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Embryonic exposure to benzo(a)pyrene inhibits reproductive capability in adult female zebrafish and correlation with DNA methylation☆

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ABSTRACT

This study was conducted to investigate the effects of embryonic short-term exposure to benzo(a)pyrene (BaP), a model polycyclic aromatic hydrocarbon, on ovarian development and reproductive capability in adult female zebrafish. In 1-year-old fish after embryonic exposure to BaP for 96 h, the gonadosomatic indices and the percentage of mature oocytes were significantly decreased in the 0.5, 5 and 50 nmol/L treatments. The spawned egg number, the fertilization rate and the hatching success were significantly reduced, while the malformation rate of the F1 unexposed larvae were increased. The mRNA levels of follicle-stimulating hormone, luteinizing hormone, ovarian cytochrome P450 aromatase *cyp19a1a* and *cyp19b*, estrogen receptor *esr1* and *esr2*, and hepatic vitellogenin *vtg1* and *vtg2* genes, were down-regulated in adult female zebrafish that were exposed to BaP during embryonic stage. Both 17β-estradiol and testosterone levels were reduced in the ovary of adult females. The methylation levels of the gonadotropin releasing hormone (GnRH) gene *gnrh3* were significantly increased in the adult zebrafish brain, and those of the GnRH receptor gene *gnrhr3* were elevated both in the larvae exposed to BaP and in the adult brain, which might cause the down-regulation of the mRNA levels of *gnrh3* and *gnrhr3*. This epigenetic change caused by embryonic exposure to BaP might be a reason for physiological changes along the brain–pituitary–gonad axis. These results suggest that short-term exposure in early life should be included and evaluated in any risk assessment of pollutant exposure to the reproductive health of fish.

1. Introduction

The toxic effects of environmental contaminants on fish is one of the reasons for fish population decline (Blewett et al., 2016; Duffy et al., 2014; Johnson-Restrepo et al., 2008). Although the production and application of some persistent organic pollutants have been prohibited, there are still many endocrine disrupting chemicals (EDCs) present in the environment. Aquatic organisms in contaminated sites, including fishes, are likely to be exposed to chemicals possessing endocrine disrupting effects during their life. The early life stages of fish are vulnerable to EDCs (van Aerle et al., 2002). For example, adult fathead minnows (*Pimephales promelas*) exposed to 10 ng/L ethinylestradiol (EE_2) during early life exhibited changes in gonadal development (van Aerle et al., 2002). In three-spined sticklebacks (*Gasterosteus aculeatus*), short-term exposure as juveniles to EE_2 clearly influences their reproductive performance as adults (Maunder et al., 2007). However, few studies concerning long-term effects on the reproductive system in fish and the mechanisms involved following the exposure of early life stages to environmental pollutants are available.

One mechanism by which early exposure is known to produce later outcomes is epigenetic modifications (Vandegehuchte and Janssen, 2011). There is increasing evidence that pollutants may cause adverse health outcomes, such as embryonic lethality, infertility, diabetes, pulmonary disease, and cancer via epigenetic modifications (Leclercq et al., 2017; Pierron et al., 2014). Epigenetics is the study of molecular mechanisms altering gene transcription

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without the alteration of the primordial DNA coding sequence (Vandegehuchte and Janssen, 2011). One of the most researched epigenetic mechanisms is DNA methylation, which transfers a methyl group to the cytosine bases of DNA to form 5-methylcytosine (Liu et al., 2014). The hypermethylation and hypomethylation of the promoter DNA usually causes gene silencing and transcriptional activation respectively (Suzuki and Bird, 2008). Early development is peculiarly sensitive to chemically caused epigenetic disruption, since embryo development can be regulated by epigenetic procedure (Feng et al., 2010; Groh et al., 2015; Morgan et al., 2007), and epigenetic alteration during embryo development may persist into adulthood (Groh et al., 2015). There have been studies in fish on the toxic effects caused by exposure to pollutants during their early-life stages (Hicken et al., 2014; Huang et al., 2014; Maunder et al., 2007; van Aerle et al., 2002). At the same time, some investigations showed that pollutants can change DNA methylation levels in fish (Corrales et al., 2014a; Fang et al., 2013a, 2013b; Pierron et al., 2014). For example, the DNA methylation level of *fshr* (the follicle stimulating hormone receptor gene) in female silver eels (*Anguilla anguilla*) from a contaminated site was significantly correlated with the pollutant content of the gonads (Pierron et al., 2014). However, the linkage between toxic effects and chemically induced epigenetic changes is still lacking. In addition, there is little knowledge about the toxic effects caused by promoter-specific methylation in zebrafish *(Danio rerio)* (Kamstra et al., 2015). To investigate the effects on fertility in fish after exposure to low concentrations of environmental pollutants during embryo development, benzo[a]pyrene (BaP), a model polycyclic aromatic hydrocarbon (PAH), was used in the present study as a harmful and ubiquitous environmental pollutant.

Because their natural and human source included volcanic eruptions, oil production and shipping, vehicle exhausts and the incomplete combustion of organic compounds in industrial production and cooking, PAHs are ubiquitously distributed and are detected in diverse environmental substances such as air, water, soil and food. Even if the concentrations of PAHs in the aquatic environment are sometimes low, fishes and other aquatic organisms are inevitably exposed to PAHs during their embryonic or entire life cycles. Numerous studies have revealed that PAHs possess a diversity of toxicities to organisms, including carcinogenicity, developmental toxicity, neurotoxicity and reproductive toxicity. Although there are detrimental influences on the reproduction in wild fish from PAH polluted environments (Nicolas, 1999), whether exposure to PAHs in critical developmental periods would cause adverse effects on adult fish remains unclear. In zebrafish, transient exposure of embryo to a very low dose of crude oil resulted in alterations of cardiac morphology and cardiovascular function in adult (Hicken et al., 2014). Additionally, embryonic short-term exposure (for 96 h) to low dose BaP caused symptoms similar to cardiac hypertrophy in adult fish (Huang et al., 2014). Adult zebrafish from embryonic exposure to BaP exhibited neurobehavioral deficits as well as neurodegenerative syndromes, accompanied by altered DNA methylation levels (Knecht et al., 2017; Gao et al., 2017). BaP exposure via adult zebrafish diet impacted the development of the F1 and F2 generation (Corrales et al., 2014b). Exposure of adult *Fundulus heteroclitus* to waterborne BaP significantly decreased the egg fertilization and hatching success of F1 embryos (Booc et al., 2014). BaP exposure to zebrafish embryos decreased the global methylation, suggesting that aberrant DNA methylation can relate with BaP-induced toxicity (Fang et al., 2013b; Gao et al., 2017; Knecht et al., 2017). However, the potential mechanisms for these toxic effects are not adequately studied.

In the present study, BaP is used to investigate the reproductive toxicity during embryonic exposure in adult zebrafish and its mechanism. The concentrations of BaP in surface seawater range from 0.004 to 0.09 nmol/L (1.0–23.4 ng/L) in Maluan Bay in Xiamen, China (Tian et al., 2004) and 2–10 nmol/L (0.56–3.32 μg/L) in the Jiulong River Estuary and the western Xiamen Sea, Xiamen, China (Maskaoui et al., 2002). The concentrations of BaP used in our study were 0.05–50 nmol/L (0.0126–12.6 μg/L), which coincided with the environmentally relevant concentrations of BaP. Long-term reproductive effects in later-life resulting from embryonic exposure should provide new ecotoxicological information for risk evaluation of pollutants on fish populations.

2. Materials and methods

2.1. Zebrafish culture and exposure

All fish experiments were performed following the guides of Animal Ethics Committee of Xiamen University. To get enough embryos for the experiment, we chose wild-type TU zebrafish without any signs of disease as breeders. They were raised in a condition as: a 14 h light/10 h dark cycle, water temperature 28 ± 1 °C, pH 7.2–7.3, and dissolved oxygen 7–8 mg/L. Fish were fed twice daily with live brine shrimp and supplemented with a commercial diet. BaP (purity > 99%) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and was dissolved in dimethylsulfoxide (DMSO) to get a series of stock fluids at concentrations of 0.001, 0.01, 0.1 and 1 mg/mL. Fertilized embryos [from 0.5 to 1.5 h post-fertilization (hpf)] were collected after spawning and were exposed to BaP at concentrations of 0.05, 0.5, 5 and 50 nmol/L. Exposure solutions, freshly made up with stock solutions, were collected three times at random for the determination of the actual BaP concentrations. Employing the method described by Huang et al. (2014), the actual concentrations of BaP in the test solutions measured using an Agilent 6890 gas chromatograph linked with an Agilent 5975B mass spectrometer (GC/MS) (Agilent Technologies, Palo Alto, CA, USA) were 0.05 ± 0.004, 0.57 ± 0.018, 5.57 ± 0.217, and 53.93 ± 2.511 nmol/L. The experimental scheme is shown in Fig. 1. Approximately 100 embryos were cultured in 100 mL exposure solution in each petri dish, and there were 6–7 replicates in each treatment. The exposure solutions were renewed twice daily. The embryos in the control group received only 12.5 μl/L DMSO, which was same as that in each treatment. After exposure for 72 h and 96 h, the malformation rate and hatching success rate of the embryos were assessed according to the reported method (Zhang et al., 2008): malformation rate (%) = malformed embryos number/survival embryos number × 100%; hatched rate (%) = hatched embryos number/fertilized eggs number × 100%. Malformation includes dorsal curvature, yolk sac and pericardial edema, which were identified according to the previous methods (Westerfield, 2000). Except for a significant increased pericardial edema rate at the 50 nmol/L group, the hatching success, mortality and dorsal curvature rate of the F0 embryos showed no significant alteration compared to the control [Supporting Information (SI), Fig. S1]. The larvae after exposure for 96 h were transferred to clean water. Larvae at 30-day post-fertilization (dpf) occupied 200 mL water per fish; and at 60 dpf, 300 mL. Larvae at 90 dpf were maintained in the Aquatic Habitats Zebrafish System up to 365 days and occupied 600 mL water per fish. The culture conditions were the same as those mentioned above.

Another batch of embryos exposed to BaP for 96 h at the same conditions were collected for the analysis of mRNA levels and CpG methylation of genes.

2.2. Evaluation of reproductive capability

During the last two weeks of culture, five F0 female adult