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Nonlinear responses to waterborne cadmium exposure in zebrafish. An *in vivo* study

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ABSTRACT

Cadmium (Cd) has proved to be associated with numerous toxic effects in aquatic organisms via waterborne exposure. With a view to investigate Cd toxicity along a broad spectrum of exposures reaching from environmental to toxic, we employed adult zebrafish (*Danio rerio*) for an in vivo study. A number of 10 fish per tank were placed in 40 L tanks and were exposed for 30 days to 0.0, 5.0, 25, 50, 75, 100 and 1000 μg Cd per liter. There were 2 tanks for each Cd exposure (duplicate experiment). Mortality was recorded daily, dead fish were collected and tissue samples were obtained for histologic observation, whereas remaining tissues were stored for Cd burden determination. Surviving fish were collected at the end of the experiment. Median overall survival (OS) in days was found to be 9.0, 11.0, 8.0 and 7.0 for 25 μg/L, 50 μg/L, 75 μg/L and 100 μg/L respectively, with all of them showing mortality greater than 50%. Remarkably, fish exposed to the highest Cd concentration (1000 μg/L) survived the longest exhibiting a mean OS of 29.2 days. Cd determination in fish tissue was conducted with an in house ICP-MS method and levels ranged from 3.1 to 29.1 ng/mg. Log Cd tissue levels were significantly correlated with the log Cd exposure levels ($r = 0.535$, $p < 0.001$). The highest Cd burden was determined for fish exposed to 1000 μg Cd /L (mean = 12.2 ng/mg). Histopathology supported these results. Our findings disclose a deviation in toxic responses through the range of Cd concentrations, leading to nonlinear responses. These differentiated responses, could be linked to hormesis phenomena.

1. Introduction

Cadmium (Cd) toxicity in aquatic organisms mainly via waterborne exposure, is rising to a major topic, being associated with parameters both of environmental pollution and human health risks. Cd is a non-essential heavy metal widely present in aquatic environments as a result of industrial and mining activities (Hsu et al., 2013) which tends to bioaccumulate in fish tissues (Arini et al., 2015). It can be eventually hazardous to humans with fish consumption being one of the important sources of Cd exposure. (Copat et al., 2013; Kalantzi et al., 2013; Renieri et al., 2014).

Even trace amounts of Cd can be toxic for fish, and its toxicity is dependent on the concentration of Cd^{2+}, which is the most bioavailable form (McGeer et al., 2011). Cd toxicity is affected by the timing of exposure and more specifically life stage, in addition to other factors such as internal biodynamics, temperature, salinity and genetics. The primary route of aqueous Cd exposure in fish is branchial and secondary the olfactory epithelium. Mechanisms of Cd toxicity involve disruption of ion regulation, oxidative damage, endocrine disruption, genotoxicity, olfactory and renal impairments, histopathological effects and adverse effects on behavior, survival, reproductive parameters and growth (Alsop and Wood, 2011; McGeer et al., 2011; Arini et al., 2015; Sfakianakis et al., 2015; Buha et al., 2013; Acosta et al., 2016).

Zebrafish (*Danio rerio*) has served as a vertebrate model for Cd toxicity studies, however, most literature is focused on its early life stages (embryo and larvae) and few research studies have utilized adult fish. Primary acute toxic effect of Cd in zebrafish appears to be ion loss, especially Ca^{2+} and Na^{+}(Aslop and Wood, 2011) both for larvae and adults. It is stated that Cd^{2+} antagonizes Ca^{2+} for gill binding sites leading to various adverse effects and eventual death (McGeer et al.,

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2011). Alsop and Wood (2011) also suggest that ion loss is due to the endocrine stress response which highlights the role of Cd as an endocrine disrupting (ED) chemical. Studies with different fish species, support that Cd behaves as an antiestrogenic chemical through several signaling pathways (Denslow, and Sepúlveda, 2007), reduces thyroid hormone levels and disrupts growth hormone expression (Sfakianakis et al., 2015). Moreover, oxidative stress caused by Cd in zebrafish is reported, via alterations in activities of catalase (CAT) and superoxide dismutase (SOD) (Wang and Gallagher, 2013), liver oxidative damage to proteins (Vergauwen et al., 2013a) and alteration of antioxidant capacity in zebrafish brain (Richetti et al., 2011). Exposure of adult zebrafish to Cd oxide nanoparticles also showed liver tissue damage and oxidative stress induction (Balmuri et al., 2017). There is also evidence of over-expressions of genes involved in protection against oxidative stress, following Cd exposure (Arini et al., 2015). With respect to olfactory effects, cell death, structural alterations, potential disruption of foraging, predator avoidance, and altered behavior are noted (Wang and Gallagher, 2013; Matz and Krone, 2007). Cd-induced deformities are also described not only in zebrafish but other species as well (Sfakianakis et al., 2015).

Tissue accumulation also plays an important part in Cd toxicity, with gills, kidney, liver, and brain exhibiting the highest Cd levels after waterborne exposure (Cambier et al., 2010), although levels appear to be modified after decontamination (Arini et al., 2015). Accumulation is profoundly affected by acclimation responses as well, such as temperature (Vergauwen et al., 2013a, b), or genetic ones (Arini et al., 2015; Gonzalez et al., 2006; Cambier et al., 2010) which subsequently affect short- and long-term physiological effects.

Each study conducted on zebrafish investigates different Cd effects hence, different LC_{50}s are reported, rendering it difficult to compare and assimilate the results. In order to better elucidate the mechanisms of Cd toxicity, fish responses should be investigated in a broader spectrum of exposure levels, as it is becoming more and more evident that there is a modulation of toxic responses depending on the concentration of exposure as well as tissue accumulation.

In this study, we employed adult zebrafish as means of investigating Cd ecotoxicity through an in vivo study. The aim was to investigate the Cd burden in zebrafish exposed to concentrations ranging from environmental to toxic and its association with fish mortality. We hypothesize that there is a linear survival response to exposure levels and attempt to investigate the response features.

2. Materials and methods

2.1. Chemicals – Reagents

Cadmium chloride hydrate ($CdCl_2{\cdot}H_2O$) 99.995% trace metals basis was purchased from Sigma Aldrich. Multielement standard solution for ICP was purchased from Target Analysis (CPA Chem). Tune A (Multielement standard solution) and Tune F (Cross calibration solution) were purchased from Target Analysis (CPA Chem). Nitric acid (HNO_3) trace SELECT, for trace analysis ≥ 69%, hydrogen peroxide solution (H_2O_2) for ultratrace analysis ≥ 30% and hydrochloric acid (HCL) ≥ 37%, trace SELECT, for trace analysis, were purchased from Sigma Aldrich. Type 1 (18.2 MΩ cm at 25 °C) ultrapure water was used (produced by a Direct-Q® Water Purification System). Dorm 4 (Fish protein CRMs, National Research Council of Canada; Joint Research Centre of European Commission) was used for quality control measures. All glassware and polyethylene vials were kept in 10% HNO_3 solution overnight and rinsed thrice with ultrapure water prior to use.

2.2. Experimental set-up and Cd exposures

2.2.1. Zebrafish maintenance

Wild type adult zebrafish (*Danio rerio*: 0.2–0.8 g), about 12 months old, were used for the study (ZF WT2 F5, Wageningen Agricultural University, The Netherlands). At the beginning of the experiment, fish were placed in 40 L tanks at the average temperature (28 ± 0.5 °C) and photoperiod (artificial 14 h light: 10 h dark) conditions, while oxygen saturation was constantly > 99% (through the use of air pump). Fish were fed twice a day (ad libitum) with industrial dry food in flakes (Sera Vipan, Germany), whereas the freshwater medium of the tanks was not renewed during the experiment due to its short final duration.

2.2.2. In vivo experimental set-up

A series of seven tanks with increasing Cd levels (including blank) were used for exploring the toxic response and the estimation of dose-death response curves. The experiment outline consisted of duplicates of aquaria for each Cd exposure level. Ten adult zebrafish were placed in each aquarium containing 36 L freshwater, after applying Cd ($CdCl_2$) exposures. Subsequently, fish were kept for 30 days in the aquaria or until fish death. Water temperature was monitored daily.

2.2.3. Cd exposures

A wide range of Cd exposure concentrations was selected: 0.0(control), 5.0, 25, 50, 75, 100 and 1000 μg Cd per liter. Cd was administered as $CdCl_2{\cdot}H_2O$, on account of being the most bioavailable species. Levels were selected to cover the range from environmental to polluted and finally toxic. (Cambier et al., 2010; Alsop and Wood, 2011;Vergauwen et al., 2013a, 2013b; Wang and Gallagher, 2013; Wang et al., 2015; Arini et al., 2015; Copat et al., 2013).

2.3. Sample collection, preparation, and digestion

Deaths for each Cd exposure level for both aquaria duplicates were recorded daily. Dead fish were collected from the aquaria and before sample storage, tissue samples of muscle, liver, gills and intestinal tube were obtained in formaldehyde solution 10% for histologic observation, using a pre-cleaned, stainless steel lancet. Remaining tissues were subsequently collected in polyethylene vials and stored at −20 °C until use. Fish which survived until the end of the experiment were sacrificed in liquid nitrogen and stored in polyethylene vials at −20 °C until use. The whole fish body was homogenized with liquid nitrogen, and 200 mg wet weight (w.w.) of each sample was weighed and placed in acid-cleaned borosilicate glass vials. 6 ml HNO_3 ≥ 69% 1:1 UP H_2O was added to each vial and left overnight for pre-digestion. For total dissolution, the predigest with the addition of 0.5 ml H_2O_2 and 1 ml HCl were placed in Teflon digestion vessels, sealed and placed in a high-pressure microwave digestion system. A speedwave MWS- 3^+, BERGHOF microwave digestion system with built in, non- contact temperature and pressure measurement was used for the digestion of the samples in PFA Teflon DAP-60 + pressure vessels. Digested samples were stored in borosilicate glass vials at 4 °C until further analysis. Each sample was diluted with ultrapure water up to HNO_3 2% final concentration prior to Inductively Coupled Plasma – Mass Spectrometer (ICP-MS) analysis. All method blanks and spiked samples were prepared using the same protocol. Spiking was conducted before the addition of acids and immediately after homogenization.

2.4. Analysis

2.4.1. Instrumentation and Cd analysis

A Thermo Fischer Scientific, XSERIES 2 ICP-MS equipped with an autosampler was used for the determination of Cd at the Laboratory of Toxicology, Medical School, University of Crete. Plasma lab software was used for sample analysis. The instrument was tuned (Tune A) and performance checks (Tune F) were run daily. The adjustment was performed by optimizing the nebulizer gas flow to achieve a high indium (In115) sensitivity while maintaining a low oxide and double charged ions sensitivity (156CeO + /140Ce + < 2% and $138Ba^{++}$/ 138Ba < 3%) and by lenses adjustment for achieving equal sensitivity for both the low mass (Li) and high mass (U) ions. Concentrations were