Effects of sodium para-aminosalicylic acid on chelation treatment in Pb-exposed mice

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Abstract

Lead (Pb) is a corrosion-resistant, heavy, non-ferrous metal with widespread environmental pollution. Several metal chelators have been used in the treatment of Pb poisoning. However, the effect of sodium para-aminosalicylic acid PAS-Na on Pb excretion has yet to be reported. To investigate the effects of PAS-Na on Pb excretion, a mouse model of acute lead exposure was established. Healthy mice (90) received abdominal injection (i.p.) of 120 mg/kg Pb acetate, and 4 h later 80, 160, 240 mg/kg PAS-Na, or 240 mg/kg edetate calcium disodium (CaNa$_2$EDTA) were injected subcutaneously (s.c.) once per day for 6 days into the corresponding groups, respectively. The Control, Pb-exposed, PAS-Na and CaNa$_2$EDTA groups were evaluated simultaneously. After collecting 24 h urine samples, the animals were sacrificed in batches on the 2nd, 4th, 6th day. Levels of Pb and other metal elements [including manganese (Mn) and copper (Cu)] in the urine, whole blood and brain tissues were analyzed by graphite furnace atomic absorption spectrometry (AAS). The results showed that lead exposure increased Pb levels in urine and blood of mice, and PAS-Na treatment afforded antagonistic effect against Pb-induced toxicity. While additional studies will be needed, these novel results establish PAS-Na as a potential efficacious treatment for mitigating Pb-induced toxicity.

Introduction

Lead (Pb), a corrosion-resistant heavy metal, has been widely used in metal soldering, paint manufacturing, battery manufacturing, maritime industry, nuclear industry, and other industrial departments.$^1$-$^6$ As a toxic heavy metal, Pb readily enters the human body by various routes, such as skin, the respiratory tract and digestive tract. Pb has deleterious effects on several major systems, adversely affecting primarily the nervous system and kidneys.$^7$-$^11$

Chelation therapy has been used for more than fifty years as a treatment for lead excretion in the body. In 1950, The US Food and Drug Administration (FDA) approved CaNa$_2$EDTA chelation therapy for patients with lead poisoning.$^{12}$ CaNa$_2$EDTA is not metabolized and it may redistribute lead to the brain in a minor way following acute Pb poisoning.$^{13}$ CaNa$_2$EDTA has traditionally been used for the treatment of lead poisoning, and meso-2,3-dimercaptosuccinic acid (DMSA) has also been showed to be an effective lead chelator.$^{14}$-$^{17}$ CaNa$_2$EDTA and DMSA have both been approved as standard treatments for moderate lead poisoning and registered on the “WHO Model List of Essential Medicines”.$^{18}$ Both DMSA and CaNa$_2$EDTA have been shown to be well tolerated. However, transient increases in liver enzymes were observed in the treatment of both chelating agents.$^{17}$

Nonsteroidal anti-inflammatory drugs (NSAIDs) have been shown to be neuroprotective in neurodegenerative diseases by interfering with inflammatory processes.$^{19}$ Para-aminosalicylic acid (PAS-Na), a NSAID, has been widely used to treat tuberculosis patients in the 1940s and 1950s. Indian researchers found that PAS-Na could promote the excretion of manganese (Mn) in Mn-infected animals,$^{20}$ and after chelating brain Mn, the chelate could be excreted in the urine for the treatment of
chronic Mn poisoning.\textsuperscript{21-23} Furthermore, PAS-Na has been proven to be effective in the treatment of Mn-induced occupational Parkinson’s disease.\textsuperscript{24} In addition, our earlier study found that Mn levels in the brain of Mn-poisoned rats decreased after PAS-Na treatment, that brain copper (Cu) levels were constrained by Mn, and that PAS-Na had a chelating effect on Cu.\textsuperscript{25} Early studies by Anetor et al. (2002) suggest increased lead burden will increase Cu levels.\textsuperscript{26} In light of these studies, we were interested in investigating the relationship in Cu and Mn levels in mice after PAS-Na treatment of lead-poisoned mice.

Our earlier study found that PAS-Na mitigated Pb-induced neurodegeneration in hippocampal neurons in rats.\textsuperscript{27} However, the effect of PAS-Na on Pb excretion has not been fully characterized. Detailed investigations of PAS-Na are underway to determine their therapeutic significance in lead poisoning and their possible effectiveness in preventing the onset of poisoning. Therefore, we conducted an \textit{in vivo} study with Pb exposure to investigate the effects of PAS-Na on Pb excretion. Moreover, we measured Cu, Mn levels in the brain, urine and whole blood. Finally, we conducted a systematic analysis of the relationships between Pb levels in biological samples to confirm their predictive value of PAS-Na for Pb excretion and their effect on Cu and Mn levels \textit{in vivo}.

**Materials And Methods**

**Instrument.** Pb assays were performed by electrothermal atomic absorption spectrometry (ETAAS) with ICE 3000 spectrometers (Thermo, USA) using a Zeeman background correction. The light source consisted of a hollow lead cathode lamp (analysis wavelength: 283.3 nm). Furnaces with integrated platform were used.

**Blood.** Whole blood was diluted 1:51 in 0.125\% Triton X-100 (Rohm & Haas, USA) solution. Calibration was performed by the addition-calibration technique. Palladium nitrate was used as matrix modifier. Five internal quality controls were analyzed in each series: Lead in Frozen Bovine Blood Level 1 and 2 (Chinese Center for Disease Control, China). The limits of detection (LOD defined as three standard deviations (SD) from the blank) and quantitation (LOQ defined as 10 SD from the blank) were 0.6 and 1.9 $\mu$g/L, respectively. Reproducibility determined at four concentration levels (20, 40, 80 and 120 $\mu$g/L, respectively) was 3.1, 3.7, 4.2 and 5.1\%, respectively.

**Urine.** Urine samples were diluted 1:10 in 0.05\% Triton X-100 to analysis. Calibration was performed by the standard addition technique applied to each sample. Palladium nitrate was used as matrix modifier. LOD and LOQ were 1.5 and 5.1 $\mu$g/L, respectively. Toxic Elements in Frozen Human Urine Level 2 (National Institute of Standards and Technology, USA) was used as internal quality controls.

**Brain.** Brain samples were diluted 1:5 in 0.05\% Triton X-100 to analysis. Calibration was performed by the standard addition technique applied to each sample. Palladium nitrate was used as matrix modifier. LOD and LOQ were 1.2 and 3.1 $\mu$g/L, respectively. Recovery rates of 15 and 30 $\mu$g/L were used as internal quality controls, with matrix spiked recoveries exceeding 95\%.
Animal. A total of 90 CL grade mice (weighing 18 ~ 25 g) were purchased from the Experimental Animal Center of Guangxi Medical University (Nanning, China). All the experimental procedures were approved by the Animal Ethics Committee of Guangxi Medical University and performed following their guidelines. Mice were maintained in conditions of adequate temperature (22 ± 3°C) and humidity (50 ± 10%) with a 12 h light/12 h dark cycle. Food and distilled water were available ad libitum.

Animal Experiments. Pb is injected intraperitoneally and enters the urine and blood, circulating throughout the body and then stored mainly in the bones. After a 7-day acclimatization period, the animals were divided into 6 groups, 5 mice per group: Control, Pb-exposed group, Pb + 80 PAS-Na, Pb + 160 PAS-Na, Pb + 240 PAS-Na and Pb + CaNa$_2$EDTA control groups. The mice in the Pb-exposed group, Pb + (80,160,240) PAS-Na groups and Pb + 240 CaNa$_2$EDTA control groups were given (CH$_3$COO)$_2$Pb·4H$_2$O (120 mg/kg) via the intraperitoneal (i.p.) route, while mice in the Control group were i.p. injected with sterile physiological saline. Subsequently, mice in the Pb + 80 PAS-Na, Pb + 160 PAS-Na, Pb + 240 PAS-Na L-, M-, H-PAS), and Pb + 240 CaNa$_2$EDTA control groups were subcutaneously (back) injected (s.c.) with physiological saline with PAS-Na or CaNa$_2$EDTA (80, 160, and 240 mg/kg PAS-Na in the PAS-Na treatment group and 240 mg/kg CaNa$_2$EDTA in the C-CaNa$_2$EDTA group, respectively), once a day, for 6 days, while mice in the other groups were administered the same volume of sterile physiological saline alone injected subcutaneously. Body weights were recorded daily. The selected Pb exposure and PAS-Na treatment doses were based on our previously published studies.

The Collections of Animal Blood, Urine and Brain Tissues. At the end of the experiment, mice were placed in the metabolic cage, food and distilled water were available ad libitum. After collecting the 24 h urine of mice, the animals were anesthetized with 5% chloral hydrate (0.1 ml/10 g body weight, i.p.) and sacrificed in batches on the 2nd, 4th, 6th day. Next, the whole blood and brain tissues were collected. Brains were weighed and stored at -80°C for metal content analyses. To prevent metal contamination, the samples were stored in trace element–free tubes.

Analysis of Pb, Cu, Mn in Blood, Urine and Brain Tissues. Brain tissue samples were digested with 5 mL ultrapure 65% HNO$_3$ with a microwave digestion technique at 190°C. Subsequently, the samples were placed on 150°C graphite heating plates to remove the acid for 90 min. After the samples were evaporated to approximately 0.5 mL, the samples were cooled down to room temperature. All brain samples were diluted to 5 mL with distilled water. The samples of blood and urine were analyzed absent digestion. Lastly, metal levels (including Pb, Mn, and Cu) in the samples were analyzed with ice 3000 AAS (Thermo, USA). Blank controls and quality controls were also processed simultaneously.

Statistical analysis. All data analysis was performed with the Statistical Package for Social Sciences version 26.0 (SPSS Inc.). Results are expressed as means ± SD of at least three independent experiments. Normality of distribution was assessed by the Lilliefors test, and homogeneity of variance was tested with the Levene’s test. Statistical comparisons were performed by one-way analysis of variance (ANOVA).
Results

Effects of PAS-Na treatment on Pb levels in urine of Pb-exposed mice. As shown in Figure 1, Pb levels were significantly different between the Pb-exposed and three of the groups administered PAS-Na. L-PAS $p < 0.05$, M-PAS $p < 0.01$, H-PAS $p < 0.001$ on the 2nd day. L-PAS $p < 0.001$, M-PAS $p < 0.01$, H-PAS $p < 0.01$ on the 3rd day. L-PAS $p < 0.001$, M-PAS $p < 0.01$, H-PAS $p < 0.01$ on the 4th day. However, the changes in urinary Pb levels in the L-, M-, and H-PAS group were statistically indistinguishable from the Pb-exposed group ($p > 0.05$, Fig. 1) on the 5th and 6th day. The effects in the PAS-Na group were lower than in the C-CaNa$_2$EDTA group.

Effects of PAS-Na treatment on Pb levels in whole blood of Pb-exposed mice. As shown in Fig. 2, after treatment with 80, 160, or 240 mg/kg PAS-Na, Pb blood levels of the H-PAS group were statistically different compared with Pb exposed mice ($p < 0.05$) on the 2nd day, while 80 and 160 mg/kg PAS-Na did not result in statistically distinguishable changes compared to the Pb alone treated mice. No significant differences were found in blood Pb levels between the Pb exposed group and the PAS-Na control groups on the 4th and 6th day ($p > 0.05$). Changes in blood Pb levels in the C-CaNa$_2$EDTA group were statistically indistinguishable from the Pb-exposed group ($p > 0.05$) on the 2th and 4th day, while CaNa$_2$EDTA treatment significantly reduced blood Pb levels compared with Pb alone exposed mice ($p < 0.01$) on the 6th day.

Effects of PAS-Na treatment on Mn levels in urine of Pb-exposed mice. The effects of PAS-Na treatment on Mn levels in urine were also investigated. As shown in Fig. 3, No significant differences were found in urinary Mn levels between the Pb exposed group and the PAS-Na control groups ($p > 0.05$). However, the CaNa$_2$EDTA treatment significantly increased urinary Mn levels compared with the Pb treated mice ($p < 0.001$) from the 2nd to the 6th day.

Effects of PAS-Na treatment on Cu levels in urine of Pb-exposed mice. As showed in Fig.4, Cu levels in urine ($p > 0.05$) were statistically indistinguishable from Pb-exposed group after the PAS-Na treatment. The CaNa$_2$EDTA treatment significantly reduced urinary Cu levels compared with Pb exposed mice ($p < 0.05$) on the 2nd day, while no change in that from the 3rd to the 6th day ($p > 0.05$).

Effects of PAS-Na treatment on Pb, Mn, Cu levels in brain of Pb-exposed mice. No significant differences were found in Pb, Mn, Cu levels in brain tissues between the Pb-exposed and three doses of PAS-Na groups ($p > 0.05$). These findings likely reflect difference Pb exposure doses and times.

Discussion

Pb is one of the most ubiquitous elements in the environment, and the health risks associated with Pb exposure remain a topic of major health concern. A broad range of studies have revealed that Pb can adversely affect various organs at all ages. Therefore, it is important to identify a predictive and reliable biomarker for Pb exposure. In previous studies, various biomarkers have been used to evaluate
the effects of Pb exposure on human health.\textsuperscript{34-36} After absorption, Pb mainly distributes to blood and tissues, and is excreted in the urine.\textsuperscript{37-39} Pb levels in urine\textsuperscript{40} and blood\textsuperscript{37, 41} have also been widely used as biomarkers of recent Pb exposures in vivo. Generally, Pb levels in whole blood (for recent exposure) are regarded as valuable and predictive biomarkers of long-term Pb exposure and Pb-induced neurotoxicity.\textsuperscript{42} However, only biomarkers of effect alone are not sufficiently sensitive for early detection of health damages caused by Pb exposure. To resolve Pb-induced effects, the recommended diagnostic strategy is to measure Pb in whole blood in combination with urinalysis.\textsuperscript{37} In this study, we used Pb levels in whole blood and urine to assess Pb exposure in mice.

According to the Diagnostic Criteria for Occupational Chronic Lead Poisoning (GBZ37-2002), urinary Pb $\geq 120 \mu g/L$ is one of the main diagnostic indicators of mild Pb poisoning.\textsuperscript{27} In this experiment, the mean urinary Pb level in Pb-infected mice was 716.9 $\mu g/L$, suggesting that the dose and time used in the experiment were sufficient to reach a poisoning level.

Chelation therapy is central to treatment of metal intoxications due to environmental, dietary, occupational exposures, or genetic disorders.\textsuperscript{43, 44} PAS-Na has shown efficacy as a Mn chelator both in blood and brain.\textsuperscript{22, 23} The results of our study showed that PAS-Na significantly increased murine urinary Pb levels, demonstrating a significant difference compared with the Pb-exposed group. Furthermore, PAS-Na promoted faster urinary Pb excretion within 4 days of administration. In addition, PAS-Na increased blood Pb levels within 2 days of administration. PAS-Pb chelates formed in the excretory system may be readily reabsorbed from the intestine and then into the urine, where they are excreted in more significant amounts than in whole blood. However, there were no effects of PAS-Na treatment on Pb levels in the analyzed brain regions.

Metabolic balance of essential metal elements plays an important role in maintaining healthy.\textsuperscript{45} Both Mn and Cu are the most abundant bodily minerals, playing critical roles in maintaining normal functioning of the CNS\textsuperscript{46, 47} and other tissues and organs. Cu is essential for key biological functions such as hematopoiesis and iron metabolism, and increased Cu exposure may cause neurotoxicity.\textsuperscript{48, 49} The results of our study showed that upon acute Pb poisoning, PAS-Na had no effects on either Mn nor Cu levels, neither in urine nor in brain. Our studies also found that the PAS-Na group had lower Pb and Mn excretion in urine than the CaNa$_2$EDTA group, and that CaNa$_2$EDTA treatment reduced urinary Cu levels. Moreover, CaNa$_2$EDTA treatment significantly reduced blood Pb levels compared with Pb exposed mice on the 6th day after treatment.

In summary, our novel results addressing metal homeostasis in relationship to Pb exposure and PAS-Na chelation show that the excretion of lead in poisoned mice was increased by treatment with PAS-Na, suggesting that complexes were formed between lead and these chelates. That is, PAS-Na can rapidly form a stable complex with Pb once absorbed, and it is readily excreted in urine. PAS-Na can be used alone to treat acute Pb poisoning. Therefore, the earlier PAS-Na is clinically administered, the more
pronounced is its effect on Pb excretion. However, the pro-elimination mechanism of PAS-Na on Pb is not completely clear and needs further in-depth study.

**Declarations**

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**Authors contribution**

Y.Li. conceptualization, methodology, investigation, resources, data curation, writing original draft, writing review and editing. Y.Liang. investigation, resources, data curation, methodology and formal analysis. Y.Y.F. investigation, methodology and formal analysis. S.Y.O. J.C., X.W.Z. and L.L.L. methodology, validation. W.W.Z. investigation. M.A. conceptualization, writing-review and editing. Y.M.J. conceptualization, resources and supervision. All authors reviewed the manuscript.

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**Data availability** All data generated or analyzed during this study are included in this published article.

**Ethics approval and consent to participate** All animal procedures performed in this study were performed strictly according to the international standards of animal care guidelines and have been approved by the Animal Care and Use Committee of Guangxi Medical University.

**Consent for publish** The paper has been approved by all authors for publication.

**Conflicts of interest** - The authors declare that they have no conflict of interest.

**References**


**Figures**

![Figure 1](image_url)

**Figure 1**

The effect of PAS-Na on mice urinary Pb levels. Data are mean ± SD, n = 5 for each group; *p < 0.05, **p < 0.01, ***p < 0.001 vs. Pb-exposed mice.
Figure 2

The effect of PAS-Na on whole blood Pb levels in mice. Data are mean ± SD, n = 5 for each group; *p < 0.05, **p < 0.01, ***p < 0.001 vs. Pb-exposed mice.

Figure 3

Urine manganese (µg/L)

Figure 3
The effect of PAS-Na on mice urinary Mn levels. Data are mean ± SD, n = 5 for each group; ***p < 0.001 vs. Pb-exposed mice.

Figure 4

The effect of PAS-Na on mice urinary Cu levels. Data are mean ± SD, n = 5 for each group; *p < 0.05, **p < 0.01 vs. Pb-exposed mice.