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Comparative effects of cadmium, zinc, arsenic and chromium on olfactory-mediated neurobehavior and gene expression in larval zebrafish (*Danio rerio*)

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ABSTRACT

Studies have shown that olfactory-mediated behaviors that are critical to survival can be disrupted by exposure to certain metals. Polluted waterways often contain elevated levels of metals, yet only a subset have been characterized for their potential to cause olfactory toxicity. A larval zebrafish behavioral assay was developed to characterize concentration-response curves for zinc (Zn), hexavalent chromium (Cr), and arsenate (As) olfaction inhibition. Cadmium (Cd), an established olfactory toxicant, was used as a positive control. As expected, following a 24-hour exposure to Cd, we observed a reduced response to taurocholic acid (TCA), a substrate for ciliated olfactory sensory neurons (OSNs), thus validating the behavioral assay. Zn exposure similarly decreased the olfactory response toward TCA, (IC_{50}: 36 μg/L and 76 μg/L, for Cd and Zn, respectively). The response towards a secondary odorant L-cysteine (Cys), a substrate for ciliated and microvillous OSNs, was significantly altered by both Cd and Zn exposure, although the response to Cys was not completely removed in Zn treated larvae, suggesting preferential toxicity towards ciliated OSNs. No significant changes in olfactory responses were observed following Cr and As exposures. Exposures to binary mixtures of Cd and Zn indicated that Zn had a protective effect against Cd toxicity at low Zn concentrations. QuantiGene (QDP) RNA analysis revealed Cd to be a potent inducer of metallothionein 2 (mt2) mRNA in zebrafish larvae, and Zn to be a weak mt2 inducer, suggesting a protective role of mt2 in Cd and Zn olfactory injury. By contrast, QDP analysis of eight other genes important in mitigating the effects of oxidative stress suggested an antioxidant response to Cd, but not Zn, As, and Cr suggesting that oxidative stress was not a primary mechanism of Zn-induced olfactory dysfunction. In summary, our study indicates that Zn inhibits zebrafish olfaction at environmental concentrations and may potentially mitigate Cd induced olfactory dysfunction when present in mixtures. The zebrafish behavioral trough assay incorporating the odorants L-cysteine and TCA is an effective assay to assess the effects of metals on olfactory function.

1. Introduction

A functional olfactory system is paramount for aquatic organisms for behaviors including prey selection, predator avoidance, homing, kin identification, and mate selection (Tierney et al., 2010; Cooper et al., 1976; Dittman and Quinn, 1996; Hara, 1992; Sutterlin & Gray, 1973). Teleost fishes comprise more than 90% of all fish species, and include most commercially relevant species such as salmon, trout, sablefish, pollock, and rockfish, as well as model organisms such as zebrafish. The olfactory system of teleosts is remarkably sensitive, with some species able to detect amino acids in the nanomolar range (Yamamoto et al., 2013; Bandoh et al., 2011). Olfactory sensory neurons (OSNs) present in the olfactory rosettes are exposed to the aquatic environment and are protected only by a mucosal lining which is secreted by goblet cells

Abbreviations: Nrf2, nuclear factor, erythroid 2-like 2; omp, olfactory marker protein b; dpf, days post fertilization; EM, E3 embryo medium; SRP, Superfund Research Program; Zn, zinc; As, arsenic; Cr, chromium; Cd, cadmium; TCA, taurocholic acid; OE, olfactory epithelium; OSN, olfactory sensory neuron; OR, olfactory receptor; MOR, major olfactory receptor; TRPC2, Transient Receptor Potential Cation Channel Subfamily C Member 2; OB, olfactory bulb; hmox1a, heme oxygenase 1a; gstp, glutathione *S*-transferase pi; gclc, glutamate-cysteine ligase, catalytic subunit; nqo1, NAD(P)H dehydrogenase, quinone 1; prdx1, peroxiredoxin 1; gpx1a, glutathione peroxidase 1a; sod1, superoxide dismutase 1, soluble; sod2, superoxide dismutase 2, mitochondrial; hsp70, heat shock cognate 70-kd protein, tandem duplicate 3; actb1, actin, beta 1; gapdh, glyceraldehyde-3-phosphate dehydrogenase; hprt1, hypoxanthine phosphoribosyltransferase 1; gadd45bb, growth arrest and DNA-damage-inducible, beta b

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within the olfactory epithelium (OE). Odorants are detected by OSNs within the OE, which transmit information via the olfactory nerve to the glomeruli within the olfactory bulb (OB) for odorant processing (Biechl et al., 2016).

To date, five OSN families have been identified in fish, including: ciliated, microvillous, crypt, kappe, and pear OSNs, which collectively detect six main odorant types; including amino acids, bile acids, amines, steroids, prostaglandins, and nucleotides (Ahuja et al., 2014; Koide et al., 2009; Oka et al., 2011; Sato et al., 2007; Wakisaka et al., 2017). Each of these five OSN types can express different classes of olfactory receptors (ORs), which are grouped into four major gene families: Trace Amine Associated Receptor (TAAR), Major Olfactory Receptor (MOR), Vomeronasal type 1 receptor-like (V1R), and Vomeronasal type 2 receptor-like (V2R). First described in rodents, a general rule in teleosts is that each individual OSN expresses a single OR, and that OSNs expressing the same OR converge to the same glomeruli within the olfactory bulb (OB). Violations of this rule have been demonstrated in zebrafish, with some OSNs found to express multiple ORs (Hansen et al., 2003; Hansen et al., 2004; Morita & Finger, 1998; Sato et al., 2007; Wakisaka et al., 2017; Yabuki et al., 2016). Microvillous and ciliated OSNs are the most numerous OSNs in teleosts and are preferentially activated by amino acids and bile acids, respectively. Amino acids are potential feeding cues, while bile acids can act as social and homing cues (Sato et al., 2005).

Because the fish peripheral olfactory system is in contact with the surrounding environment, it is vulnerable to the adverse effects of aquatic contaminants. Of particular concern are exposures to metals and pesticides that inhibit olfaction (Lürling & Scheffer, 2007; Sandahl et al., 2007; Tierney et al., 2010). Many of these chemicals are detected at elevated levels in the surface waters of important fish habitat and may impair or completely ablate olfactory mediated behavior at ecologically-relevant exposures (Tierney et al., 2010). In the Pacific Northwest, short-term exposure to copper (Cu) at concentrations found in surface waters and urban runoff can decrease predator detection in coho salmon (McIntyre et al., 2012). Cu and cadmium (Cd) are among the most well-studied olfaction impairing metals in teleosts (Baldwin et al., 2011; Green et al., 2010; Sandahl et al., 2007; Sloman, 2007; Tierney et al., 2010; Williams et al., 2016), while few other metals have been investigated for their ability to interfere with fish olfactory function. Furthermore, the effects of metals on olfaction have predominantly been assessed individually (Tierney et al., 2010), whereas environmental exposures often occur as mixtures (Tierney, 2016). Where addressed, metal mixtures are often identified using concentrations present in contaminated lakes; these studies have demonstrated that mixtures could impair the fish olfactory response, although the specific metals underlying these effects were often not identified (Thompson and Hara, 1977; Azizishirazi et al., 2013). Recent work by Dew et al. (2016) demonstrated that additive olfactory toxicity cannot be assumed, underscoring the need to assess metal mixtures on fish behavior.

In the present study, a larval zebrafish behavioral assay was used to assess the comparative olfactory toxicity of a common group of metals. Rapid development, powerful genetic tools and databases, optically clear embryos, ease of husbandry, and phenotype of chemical-induced olfactory injury make zebrafish an attractive model species to assess olfactory function (Sato et al., 2005; Wang & Gallagher, 2013; Ahuja et al., 2014). Even at the early stages of development, larval zebrafish respond to amino acids and bile acids that may activate, with little overlap, distinct areas of the OB, predominantly the lateral and medial OB (Li et al., 2005). Following assay validation, the olfactory toxicity of three relatively understudied metals (zinc, zrsenic and chromium) were assessed using responses to two odorants, taurocholic acid (TCA) and L-cysteine (Cys),which are preferentially recognized by ciliated and microvillous OSN types, respectively. The metals were selected based on their common occurrence in contaminated aquatic sites, and specifically in areas of the Lower Duwamish Waterway (LDW) in Seattle. The LDW comprises the lower section of the Green river, a productive river system for Chinook salmon (WDFW, 2017) and contains areas with elevated concentrations of metals, including zinc (Zn), hexavalent chromium (Cr), arsenate (As), and Cd in sediment and water (Conn et al., 2015; Paulson et al., 1989; Windward, 2010). The metals and exposure scenarios are not unique to this site, and are among the most common metal contaminants found at EPA National Priority List (NPL) sites. Our study also investigated binary mixtures of Cd and Zn using a method analogous to a chemical titration in which one metal's concentration remains the same while the other varies over successive metal mixtures. This allowed us to assess non-additive effects of binary mixtures of Zn and Cd, which commonly occurs in NPL sites. We hypothesized that the degree of metal induced oxidative stress would be an underlying mechanism of olfactory inhibition and used antioxidant and metallothionein gene expression analysis to characterize the role of these pathways in metal olfactory injury.

2. Materials and methods

2.1. Zebrafish maintenance

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Washington (IACUC). Adult wild type zebrafish were housed in re-circulating aquaria maintained at 28 ± 0.5 °C in a 14 h light/10-hr dark cycle. Fish received 2% of their body weight in flake food per day, and were provided with supplemental artemia twice daily. Source water from city municipal water was passed through a reverse osmosis filtration system and adjusted to 1000 ± 100 μS (pH 7.2) using Instant Ocean® salt and Na_2HCO_3. Critical water quality parameters (ammonia, nitrite, pH, and temperature) were checked daily. Paired male and female adult zebrafish were placed in divided spawning tanks the evening prior to spawning. Embryos were collected in the morning and placed into petri dishes containing fresh E3 embryo medium (EM; 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, and 0.33 mM $MgSO_4$, pH 7.2–7.4). Static renewal of EM occurred once daily until exposure at 5 days postfertilization (dpf).

2.2. Metal exposures

Metal salts (> 95% pure sodium arsenate dibasic hepta hydrate, zinc sulphate, cadmium chloride, and potassium dichromate) obtained from Sigma-Aldrich (St. Louis, MO) were used to make 1.0 mM stock solutions. Stock solutions of metals were verified via ICP-MS (University of Washington Environmental Health Laboratory, based on EPA 6020a Rev.1 2007). All metal stock solutions were found to be within 1 ± .09 mM of intended concentrations and the values reported reflect the measured concentrations for all metals. On the day of exposure, the stock solutions were diluted to working concentrations with EM (Table 1) and added to 90 mm petri dishes prior to transferring 4 dpf larval zebrafish (50 fish per 25 mL solution). Zebrafish larvae were exposed to metal solutions for 24-h prior to removal into fresh EM. To assess effects of binary mixtures of Zn and Cd, an approach analogous to a chemical titration of the two metals was used (Meyer et al., 2015). In this approach, one metal was held constant at its IC_{50} concentration, while the concentration of the other metal is modulated over successive metal mixtures (Table 1).

2.3. Behavior assay

An assay similar to that described by Shamchuk et al. (2018) was used to observe behavioral changes in larval zebrafish in response to the aversive odorants, TCA and L-cysteine, following metal exposures. Briefly, a clear acrylic trough (10.5 cm x 3.5 cm x 1.7 cm) was placed on top of a leveled light box for stable illumination. Two clear removable dividers divided the trough into three equally-sized zones. For each