on the neurotoxicity of ytterbium (Yb), another rare earth element, the Ca contents in tibia and serum of rats significantly decreased after exposure to 40 mg/kg Yb for 6 months, which suggested that Yb exposure would cause calcium deficiency; the impairment in spatial memory and the intracellular free calcium overload in the hippocampal cells were also found (He *et al*., 2007b).

Our views are supported by some data from the environmental and epidemiological surveys. It was reported that REEs contents in the well water in rare earth mining area was 24.8 times as high as those in the normal area (Zhu *et al*., 2002) and the bioavailability of REEs in the local soil is much higher than those in the normal regions (Gao *et al*., 2001). It was also reported that there was a significant decrease in serum calcium of the villagers living in rare earth mining areas in southern Jiangxi Province (Yuan *et al*., 2003). So the significant increase in the occurrence of arteriosclerosis of the fundus oculi among

the villagers aged 20–40 in these areas over normal regions, discovered in another epidemiological survey (Zhu *et al.*, 1997b), might be a signal of intracellular calcium overload according to the theory of calcium paradox disease. The findings in these surveys and the results from our research suggest that the calcium related alterations and the consequent neuron damage or dysfunction might be involved in the negative effect of high-REEs background on the mental age and intelligence quotients of local children.

Our results are helpful to reveal the causes for the decline in the mental age and intelligence quotients of the children living at the high-REEs regions. And meanwhile, our findings suggest REEs exposure might adversely influence the spatial learning and memory in an indirect way. Thus the penetration to the blood–brain barrier should no longer be regarded as the premise of REEs' neurotoxicity.

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