Effects of Lead and Cadmium Combined Heavy Metals on Liver Function and Lipid Metabolism in Mice

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Received: 4 June 2022 / Accepted: 10 August 2022 / Published online: 22 August 2022
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Abstract
Although a large number of studies have been conducted on lead (Pb) and cadmium (Cd) exposure individually, information regarding the toxicity of combined Pb and Cd exposure is relatively limited. The present study aims to investigate the toxicity of Pb–Cd combination exposure and the corresponding mechanism. A heavy metal exposure model was established in mice by subcutaneous intragastric administration of Pb–Cd (50:1) for 35 days. Body weight, diet, hair state, mental state, liver index, haematological index, biochemical indicators and pathological section analysis were used to comprehensively evaluate toxicity. Then, classical oxidative stress indexes and lipidomics techniques were used to explore the potential mechanism. The results showed that Pb–Cd caused the mice to have low appetite, poor spirit, significantly reduced activity, slow weight gain and irritated or drying hair. Pb–Cd also caused liver enlargement, significantly increased aspartate aminotransferase (AST) and alanine aminotransferase (ALT) enzyme activities, and resulted in pathological changes to the liver. Prolonged Pb–Cd exposure led to significantly increased thrombocyte haematocrit (PCT), white blood cell (WBC), platelet (PLT) and monocyte (MON) counts and decreased red blood cell (RBC), haemoglobin (HGB), haematocrit (HCT) and lymphocyte (LYM) counts. Pb–Cd increased oxidative stress by increasing the activity of superoxide dismutase (SOD) and lactate dehydrogenase (LDH) and the content of malondialdehyde (MDA). Finally, Pb–Cd triggered lipid metabolism disorders by regulating linoleic acid, sphingolipid and glycerolipid metabolism.

Keywords
Lead-cadmium Complex Heavy Metals · Toxicity Assessment · Liver Injury · Lipidomics

Abbreviations

| Pb  | Lead                  |
| Cd  | Cadmium               |
| Cu  | Copper                |
| Zn  | Zinc                  |
| Hg  | Mercury               |
| Ag  | Silver                |
| Mn  | Manganese             |
| KM  | Kunming               |
| SPF | Specific pathogen free|
| HESI| Heated electron spray ionization |
| RSD | Relative standard deviation |
| HE  | Haematoxylin-eosin    |
| FC  | Fold change           |
| VIP | Variable importance for the projection |
| UHPLC–MS/MS | Ultra-high performance liquid chromatography–tandem mass-spectrometry |

Pb(CH3COO)2  Lead acetate
CdCl2  Cadmium chloride
ALT  Alanine aminotransferase
AST  Aspartate aminotransferase
SOD  Superoxide dismutase
MDA  Malondialdehyde
LDH  Lactate dehydrogenase
GSH-Px  Glutathione peroxidase
RBC  Red blood cell
WBC  White blood cell
HGB  Haemoglobin
PLT  Platelet

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Introduction

The term heavy metals, which have a specific gravity greater than 4 or 5, refers to approximately 45 species, such as copper (Cu), lead (Pb), zinc (Zn), cadmium (Cd), mercury (Hg) and silver (Ag) [1]. Although manganese (Mn), Cu, Zn and other heavy metals are trace elements needed for life activities, most of them, such as Pb and Cd, are not necessary, and all heavy metals are toxic to the human body above a certain concentration [2]. For example, after Pb enters the body, part is excreted through faeces and sweat whilst the rest dissolves the blood over several hours, hindering blood synthesis and leading to anaemia, headache, dizziness, fatigue, sleepiness, constipation and limb soreness [3]. Cd enters the human body and forms Cd-thionein, which reaches the entire body through blood circulation and is selectively stored in the kidney and liver [4]. Since heavy metals are extremely harmful to the human body, scientists have carried out a substantial amount of research on the damage caused by heavy metals. Mengjiao Bi et al. [5] reported the molecular mechanisms of Pb-induced changes in selenium status in mouse livers through interactions with phosphorus from selenoproteins. The results of Debashis Reja et al. [6] showed that blood Pb levels were associated with advanced liver fibrosis in patients with nonalcoholic fatty liver disease. Damir Suljevic et al. [7] suggested that chronic Cd exposure in vivo could cause damage to bone marrow haematopoietic cells, followed by severe red blood cell (RBC) damage and liver necrosis. Eunjung Park et al. [8] conducted a study on the relationship between environmental Cd exposure and the risk of suspected nonalcoholic fatty liver disease.

Thus, although a large number of studies have been conducted and positive results after Pb or Cd exposure individually, information regarding the toxicity of combined Pb and Cd exposure is relatively limited, and the effects of combined exposure to Pb and Cd on human health are not clear. However, in some special environments, such as Pb and Zn mines, there is a large amount of heavy metal pollution, and Pb and Cd are associated. Therefore, mice were used as the research subject in this study to examine the damage of combined exposure to Pb and Cd on the liver to provide a basis for toxicity evaluations of combined heavy metal exposure.

Materials and Methods

Materials

Enzyme-linked immunosorbent assay (ELISA) kits for alanine aminotransferase (ALT), aspartate aminotransferase (AST), superoxide dismutase (SOD), malondialdehyde (MDA), lactate dehydrogenase (LDH) and glutathione peroxidase (GSH-Px) were obtained from Shanghai Guduo Biotechnology Co., Ltd. Lead acetate (Pb(CH$_3$COO)$_2$) and cadmium chloride (CdCl$_2$) were obtained from Tianjin Komil Chemical Reagent Co., Ltd. All other reagents were of analytical grade.

Animals

Kunming (KM) species specific pathogen-free (SPF) grade male mice (body weight 18–22 g) were purchased from Changhai Tianqin Biotechnology Co., Ltd. (Licence No. SCXK-2019–0014). They were bred in the Guizhou Engineering Laboratory for Quality Control and Evaluation Technology of Medicine with a relative temperature of 20–25 °C and humidity of 60–75%. All animal experiments in this study were conducted according to the Guide of the Care and Use of Laboratory Animals, Eighth Edition [9] and approved by the Guizhou Normal University Animal Care and Use Committee.

Modelling and Group Administration

In the process of this research, we investigated the concentration of Pb and Cd pollution produced by Pb and Zn mining, and the results showed that the ratio is diverse but, in most cases, is closest to 50:1. Therefore, in this study, 50:1 was selected as the final test ratio. Therefore, 4.66 and 0.046 g of Pb(CH$_3$COO)$_2$ and CdCl$_2$, respectively, were accurately weighed in a 50-mL volumetric flask and dissolved in pure water to prepare 250 mmol/L and 5 mmol/L Pb(CH$_3$COO)$_2$ and CdCl$_2$ solutions.

Twenty KM male mice were fed adaptively for 3 days and randomly divided into 2 groups with 10 mice in each group: the blank control group and the Pb–Cd group. The blank control group was intragastrically administered normal saline twice a day at an interval of 2 h. The Pb–Cd model group was intragastrically administered Pb–Cd solutions (250 mmol/L and 5 mmol/L Pb(CH$_3$COO)$_2$ and CdCl$_2$ solutions, respectively) once a day and then intragastrically administered normal saline once a day. The whole observation period was 5 weeks, and the intragastric dose was 0.01 mL per gram of mouse.
weight. During the experiment, the mice were free to eat and drink water.

**Observation and Recording of the Routine Indexes**

During modelling and administration, food and drinking water were provided as usual. The hair state, dietary status, urine, faeces and mental status of the mice were observed and recorded. Additionally, the mice were weighed every 3 days until the end of the fifth week of the experiment. After the last modelling and administration, the mice were fasted without water for 12 h, and the final body weights of the mice were recorded.

**Collection of Tissue Samples**

After the last administration, the mice were fasted and watered. Twenty-four hours later, blood and tissue samples were collected. Blood was collected from the ipsilateral orbit into a centrifuge tube with the anticoagulant heparin sodium. Each blood sample was shaken back and forth to prevent blood coagulation and then stored in a 4 °C refrigerator for later use. Then, the mice were sacrificed by cervical dislocation, and the livers were quickly dissected. The blood on the surface of the organs was washed away with normal saline and drained with filter paper. The organs were weighed on an analytical balance to calculate the organ index [10].

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\text{Liver index} = \frac{\text{liver weight}}{\text{body weight of mice}} \times 100\%
\]

Then, small pieces of liver tissue from the same part of the liver were cut and fixed in 10% formaldehyde fixation solution and refrigerated at 4 °C for pathological sectioning. The remaining tissues were divided into two parts and stored in a −80 °C freezer for the determination of biochemical indexes and lipid analysis.

**Determination of Blood Indicators**

RBCs, white blood cells (WBCs), haemoglobin (HGB), platelets (PLTs), thrombocyte haematocrit (PCT), haematocrit (HCT), lymphocytes (LYMs) and monocytes (MONs) in the blood samples were detected and analysed with a haematology analyser (Animal-6008, Jinan Glitter Technology Co., Ltd., China). Briefly, the blood sample was combined with ethylenediaminetetraacetic acid (EDTA) dipotassium (2.0 mg/mL) as an anticoagulant and was fully shaken. Then, a sampling needle was inserted into the blood sample for counting. When the sampling needle was lifted, the blood sample was removed. Approximately 1 min later, the instrument showed the counting results.

**Determination of AST and ALT Activity**

After the liver tissues from the mice in each group were removed from the refrigerator and thawed to room temperature, 0.10 g of each sample was accurately weighed and placed in a 5-mL centrifuge tube. Next, 0.9 mL of normal saline was added for homogenization in a homogenizer followed by centrifugation at 4 °C and 3000 r/min for 10 min. The supernatant was removed and placed in a low-temperature environment (4 °C) for later use [11]. Finally, the activities of AST and ALT were measured according to the instructions of the kits.

**Histopathological Analysis**

Liver samples fixed with 10% formaldehyde were embedded in paraffin, sectioned, stained with haematoxylin and eosin (HE) and sealed with neutral gum [12]. The morphological changes in the liver tissues from each group were observed under a light microscope (MF53, Guangzhou Mingmei Technology Co., Ltd. China).

**Determination of Oxidative Stress Indicators**

A total of 0.1 g of liver sample was accurately weighed into a 5-mL centrifuge tube, and 0.9 mL of normal saline was added for homogenization. The mixture was centrifuged at 4 °C and 3000 r/min for 10 min [13]. The supernatant was taken to determine the activities of SOD, GSH-Px and LDH and the content of MDA according to the instructions of the kits.

**Lipidomic Analysis**

**Lipid Extraction**

Liver tissue samples from each group were thawed stepwise, 0.2 g of liver tissue was accurately weighed into a centrifuge tube, and 600 μL of ice-cold methanol (stored overnight at −20 °C) and 150 μL of pure water were added [14]. After the mixture was homogenized in a homogenizer, 450 μL of dichloromethane was added, the mixture was swirled for 30 s at 3000 r and then 150 μL of pure water was added for an additional 1 min of swirling. The final mixture was placed at room temperature for 5 min and then centrifuged at high speed (12,000 r/min) at −6 °C for 15 min. The lower liquid was placed into a 1.5-mL centrifuge tube and dried with nitrogen. Before detection and analysis, a mixture of 200 μL acetonitrile:isopropanol:water (65:30:5) was added followed by centrifugation at 4 °C and 8000 r/min for 5 min [15]. The supernatant was filtered through a 0.22-μm
microporous filter membrane and placed in an injection flask for ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC–MS/MS) analysis.

**Quality Control Sample Preparation**

Liver tissue homogenates from each group were mixed in equal volumes and treated as quality control (QC) samples according to the same method in the lipid extraction section to evaluate the stability and repeatability of the system [16].

**Chromatographic Separation Conditions**

The samples were separated using a DiONEX™ Ultimate™ 3000 UHPLC system and connected to an ACE Excel 1.7C18-AR (2.1 x 100 mm, 1.7 μm) column. The mobile phase consisted of A (0.1% acetic acid in water) and B (acetonitrile containing 0.1% acetic acid), and the elution conditions were as follows: from 0 to 2 min, the proportions of A and B were maintained at 50%; from 2 to 25 min, mobile phase A decreased from 50 to 10%, and mobile phase B increased from 50 to 90%; from 25 to 30 min, mobile phase A returned from 10 to 50%, and mobile phase B decreased from 90 to 50%. The flow rate, injection volume and column temperature were set at 300 μL/min, 4 μL and 40 °C, respectively.

**Mass Spectrometry Analysis Conditions**

The samples were detected in positive and negative ion modes by Q Exactive mass spectrometry. The ion source was heated electrospray ionization (HESI), and the parameters of the ion source were as follows: the spray voltage was set to 3.5 kV (+)/3.2 kV (−), the capillary temperature was set to 320 °C, the probe heating temperature was set to 350 °C and the sweep gas was set to 0 arb. The sheath gas and AUX gas were 35 arb and 10 arb, respectively. The primary mass spectrometry full scan was 100–1000 mass-to-charge ratio (m/z), and the mass resolution was 70,000.

**Nontargeted Lipidomic Data Processing**

Progenesis QI software was used to perform peak alignment and peak extraction on the original mass spectra to obtain lipid data. After standardized pretreatment, the retention time (RT), m/z and peak intensity were obtained. Lipid data with a deletion value of more than 50% in the group and with a relative standard deviation (RSD) of more than 30% in the quality control samples were deleted, and then the total peak area was normalized. SIMCA 14.1 software was used for pattern recognition of the mass spectral data. After pre-processing with UV and Pareto scaling modes, unsupervised principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were performed. Then, SPSS 21.0 and MetaboAnalyst 5.0 were used for t test, multiple of variation and other one-dimensional statistical analyses.

**Results**

**Routine Index Analysis**

There were no significant differences in body weight, diet, hair state or mental state during adaptive feeding. During the experimental period of 5 weeks, mice in the blank control group had a flexible response, good appetite and bright fur. Compared with the blank control group, mice in the Pb–Cd model group showed low appetite, poor spirit, significantly reduced activity, slow weight gain (Fig. 1) and irritated or drying hair. The results showed that combined exposure to Pb and Cd had negative impacts on body weight, mental state and hair in mice.

In addition, when the body is damaged by Pb combined with Cd, a series of changes in cell morphology, substance

**Fig. 1** Changes in mouse body weights. * Compared with the blank control group, **P < 0.01
metabolism and so on occur, leading to changes in the organ indexes. Therefore, organ index analysis can reflect liver injury in mice. In this study, the liver index of each group was measured. As shown in Table 1, the liver index of the Pb–Cd model group was 4.15 ± 0.33%, which was significantly higher than that of the blank control group (P < 0.01). The results showed that combined exposure to Pb and Cd could cause liver enlargement in mice.

**Haematological Index Analysis**

Long-term exposure to Pb or Cd pollution will lead to decreased blood quality; therefore, haematological indicators are often used to evaluate the degree of harm caused by heavy metals to the body and the efficacy of therapeutic agents [17]. In the present study, haematological indexes (RBC, WBC, HGB, PLT, PCT, HCT, LYM and MON) were monitored. As shown in Table 2, compared with the blank group, PCT in the Pb–Cd group was significantly increased (P < 0.05), WBC, PLT and MON showed an upwards trend, and RBC, HGB, HCT and LYM showed a downwards trend.

**ALT and AST Analysis**

Liver function examination reflects the physiological function of the liver [18]. There are many liver function indicators, including ALT, AST, total bilirubin, direct bilirubin, indirect bilirubin, alkaline phosphatase, glutamyl transpeptidase, albumin, globulin, prealbumin, total bile acid, etc. These indicators can reflect whether there is damage to liver cells or an obstruction of the biliary tract and also the synthetic function of the liver [19]. For example, in humans, the normal value of AST is 8–40 U/L, and when ALT is significantly elevated and the AST/ALT ratio is greater than 1, damage to the liver parenchyma is indicated. In the present study, the ALT and AST indexes of different groups of mice were systematically observed, and the results are shown in Table 2. Compared with the blank control group, the AST and ALT activities in the Pb–Cd group were significantly increased (P < 0.01), indicating that Pb combined with Cd caused significant damage to the liver.

**Pathological Analysis**

To explore the disease process in the organs, tissues and cells, pathomorphological examination methods can be used to examine lesions, discuss the causes, pathogenesis and development of the lesions and finally make pathological diagnoses [20]. Pathological examination has been widely used in clinical work and scientific research [21]. In this study, to explore the liver injury of the mice exposed to Pb and Cd, HE staining was performed to evaluate the histopathological changes in the mouse livers. Prepared sections were observed under a microscope, and the results are shown in Fig. 2. The liver tissue structure of the mice in the blank control group was complete with clear morphology.

**Table 1** Changes in liver indexes in mice of each group (x±SD, n=10)

| Group            | Liver index (%) |
|------------------|-----------------|
| Blank control group | 3.51 ± 0.19    |
| Pb–Cd group       | 4.15 ± 0.33**   |

* Compared with the blank control group, **P < 0.01

**Table 2** Changes in the blood and biochemical indexes in mice (x±SD, n=10)

| Index       | Blank control group | Pb–Cd group |
|-------------|---------------------|-------------|
| WBC         | 5.71 ± 1.72         | 6.35 ± 0.36 |
| RBC         | 7.31 ± 0.27         | 7.07 ± 0.35 |
| HGB         | 151.56 ± 4.38       | 149.24 ± 8.18 |
| PLT         | 222.60 ± 28.42      | 330.90 ± 47.61* |
| PCT         | 0.25 ± 0.04         | 0.47 ± 0.15** |
| HCT         | 37.33 ± 0.96        | 36.77 ± 2.12 |
| LYM         | 2.86 ± 0.51         | 1.99 ± 0.25 |
| MON         | 1.24 ± 0.26         | 2.01 ± 0.14* |
| AST (U/L)   | 33.25 ± 1.46        | 38.25 ± 3.89** |
| ALT (U/L)   | 54.20 ± 4.69        | 60.72 ± 4.62** |

* Compared with the blank control group, **P < 0.05; ***P < 0.01

**Fig. 2** Liver tissue pathological sections from each group of mice (40×) (A: blank control group, B: Pb–Cd group)
and orderly arranged liver cells with normal structure and distinct hierarchy. The liver cells of the Pb–Cd group showed extensive necrosis, an uneven arrangement, vacuolar degeneration and congestion (Representative areas or phenomena with significant pathological changes are shown by arrows in Fig. 2B).

**Analysis of the Oxidative Stress Index**

There have many studies on the toxicological mechanism by which heavy metals damage the body [22]. Studies have shown that long-term exposure to Pb or Cd can cause oxidative stress damage [23]. In this study, classical oxidative stress targets, such as SOD, GSH-Px, MDA and LDH, were used to evaluate the toxicology mechanism of Pb–Cd-induced liver injury in mice. The results are shown in Table 3. Compared with the blank group, the activities of SOD and LDH in the Pb–Cd group were significantly increased (P < 0.01), and the content of MDA was significantly increased (P < 0.01), whilst the activity of GSH-Px was decreased. These results indicated that Pb–Cd could indeed cause disorder to the oxidative stress indexes in the body, leading to body damage.

**Lipidomics Analysis**

In recent years, omics technology has been developed rapidly with the development of analytical chemistry technology [24]. Metabolomics is a discipline that examines the endogenous and exogenous changes in the body to provide further understanding of a disease [25]. Lipidomics is an important branch of metabolomics. The changes in lipid metabolism under different physiological and pathological conditions are analysed and compared to determine changes in physiology and pathology [26]. Therefore, in addition to studying the toxic mechanism of lead and cadmium from the perspective of classical oxidation targets, this study also used nontargeted lipidomics to analyse the changes in the liver tissues of the mice in each group and to search for possible lipid regulatory pathways.

**Quality Control of the Nontargeted Lipidomics Data**

In the process of this study, 5 QC samples were randomly inserted during liver sample detection from each group of mice to verify the reliability of the experimental method and the stability of the instrument. Unsupervised PCA was conducted on the preprocessed QC sample data from the five samples, and the results are shown in Fig. 3.

The results showed that the QC samples were all within 2 std, indicating that the experimental method was reliable and that the instrument had good stability.

**Table 3** Changes in the SOD, GSH-PX, LDH activities and MDA content in liver tissues of mice in each group, x ± SD (n = 10)

| Group | Blank control group | Pb–Cd group |
|-------|---------------------|-------------|
| SOD (U/mL) | 146.62 ± 4.14 | 180.83 ± 7.57* |
| GSH-PX (U/L) | 442.42 ± 23.81 | 421.82 ± 36.15 |
| MDA (nmol/mL) | 3.60 ± 0.21 | 4.20 ± 0.28** |
| LDH (IU/L) | 4.53 ± 0.21 | 5.17 ± 0.16** |

*Compared with the blank control group, **P < 0.01

Fig. 3   PCA score chart of QC sample (A: negative ion mode, B: positive ion mode)
Multivariate Statistical Analysis of the Lipid Data

After pretreatment of the original lipid data from each group, Metabo Analyst 5.0 (https://www.metaboanalyst.ca/) was used for data processing, such as considering missing values and normalizing total peak area, and SIMCA 14.1 software was used for multivariate statistical analysis of the processed data.

First, PCA was performed on the lipid data from the liver tissue of the mice in each group to obtain PCA scores from both positive and negative ion modes. As shown in Fig. 4, the lipid data from the blank group and the Pb–Cd group were well separated. The results showed that the two groups of mice could be distinguished normally, suggesting that Pb–Cd had a significant effect on lipid disorders.

To further screen out the different lipids, the mass spectrometry data from the blank control group and Pb–Cd model group were further analysed by supervised OPLS-DA. As shown in Fig. 5A, B, parameter $R^2$ from the OPLS-DA model represents the fitting degree of the model, and parameter $Q^2$ represents the prediction ability of the model. In Fig. 5A, $R^2 = 0.564$ (cum) and $Q^2 = 0.9$ (cum) in the blank control group and Pb–Cd group in negative ion mode, $R^2$ was greater than 0.5, the difference between $R^2$ and $Q^2$ was less than 4 and $Q^2$ was close to 1, indicating that the model had good fitting and prediction ability in negative ion mode. Similarly, as shown in Fig. 5B, in positive ion mode, $R^2 = 0.515$ (cum), $Q^2 = 0.88$ (cum), $R^2 > 0.5$, the difference between $R^2$ and $Q^2$ was less than 3 and $Q^2$ was close to 1, indicating that the model of the lipids in the liver tissues had good fitting and prediction ability in positive ion mode. To prevent overfitting of the OPLS-DA model, 200 displacement tests were carried out for the establishment of the OPLS-DA model, as shown in Fig. 5C, D. Both the y-intercepts of $Q^2$ in positive and negative ion modes were less than 0, indicating that no overfitting occurred in the model and that the results were reliable.

A volcano map is a combination of multiple variation analysis and t tests, which can intuitively show the significance of lipid changes between two groups of samples. Through experimental screening of the fold change (FC) values in the blank group and Pb–Cd group, a volcano diagram was made for the screened differential lipids, as shown in Fig. 6. Here, Log2 (FC) was the abscissa, and $-\log_{10}(P)$ was the ordinate.

As shown in Fig. 6, there were clear differences in the lipids in the blank and Pb–Cd groups, in which blue indicates downregulation, and the red indicates upregulation, indicating that the lipid metabolites in the mouse liver tissues had undergone substantial changes after poisoning with lead and cadmium.

Screening of Differential Lipids

To further screen differential lipids, variable importance in the projection (VIP) values $>1$, $P < 0.05$, FC $>2$ or FC $<0.5$ were used as the parameters. According to LIPID MAPS, HMDB, MassBank of North America (MONA) and other online datasets, the LIPID ratio and secondary mass spectrometry data were compared. A total of 24 differential lipids were identified, and the specific substances are shown in Table 4.

Cluster analysis was performed on the 24 differential lipid metabolites screened, which can more comprehensively and intuitively display the relationship between the samples and the expression patterns of different groups of lipids. As
shown in Fig. 7, there were significant differences in metabolites between the Pb–Cd group and the blank group. The experimental results showed that Pb–Cd exposure caused significant lipid metabolism disorder.

Pathway Analysis and Biological Interpretation

Amongst the 24 differential lipid metabolites, 12 were downregulated in the Pb–Cd model group compared with the blank control group, including PC (P-18:1/P-18:1), PC (16:0–18:1), SM (d18:1/18:0), ginkgolide B, PC (16:0/20:5), stearoyl-L-carnitine, pentaethylene glycol, aminocaproic acid, LPE (16:1), LPE (14:1), sphingosine-1-phosphate and linoleic acid. PG (22:6/18:0), LPE (18:2), LPE (18:0), agnuside, PE (20:5/22:6), PE (18:1/22:6), PE (16:0/20:5), bexarotene, SM (d18:0–16:0), LPE (20:3), LPE (20:5) and abietic acid were upregulated. The 24 selected differential lipids included four major categories.
H. Chen et al.

(fatty acyls, glycerol phospholipids, prenol lipids and sphingolipids) and 11 subcategories (fatty acid esters, fatty acid conjugates, linear acid derivatives, glycerol phospholipid ethanolamine, glycerol phospholipid choline, glycerol phosphate glyceride, vitamin A-like, terpene glycoside, terpene lactone, diterpene and sphingolipids). According to the HMDB ID numbers, the pathways of the 24 differential lipids were analysed using the MetaboAnalyst 5.0 site.

Table 4  Differential lipids between the blank group and Pb–Cd group

| Compound name          | Formula       | Class | RT  | m/z    | VIP | FC  | P value |
|------------------------|---------------|-------|-----|--------|-----|-----|---------|
| Stearoyl-L-carnitine   | C_{26}H_{49}NO₄ | FA    | 6.56| 426.342| 1.022| 8.800| 0.016   |
| Pentaethylene glycol   | C_{26}H_{49}NO₄ | FA    | 5.96| 426.342| 1.307| 7.872| 0.017   |
| 6-Aminohexanoate       | C_{6}H_{13}NO₂ | FA    | 15.62| 392.316| 1.504| 2.633| 0.002   |
| Linoleic acid          | C_{18}H_{32}O₂ | FA    | 11.60| 456.332| 1.210| 3.703| 0.003   |
| LPE 20:5               | C_{25}H_{42}O₂P | GP    | 5.64| 500.276| 4.172| 0.443| 0.002   |
| PE (18:1/22:6)         | C_{26}H_{42}O₂P | GP    | 23.69| 790.536| 1.04 | 0.387| 0.004   |
| LPE 18:0               | C_{26}H_{42}O₂P | GP    | 9.87 | 480.308| 7.000| 0.484| 0.005   |
| PC (P-18:1/P-18:1)     | C_{24}H_{44}O₂P | GP    | 21.01| 340.356| 1.373| 2.310| 0.006   |
| PC (16:0/18:1)         | C_{24}H_{44}O₂P | GP    | 19.78| 703.468| 1.130| 6.477| 0.045   |
| LPE (20:3)             | C_{25}H_{42}O₂P | GP    | 11.56| 580.361| 1.538| 0.394| 0.000   |
| PG (22:6/18:0)         | C_{29}H_{50}O₈P | GP    | 23.76| 738.507| 1.795| 0.487| 0.020   |
| PC (16:0/20:5)         | C_{25}H_{44}O₂P | GP    | 24.40| 764.548| 2.263| 2.889| 0.025   |
| LPE (18:2)             | C_{25}H_{44}O₂P | GP    | 23.17| 712.491| 1.027| 0.478| 0.042   |
| LPE 14:1               | C_{25}H_{44}O₂P | GP    | 15.93| 370.367| 2.846| 2.170| 0.001   |
| PE (16:0/20:5)         | C_{26}H_{44}O₂P | GP    | 23.36| 738.505| 5.256| 0.314| 0.001   |
| PE (20:5/22:6)         | C_{24}H_{42}O₂P | GP    | 20.67| 808.491| 1.677| 0.450| 0.001   |
| Bexarotene             | C_{20}H_{20}O₂ | PR    | 23.45| 762.507| 4.048| 0.371| 0.000   |
| Agnuside               | C_{20}H_{20}O₂ | PR    | 19.75| 465.304| 5.365| 0.416| 0.000   |
| Ginkgolide B           | C_{20}H_{20}O₂ | PR    | 17.86| 572.480| 3.202| 2.321| 0.000   |
| Abietic acid           | C_{20}H_{20}O₂ | PR    | 5.62 | 498.262| 4.345| 0.426| 0.001   |
| SM (d18:1/18:0)        | C_{18}H_{20}O₄P | SP    | 9.09 | 762.556| 1.362| 17.399| 0.014   |
| SM (d18:0/16:0)        | C_{18}H_{20}O₄P | SP    | 22.04| 618.476| 1.006| 0.498| 0.003   |
| Sphingosine-1-phosphate| C_{18}H_{20}O₄P | SP    | 11.57| 366.336| 4.498| 2.834| 0.002   |

The results are shown in Fig. 8, where red represents a low P value, the circle size represents the path influence value; the larger the influence value is, the greater the influence. A total of 8 pathways were identified, including linoleic acid metabolism, sphingolipid metabolism, glycerophospholipid metabolism, alpha-linolenic acid metabolism, arachidonic acid metabolism, glycosylphosphatidylinositol anchor biosynthesis, biosynthesis of unsaturated fatty acids and fatty acid degradation. Amongst them, linoleic acid metabolism was the most influenced pathway, followed by

Fig. 6  Volcano map of differential metabolites (A: negative ion mode, B: positive ion mode)
The results showed that Pb–Cd could cause lipid disorders, possibly by regulating linoleic acid, sphingolipid and glycerolipid metabolism.

**Discussion**

Heavy metals in the environment are very difficult to biodegrade but can be enriched by hundreds or thousands of times by biological amplification in the food chain and eventually enter the human body [27]. Heavy metals can interact strongly with proteins and enzymes in the human body to inactivate them. Heavy metals may also accumulate in some organs of the human body and cause chronic poisoning [28]. Therefore, the harmful effects of heavy metals on the human body have been widely studied. Environmental Pb and Cd are not only nonessential metal elements but also harmful [29]. Pb mainly enters the body through the digestive system, respiratory system and skin and circulates in the blood,
causing functional disorders, including those of the central nervous system, haematopoietic function, gastrointestinal tract, liver and kidney [30]. Cd mainly accumulates in the body through drinking water, ingestion and inhalation and can accumulate in tissues and organs such as the liver, kidney and lung for a long time, resulting in disease [31].

In addition to a single type of heavy metal pollution bringing great harm to human health, there may be pollution from two or more heavy metals in the environment at the same time. Therefore, it is of great significance to study the toxicity of heavy metal combinations to suggest environmental protection and human health guidelines. Yuan G et al. [32] conducted acute and 90-day subchronic toxicity tests with Pb and Cd, and the results showed that the low-dose Pb and Cd exposure combination significantly changed the physiological and biochemical parameters of rat blood in a dose-dependent manner and had additive toxicity to SD rats. Zou H et al. [33] studied liver damage caused by Cd and Pb exposure and found that Cd and Pb accumulate in large quantities in the liver, reduce liver weight and the liver coefficient, and damage the liver structure and function. Our study also showed that the combined exposure of Pb and Cd (in a 50:1 ratio) had negative effects on body weight gain, the liver index and liver function in mice. Thus, these results are basically the same. In addition, combined treatment with Pb and Cd upregulated the contents of antioxidant enzymes and related trace elements and the expression of autophagy-related proteins and mRNA (ATG5, ATG7, Beclin-1, P62 and LC3) and affected the levels of oxidative stress and autophagy in hepatocytes. Compared with the single-exposure group, the combination of Cd and Pb tended to increase these parameters, and the toxicity increased. In contrast, Nation et al. [34] exposed rats to Pb and Cd alone and in combination. After 60 days, it was found that lead exposure could increase rat activity, whilst Cd exposure could reduce exercise. These results showed that toxicity caused by the combination of Pb and Cd showed antagonistic effects. Haneef et al. [35] studied the pathological changes in renal function after injury in goats and observed that lead poisoning occurred earlier than Cd or Pb + Cd poisoning in goats, indicating that Cd reduced the toxicity of Pb to a certain extent and that there was an antagonistic relationship. Additionally, no changes in the reproductive cycle were found after subcutaneous injection of lead and cadmium into adult female rats during pregnancy and lactation.

Lipids are hydrophobic or amphipathic compounds formed by the carbon anion condensation of a ketoethylthiolate or the carbon cation condensation of isoprenyl [36]. Lipids play an important role in various life activities as intermediates involved in the cell membrane, the cell barrier and signal transduction and as an energy source and are crucial biological small molecules for life activities [37]. Lipidomics, which was proposed by Han Xianlin et al. [38] in 2003, is a discipline derived from metabolomics that mainly studies the changes in and interactions between lipids in biological systems. With the development of modern analytical techniques, lipidomics has become widely used to study various biological models and human diseases due to the important roles of lipids in biological processes and has achieved good results. To the best of our knowledge, this study is the first to investigate the effects of combined lead and cadmium exposure on health from the perspective of lipid metabolism. Herein, it was found that combined exposure to Pb and Cd resulted in significant changes in at least 4 categories and 11 subcategories of lipid metabolism. These four categories of lipids are fatty acyls, glycerol phospholipids, prenol lipids and sphingolipids. Fatty acyls, which include fatty acids and aliphaloacylates, contain a series of repeating methylene groups that confer hydrophobicity to these lipids [39]. Fatty acyl lipids have a variety of physiological functions, such as maintaining the relative mobility of the cell membrane to ensure the normal physiological function of cells, reducing cholesterol and triglyceride contents in the blood by cholesterol esterification and improving the activity of brain cells to enhance memory and thinking ability [40]. Glycerol phospholipids are one of the most abundant phospholipids in the body. In addition to forming biofilms, they are one of the components of bile and membrane surface active substances and participate in the recognition and signal transduction of proteins by the cell membrane [41]. Prenol lipids are synthesized from the five-carbon precursors isoprene diphosphate and p-dimethylpropenyl diphosphate. Prenol lipids and their phosphorylated derivatives play important roles in the transport of oligosaccharides across cell membranes [42]. Prenol lipids are also prohormones involved in the production of steroid hormones such as progesterone, halocorticoids, glucocorticoids, androgens and oestrogens [43]. Sphingolipids have long been considered as only a component of cellular structure [44]. With the deepening of research, it has been found that sphingolipids are important signalling molecules that are closely related to cell growth, ageing, meiosis, maturation and death and are also involved in tumour invasion, the heat shock response and the genotoxic stress response [45].

Conclusion

In this study, mice were exposed to both Pb and Cd. Long-term exposure to Pb and Cd resulted in loss of appetite, poor mental performance, significantly reduced activity, slow weight gain and irritated or drying hair. Combined exposure also led to liver enlargement, changes in the liver tissues and cells, and elevated ALT and AST levels. Pb–Cd indeed caused disorders of the oxidative stress indexes in the body,
thus damaging health. Combined Pb and Cd exposure can also lead to disordered lipid metabolism in the body and induce a variety of diseases.

The representative total ion current (TIC) spectra of the samples from the mice in the blank group, QC sample and Pb–Cd group samples are provided in the Supporting Information. These materials are available free of charge on the internet at https://www.springer.com/journal.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12011-022-03390-5.

Author Contribution All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [HC], [CZ] and [XZ]. The first draft of the manuscript was written by [HC], and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding This work was supported by the National Natural Science Foundation of China (No. 81860738) and the Special Project for the Cultivation and Innovation of Academic New Seedlings of Guizhou Normal University ([2018]5769–27).

Data Availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing Interests The authors declare no competing interests.

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