Toxicological study of injuries of rat's hippocampus after lead poisoning by synchrotron microradiography and elemental mapping

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Abstract

The hippocampus, a major component of the brain, is one of the target nervous organs in lead poisoning. In this work, a rat's hippocampal injury caused by lead was studied. The lead concentrations in blood and hippocampus collected from rats subject to lead poisoning were quantified by Inductively Coupled Plasma Mass Spectrometry while morphological information and elemental distributions in the hippocampus were obtained with synchrotron radiation X-ray phase contrast imaging and synchrotron radiation micro-beam X-ray fluorescence, respectively. For comparison, identical characterization of the specimens from the rats in the control group was done in parallel. Results show that the ratios between the lead content in the treated group and that in the control group of the hippocampus, bone, and blood are about 2.66, 236, and 39.6, respectively. Analysis also revealed that some health elements such as S, K, Cl and P increase in the regions with high lead content in the treated hippocampus. Morphological differences between the normal and lead-exposed hippocampus specimens in some local areas were observed. Explicitly, the structure of the lead-exposed hippocampus was tortuous and irregular, and the density of the neurons in the Dentate Gyrus was significantly lower than that from the control group. The study shows that the synchrotron radiation methods are very powerful for investigating structural injury caused by heavy metals in the nervous system.

1. Introduction

The adverse effects of health caused by lead are of particular concern because it is a toxin widely distributed in the environment and may induce a broad range of toxic effects, particularly in the central nervous system such as the hippocampus. After long term exposure to lead, the nervous system, blood cardiovascular, endocrine and immune systems can be damaged in the human body and in animals [1]. Moreover, during development, lead poisoning caused severe consequences such as learning impairment, declined hearing, and impaired cognitive functions and behavioral abnormalities [2]. The hippocampus, composed of multi functional regions including CA1, CA2, CA3, CA4 and Dentate Gyrus, is a part of the telencephalon (forebrain), belonging to the limbic system, and plays major roles in short term memory and spatial navigation. The lead distribution in the central nervous system is homogenous. It is found that the lead concentration in the hippocampus is high after lead passing the blood–brain barrier [3]. A few observations from pathological studies suggest that neuronal cells are degenerated and undergo apoptosis induced by lead in hippocampus [4,5].

Many traditional methods including optical microscopy, computer tomography (CT), positron emission tomography (PET), magnetic resonance imaging (MRI), as well as scanning proton microprobe and ICP-MS equipped with laser ablation introduction system for elemental mapping in toxicological studies [6,7], have become popular in medical diagnosis and treatment. However, these methods are limited to spatial resolution, contrast, sensitivity, etc. and not enough in many cases [8]. Synchrotron radiation is an intense type of X-ray source that has advantages of high flux, tunable energy range, highly collimating beam, etc. compared to X-ray tube sources. Synchrotron radiation has been employed in the vivo and in vitro studies of health effects induced by toxins [9]. In Refs. [10,11], acute adverse effects on the pulmonary system for mice after being instilled intratracheally for PM2.5 into their lungs was monitored in vivo by synchrotron radiation X-ray phase...
contrast imaging (SR-XPCI) microradiographs. By that non-uniformity of appearance of the lung texture, hemorrhage spots with a size less than 0.5 mm can be found. This spatial resolution is even much higher than the one obtained by PET. SR-XPCI has also been applied to study, for example, tumor angiogenesis in a nude mouse model [12], and Lewy bodies in Parkinson disease [13]. Synchrotron radiation micro-beam X-ray fluorescence (SR-µXRF) is able to detect trace element distribution in biology samples with a sensitivity of order μg g⁻¹ [14]. SR-µXRF has been applied in tumor studies [15]. In the present work, SR-XPCI was employed to observe the morphological change in the hippocampus specimens induced by a lead toxin, and SR-µXRF was employed to obtain the element distribution in the same samples. The results are correlated to the concentrations of lead in blood, bone and hippocampus that were inspected by ICP-MS.

2. Experiment methods

2.1. Animal experiment

Twelve male sprague dawley rats, weighting 120–150 g, 5 weeks old, were provided by Animal House Center at the Chinese Academy of Sciences (Shanghai, China). These rats were randomly split into two groups. One group as control group was fed distilled water, and another was fed 0.5% lead acetate as the lead-treated group. All rats were housed in plastic micro-isolator cages at room temperature (20 ± 2 °C), relative humidity of 60 ± 10% and a 12 h light/dark cycle. The same food was fed to all animals. After being anesthetized, the rats were sacrificed by exsanguinations, and blood samples were obtained from the carotid artery in anti-coagulative tubes. The hippocampus and bone were fixed with 4% paraformaldehyde fixative for ICP-MS analysis. After being quickly frozen, the hippocampus pieces were cut along the long axis of hippocampus with 40 μm in thickness, and then held in mylar membrane for SR-XPCI and SR-µXRF measurements. All surgical procedures were performed in accordance with guidelines of the Animal Care and Use Committee at School of Medicine of Shanghai Jiaotong University.

2.2. Lead concentration analysis by ICP-MS

Blood sample with a volume of 0.5 mL was diluted with 9.5 mL dilution buffer containing 0.5 mg mL⁻¹ Triton X-100, 0.5 mg mL⁻¹ EDTA and 1.25% ammonia. The blood sample was inspected by ICP-MS. About 0.5 g hippocampus was mixed with 2.5 mL of concentrated HNO₃ and 0.5 mL H₂O₂ in a clean and dry microwave digestion vessel. The mixtures were then put into a microwave oven. In less than 10 min, the microwave system reached 190 °C and stayed at this temperature for 15 min. The digestate was dissolved with ultrapure water to a final volume of 5 mL after cooling, and then analyzed by ICP-MS. Approximate 0.5 g bone was completely incinerated at 600 °C, and then analyzed by ICP-MS. For all data obtained above the background was corrected with the blank background.

A quadruple ICP-MS (Thermo X 7, USA) was employed for quantifying lead levels in all samples. The operating parameters were as follows: RF power of 1250 W was applied to the plasma, and flow rates of 14, 0.7 and 0.85 L min⁻¹ were used for cool, auxiliary and nebulizer gases, respectively. Thallium with an elemental concentration of 10 ng mL⁻¹ was added online by a T type interface as an internal standard subjected to lead concentration measurement. The mass 202,203, 206, 207 and 208 were monitored and the lead concentration was calculated by the average of each value on 206,207 and 208.

2.3. Micrographs with SR-XPCI

Traditionally, the neurodegeneration in the hippocampus induced by lead is inspected with optical microscopes. Sample preparation involves immunohistochemical or histochemical staining such as hematoxylin and eosin stain, and other complicated biological/chemical procedures. The spatial resolution of this approach is limited to 0.2 μm [4,5]. Compared to optical microscopy, it is much easier to observe the neurodegeneration and structure damage in the hippocampus induced by lead with SR-XPCI, and it is possible to have higher resolution and does not need any chemical or immune antibody staining. Hippocampus specimens were imaged at the micro-tomography beamline 2-BM at the Advanced Photon Source (APS), Argonne National Laboratory, USA. The X-ray beam emitted from an electron storage ring with electron energy of 7 GeV and beam current of 102 mA, passes through two beryllium windows and reaches the specimen. In the experiments, the radiograms of the hippocampus specimens were taken with a monochromatic synchrotron X-ray beam of 9.3 keV delivered from a double-bounce multilayer monochromator. The bandwidth of the X-ray beam is 1.5% and the flux is 1.5 × 10¹³ photons/mm²/s. The detector system is composed of a scintillator, a microscope objective lens, and a Coolscan 4K CCD camera. The X-ray image of a specimen is converted by the scintillator into a visible-light image, which is subsequently magnified by the objective lens and registered in the camera. The samples were imaged with two different objectives lenses, which give 5.7 and 2.9 μm pixel spatial resolution, respectively. With the low-magnification lens, each specimen was scanned over and the images were stitched to obtain an overall image of the specimen. High-resolution images were only taken for a few regions of interest in the specimens. The hippocampus specimens were placed 150 mm from the scintillator to optimize the detection of the phase contrast effects. In contrast with the conventional X-ray absorption images of soft tissues this method can achieve a dramatic improvement for the hippocampus microradiograph [16]. As is well known that the refractive index of X-rays in materials is \( n = 1 - \delta - i\beta \), where \( \delta \) and \( \beta \) are related with phase shift and absorption coefficient, respectively. For hard X-rays, \( \delta \) is about three orders of magnitude higher than \( \beta \) [17] for the soft tissue with light elements. Therefore the method of SR-XPCI could provide more clear contrast than conventional X-ray absorption [18].

2.4. Elemental mapping with SR-µXRF

The elemental mapping of the same specimens in the SR-XPCI experiments was carried out at beamline 2-ID-E at APS. The pink undulator beam is monochromatized using a double crystal monochromator. The X-ray beam of energy 14 keV was focused down to about 0.2 μm spot size with a Fresnel zone plate. The incident photon flux is \( 1 \times 10^{10} \text{ph/s} \) at the focal spot. The regions of hippocampus with morphological changes detected by SR-XPCI were raster scanned through the focal spot, and at each scan position, illuminated with X-rays. Scanning was carried out in two dimensions using a step size of 0.2 μm and a dwell time of 1 s/position. The SR-µXRF was collected up to 10 s for each point by a three-element germanium energy dispersive detector (3 × 100 mm²) (Canberra Ultra-LEGGe detector), positioned at 90° to the beam line, 6 cm from the target. The fluorescence intensity maps of P, S, Cl, K, Co, Cr, Mn, Fe, Ni, Cu, Zn, Se, Br, Pb were generated with Maps software (Stefan Vogt, Advanced Photon Source) [19].

In order to correct the effect of the SR beam flux variation on the signal intensity, the fluorescence was normalized to the incident X-ray intensity, which was monitored by an ionization chamber located behind the sample. The relative fluorescence intensity was
used to compare the elemental content in experimental sample with the control sample.

3. Results and discussion

3.1. Lead content in blood, bone and hippocampus

Table 1 shows the concentrations of lead in blood, bone and hippocampus in normal rats and lead treated rats, together with the value of $R_{LC}$. The concentration of lead in bone of $119 \pm 5 \mu g L^{-1}$ is the highest compared to the values $396 \pm 118 \mu g L^{-1}$ in blood, and $0.44 \pm 0.06 \mu g g^{-1}$ in the hippocampus. The blood is the initial site of lead storage and distributor source to other tissues in the body, though lead in blood is only a small fraction of the total body lead burden. In China, the current reference range for acceptable blood lead concentration in healthy children without excessive exposure to environmental sources of lead is less than $100 \mu g L^{-1}$.

In a recent evaluation of all available information, Skerfving concluded that neurobehavioral effects may occur at a blood lead level of $311–414 \mu g L^{-1}$ [20]. It was shown in the epidemiologic results that there is an association between electrocardiographic heart rate variability and blood lead concentration at mean level of $360 \mu g L^{-1}$ [21]. In our experiments, the lead concentration level in blood is $396 \pm 118 \mu g L^{-1}$. It suggests that there might be neuro-degeneration in the rats’ nervous system.

Table 1

<table>
<thead>
<tr>
<th>Samples</th>
<th>Control group (\mu g g^{-1}/\mu g L^{-1} for blood)</th>
<th>Lead poisoning group (\mu g g^{-1}/\mu g L^{-1} for blood)</th>
<th>$R_{LC}$ (lead poisoning group to control group)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>&lt;10</td>
<td>396 ± 118</td>
<td>39.6 ± 11.8</td>
</tr>
<tr>
<td>Bone</td>
<td>0.50 ± 0.03</td>
<td>119 ± 5</td>
<td>236 ± 24.5</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>0.16 ± 0.03</td>
<td>0.44 ± 0.06</td>
<td>2.66 ± 0.85</td>
</tr>
</tbody>
</table>

Considering the background of lead in different tissues, the value of $R_{LC}$ may better reflect the lead level rather than the absolute concentration. Although the absolute values in blood and hippocampus for the poisoned rats are quite similar, $R_{LC}$ for blood and hippocampus are quite different, which are 39.6 and 2.66, respectively. The $R_{LC}$ in blood is 15-fold higher than that in the hippocampus. Bone, which is the main pool for lead storage containing about 95% lead of the total body burden [20], has the highest $R_{LC}$ value of 236. It has been reported that lead dose reaching to some extent could pass the blood–brain barrier [22], the blood–brain barrier prevents most of lead in blood from entering the nervous system. As a consequence, smaller $R_{LC}$ could occur for hippocampus in contrast with blood that was proved by our experiment. Some studies show that the blood brain barrier prevents > 98% of small molecules and 100% of all large molecules from entering the brain from blood [23]. After lead entering brain, it may cause lesions in some parts, particularly in the hippocampus. Thus, it is necessary to study the relationship between the morphological change and lead concentration in hippocampus.

3.2. Morphological changes in the hippocampus

From SR-XPCI results, the micro-structure has significant change only in the Dentate Gyrus area of the hippocampus. The Dentate Gyrus has high rates of neurogenesis [24] and is thought to contribute to new memories as well as other functional roles [25]. Fig. 1(a and b) show the area of Dentate Gyrus in the hippocampus taken from a rat in the control group, and Fig. 1(c and d) show the same area of the hippocampus of a rat in the lead-treated group. Fig. 1(a and c) are low-resolution images, and Fig. 1(b and d) are high-resolution images. Obviously, the density of the neurons in the lead-poisoned hippocampus is lower than that in the control one, and the structure of the poisoned hippocampus is more tortuous and irregular in comparison to the normal one. Several lines of evidence show that neuronal damage and apoptosis caused by lead in hippocampus can be observed by histological analysis [4,5].
neuronal cell patterns in Fig. 1(c and d) are more irregular, while they are quite smooth and regular in normal tissue as shown in Fig. 1(a and b). It suggests that in lead treated hippocampus, more neuronal cells are degenerated, and apoptosis appears. As is well known, apoptosis is characterized by distinct morphological features such as nuclear shrinkage, membrane bleb, chromatin con-

**Fig. 2.** Multi-elemental mapping of the hippocampus from lead poisoned rats and normal rats with SR-XRF. (a) Normal group and (b) lead poisoned group.


densation as well as oligonucleosomal DNA fragmentation, and regulated by gene in pathological condition [26,27]. In previous studies, most of the findings of lead inducing wide spread neurodegeneration in the hippocampus used immunohistochemical or histochemical staining methods such as hematoxylin and eosin stain with a complex sample preparation, then observed by traditional optical microscopy with a limited resolution (0.2 μm) [4,5]. Compared with traditional research technique, it is much easier to observe the neurodegenetion and structure damage in hippocampus by using synchrotron X-ray phase contrast imaging, and moreover it presents high resolution and does not need any chemical or immune antibody staining.

3.3. Elemental distributions in hippocampus**

Fig. 2 shows a two-dimensional SR-μXRF scanning of the particular regions shown in Fig. 1. The SR-μXRF intensity is normalized to the intensity detected by an ionization chamber, and represented by a color scale from blue to red corresponding to the XRF intensity from lowest to highest. In Fig. 2a, the endogenous elements in normal rats such as P, S, Cl, K and Se are distributed homogenously in the tissue. Nevertheless, in the lead-poisoned hippocampus specimen these elements are obviously distributed in the regions rich in lead as shown in Fig. 2b. As is well known, thiol groups locate mainly within the active catalytic sites of proteins and play fundamental structural and functional roles in protein. Moreover, the metallothioneins (MTs) containing high contents of metals and cysteine residues that are rich in thiol groups, play an important role in regulation of detoxification of heavy metal, regulation of metal concentration and antioxidation [28]. Many evidences have shown that MTs may protect central nervous system by means of decreasing oxidative stress, inflammation and apoptosis in central nervous system when the brain is exposed to heavy metals [28,29]. Fig. 2b shows a positive correlation between S and lead distribution in the lead rich regions. This suggests that, when the hippocampus is exposed to lead, the level of MTs in the hippocampus increases in order to elicit neuroprotective effects and to protect the hippocampus from being injured by lead. Thus, S accumulation in response to lead content may be a pathway to protect the hippocampus from damage by lead.

Fig. 2b also shows a strong positive distribution correlation among K, Cl and Pb. It indicates that the increases of K and Cl may be induced by a series of responses in the hippocampus during lead poisoning. K+ currents are important in the regulation of neuronal excitability and have a major impact on the overall neuronal response [30]. In addition, K+ is also related to apoptosis and cell death [31]. The intracellular Cl− concentration in neurons of the hippocampus is regulated by cation chloride co-transporters, such as potassium chloride, to maintain the low intracellular Cl− concentration. Other evidence [32] shows that the increase of potassium currents in CA1 neurons in hippocampus might be associated with the neuronal damage after ischemia. In Fig. 2b, when the neurons in the hippocampus was damaged by Pb, K+ and Cl− transport out of neurons in cortical neurons may be induced by the potassium chloride co-transporter, that is in agreement with what Payne suggested in ref [26]. Therefore the XRF mapping result in this work indicates that the neurons in the areas with a high concentration of K and Cl in the hippocampus for lead treated rats are damaged more severe than in other regions.

In the treated group, the P distribution is not homogenous and positively correlated with Pb as shown in Fig. 2b. This phenomenon may be caused by consuming ATP (Adenosine-5′-triphosphate) when K+ and Cl− are transported out of neurons in the lead-poisoned hippocampus.

4. Conclusions

Based on the ICP-MS measurements, exposed lead is mainly stored in bone, and then a small fraction of lead in blood could enter into hippocampus in rat’s brain across the blood brain barrier. The interesting finding by synchrotron X-ray phase contrast imaging is that the structure changes due to the lead toxicity occurred mainly in the area of Dentate Gyrus of the hippocampus. Those structure changes may be as a consequence of neurodegeneration and neuronal cells apoptosis caused by lead. Lead concentration measured by micro-beam X-ray fluorescence in the Dentate Gyrus for the exposed rats is 2.66 times as high as for normal rats. Meanwhile it is found that S, P, Cl and K elements in that area are positively correlated with lead. The study also shows that synchrotron radiation combined with other techniques is a powerful tool to study nervous system structure and micro-injury for the hippocampus and other organism caused by heavy metals.

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