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Modulations of TCDD-mediated induction of zebrafish cyp1a1 and the AHR pathway by administering Cd^{2+} *in vivo*

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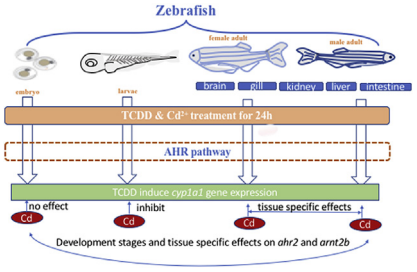
HIGHLIGHTS

- Combined effects of Cd^{2+} and TCDD on zebrafish were studied *in vivo*.
- Zebrafish embryo, larvae, and adults were investigated.
- Inhibitory effects of Cd^{2+} on TCDD-induced genes in larvae and some adult organs.
- Female fish are more sensitive than males to show cyp1a inductions.

GRAPHICAL ABSTRACT



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ABSTRACT

Trace metal ions such as cadmium (Cd^{2+}) and trace organics typified by 2,3,7,8- tetrachlorodibenzo-p-dioxin (TCDD) are common co-contaminants in the environment and cause toxic effects in aquatic organisms that pose serious health risks. We studied the effects of Cd^{2+} on the regulation of cytochrome P450 1A1 (*cyp1a1*) gene-induction by TCDD using zebrafish embryos and larvae and adult zebrafish tissues. Our results showed that TCDD induced the *cyp1a1* gene in all developmental stages and tissues of zebrafish, and the induction was higher in females than males. However, for the upstream genes (*ahr2* and *arnt2b)* that mediate *cyp1a1* gene induction in the zebrafish liver cell line was not induced by TCDD similar to the pattern of *cyp1a1* in all investigated groups. After co-treatment with Cd^{2+}, induction of the aryl hydrocarbon receptor pathway by TCDD was inhibited in the zebrafish larvae and the livers, intestines, kidneys and gills of adult zebrafish, but not in the embryos or brains of adult zebrafish, indicating that the toxicological effects of Cd^{2+} on TCDD are dependent on the developmental stages and tissue types. The present study confirms that Cd^{2+} blocks the TCDD-induced *cyp1a1* gene *in vivo* but emphasizes that the effects are specific to the developmental stage, type of tissue and sex. The combined effects of Cd^{2+} and TCDD must be taken into consideration together with these parameters to accurately predict and assess cadmium and TCDD-induced toxicity in fish and carcinogenesis in animals in general.

1. Introduction

Cytochrome P450 (CYP) 1A1 is an important phase 1 enzyme that metabolizes several drugs and compounds that are widely used in pharmacotherapy or present in the diet. It is a useful biomarker for analyzing the cellular responses and actions of planar halogenated aromatic hydrocarbon (PHAHs), as typified by 2,3,7,8-tetrachlorodibenzo -*p*-dioxin (TCDD), in both mammals and fish

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(Cao et al., 2000; Teramitsu et al., 2000; Zeruth and Pollenz, 2005). Among the various reactions catalyzed by CYP1A1, hydroxylation at an aromatic ring's vacant position is considered the hallmark of the initiation of carcinogenesis through the formation of highly reactive conversion products (such as epoxides) that have been shown to cause oncogenic mutations in experimental animals and humans (Androutsopoulos et al., 2009). The expression of CYP1A1 is transcriptionally up-regulated through the ligand-activated aryl hydrocarbon receptor (AHR), that can form a heterodimer with the aryl hydrocarbon receptor nuclear translocator (ARNT) during activation of gene transcription (Swanson, 2002). AHR plays an important role in mediating an adaptive response to xenobiotics, as well as in normal physiology and embryonic development (Stejskalova and Pavek, 2011).

Many sources of environmental exposure to AHR ligands involve metal co-exposure, as they are common co-contaminants in hazardous waste sites and are co-released from activities such as fossil fuel combustion and municipal waste incineration (Vakharia et al., 2001). Among these contaminants, TCDD is the most toxic PHAH (Fowles et al., 2009) and Cd is one of the most toxic trace elements (Asgary et al., 2017; Huo et al., 2017). Both of these chemicals are widespread in the environment and cause numerous adverse effects. Several reports have implicated trace metal ions as modifiers of CYP1A1 function and regulation, which implies that such metals could alter CYP1A1-mediated PHAH mutagenicity and carcinogenicity. For instance, Cd^{2+} in concentrations from 1.25 to 15 μM caused the significant dose-dependent induction of CYP1A1 in hepatoma (HepG2) cell lines (Tully et al., 2000). Elbekai and El-Kadi (2004) co-administered Cd^{2+} with an AHR ligand to inhibit the induction of CYP1A1 activity. The observed increases in mRNA levels and protein expression in Hepa 1c1c7 cells suggested that the inhibition was exclusively at the enzyme activity level (Elbekai and El-Kadi, 2004). We have recently shown that Cd^{2+} can significantly reduce the induction of *cyp1a1*, *arh2* and *arnt2b* by TCDD at the level of gene transcription in a zebrafish liver cell line (ZFL) (Chen and Chan, 2018). However, all of these studies were performed using cell lines *in vitro* which cannot replace the *in vivo* systems in natural cellular conditions. Compared with the *in vitro* study of cell cultures, using the *in vivo* model of zebrafish data allowed us to capture the deregulated biological processes that involve interactions between various cell types and whole-organism physiological metabolic processes at different life stages (Ung et al., 2010). Therefore, it would be important to further investigate those inhibition effects using a zebrafish model *in vivo*.

In this study, zebrafish (*Danio rerio*) embryos, larvae and adults were used to study the combined toxic effects of Cd^{2+} and TCDD. Zebrafish are effective for studying vertebrate toxicology due to their rapid development, conserved molecular pathway and potential for high through-put screening (Hill et al., 2003). More importantly, the pathways responsible for the developmental effects of TCDD in zebrafish is similar to that in mammalian models (Prasch et al., 2003). The genetic homology between human and zebrafish is approximately 70%, and ~84% of human genes known to be associated with human diseases are also present in zebrafish (Howe et al., 2013; Beaver et al., 2017). Unlike the limitation of *in vitro* cell line studies, toxic effects can be tested in different developmental stages, organs, and sexes of zebrafish. Previous studies have reported that stress responses to outside environmental stimuli differ between life stages, tissues and sex of zebrafish (Pavagadhi et al., 2012; Jeon et al., 2016; Li et al., 2017; Zhu et al., 2017). Therefore, the combined toxic effects of cadmium and TCDD were systematically investigated in the embryos, larvae and various organs of male and female adult zebrafish.

2. Materials and methods

2.1. Zebrafish husbandry and chemical exposure

AB strain zebrafish were maintained in an aquarium with the temperature controlled at 25 °C and a light: dark cycle of 16: 8 h, with licenses from the Department of Health, Hong Kong Special Administrative Region Government, and permit under the Animals (Control of Experiments) Regulations and the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong. All of the experimental protocols and procedures involving zebrafish were also performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals (no. 8023, revised in 1996). All surgery was performed under cold anesthesia, and all efforts were made to minimize the suffering of the animals.

The eggs were obtained from the natural spawning of adult zebrafish maintained according to the procedures described previously (Chan and Chan, 2012), collected within 4 h of spawning, transferred to petri dishes and incubated at 28 °C in an embryo medium (19.3 mM NaCl, 0.23 mM KCl, 0.13 mM MgSO4·7H2O, 0.2 mM $Ca(NO_3)_2$, 1.67 mM Hepes pH 7.2). At 5 h post fertilization (hpf), the embryos were examined under a dissecting stereomicroscope at ×6 magnification (Nikon, Tokyo, Japan). Only the embryos that had developed normally and reached the blastula stage (30% epiboly) were selected for subsequent experiments. The embryos were then incubated at 28 °C for 4 days to obtain 96-hpf larvae.

The selected 5-hpf zebrafish embryos with chorion and 96-hpf larvae were exposed to solvent control and different nominal test concentrations of $CdCl_2$ (Sigma-Aldrich, St. Louis, USA). Six replicates were set up for each concentration, each comprising 30 embryos/25 larvae exposed to 10 ml of test medium in a 50-ml beaker. The numbers of dead embryos and larvae were recorded after 24-h exposure, and the 24-h median lethal concentration (24 h-LC50) was calculated. For the adult zebrafish, the 24 h-LC50 was determined through exposure to six concentrations of Cd^{2+} and a fresh water control. Three replicates were set up for each concentration, comprising 12 fish (6 female and 6 male) exposed to 2 L of test medium in a 4-L glass container.

For the RNA assay and observation of deformity or dysmorphorgenesis, 30 individuals of 5-hpf zebrafish embryos, 25 of 96-hpf larvae and adult zebrafish comprising 12 fish (6 female and 6 male) exposed to 3- and 30-nM TCDD (Cambridge Isotope Laboratories, MA, USA) with or without Cd^{2+} at 10%, 50% and 100% of the 24 h-LC50 values were collected. Fresh water controls and individual exposure to Cd^{2+} were also set up. The exposure conditions were the same as those described above, and after 24-h exposure, the embryos (~12) and larvae (~6) were collected and washed once in phosphate-buffered saline. The samples were then stored at −80 °C for RNA extraction. After treatments, the adult male and female fish were separated and euthanized in ice water. Then, the brains, gills, kidneys, livers and intestines were immediately removed and individual organs were pooled from at least four fish for RNA isolation. The number of embryo or larvae that showed typical deformity were counted and one or two of them were imaged. All experiments were run in three replicates.

2.2. Determination of Cd^{2+} uptake in zebrafish

Embryos (30 individuals), larvae (25 individuals) and adult zebrafish (12 individuals) were co-treated with 3 nM TCDD and Cd^{2+} (50% LC50 for different stages of zebrafish) for 24 h. Each treatment was performed in triplicate. All samples were harvested,