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Thyroid endocrine disruption in male zebrafish following exposure to binary mixture of bisphenol AF and sulfamethoxazole

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ABSTRACT

Thyroid endocrine disruption by bisphenol AF (BPAF) alone or in combination with sulfamethoxazole (SMX) exposure was evaluated in adult male zebrafish. Changes in thyroid gene transcription were examined using microarrays and were linked to effects on thyroxine hormone production and transcription of genes related to the hypothalamic-pituitary-thyroid axis. BPAF alone or in combination with SMX affected genes related to thyroid hormone production and receptor activity, thyroid gland development, and deiodinase activity. Increases in thyroxine levels, and gene transcription were more pronounced in the BPAF and SMX mixture group than in the BPAF group. Significant down-regulation of *trh* and *tshβ* genes in the brain suggested a negative feedback response resulting in increased thyroxine levels. The present study indicated that BPAF exposure alone alters transcription of genes associated with the thyroid endocrine system, and combination with SMX could increase the endocrine disrupting effect of BPAF.

1. Introduction

Bisphenol AF (BPAF) is an analogue of bisphenol A (BPA) in which the methyl (−CH3) groups are replaced by trifluoromethyl (−CF3) groups. A fluorinated derivative of BPA has been widely used in the manufacturing of polycarbonate copolymers in high-temperature composites, electronic materials, and gas-permeable membranes (Li et al., 2012; Matsushima et al., 2010). Approximately 10,000–500,000 pounds (4,536–226,796 kg) of BPAF are produced annually in the United States (Stout, 2008). An annual production capacity of about 100,000 kg was reported in the largest manufacturer of BPAF in China (Song et al., 2012). The high production and numerous applications of BPAF facilitate its release into the environment. BPAF has been found in various environmental samples, e.g., indoor dust (Liao et al., 2012a; Song et al., 2012; Wang et al., 2015), sludges (Lee et al., 2015; Song et al., 2014a; Yu et al., 2015), sediments (Liao et al., 2012b; Song et al., 2012), surface water and sewage (Feng et al., 2012; Song et al., 2012; Yang et al., 2014a), and foodstuffs (Liao and Kannan, 2013). This compound has been detected in river water at concentrations of up to 15.3 $\mu g\,L^{-1}$ in China (Song et al., 2012).

BPAF has been reported to cause adverse reproductive and developmental effects in several *in vitro* (Akahori et al., 2008; Bermudez et al., 2010; Kitamura et al., 2005; Li et al., 2012; Matsushima et al., 2010; Okada et al., 2008; Song et al., 2014b) and *in vivo* studies (Shi et al., 2015; Song et al., 2014b; Tang et al., 2015; Yang et al., 2016). BPAF induces estrogen-dependent responses by binding to estrogen receptor α (ERα) or ERβ with a binding affinity that is 20–48 times stronger than that of BPA in human cell lines (Kitamura et al., 2005; Matsushima et al., 2010; Okada et al., 2008; Song et al., 2014b). The greater estrogenic activity of BPAF compared to that of BPA was attributed to the trifluoromethyl group because the CF3 group has greater electronegativity than the CH3 group, and therefore, is potentially more reactive (Bermudez et al., 2010; Kitamura et al., 2005; Matsushima et al., 2010). Song et al. (2014b) reported 100% mortality of embryos and larvae exposed to 2 $mg\,L^{-1}$ BPAF for 144 h. Previous experiments confirmed that BPAF exposure could disrupt sex hormone concentrations, as well as *vitellogenin* (*vtg*) transcription in male zebrafish (Yang et al., 2016). One study showed that exposure to low levels of BPAF (25 $\mu g\,L^{-1}$) could affect the feedback regulatory circuits of the hypothalamic-pituitary-gonad (HPG) axis in zebrafish and impair development of offspring (Shi et al., 2015). However, there is only limited information available on their endocrine disrupting effects on the thyroid system.

In fish, thyroid hormones play an important role in the regulation of development, growth, reproduction, and metabolism

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(Jugan et al., 2010). The hypothalamic-pituitary-thyroid (HPT) axis is responsible for maintaining homeostasis of the thyroid hormone (Zhu et al., 2014). Therefore, environmental contaminants that can affect the transcription of genes and concentrations of hormones involved in this axis could affect the function of the endocrine system and consequently affect development. An investigation of the effects of 168-h exposure on zebrafish embryos found that 50 $\mu g\,L^{-1}$ BPAF disrupted thyroid hormone levels, as well as the transcription of the representative genes along the HPT axis, and consequently, interfered with embryonic development (Tang et al., 2015). However, the potential effects of BPAF on the HPT axis in adult zebrafish have not yet been elucidated.

Like other contaminants, bisphenols occur as mixtures in aquatic environments; hence, adverse effects on biota associated with chemical interactions are also of concern. Previous studies have revealed increasing toxicity to fish exposed to BPA and other pollutants in mixtures (Kwak et al., 2001; Song et al., 2014c; Wu et al., 2011). To our knowledge, no study has previously investigated the toxic potential of BPAF in fish *in vivo* in combination with other environmental contaminants. Therefore, we investigated the effects of combined exposure to BPAF and the well-known endocrine disrupting chemical sulfamethoxazole (SMX), which was chosen because of its extensive applications and numerous indications of its endocrine effects in fish (Madureira et al., 2011; Thienpont et al., 2011). SMX is an antibiotic used for both humans and in veterinary practices, especially in aquaculture (Trovó et al., 2009). SMX has been detected at concentrations as high as 0.520 $\mu g\,L^{-1}$ in streams in the United States (Kolpin et al., 2002), 0.435 $\mu g\,L^{-1}$ in major rivers in Korea (National Institute of Environmental Research (NIER), 2007), and 248 $\mu g\,L^{-1}$ in wastewater treatment plant effluent water in Korea (Sim et al., 2011).

In the present study, the effects of exposure to BPAF and a combination of BPAF and SMX on the thyroid endocrine system in male zebrafish (*Danio rerio*) were investigated. Microarray and parallel experiments with quantitative real-time polymerase chain reactions (qPCR) were performed to characterize gene transcription profiles related to thyroid and metabolism by mixture exposure. To gain better understanding of the mechanism, plasma thyroxine (T4) levels and transcription of genes related to the HPT axis were also analyzed. The results of this study will provide valuable information for assessing the risk of BPAF and their mixtures to aquatic species.

2. Materials and methods

2.1. Test chemicals and chemical analysis

BPAF (>97% purity, CAS no. 1478-61-1) and SMX (>98%, CAS no. 723-46-6) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Because these chemicals are water soluble, no solvent was used for preparation of fish test solutions.

The actual concentrations of BPAF and SMX in exposure media were measured 24 h before and after exposure using high performance liquid chromatography (Series 1100, Agilent Technologies, Palo Alto, CA, USA) with triple quadrupole mass spectrometry (MS/MS) (Table S1). Analyzed compounds were separated on a 2.0 × 75 mm Cadenza C18 column (Imtakt USA). The injection volume was 5 μL and the flow rate was 200 $\mu L\,min^{-1}$. Analytes were separated with mobile phases of 90% of A (5 mM ammonium acetate with 0.02% formic acid) and 10% of B (methanol) (v/v). Analytes were identified and quantified using an API 4000 triple MS/MS system (Applied Biosystems, Foster City, CA, USA), operated in electrospray ionization (ESI) mode (negative mode for BPAF and positive mode for SMX) (Table S2; for details, see Supplementary data). The detection limits for BPAF and SMX were 0.25 $ng\,mL^{-1}$ and 0.78 $ng\,mL^{-1}$, respectively. The acceptance criterion for quality control was a coefficient of variation <10%. Herein, the average measured concentrations (24.7 $\mu g\,L^{-1}$ BPAF and 5.6 $\mu g\,L^{-1}$ SMX) were used for presentation of the results (Table S3).

2.2. Zebrafish culture and exposure design

Adult male zebrafish (*D. rerio*) were acclimated in tanks containing dechlorinated tap water for 6 weeks prior to the experiment. The fish were maintained at 26 ± 2 °C under a photoperiod of 16:8 h light/dark in the Molecular and Environmental Toxicology Laboratory at Yongin University (Yongin, Korea). The fish were fed *ad libitum* with commercially available mosquito larvae and *Artemia* (Green Fish, Seoul, Korea) twice daily. Thirty minutes after feeding, the remaining food and feces were removed.

Four male zebrafish each were exposed to the control, 24.7 μg BPAF L^{-1}, 5.6 μg SMX L^{-1}, and a binary mixture of 24.7 μg BPAF L^{-1} and 5.6 μg SMX L^{-1} for 21 d, for a total of 12 fish. Test concentrations were chosen based on the effective concentration in fish endocrine system and the environmental exposure relevance. Three replicates of four adult male fish for each treatment group were placed in aquarium tanks (10-L tanks with 8.5-L exposure media). Exposure media were renewed daily, and mortality was determined daily. Fish were fed twice daily with mosquito larvae and *Artemia*. The pH, conductivity, temperature, and dissolved oxygen of the exposure media were monitored regularly.

At the end of the exposure period, all surviving fish were euthanized using 2-phenoxyethanol (Sigma-Aldrich). For the measurement of T4 hormone, plasma samples (5 μL with three replicates) were collected. Thyroid gland in adult zebrafish is composed of loosely arranged follicles that are not encapsulated (Harper and Lawrence, 2011). Thyroid follicles are distributed surrounding the ventral aorta, and found between the first gill and the heart (McGonnell and Fowkes, 2006; Wendl et al., 2002). Therefore, it is not physically easy to dissect thyroid follicles in adult zebrafish. To overcome these limitations, we dissected gill region in adult zebrafish since thyroid gland is scattered in this reason. Thyroid tissue samples were used for the cDNA microarrays (n = 6 for each treatment group), and two tissues (brain and thyroid) were used for qPCR (n = 4 for each treatment group).

2.3. Thyroid hormone measurement

Total thyroxine (T4) level was measured using an enzyme-linked immunosorbent assay (ELISA). Briefly, blood samples were collected from the caudal vein of each zebrafish and centrifuged at 2,000 × *g* for 15 min. Plasma samples (5 μL with three replicates per treatment group) were stored at −20 °C until further analysis. T4 levels were quantified using an ELISA kit (Cat No. CEA452Ge, USCNlife, Wuhan, China) following the manufacturer's recommendations.

2.4. cDNA microarray

Thyroid tissues were collected from each zebrafish, and total RNA was extracted from the tissue using Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA quality and quantity were assessed by the Agilent 2100 bioanalyzer (Agilent Technology, Palo Alto, CA, USA) and ND-2000 Spectrophotometer (Thermo Inc., DE, USA), respectively. Isolated intact and high-quality RNA was further processed for microarray analysis. Three RNA samples per treatment group were pooled for cDNA synthesis.

Total RNA was linearly amplified and labeled using an Agilent Low RNA Input Linear Amplification kit PLUS (Agilent Technology) according to the manufacturer's instructions. In brief, 1 μg total RNA was reverse transcribed into cDNA using oligo dT primers.