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Effects of exposure to BPF on development and sexual differentiation during early life stages of zebrafish (*Danio rerio*)

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ABSTRACT

Bisphenol F (BPF) has become a predominant bisphenol contaminant in recent years. It has significant estrogenic properties in both in vivo and in vitro studies. We have previously studied the disrupting mechanisms of BPF on the hypothalamic–pituitary–gonadal axis of adult zebrafish. However, the effects of BPF exposure on development and sexual differentiation of zebrafish embryos/larvae remain unclear. To determine the effects of BPF on the critical stage of sex differentiation in zebrafish, zebrafish embryos/larvae were exposed to 1, 10, 100, and 1000 μg/L BPF from fertilization to 60 days post-fertilization (dpf). Developmental malformations were induced by exposure to BPF from 2 h post-fertilization (hpf), with a LC_{50} of 10,030 μg/L at 96 hpf and 9391 μg/L at 120 hpf. Long-term exposure during sex differentiation tended to result in a female sex ratio bias. Histological analyses at 60 dpf indicated that the development of ovo-testes and immature ovaries was induced by 100 and 1000 μg/L BPF. Homogenate testosterone levels decreased and 17β-estradiol levels increased in zebrafish in a concentration-dependent manner. BPF exposure suppressed gene expression of double sex, Mab3-related transcription factor 1(*dmrt1*), fushi tarazu factor 1d (*ff1d*), sry-box containing gene 9a (*sox9a*) and anti-Mullerian hormone (*amh*); induced expression of the forkhead box L2 transcription factor (*foxl2*), leading to increased expression of aromatase (*cyp19a1a*), which promoted production of estrogens, and further caused phenotypic feminization of zebrafish. These results suggest that developmental exposure to BPF has adverse effects on sexual differentiation, and the results were useful for a BPF risk assessment.

1. Introduction

Bisphenol A (BPA) is widely used in the manufacturing of epoxy plastics and polycarbonate, which are used in various food contact materials. Increasing evidence has shown that BPA acts as an endocrine disrupting chemical (EDC) and may be harmful to human and wildlife health (Chen et al., 2016; Rochester and Bolden, 2015). In addition, it was assigned to the third highest Toxicological Priority Index among 309 environmental chemicals (Reif et al., 2010). BPA has been prohibited in production of infant feeding bottles (European Commission, 2011; FDA, 2008). It is also listed on the candidate list of substances of very high concern for authorization (https://echa.europa.eu/candidate-list-table). Because of these restrictions, a number of substitutions that are structurally similar to BPA have been developed to manufacture polycarbonate and epoxy resins. Bisphenol analogues have been found in environmental compartments, consumer products, and food and food containers, leading to increasing exposure to humans and other organisms. The toxic effects of these substances have been reported, and some substitutes were reported to act via endocrine disruption similar to BPA.

Among these substitutions, bisphenol F (4,4′-dihydroxydiphenylmethane) is widely used as a raw material in the plastics industry and has gradually replaced BPA. BPF was reported to be the second-most abundant analogue in foodstuffs and environmental samples after BPA (Chen et al., 2016). It has been detected in indoor dust, river and seawater, sediments, municipal sewage sludge, and surface water (Liao et al., 2012a; Yamazaki et al., 2015; Liao et al., 2012b; Lee et al., 2015; Song et al., 2014b; Chen et al., 2016). It was reported as a predominant bisphenol analogue in sludge from wastewater treatment plants (Song et al., 2014a; Lee et al., 2015). Rivers and seawater collected from some Asian countries showed that BPF was the major contaminant and the concentrations were one to two orders of magnitude higher than those of BPA in corresponding samples. BPF concentrations exceeded 1 μg/L in several surveyed locations, and ranged up to 2.85 μg/L in the Tamagawa River in Tokyo (Yamazaki et al., 2015), which have exceeded the predicted no effect concentration

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suggested by the European Union for BPA (1.5 μg/L) at some study sites (EU, 2008). As a result, the toxicity of BPF in the aquatic environment is a concern.

Recent studies indicate that BPF possesses estrogenic activities similar to those of BPA. BPF was confirmed as an EDC by the US Environmental Protection Agency (Zhang et al., 2013a). Previous studies have demonstrated that BPF could activate both human estrogen receptors, it is also a strong human androgen receptor antagonist (Molinamolina et al., 2013). Yang et al. (2016) found the anti-androgenic activity of BPF was similar to that of BPA by developing a quantitative structure-activity relationship model. BPF decreases basal testosterone (T) secretion by human fetal testes (Eladak et al., 2015) and induces the production of 17β-estradiol (E2) at the lowest observed-effect-concentration in the H295R steroidogenesis assay (Goldinger et al., 2015), indicating anti-androgenic and estrogenic activities. We have previously studied the effects of BPF on the endocrine system of adult zebrafish and reported that BPF impairs reproductive functions, including gonadal development and induces increases in E2 levels and decreases T levels. We speculate that these changes might be caused by disrupted gene expression along the hypothalamic–pituitary–gonadal (HPG) axis. However, the adverse effects of BPF on early life stages of zebrafish remain unclear, and the similarities and differences need to be illustrated.

Zebrafish (*Danio rerio*) is an important model species for developmental biology over the past few decades. Sex in teleosts, including zebrafish, is determined in multiple ways, including by genetic and environmental conditions (most often temperature, pH, and social factors) and steroid hormones. Steroid hormones are extremely important in sex determination and sexual differentiation in fish, and any change created by exogenous influences, including EDCs will partially or completely redirect development and functionally to the opposite genotypic sex (Scholz and Klüver, 2009). Exposure to EDCs, including BPA and BPS, leads to a series of adverse effects on fish after the embryonic stage, e.g., altered development (developmental malformations), disruption of gametogenesis, and changed sex ratios (Crane et al., 2007; Drastichová et al., 2005; Naderi et al., 2014). We have previously studied the effects of BPF on the endocrine system of adult zebrafish and reported that BPF impairs reproductive functions, including gonadal development, and induces increases in E2 levels and decreases in T levels. We speculate that these changes might be caused by the disrupted gene expressions along the HPG axis. However, stage-specific outcomes of BPF exposure in fish development remain to be illuminated. Zebrafish embryos/larvae were exposed to different concentrations of BPF for 60 days to determine the effects of BPF on embryos/larvae and the critical stage of sex differentiation in zebrafish. The main target of this study was to investigate the effects and mechanism of BPF on sex differentiation and sexual differentiation in zebrafish. In addition, the lethal and developmental toxicity of BPF in zebrafish embryos/larvae were also studied.

2. Materials and methods

2.1. Materials and reagents

BPF (CAS No. 620-92-8) was purchased from J&K Scientific Ltd. (Shanghai, China) and dissolved in dimethyl sulfoxide (DMSO) to form a stock solution (10^4 mg/L). All solvents used in this study were of analytical grade and purchased from Merck (Darmstadt, Germany). All chemicals had purity ≥98%. The water was deionized and purified by a Milli-Q plus system (Millipore, Billerica, MA, USA).

2.2. Fish maintenance and experimental design

Zebrafish (*Danio rerio*, AB strain) has been raised at least 3 generations in our laboratory. The fish were maintained on a 16 h:8 h light/dark cycle and were fed twice daily with fresh Artemia sp. (*nauplii*). To obtain eggs, four males and four females were independently housed in cages with a net bottom in the afternoon before breeding. The following day, spawning occurred after the light period, and eggs were collected and cleaned 2 h post fertilization (hpf) and examined under a stereomicroscope (S8APO; Leica, Jena, Germany). Those observed to be developing normally were used in subsequent experiments. All animal experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

2.2.1. Acute toxicity test

To determine LC_{10} and LC_{50} values of BPF on zebrafish embryos, 20 eggs were randomly distributed into 6-well cell culture plates (Eppendorf, Hamburg, Germany) with lids. Each well contained 10 mL of BPF solution in triplicate (0, 1, 10, 100, 1000, 2000, 4000, 6000, or 8000 μg/L) and were exposed for 2–120 hpf. All solution in each well was replaced by fresh solution every 48 h. An incubator (Climacell; MMM, Munich, Germany) was used to maintain a stable environment (photoperiod: 16 h: 8 h light/dark; temperature: 28 ± 0.5 °C). All exposure groups received 0.01% (v/v) DMSO. Morphological changes in the zebrafish embryos and larvae were observed at several developmental stages (12, 24, 36, 48, 54, 60, 72, 96, and 120 hpf) using a multipurpose light microscope (DMIL; Leica).

2.2.2. Long-term exposure

Approximately 500 eggs were placed randomly into separate glass beakers containing 500 mL of BPF solution in triplicate (0, 1, 10, 100, or 1000 μg/L), according to the acute toxicity test (the maximum concentration was about one-tenth of the LC_{50}) and environmental concentration (Chen et al., 2016). During the exposure period, 75% of the solution in each beaker was renewed with fresh solution every 48 h from 1 to 60 dpf. The larvae were fed an *Artemia* (sp. *nauplii*) homogenate twice daily from 4 to 9 dpf. After 10 dpf, the larvae were fed fresh *Artemia*. At 10 dpf, the larvae were transferred to 10 L glass aquaria kept in a water bath to maintain a constant temperature. At 30 dpf, the number of fish was adjusted to 100/aquarium, and this number was reduced to 40 fish/tank at 30 dpf. During the exposure period, fish were sampled regularly, i.e., 10 dpf (n = 20), 20 dpf (n = 20), 30 dpf (n = 10), 42 dpf (n = 5) for analysis of sex determination and differentiation genes (Supplementary file 1). At 7 and 60 dpf, 20 fish were randomly selected for body length and weight measurements, to calculate specific growth rate (SGR, % day^{-1}) as reported previously (Wang et al., 2017):

$$SGR_{BW} = 100 \times [\ln BW_{t7} - \ln BW_{t60}]/days \quad (1)$$

$$SGR_{BL} = 100 \times [\ln BL_{t7} - \ln BL_{t60}]/days \quad (2)$$

where BW_{t7} and BW_{t60} are the body weight (fresh weight, g) of 7 dpf and 60 dpf zebrafish, and BL_{t7} and BL_{t60} are body length (cm) of 7 dpf and 60 dpf zebrafish, respectively.

Hatching rates, developmental malformation and survival rates were recorded. Steroid hormones were also measured after 60 d of exposure.

2.3. Determination of BPF in the exposure solution

As 75% of the solution in each beaker was renewed every 48 h, the concentrations of BPF were determined at the beginning of exposure and after 24 and 48 h exposure. The samples were analyzed as previously describe (Yang et al., 2017). The main operations are as following: Water samples were filtered to remove particulates and then injected into Oasis HLB cartridges (Waters, Milford, MA, USA), which were conditioned with 5 mL methanol and 5 mL ultrapure water. Then, the filtrates were passed through the cartridges at a flow rate of about 5 mL/min. The extracts were eluted with 10 mL methanol/ultrapure water (99/1, v/v) after drying the solvent for 30 min under a gentle stream of N2. The residue was concentrated to 1 mL methanol for high