Aquatic Toxicology 148 (2014) 16–26

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Multigenerational effects of benzo[a]pyrene exposure on survival and developmental deformities in zebrafish larvae

Jone Corrales, Cammi Thornton, Mallory White, Kristine L. Willett*

Department of Pharmacology and Environmental Toxicology Research Program, School of Pharmacy, University of Mississippi, University, MS 38677, USA

ARTICLE INFO

Article history:
Received 18 October 2013
Received in revised form
18 December 2013
Accepted 24 December 2013

Keywords:
Multigeneration
Survival
Hatching
Deformities
Zebrafish
Benzo[a]pyrene

ABSTRACT

In the aquatic environment, adverse outcomes from dietary polycyclic aromatic hydrocarbon (PAH) exposure are poorly understood, and multigenerational developmental effects following exposure to PAHs are in need of exploration. Benzo[a]pyrene (BaP), a model PAH, is a recognized carcinogen and endocrine disruptor. Here adult zebrafish (F0) were fed 0, 10, 114, or 1012 μg BaP/g diet at a feed rate of 1% body weight twice/day for 21 days. Eggs were collected and embryos (F1) were raised to assess mortality and time to hatch at 24, 32, 48, 56, 72, 80, and 96 h post fertilization (hpf) before scoring developmental deformities at 96 hpf. F1 generation fish were raised to produce the F2 generation followed by the F3 and F4 generations. Mortality significantly increased in the higher dose groups of BaP (2.3 and 20 μg BaP/g fish) in the F1 generation while there were no differences in the F2, F3, or F4 generations. In addition, premature hatching was observed among the surviving fish in the higher dose of the F1 generation, but no differences were found in the F2 and F3 generations. While only the adult F0 generation was BaP-treated, this exposure resulted in multigenerational phenotypic impacts on at least two generations (F1 and F2). Body morphology deformities (shape of body, tail, and pectoral fins) were the most severe abnormality observed, and these were most extreme in the F1 generation but still present in the F2 but not F3 generations. Craniofacial structures (length of brain regions, size of optic and otic vesicles, and jaw deformities), although not significantly affected in the F1 generation, emerged as significant deformities in the F2 generation. Future work will attempt to molecularly anchor the persistent multigenerational phenotypic deformities noted in this study caused by BaP exposure.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs), such as benzo[a]pyrene (BaP), are ubiquitous environmental contaminants derived from the incomplete combustion of organic compounds. While forest fires and ocean floor seeps are natural sources of PAHs (Latimer and Zheng, 2003), the more numerous and damaging anthropogenic sources include diesel- and gasoline-powered vehicles, coal-fired power plants, residential heating, cooking, and tobacco smoking. PAHs enter the aquatic environment via urban runoff and dry and wet depositions of atmospheric PAHs (Boström et al., 2002; Latimer and Zheng, 2003). Once in the aquatic environment, PAHs are taken up by organisms from the water or through the diet (Hylland, 2006). In the 2011 CERCLAs Priority List of Hazardous Substances, BaP was ranked #8 in front of PAHs as a mixture (#9) and benzo(b)fluoranthene (#10), an increase in priority since CERCLA's 2007 report. Moreover, in the 2012 IARC Monographs, BaP was classified as Group 1 (an animal and human carcinogen) (http://monographs.iarc.fr/ENG/Classification/).

Waterborne PAH exposure caused toxicity and altered the rate of development in fish early life-stages (Barron et al., 2004; Hawkins et al., 2002; Incardona et al., 2004; Colavecchia et al., 2004). *Fundulus heteroclitus*, a model fish species used in ecotoxicological studies, had elevated deformity indices (heart elongation, pericardial edema, tail shortening, and hemorrhaging) after exposure to binary mixtures of PAHs including BaP, β-naphthoflavone (BNF), α-naphthoflavone (ANF), fluoranthene (FL), piperonyl butoxide (PBO), and 2-aminoanthracene (AA) (Wassenberg and Di Giulio, 2004). Cardiac deformities were also apparent after BaP exposure in the absence of other PAHs (Wills et al., 2009). In pink salmon (*Oncorhynchus gorbuscha*) and rainbow trout (*O. mykiss*), dissolved PAHs from crude oil caused higher mortality, alterations in time to hatch, growth reduction, spinal deformities, jaw deformities, yolk sac edema, and impaired swimming (Carls and Thedinga, 2010; Barron et al., 2004; Hawkins et al.,

* Corresponding author at: Box 1848, 305 Faser Hall, Department of Pharmacology, University of Mississippi, University, MS 38677, USA. Tel.: +1 662 915 6691; fax: +1 662 915 5148.
E-mail addresses: jcorrale@olemiss.edu (J. Corrales), cehickm2@olemiss.edu (C. Thornton), kwillett@olemiss.edu (K.L. Willett).

0166-445X/$ – see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.aquatox.2013.12.028

2002). In *Sebastiscus marmoratus*, a teleost of the scorpion fish family, skeletal deformities including incidence of spinal curvature and craniofacial defects were caused by BaP as well as altered expression of genes involved in bone formation (He et al., 2011).

Recently zebrafish (*Danio rerio*) has become a preferred toxicity model due to its rapid life cycle, high fecundity, transparent development, and because the embryos are amenable to genetic manipulation using transgenic approaches and morpholino gene knockdowns (Sipes et al., 2011). Cardiac dysfunction resulting in pericardial edema and heart looping after exposure to PAHs has been extensively examined in zebrafish by Incardona et al. (2004, 2005, 2006, 2011). Zebrafish larvae treated with dibenzothiophene, phenanthrene, or fluorene not only displayed curvature of the trunk and tail and growth reduction, but also showed pericardial edema (Incardona et al., 2004). Moreover, atrioventricular conduction block was the primary effect of dibenzothiophene and phenanthrene with secondary defects on cardiac morphology, kidney development, neural tube structure, and craniofacial skeleton (Incardona et al., 2004). BaP exposure also caused defects in heart morphology and dysfunction (Incardona et al., 2011).

However, none of the studies described above examined developmental malformations across generations. In fish, multigenerational studies have primarily focused on reproductive repercussions associated with endocrine disruptors [e.g. bisphenol A (Sohoni et al., 2001; Staples et al., 2011), nonylphenol (Holdway et al., 2008), 2,3,7,8-tetrachlorodibenzo-p-dioxin (King-Heiden et al., 2005), 17α-ethinylestradiol (Zha et al., 2008), and pulp and paper mill effluents (Parrott et al., 2010)] with little emphasis on multigenerational developmental deformities. Moreover, all the previously mentioned studies were waterborne exposures. Alternatively, dietary exposures provide an avenue to investigate this environmentally relevant exposure route (Peterson et al., 2003). Although some work on the reproductive impacts of dietary persistent organic pollutants and brominated flame retardants (Berg et al., 2011; Halden et al., 2011; Nourizadeh-Lillabadi et al., 2009; Chou et al., 2010) has been done, studies looking at dietary and multigenerational effects of PAH exposure on fish developmental deformities are lacking.

In the work presented here, multigenerational developmental defects (F1, F2, F3) were measured subsequent to a parental dietary BaP exposure (F0). We found that (1) a parental dietary exposure to BaP had adverse effects on the immediate offspring and (2) some of these adverse effects persisted across generations.

2. Materials and methods

2.1. Zebrafish care

AB line wild-type zebrafish were purchased from Zebrafish International Resource Center (ZFIN, Eugene, OR) and raised under the approved IACUC protocol. Fish were kept in Aquatic Habitats ZF0601 Zebrafish Stand-Alone System (Aquatic Habitats, Apopka, FL) with zebrafish water (pH 7.0–7.5, 60 parts per million (ppm), Instant Ocean, Cincinnati, OH) at 25–28 °C, 14:10 light-dark cycle. Fish were fed twice daily with TetraMin® Tropical Flakes and live brine shrimp. Sexually mature fish without any deformities or signs of disease were selected as breeders. Their eggs were collected and larvae were raised to 120 days post fertilization (dpf) to obtain the F0 generation for the dietary exposure described below.

2.2. Parental dietary exposure

Sexually mature (120 dpf) zebrafish were fed either acetone-treated (control) or BaP-treated (0.25, 2.5, or 25 μg/g fish nominally equivalent to 12.5, 125, or 1250 μg/g food, respectively) flake food; for actual BaP concentrations measured in the diet, see section 2.3. Comparable BaP concentrations have previously been used in fish including zebrafish, although we also extended the dose range to include a higher concentration (Alsop et al., 2007; Bailey et al., 2009). A preliminary exposure in our lab showed that feed rate and egg production did not vary between fish fed acetone-treated and non-treated food, therefore acetone was used as the BaP carrier solvent. Acetone was purchased from Fisher Scientific (Fair Lawn, NJ) and BaP from Supelco Analytical (Belfonte, PA). To prepare the treated flake food, 24 g of flake food were spiked with 18 mL of acetone containing BaP (0, 0.01667, 0.1667, or 1.667 μg/μL). The spiked flakes were immediately rotary evaporated to dryness and stored in amber vials at room temperature. Paired (2 × 2) zebrafish in ten replicate tanks per treatment group ($N = 10$ replicate tanks for a total 40 fish/group) were allowed to acclimate for a week while maintained at 25.5–28 °C and fed twice daily with TetraMin® Tropical Flakes and live brine shrimp. During the exposure, fish were fed 1% body weight twice daily of the corresponding dose of BaP-treated flake food and once daily live brine shrimp for 23 days. On days 20 and 21, eggs were collected to produce the F1 generation. On day 22, one fish per tank (five females and five males per group) were euthanized for histological examination. On day 23, the exposure was terminated and remaining fish were euthanized.

2.3. Extractions and chemical analysis

Treated flake food was extracted with methylene chloride to confirm the nominal concentrations of BaP. Approximately 10 mg of flakes were extracted right before day 0 and on days 7 and 14 with 2–3 mL of methylene chloride. A known concentration of a surrogate standard, benzo[a]pyrene-d12, was added to each sample to yield a final concentration of 0.2 μg/mL. Samples were vortexed for 30 s and centrifuged for 7 min at 2000 rpm (668 × *g*). Samples were then blown to dryness with N_2 and brought back up with a known volume of hexane. A known concentration of internal standard, fluorene-d10, was added to each sample to determine extraction efficiency. Samples were run on the GC/MS under the selected ion mode to quantitate the concentration of BaP in each sample. BaP was not identified in the acetone-treated samples. Actual BaP concentrations of the treated flakes were: 10.44 ± 0.4, 113.6 ± 2.3, and 1012 ± 30.7 μg BaP/g flake equivalent to 0.209 ± 0.008, 2.27 ± 0.046, and 20.25 ± 0.614 μg BaP/g fish, respectively. Percent recoveries ranged from 70 to 145%.

In addition to the BaP-treated flake food, chemical analysis was done in F1 fertilized eggs to assess potential BaP transfer into the embryos. Approximately 110 F1 fertilized eggs (8 hpf) collected on days 20 and 21 were stored at −80 °C until chemical analysis. Eggs from the four treatment groups in triplicate were extracted 3 times with 3 mL of methylene chloride each time. A known concentration of the surrogate standard, benzo[a]pyrene-d12, was added to each sample to yield a final concentration of 0.2 μg/mL. Samples were vortexed for 15 s and centrifuged for 7 min at 3000 rpm (1509 × *g*) and passed over an alumina and sodium sulfate column to remove fat and water from the extract. Samples were then blown to dryness with N_2 and brought back up with a known volume of hexane. A known concentration of the internal standard, fluorene-d10, was added to each sample to yield a final concentration of 0.2 μg/mL to determine extraction efficiency. Samples were run on the GC/MS under the selected ion mode to quantitate the concentration of BaP in each sample. BaP was not detected in the eggs despite good recovery concentrations (50–105%). This could be due to multiple reasons including but not limited to insufficient number of eggs, small amount of BaP deposition in the eggs, or BaP being rapidly metabolized by the mothers.