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Adverse morphological development in embryonic zebrafish exposed to environmental concentrations of contaminants individually and in mixture

Cassandra D. Kinch [a,b,c], Deborah M. Kurrasch [b,c,*], Hamid R. Habibi [a]

[a] Department of Biological Sciences, University of Calgary, 2500 University Dr. NW, Calgary, Alberta, T2N 1N4, Canada
[b] Department of Medical Genetics, Cummings School of Medicine, University of Calgary, 3330 Hospital Dr. NW, Calgary, Alberta, T2N 4N1, Canada
[c] Alberta Children's Hospital Research Institute, Heritage Medical Research Building, 3330 Hospital Dr. NW, Calgary, Alberta, T2N 4N1, Canada

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ABSTRACT

Exposure to environmental contaminants has been linked to developmental and reproductive abnormalities leading to infertility, spontaneous abortion, reduced number of offspring, and metabolic disorders. In addition, there is evidence linking environmental contaminants and endocrine disruption to abnormal developmental rate, defects in heart and eye morphology, and alterations in behavior. Notably, these effects could not be explained by interaction with a single hormone receptor. Here, using a whole-organism approach, we investigated morphological changes to developing zebrafish caused by exposure to a number of environmental contaminants, including bisphenol A (BPA), di(2-ethylhexyl)phthalate (DEHP), nonylphenol, and fucosterol at concentrations measured in a local water body (Oldman River, AB), individually and in mixture. Exposure to nanomolar contaminant concentrations resulted in abnormal morphological development, including changes to body length, pericardia (heart), and the head. We also characterize the spatiotemporal expression profiles of estrogen, androgen, and thyroid hormone receptors to demonstrate that localization of these receptors might be mediating contaminant effects on development. Finally, we examined the effects of contaminants singly and in mixture. Combined, our results support the hypothesis that adverse effects of contaminants are not mediated by single hormone receptor signaling, and adversity of contaminants in mixture could not be predicted by simple additive effect of contaminants. The findings provide a framework for better understanding of developmental toxicity of environmental contaminants in zebrafish and other vertebrate species.

1. Introduction

The impact of environmental contaminants with hormone-like activity (endocrine disrupting chemicals; EDCs) is a growing concern. Indeed, a number of contaminants have been found to interfere with estrogen and androgen mediated pathways, and are suspected to disrupt fertility, reproductive success, onset of puberty, gonadal development, and gamete production in invertebrates (Waye and Trudeau, 2011), vertebrates (Adewale et al., 2009; Gore et al., 2014), including humans (Diamanti-Kandarakis et al., 2009). Recent evidence suggests that EDCs can modulate multiple endocrine functions, including thyroid and glucocorticoid actions (Nakamura et al., 2006; Xi et al., 2013; Zoeller, 2005). Furthermore, EDC exposure has been linked to altered neurodevelopment (Kinch et al., 2015), behavior (Braun et al., 2011, 2009; Harley et al., 2013), body size (Jeffries et al., 2008; Lema et al., 2007), and morphological abnormalities of pericardium (Antkiewicz et al., 2005), head (Orlando et al., 2004; Yamauchi et al., 2006) and eye (Orlando et al., 2004).

Early development is dependent on hormone signaling originating from maternal sources (McCormick, 1999; Power et al., 2001). Evidence in teleosts and mammals suggests that maternal hormones are likely deposited in the yolk sac to modulate early development via endocrine and paracrine signaling (Gore et al., 2014; McCormick, 1999; Patel et al., 2011). In zebrafish, differentiation of the first endocrine gland, the thyroid gland, occurs at 3 dpf (days post fertilization) (Alt et al., 2006). Although, hormone receptors are present earlier, to potentially bind these maternally-derived hormones (Nesan and Vijayan, 2013; Pikulkaew et al., 2010), as also occurs in humans and other viviparous animals

* Corresponding author at: Department of Medical Genetics Cummings School of Medicine, T2N 4N1, Canada.
E-mail addresses: cdmaier@ucalgary.ca (C.D. Kinch), kurrasch@ucalgary.ca (D.M. Kurrasch), habibi@ucalgary.ca (H.R. Habibi).

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(Feldt-Rasmussen and Mathiesen, 2011). This early expression of hormone receptors opens the possibility that a window of vulnerability exists during early development, whereby EDC exposure might interfere with normal hormone-mediated developmental events. Previous studies have shown that minute changes in maternal thyroid hormone level during early pregnancy have resulted in decreased fetal head circumference, in addition to later-life neurological impairment including deafness and mental retardation (Patel et al., 2011; van Mil et al., 2012). These clinical manifestations suggest that small (picomolar) changes in hormone levels prenatally can result in lasting effects later in life.

Historically, the effects of EDCs were thought to be limited to hormone signaling via estrogen (ER), androgen (AR) and thyroid hormone (ThR) receptors. Yet, EDCs have been shown to bind other nuclear hormone receptors such as the glucocorticoid receptor, as well as membrane-bound ERs, membrane-bound ARs, and thyroid hormone-specific integrin $\alpha\nu\beta3$ that initiate rapid-acting signal transduction cascades (Cheng et al., 2010; Prossnitz et al., 2008; Thomas and Doughty, 2004). Further, EDCs have been shown to modulate alternative receptors, such as G-protein coupled receptors (GPCRs), kinase-linked receptors, and ionotropic receptors, which do not usually bind hormones as ligands (Aoshima et al., 2001; Bisset et al., 2011; Gao et al., 2013). The potential for EDCs to bind multiple receptors might explain adverse physiological effects from EDC exposure that cannot be explained by signaling via a single hormone receptor such as ER, AR, and ThR.

Previously, we have identified the presence of phenols (bisphenol A; BPA), nonylphenol ethoxylates (nonylphenol), phthalate esters (DEHP), phytoestrogens (fucosterol) and other anthropogenic chemicals related to municipal, industrial, and agricultural activities in the Oldman River, AB (Evans et al., 2012; Jeffries et al., 2010, 2008). These chemicals demonstrate affinity to bind multiple receptor targets, including nuclear hormone receptors, and a robust body of evidence links exposure to these contaminants with adverse endocrine and extra-endocrine effects (Diamanti-Kandarakis et al., 2009; Gore et al., 2014; Jordan et al., 2012; Kinch et al., 2015). In the Oldman River, contaminant concentrations of BPA, nonylphenol, DEHP, and fucosterol were elevated downstream of a wastewater treatment plant compared to upstream sites, and is likely linked to adverse reproductive health observed in a resident fish population (Jeffries et al., 2010, 2008). Furthermore, laboratory results show exposure of fish to a select number of contaminants separately and in mixture at the same concentrations measured in the Oldman River, AB, caused significant metabolic disruption in a manner not predicted from exposure to individual contaminants alone (Jordan et al., 2012). This is consistent with other studies using *ex vivo* approaches to study contaminants in mixture, and suggests that physiological affects from exposure to contaminant mixtures cannot consistently be predicted by the effects of these contaminants when exposed singly (Chen et al., 2007; Hu et al., 2014; Sumpter and Jobling, 1995).

Herein, we detail the effects of exposure to BPA, DEHP, nonylphenol and fucosterol at concentrations found in the Oldman River singly and in mixture on gross morphological development in growing zebrafish embryos and larvae. Using the principle of Effects Summation, we experimentally investigate whether contaminant effects in mixture can be predicted by additive effects of individual contaminants. In addition, by exposing embryonic zebrafish to physiological concentrations (Nelson and Habibi, 2008) of individual endogenous hormones, including estradiol, testosterone, and thyroid hormone (triiodothyronine) we provide a basis for comparison between contaminant effects and the effects from agonism of hormone receptors by their natural ligands. Given the impact of environmental contaminants at high (micromolar to millimolar) concentrations (Antkiewicz et al., 2005; Fraysse et al., 2006; Power et al., 2001; Raldua et al., 2008), together with the effect on sentinel fish populations, we hypothesize that exposure to environmental concentrations of BPA, nonylphenol, DEHP and fucosterol alters morphology, and that effects are not mediated by single hormone receptor binding. Moreover, we hypothesize that the effects of contaminants individually are not predictive of the effects of contaminants in mixture.

2. Materials and methods

2.1. Zebrafish husbandry and contaminant preparation

All protocols and procedures were approved by the Biological Science Animal Care Committee (protocol #BI11R-25) for morphometric experiments and by the Health Science Animal Care Committee (protocol #M10079) for in situ experiments in compliance with the Guidelines of Canadian Council of Animal Care. Wild-type TL (Tail Long) zebrafish embryos were maintained on a 14-h light: 10-h dark cycle at 28 °C in embryo medium (E3) as described by Westerfield (2000). Parental zebrafish were fed twice daily with a 1:1 mixture of hatched brine shrimp (Brine Shrimp Direct, Ogden, UT) and Nutrafin® Max Tropical Fish Flakes (Hagen, Mandsfield, MA), daily and set up in mating broods of 3–5 fish with females separated from males in breeding tanks (SBTANK, Aquatic Habitats, Apopka, FL) the evening preceding breeding. Embryos were collected using a sieve within two hours post fertilization (hpf). Following collection, embryos were immersed in hormone or contaminant treatments within three hpf until sacrifice at 24 hpf, 48 hpf or 72 hpf in glass petri dishes and maintained at 28 °C in an incubator on a 14-h light: 10-h dark cycle. Immediately prior to sacrifice, 24 hpf embryos and unhatched 48 hpf larvae were manually dechorionated using Dumont No. 5 forceps (F6521, Sigma-Aldrich, Oakville, CA) (72 hpf larvae had hatched naturally and did not require dechorionation). All contaminant treatments were prepared in a 1:3 ratio of 0.002% (vol/vol) 1 M NaOH to 95% EtOH (vehicle) in E3 the day of fertilization and stored at room temperature protected from light. Estradiol (E2; 17β-Estradiol) (E8875, Sigma–Aldrich, prepared at a final concentration of 1.8 nM), testosterone (T; 17β-Hydroxy-3-oxo-4-androstene) (T1500, Sigma-Aldrich; 1.7 nM), thyroid (T_3 3,3′,5-triiodo-L-thyronine) (T2877, Sigma-Aldrich; 0.8 nM) hormone treatments and bisphenol A (4,4′-(propane-2,2-diyl)diphenol) (BPA; 239658, Sigma-Aldrich; 6.8 nM), DEHP (Di(2-ethylhexyl)phthalate) (80030, Sigma Aldrich; 5.2 nM), nonylphenol (4-nonylphenol) (442873, Sigma-Aldrich; 0.8 nM), and fucosterol (3β-hydroxy-5,24(28)-stigmastadiene) (F5379, Sigma-Aldrich; 2.3 nM) contaminant treatments were replaced daily via multiple washes. In addition, a cohort of embryonic zebrafish were exposed to a contaminant mixture (hereafter, referred to as experimental mixture) consisting of DEHP (5.2 nM) + BPA (6.8 nM) + nonylphenol (0.8 nM) + fucosterol (2.3 nM), also refreshed daily. The concentrations of contaminants used in these studies are similar to reported concentrations sampled from the Oldman River (AB, Canada) (Sosiak and Hebben, 2005) in 2003.

2.2. Morphological measurements

A minimum of 10 hormone, contaminant or control exposed embryos or larvae were anesthetized by immersion in 0.02% tricaine methanesulfonate (3-aminobenzoic acid ethyl ester methanesulfonate) (A5040, Sigma-Aldrich) in E3 then sacrificed by immersion in 4% paraformaldehyde (PFA; P6148, Sigma-Aldrich) and fixed overnight at 4 °C. Embryos and larvae were then placed in phosphate buffered saline (PBS) (pH 7.4) containing 0.1% Tween (P2287, Sigma-Aldrich) (PBT) for morphological analyses.