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Comparative analysis of the transcriptome responses of zebrafish embryos after exposure to low concentrations of cadmium, cobalt and copper

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ABSTRACT

Metal toxicity is a global environmental challenge. Fish are particularly prone to metal exposure, which can be lethal or cause sublethal physiological impairments. The objective of this study was to investigate how adverse effects of chronic exposure to non-toxic levels of essential and non-essential metals in early life stage zebrafish may be explained by changes in the transcriptome. We therefore studied the effects of three different metals at low concentrations in zebrafish embryos by transcriptomics analysis. The study design compared exposure effects caused by different metals at different developmental stages (pre-hatch and post-hatch). Wild-type embryos were exposed to solutions of low concentrations of copper ($CuSO_4$), cadmium ($CdCl_2$) and cobalt ($CoSO_4$) until 96 h post-fertilization (hpf) and microarray experiments were carried out to determine transcriptome profiles at 48 and 96 hpf. We found that the toxic metal cadmium affected the expression of more genes at 96 hpf than 48 hpf. The opposite effect was observed for the essential metals cobalt and copper, which also showed enrichment of different GO terms. Genes involved in neuromast and motor neuron development were significantly enriched, agreeing with our previous results showing motor neuron and neuromast damage in the embryos. Our data provide evidence that the response of the transcriptome of fish embryos to metal exposure differs for essential and non-essential metals.

1. Introduction

Metal contamination is a global environmental challenge because metals are neither chemically nor biologically degradable so they persist once they have entered the environment. In addition to natural sources of metals, anthropogenic sources metal-containing consumer products and metal nanoparticles add to the burden (AMAP, 1998). Millions of tons of electronic waste materials containing metals such as cadmium, copper, chromium, silver, nickel and cobalt end up in the environment every year (Robinson, 2009). Metals leach from landfills and to a much larger degree, become remobilized from sediments, e.g., during flooding events (Redelstein et al., 2015) and enter the aquatic environment, where they harm aquatic organisms, causing sub lethal effects on activity, growth, metabolism and reproduction. Especially fish are important indicators of metal pollution in fresh water since they can accumulate metals through respiration and food uptake. The main reason for fish' high sensitivity to metal toxicity is the constant exposure of the large respiratory epithelium of the gills, where the metal ions cause topic cell toxicity and interfere with ion exchange and transport processes. However, early developmental stages of fish, which have not yet developed gills, are also prone to metal toxicity, causing mortality or sublethal developmental impairments. The molecular basis of these adverse effects is still far less understood than metal toxicity in adult fish.

Environmentally important metals such as copper, have been studied extensively due to the high toxicity to aquatic organisms like daphnids and fish. For example, hair cell death has been studied in the lateral line neuromasts of 76 hpf zebrafish embryos exposed to copper (George et al., 2006; Hernandez et al., 2011). The *hsp70.1* promoter was shown to mediate a tissue-specific stress response in the presence of waterborne copper (Hernandez et al., 2011). Cadmium is also an environmental priority metal according to the European Water

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Framework Directive (EWFD) and has been shown to cause lethal effects in 48 h zebrafish embryos (LC50 range of 5.5 to ~30 mg/L) (Hallare et al., 2005; Weil et al., 2009) and to affect their hatching at < 0.2 μM (Fraysse et al., 2006). The commitment of neural progenitor cells is inhibited during brain development in zebrafish embryos concomitant with the regulation of the neurogenic genes *ngn1* and *neuroD* (Chow et al., 2008). More recently, the importance of cobalt has increased because it is regarded as a potential carcinogen according to REACH (directive 1970/2006/EEC) despite its low general toxicity. In a previous study, we found that exposure to cobalt, cadmium and copper affected the neuromast cells of the lateral line organ of zebrafish embryos (Sonnack et al., 2015). In order to improve our mechanistic understanding of the observed effects, we combined this analysis of toxicity with transcriptomics data. We have demonstrated in earlier studies that gene expression analysis can help determine underlying mechanisms of toxicity (Turner et al., 2012; Schiller et al., 2013b; Bluhm et al., 2014). Copper is known to affect neuromasts, and transcriptomics analysis of the inner ear of one-year-old zebrafish showed that the *stat3* and *socs3* pathway is involved and important for hair cell regeneration (Liang et al., 2012).

Cadmium toxicity in common carp involves the *beclin 1* gene (Gao et al., 2014). The effect of cadmium exposure on gene expression in different organs of adult zebrafish after 7 and 21 days revealed that *mt1*, *mt2* and *c-jun* were upregulated in the brain after 21 days, and that the *hsp70.1* gene, which is a marker of oxidative stress responses, was expressed in gills (Gonzalez et al., 2006).

Despite these numerous studies, there has been no comparative transcriptome-wide analysis of exposure to different metals at different time points. We therefore investigated the molecular basis of metal toxicity on a broader scale by applying transcriptomics analysis in wild-type zebrafish embryos at different developmental stages (pre-hatch and post-hatch), following exposure to three different metal solutions: copper ($CuSO_4$), cadmium ($CdCl_2$) and cobalt ($CoSO_4$). Specifically, we used the zebrafish embryo toxicity test to study metal toxicity during very early developmental stages and extended the scope of the assay by including transcriptome analysis endpoints. The zebrafish embryo toxicity test is used as a regulatory test for acute toxicity and complies with the principles of the 3Rs of replacement, reduction and refinement of animal tests (Russell et al., 1959). This approach has many advantages, including its simplicity, convenience, and short duration, the small size of the eggs, the rapid embryonic development of zebrafish and the transparency of the chorion, to facilitate observation. The zebrafish genome is also completely sequenced, which facilitates genetic and genomic analysis (Busch et al., 2011). Despite the limitations of microarray-based over high-throughput sequencing approaches to transcriptome analysis in terms of e.g. transcript coverage or probe specificity (Zhao et al., 2014), we favored economical microarrays for our study to suit the convenience of the zebrafish embryo toxicity test and its broad application in academic research and regulatory context alike.

2. Materials and methods

2.1. Metal exposure of zebrafish embryos

The exposure concentrations of copper, cadmium and cobalt were selected based on the lowest observed effect concentration (LOEC) for neuromast damage and were below the EC_{10} value for sublethal morphological effects as determined by one of our previous studies (Sonnack et al., 2015). The nominal concentrations of cadmium as cadmium chloride ($CdCl_2$, Sigma-Aldrich) were therefore 4.2 mg Cd/L, of cobalt as cobalt(II) sulfate heptahydrate ($CoSO_4.7H_2O$, Sigma-Aldrich) 6.4 mg Co/L and of copper as copper sulfate ($CuSO_4$, Sigma-Aldrich) 11.0 μg Cu/L. The actual dissolved metal concentrations were analytically determined (as described in the following section) only after termination of the study. Due to considerable deviations of the actual from the nominal exposure concentrations (see Table 2), the actual concentrations are reported throughout results and discussion.

Zebrafish rearing and embryo toxicity tests were carried out as described previously by Sonnack et al. (Sonnack et al., 2015; Sonnack et al., 2017). Each test was conducted in 96-well plates with one embryo per well, consisting of four replicates for each treatment (chemical exposure or ISO-water control). Each replicate, for controls and exposures, contained 24 embryos and was run on a separate plate. All metal test solutions were prepared in ISO-standard water (prepared according to OECD guideline 203, Annex 2) diluted 1:5. After harvesting the eggs, embryos were quickly transferred to individual wells and incubated until 48 hpf and 96 hpf at 26 ± 1 °C with a 14-h photoperiod. The test solutions were replaced with fresh test solutions (oxygen saturation of 100%) after 48 hpf to ensure stable exposure. In all tests, the dissolved oxygen remained above 90% over the whole test duration and the pH between 7.5 and 8.5.

The assessment time points refer to the incubation time but for simplification, were recorded as hpf, disregarding that the embryos at the start of exposure were already ≤ 1 hpf.

After 48 and 96 hpf of exposure, 20 embryos per replicate were sampled, pooled in 1.5 mL Eppendorf tubes, snap-frozen over dry ice and stored at − 80 °C for RNA extraction.

2.2. Analysis of metals

Metal concentrations were analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES) using an Agilent 720 instrument. Commercially available metal ICP standard solutions (Merck) were used to prepare appropriate stock solutions and subsequently calibration solutions. All calibration standard solutions and samples were acidified (nitric acid, Rotipuran® quality, Carl Roth) for stabilization (e.g. as suggested in ISO 11885:2007) and were diluted to a target concentration of approximately 0.5 M HNO_3. The method was validated by the analysis of certified reference water TMDA-53.3 (certified for Cu, Cd and Co by Environment Canada) and the multi-element standard IV (Merck) alongside experimental samples. The recovery of recalibration samples was in the range of 100 ± 15%.

For the determination of dissolved metal concentrations in experimental samples, two series of measurements were taken at different wavelengths. The first series used 226.502 nm for Cd, 238.892 nm for Co and 324.754 nm for Cu, whereas the second series used 214.439 nm for Cd, 235.341 nm for Co and 327.395 nm for Cu. The limits of detection (LOD) and quantification (LOQ) were calculated for each series prior to the measurement of samples according to DIN 32645:2008–11 and Geiss and Einax (2001). The LODs were 0.010–0.031 μg Cd/L, 0.400–0.719 μg Co/L and 0.274–0.821 μg Cu/L. The corresponding LOQs were 0.031–0.686 μg Cd/L, 1.200–2.156 μg Co/L and 0.821–1.255 μg Cu/L.

2.3. Microarray analysis

2.3.1. RNA Extraction

Total RNA was extracted from the frozen, pooled embryos using the Trizol method followed by homogenization with motor-driven plastic micro-pistils, and the RNA was cleaned using RNeasy Mini Spin Columns (Qiagen) according to the manufacturers' protocol. The quality and quantity of the total RNA was verified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) and an Agilent 2100 Bioanalyzer (Agilent Technologies). For all RNA samples, the absorbance ratios 260/280 and 260/230 were > 2.0 and 1.8, respectively, and the RNA integrity number was above 8.7.

2.3.2. Microarray experiment

Microarray experiments were performed using the Zebrafish (V3) Gene Expression Microarray 4 × 44 K Kit (Agilent Technologies), with 43,803 *Danio rerio* probes per array, representing ~17,000 genes. The