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Effects of short term lead exposure on gut microbiota and hepatic metabolism in adult zebrafish

Jizhou Xia, Liang Lu, Cuiyuan Jin, Siyu Wang, Jicong Zhou, Yingchun Ni, Zhengwei Fu, Yuanxiang Jin*

College of Biotechnology and Bioengineering, Zhejiang University of Technology, Hangzhou 310032, China

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ABSTRACT

Lead (Pb) is one of the most prevalent toxic, nonessential heavy metals that has been associated with a wide range of toxic effects in humans and environmental animals. Here, effects of short time exposure to 10 and 30 μg/L Pb on gut microbiota and hepatic metabolism were analyzed in adult male zebrafish. We observed that both 10 and 30 μg/L Pb increased the volume of mucus in the gut. At phylum level, the abundance of *α-Proteobacteria* decreased significantly and the abundance of *Firmicutes* increased significantly in the gut when treated with 30 μg/L Pb for 7 days. In addition, the 16S rRNA gene sequencing for V3-V4 region revealed a significant change in the richness and diversity of gut microbiota in 30 μg/L Pb exposed group. A more depth analysis, at the genus level, discovered that 52 gut microbes identified by operational taxonomic unit analysis were changed significantly in 30 μg/L Pb treated group. Based on GC/MS metabolomics analysis, a total of 41 metabolites were significantly altered in 30 μg/L Pb treatment group. These changed metabolites were mainly associated with the pathways of glucose and lipid metabolism, amino acid metabolism, nucleotide metabolism. In addition, we also confirmed that the transcription of some genes related to glycolysis and lipid metabolism, including *Gk*, *Aco*, *Acc1*, *Fas*, *Apo* and *Dgat*, decreased significantly in the liver of zebrafish when exposed to 30 μg/L Pb for 7 days. Our results observed that Pb could cause gut microbiota dysbiosis and hepatic metabolic disorder in zebrafish.

1. Introduction

Heavy metals induced the toxicity are increasing in recent years (Winneke, 2011; Nadella et al., 2013; Jin et al., 2016b). Lead (pb), one of the essential trace elements, is widely used in industrial activities, and it entered into the environment through different pathways. According to previous studies, Pb is a commonly detected heavy metal and is present in diverse environmental matrices, especially in the e-waste area at high concentrations (Wong et al., 2007; Leung et al., 2008). Undoubtedly, Pb can reach aquatic systems via the effluents of industrial, urban and mining sources (Senger et al., 2006). In particular, in water samples from e-waste recycling sites in Guiyu of China, Pb concentration even reached as high as 400 μg/L (Wang and Guo, 2006). More importantly, Pb has been associated with a wide range of toxic effects in different experimental animals. According to previous reports, in addition to its major neurotoxicity (Zhang et al., 2011; Lee and Freeman, 2014; Wang et al., 2016), the toxicity of Pb was also associated with hepatotoxicity (Hasanein et al., 2016), oxidative stress (Liu et al., 2015), endocrine disruption (He et al., 2017), and cardiovascular toxicity (Gump et al., 2011) as well as immune toxicity (Kasten-Jolly and Lawrence, 2017). Thus, high concentrations of Pb in aquatic system would cause the serious toxicity to aquatic organisms directly. However, till date, studies describing the mechanisms of Pb-induced gut microbiota dysbiosis and metabolism disorder in zebrafish are still remained unclear.

As a frequently used experimental model, zebrafish (*Danio rerio*) has emerged as an ideal experimental model to study the aquatic toxicity of environmental chemicals including heavy metals (Jin et al., 2010; Lee and Freeman, 2014; Jin et al., 2015; Liu et al., 2016; Chen et al., 2017; Z.Z. Liu et al., 2017). As for Pb, a previous study reported that oral exposure to 10 mg/L Pb supplied in drinking water for 13 weeks could induce the gut microbiota dysbiosis and metabolism disorder in mice (Gao et al., 2017). Because Pb was often detected in the aquatic system, however, no study was focused on the effects of Pb on the gut microbiota and metabolism in fish.

In this study, we hypothesized that Pb could affect the composition of gut microbiota, influence the gut function and cause metabolism disorder in adult zebrafish. Because egg protein transcripts are

* Corresponding author at: 18, Chaowang Road, College of Biotechnology and Bioengineering, Zhejiang University of Technology, Hangzhou, China.
E-mail address: jinyx@zjut.edu.cn (Y. Jin).

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extremely abundant in the livers of breeding females and might confound the metabolic analysis, male fish were selected in the present study (Wang et al., 2005; Jin et al., 2008). For this purpose, male adult zebrafish was exposed to various concentrations of Pb for 7 days and determined whether or not they could induce microbiota dysbiosis in the gut and metabolism in the liver. We thought that the results acquired here will provide some new information regarding Pb-induced aquatic toxicity.

2. Materials and methods

2.1. Fish husbandry and exposure protocol

Male adult wide type AB strain zebrafish (*Danio rerio*) were kept at standard laboratory conditions with temperature at 28 °C, a 14:10 dark/light cycle according to the zebrafish breeding protocol (Westerfield, 1995). During the experiment, the fish were feed twice daily, and the water was changed every day.

The selected male adult fish were exposed to 10 and 30 μg/L Pb (PbAC) for 7 days, respectively. In each group, 6 male adult fish were reared in 2 L of each solution in a glass tank, and 3 separate tanks was carried out in each group. A total of 18 fish was used in each group. The livers excised from 3 fish were collected as 1 sample, resulting in 6 pooled samples for mRNA transcription analysis. Additionally, 2 guts selected randomly in each glass tank (and total 6 samples in each group) were used for DNA extraction, and each gut excised from each fish was collected as one sample. Additionally, 2 guts selected randomly in each tank were used for histopathological analysis. No died fish was observed during the Pb exposure.

For the hepatic metabolomic analysis, a total of 72 male adult fish (in 12 separate glass tanks, and 6 fish in each tank) were exposed to 30 μg/L Pb for 7 days. The same number of fish were reared in water as a control. After Pb exposure, the livers excised from 12 fish, from 2 tanks, were collected as one sample, and 6 pooled samples were collected both in control and 30 μg/L Pb treated groups, respectively.

In all experiments, fish were anesthetized on ice before dissection. The livers and guts were kept on dry ice during preparation and then were stored at −80 °C until they were further analyzed.

2.2. Histopathological analysis

After 7 days of exposure with 10 and 30 μg/L Pb, a portion of the middle gut was fixed in 10% formalin at 4 °C for 24 h. Then, the fixed gut tissues were dehydrated in gradient ethanol, hyalinized in xylene, and embedded in paraffin wax. Next, the paraffin blocks were sectioned at 5-μm thickness. The sections were collected on glass slides and stained with hematoxylin and eosin (H&E) or Alcian Blue-Periodic Acid Schiff (AB-PAS). And the pictures of the guts were examined by a microscope (Olympus).

2.3. DNA extraction, PCR amplification, and 16S rRNA gene sequencing

The genomic DNA (gDNA) was extracted from each gut using a commercial magnetic bead DNA isolation kit provided by Hangzhou Foreal Nanotechnology (Hangzhou, China). All the extracted gDNA was quantified by ultraviolet spectroscopy and electrophoresis for further analysis. Then, the gDNA samples were amplified by specific primers (Forward primer: 5′-ACTCCTACG GGAGGCAGCAG-3′; Reverse primer: 5′-GGACTA CHVGGGTWTCTAAT-3′) targeting the V3 and V4 regions of the bacterial 16S rRNA gene. Furthermore, the composition of the gut microbiota was detected using dual-indexing amplification and sequencing on the Illumina MiSeq platform followed by QIIME (vision 1.9.0) bioinformatics analysis. In addition, partial of the gDNA in each sample was amplified by real-time qPCR with following protocol: 50 °C for 2 min, 95 °C for 10 min, 95 °C for 15 s, 56 °C for 30 s, and 72 °C for 1 min, repeated for 40 cycles, followed by 72 °C for 10 min.

2.4. RNA extraction and gene transcription analysis

The livers from three zebrafish were dissected as one sample for total RNA extraction using TRIzol reagent (Takara, China). Six parallel total RNA samples were prepared for each Pb treated group. Then, a total of 500 ng RNA in each sample was used to synthase cDNA by a reverse transcription kit (Toyobo, Japan). The real-time quantitative polymerase chain reaction (RT-qPCR) was performed using the SYBR Green system (Toyobo, Japan) and Eppendorf MasterCycler® ep RealPlex2 system (Wesseling-Berzdorf, Germany). The sequences of the primers used in the present study were taken from previous studies (Freitas et al., 2010; Ballester et al., 2017; C.Y. Jin et al., 2017). The following PCR protocol was used: denaturation for 1 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The primer sequences are shown in Table S1. And, the transcription level of 18S rRNA gene was analyzed as a housekeeping gene. The quantification of relative abundances of gene transcription was performed as previously described (Livak and Schmittgen, 2001).

2.5. GC/MS-based hepatic metabolomic analysis and the differential metabolites selection

The sample preparation and GC/MS were performed according to a previous study (C.Y. Jin et al., 2017). Briefly, 30 mg accurately weighed sample was transferred to a 1.5-mL Eppendorf tube. Two small steel balls were added to the tube. Then, 20 μL of 2-chloro-L-phenylalanine (0.3 mg/mL) dissolved in methanol as internal standard and 600 μL mixture of methanol and water (4/1, vol/vol) were added to each sample, samples were placed at −80 °C for 2 min. Then grinded at 60 Hz for 2 min. Then ultrasonicated at ambient temperature for 10 min after vortexed, then placed at −20 °C for 30 min. Samples were centrifuged at 13,000 rpm, 4 °C for 15 min. 400 μL of supernatant in a glass vial was dried in a freeze concentration centrifugal dryer. And 80 μL of 15 mg/mL methoxylamine hydrochloride in pyridine was subsequently added. The resultant mixture was vortexed vigorously for 2 min and incubated at 37 °C for 90 min. A total of 80 μL of BSTFA (with 1% TMCS) and 20 μL n -hexane was added into the mixture, which was vortexed vigorously for 2 min and then derivatized at 70 °C for 60 min. The samples were placed at ambient temperature for 30 min before GC–MS analysis. In this study, six parallel samples (12 fish were sacrificed for each sample) were prepared for each treatment/control group.

The derivatized samples were analyzed on an Agilent 7890A gas chromatography system coupled to an Agilent 5975C MSD system (Agilent, CA). A HP-5MS fused-silica capillary column (30 m × 0.25 mm × 0.25 μm, Agilent J & W Scientific, Folsom, CA) was utilized to separate the derivatives. Helium (> 99.999%) was used as the carrier gas at a constant flow rate of 6.0 mL/min through the column. The injector temperature was maintained at 280 °C. Injection volume was 1 μL by splitless mode. The initial oven temperature was 60 °C, ramped to 125 °C at a rate of 8 °C/min, to 190 °C at a rate of 10 °C/min, to 210 °C at a rate of 4 °C/min, to 310 °C at a rate of 20 °C/min, and finally held at 310 °C for 8.5 min. The temperature of MS quadrupole, and ion source (electron impact) was set to 150, and 230 °C, respectively. The collision energy was 70 eV. Mass data was acquired in a full-scan mode (m/z 50–600), and the solvent delay time was set to 5 min.

The acquired MS data from GC–MS were analyzed by ChromaTOF software (v 4.34, LECO, St Joseph, MI). And Metabolites were quantified by the Fiehn database, which is linked to the ChromaTOF software. Briefly, after alignment with Statistic Compare component, the CSV file was obtained with three dimension data sets including sample information, peaks' name, retention time, m/z and peak intensities. The internal standard was used for data quality control (reproducibility). After internal standards and any known pseudo positive peaks, such as peaks caused by noise, column bleed and BSTFA derivatization