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Transcriptome analysis of the effects of Cd and nanomaterial-loaded Cd on the liver in zebrafish

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ABSTRACT

The wide application of engineered nanoparticles to remove heavy metals in aquatic environments has raised concerns over nanomaterial-adsorbed heavy metal toxicity. To ensure safe use of nanomaterial–heavy metal composites, understanding their biological effects at the molecular level is crucial. In the present study, we used the Illumina HiSeq technology to study the transcriptome changes induced by Cd^{2+} and nano-manganese dioxide- or nano-hydroxyapatite-adsorbed $CdCl_2$ composites ($nMnO_2$-Cd, $nHAP_{20}$-Cd, and $nHAP_{40}$-Cd) in zebrafish liver cells. We identified 545 differentially expressed genes (DEGs), 33 of which were in common between the $nMnO_2$-Cd, $nHAP_{20}$-Cd, and $nHAP_{40}$-Cd groups. The DEGs could be classified in four categories: hydrolases (enzymes involved in various physiological functions, including digestion, immune response, blood coagulation, and reproduction), biological binding (FMN-, actin- and metal ion-binding), metabolic enzymes (e.g., ceramidase, alpha-amylase, carboxylic ester hydrolase, and carboxypeptidase), and cell structure (cell surface, intermediate filament, and muscle myopen protein). The DEGs identified in this study are potentially useful markers to understand the physiological responses induced by Cd^{2+} and nano-Cd composites in zebrafish liver.

1. Introduction

Cadmium (Cd) is a toxic heavy metal that adversely affects the survival and growth of aquatic organisms (Chakraborty et al., 2010; Prozialeck et al., 2012). Multiple methods have been used for remediating Cd contamination in aquatic environments, such as ion exchange, reverse osmosis, adsorption, electrodialysis, and chemical immobilization. Chemical precipitation is currently considered the most effective and economic method to remove Cd in water (Wang et al., 2010). Nanomaterials have been widely used to remove heavy metals owing to their adsorption characteristics (Fernando et al., 2015; Jin et al., 2016). For example, Tamez et al. (2015) reported that the adsorption capacity of nano-sized Fe_3O_4 for Cu^{2+} and Pb^{2+} was 37.04 mg/g and 166.7 mg/g, respectively. The binding capacity of nano-sized Fe_2O_3 for Cu^{2+} and Pb^{2+} was 19.61 mg/g and 47.62 mg/g, respectively. The sorption maxima of nano-sized MnO_2 with Cu^{2+} and Cd^{2+} in an aqueous solution were determined to be 104.5 mg/g and 89.1 mg/g, respectively (Huang et al., 2016). However, relatively few studies on the safety of nanomaterial-adsorbed Cd for organisms have been conducted. Gao et al. (2018b) found that nano-hydroxyapatite-Cd (nHAP-Cd) and nano-manganese dioxide-Cd ($nMnO_2$-Cd) composites had lower toxicity than free Cd^{2+}, but they still showed acute toxicity to *Daphnia magna* of level II grade according to the Commission of the European Communities and Organization for Economic Co-operation and Development standards, and induced oxidative damage.

Zebrafish (*Danio rerio*) is one of the vertebrate model organisms that is most widely used in research studies pertaining to ontogenetic development, pharmacology, and toxicology (Xu et al., 2012). Specific features and biomarkers that have been used for estimating toxicological levels of exogenous compounds in zebrafish include LC_{50}, reproductive rate, antioxidant defense activation, and DNA and RNA damage. In general, the toxic effect of a pollutant is firstly triggered at the molecular level. RNA contains all the necessary information required to mediate the expression of all biomolecules encoded by the cells' DNA. Recently, RNA sequencing technology has been widely used to assess global transcriptome changes in target tissues or cells, including splice junctions, novel transcripts, alternative splice variants, and un-annotated genes (Conesa et al., 2005; Su et al., 2014). Zheng et al. (2016) found that parental exposure to ß-diketone antibiotics (DKAs) impacted the reproduction of F0 zebrafish and the development

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of F1 zebrafish using high-throughput miRNA sequencing. Wang et al. (2017) reported chronic effects of DKAs in zebrafish evaluated by high-throughput sequencing. They found abnormal expression of some lncRNAs, and that immune function-related genes were induced. Molecular biomarkers are a sensitive indicator of the response of an organism to chemicals. Therefore, gene expression analysis is an important approach to evaluating biological mechanisms (Khuansuwan and Gamse, 2014). Qiu et al. (2015) studied gene expression in the bacterium *Shewanella oneidensis* and the water flea *D. magna* exposed to functionalized gold nanoparticles. The results indicated that *act* and 16S could serve as potential biomarkers of nanoparticle-induced damage in *D. magna* and in *S. oneidensis*, respectively. The toxic effect of cationic dendrimers on primary human bronchial epithelial cells at the molecular level was studied using RNA sequencing by Feliu et al. (2015). They found that cell-cycle-related genes in primary human bronchial epithelial cells were downregulated under low doses of cationic poly (amidoamine) dendrimers.

A multitude of genes and signaling pathways impacted by xenobiotic chemicals have been reported; however, reports on the influences of nanomaterial-adsorbed Cd on mRNA expression in the zebrafish liver are limited. Therefore, in the current study, transcriptome sequencing was applied to analyze transcriptional responses in the zebrafish liver under Cd^{2+}, and nHAP-Cd, and $nMnO_2$-Cd pollution.

2. Materials and methods

2.1. Materials and experimental model

2.1.1. Chemicals

Rod-shaped nHAP of 20 nm and 40 nm in length ($nHAP_{20}$ and $nHAP_{40}$) (> 99% purity) was purchased from Nanjing Xian Feng Nanomaterials Technology Ltd. (Nanjing, China). $nMnO_2$ (20 nm, > 99% purity) was provided by the Agro-environmental Protection Institute, Ministry of Agriculture, Tianjin, China. The characteristics of the nanomaterials were described in our previous report (Gao et al., 2018b). Other reagents (analytical purity) were purchased from Bodi Chemical Co. Ltd. (Tianjin, China).

2.1.2. Zebrafish

The zebrafishes (*Danio rerio*) used in this study were acclimated to laboratory conditions for three weeks. During the study period, they were fed daily with freshly obtained *Artemia nauplii*. The temperature was maintained at 25 ± 1.0 °C, and the light conditions were adjusted according to OECD 203 guidelines (14-h light/10-h dark) (OECD, 1992). To avoid bacterial contamination in the enclosure, we regularly obliterated feces and other residues.

2.2. Experimental design

2.2.1. Preparation of nano-Cd composites

Twenty milligrams of the respective nanomaterials were suspended in 0.5 L of deionized water. The nanoparticles were dispersed by ultrasonication for 30 min. Then, different concentrations of $CdCl_2$ solutions were sequentially added to the suspensions. The suspensions were mixed by stirring with a magnetic stirrer. After adsorption equilibrium, the suspensions were centrifuged at 1600 × *g*, and then Cd^{2+} in the supernatant was filtered through a 0.22-μm syringe filter and analyzed with a triple quadrupole spectrometer. Auxiliary gas flow (Ar) and nebulizer gas flow (Ar) were 0.79 and 1.05 L/min, respectively. RF power was 1600 W. Detector model was single. In addition, the precipitates were lyophilized using a freeze-dryer to obtain nano-Cd composites.

2.2.2. Exposure experiments

Different amounts $CdCl_2$ or nano-Cd composites were dissolved/suspended in 5 L of reconstituted water that consisted of 0.294 g/L $CaCl_2{\cdot}2H_2O$, 0.123 g/L $MgSO_4{\cdot}7H_2O$, 0.648 g/L $NaHCO_3$, and 0.575 g/L KCl. The final concentrations of Cd^{2+} in sole Cd^{2+} treatments and nano-Cd composites were 0.64 mg/L. The suspensions were subjected to ultrasonication for 30 min before exposure experiments. Ten adult fishes (approximately 3 cm in length) were placed in 20-L tanks containing 5 L of different concentrations Cd^{2+} solutions and nano-Cd solutions, respectively. A control group (n = 10) was exposed to reconstituted water alone. All the treatments were performed in triplicate. The fishes were maintained at a temperature of 25 ± 1.0 °C under controlled lighting conditions (14-h light/10-h dark) and continuous aeration for 96 h. To maintain the quality of water in tanks, half of the test solutions were replaced every day. During the experimental period, the fishes were not fed at all, and dead fishes (if any) were immediately removed from exposure solutions. After completing the experimental exposure period (96 h), the zebrafishes were frozen using liquid nitrogen to death and the livers were collected immediately. The livers were maintained in TRIzol Reagent (Invitrogen, Carlsbad, CA) until total RNA extraction.

2.3. RNA-sequencing analysis

2.3.1. RNA isolation and Illumina HiSeq sequencing

Total RNA of each liver sample was extracted using TRIzol Reagent according to the manufacturer's protocol. RNA quality was measured by using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and the concentration was determined on a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Schwerte, Germany). For library preparation, we used 1 μg total RNA: the value of RNA integrity number (RIN) was > 7 for all samples. Next-generation sequencing libraries were prepared using NEBNext® Ultra™ RNA Library Prep Kit for Illumina®, following the manufacturer's instructions. After library purification (Beckman Agencourt AMPure XP beads), the PCR products were cleaned up using AxyPrep Mag PCR Clean-up (Axygen), validated using an Agilent 2100 Bioanalyzer, and quantified on a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). Then, libraries with different indices were multiplexed and loaded on an Illumina HiSeq instrument (Illumina, San Diego, CA, USA) according to manufacturer's instructions. Sequencing was carried out using a 2 × 150 bp paired-end configuration. Image analysis and base calling were conducted using HiSeq Control Software, Off-Line Basecaller, and GAPipeline-1.6 on the HiSeq instrument.

2.3.2. Read filtering and mapping

Reference genome sequences and gene model annotation files of relative species were downloaded from the UCSC, NCBI, and ENSEMBL genome databases. Next, Hisat2 (v2.0.1) was used to index a reference genome sequence. Finally, the clean data were aligned to the reference genome using the software Hisat2 (v2.0.1).

2.3.3. Differential expression analysis and Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses

On the basis of transcript abundance, gene expression counts were determined using HTSeq (V0.6.1). RPKM (Reads Per Kilo bases per Million reads) was applied to calculate the level of gene expression in different groups. Read counts were normalized with DESeq. 2 (V1.6.3). Differentially expressed genes (DEGs) were defined as those with ≥ 2-fold change and FDR ≤ 0.05).

Gene ontology (GO) terms describe cellular components, biological processes, and molecular functions of gene sets from differentially expressed genes (Huang et al., 2009). GO-Term Finder (V0.86) was used to annotate GO terms to enriched genes with a significant *p*-value < 0.05.

KEGG is a collection of databases dealing with genomes, biological pathways, diseases, drugs, and chemical substances (http://en.wikipedia.org/wiki/KEGG). We used in-house scripts to annotate KEGG pathways to significantly DEGs.