Exposure to Bisphenol AF Disrupts Sex Hormone Levels and Vitellogenin Expression in Zebrafish

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ABSTRACT: Bisphenol AF (BPAF) is widely used in food-contact products, electronic devices, and as a cross-linking reagent in fluoroelastomers. There are growing concerns about its toxicity and endocrine-disrupting effects based on its structural similarity with bisphenol A (BPA). The endocrine-disrupting effects of BPAF were studied by exposing 2-month-old zebrafish to 0, 0.05, 0.25, or 1 mg/L BPAF for 28 days and evaluating the effect on growth, histopathology, hormone levels, enzyme activity, and gene expression. The overall fitness was not significantly affected. There were no apparent alterations in the gills and intestine tissues of both sexes after BPAF exposure. However, exposure to 1 mg/L BPAF caused damage to the liver in the male fish, characterized by hepatocellular swelling and vacuolation. There was no obvious effect in the liver of female fish, suggesting that the hepatic toxicity of BPAF is gender dependent. Gonadal examination indicated that exposure to 1 mg/L BPAF caused induction of acellular areas in the testis and retardation of oocyte development in the ovary. BPAF exposure increased free triiodothyronine levels of females in a dose-dependent manner. In males, the testosterone levels decreased in a concentration-dependent manner. In contrast, estradiol levels increased in a concentration-dependent manner and were significantly higher in males exposed to 1 mg/L BPAF compared with the controls. In females, 0.05 and 0.25 mg/L BPAF caused an increase in testosterone levels. Furthermore, the estradiol levels increased in females exposed to 0.05 and 1 mg/L. We observed an upregulation of hepatic vitellogenin in both sexes and significantly higher levels in males exposed to 1 mg/L BPAF and females

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exposed to 0.25 mg/L BPAF, suggesting that BPAF has an estrogenic activity. Our results indicate that BPAF is an endocrine-disrupting chemical that exerts reproductive toxicity and estrogenic effects on zebrafish. © 2014 Wiley Periodicals, Inc. *Environ Toxicol* 31: 285–294, 2016.

Keywords: Bisphenol AF; endocrine disruptive effect; zebrafish; hormone; vitellogenin

INTRODUCTION

Bisphenol AF (BPAF) is a recently developed bisphenol analogue that is increasingly being incorporated into the production of fluoropolymers, fluoroelastomers, and a variety of polymers that are used to make electronic devices and plastic optical fibers (Matsushima et al., 2010; Feng et al., 2012). The annual production of BPAF in the United States is between 10,000 and 50,000 pounds (Stout, 2008). Although the total annual production of BPAF in China is unknown, the largest manufacturer of BPAF in China produces ~100 tons per annum (Song et al., 2012). If BPAF was released to the environment, it will mainly reside in soil, water or sediment based on the Cahill multispecies model (Cahill, 2008). A recent study demonstrated that BPAF could be detected in the sediments and soils around a manufacturing plant (Song et al., 2012). In addition, BPAF has been detected in surface water and sewage (Stout, 2008). No experimental degradation and bioaccumulation data of BPAF have been identified. However, the results from quantitative structure–activity relationship models suggest that BPAF biodegrades very slowly and its half-life in water is >182 days (BIOWIN, 2000). Moreover, the modeled bioconcentration factor value for BPAF in fish was 715.6 L/kg (Arnot and Gobas, 2003) and the modeled log K_{ow} value for BPAF indicates that it has higher bioaccumulative potential in the biota.

Despite the frequent use and increasing production of BPAF, there is limited information on its toxicology and endocrine-disrupting effects. The National Institute of Environmental Health Sciences in America listed BPAF for further investigation in 2008 to improve knowledge about its toxicological effects (Yang et al., 2012). Given its structural similarity with BPA, a well-known endocrine-disrupting compound (EDC), attention has also focused on understanding the potential estrogenic-like activity of BPAF. Indeed, research suggests that BPAF may have higher estrogenic activity than BPA because it contains a hydrophobic group (Kitamura et al., 2005; Bermudez et al., 2010). Other studies show that BPAF acts as an endocrine disruptor *in vitro* by binding to estrogen receptors (ERs) (Matsushima et al., 2010; Li et al., 2012). *In vivo*, testosterone levels were markedly decreased in Sprague–Dawley (SD) male rats receiving 200 mg/kg/days of BPAF, whereas the levels of luteinizing hormone and follicle-stimulating hormone increased significantly (Feng et al., 2012). Very recently, BPAF-induced estrogenic activity has been proven to be mediated through both genomic and nongenomic pathways, which involved the ER α and ERK1/2 signaling (Li et al., 2014).

Zebrafish are widely used as a model to evaluate the toxic effects of chemicals and the mechanisms by which they act as EDCs (Segner, 2009). Zebrafish have high fecundity, are small in size, have a short generation time, and a large genetic database (Goldsmith, 2004; Hill et al., 2005; Aleström et al., 2006). Given these traits, we used zebrafish to evaluate the aquatic toxicity and endocrine-disrupting effects of BPAF. In the studies to screen endocrine activity, the interactions of chemicals with fish have focused on some end points or biomarkers. Generally, the specific target of the endocrine-active chemical was identified by the hypothalamic–pituitary–gonadal (HPG) axis and liver (Ville-neuve et al., 2007), where sex hormones including T and E2 can regulate gametogenesis and maturation of oocytes (Nagahama and Yamashita, 2008). Thus, the interference with sex steroid system can directly or indirectly reveal endocrine disruption of certain substance. One of the most important biomarkers in screening assay is induction of vitellogenin (VTG), which is a major precursor of egg yolk, and is synthesized in the liver of females in all oviparous vertebrates. VTG is transported through the blood to the ovary, and ultimately absorbed in the mature oocytes (Lubzens et al., 2010). In general, VTG is primarily produced by the liver in mature female fish (Tao et al., 2006). However, the synthesis of VTG in adult male fish can be induced by the presence of estrogenic compounds (Jin et al., 2008). Thus, the induction of VTG gene expression in males or larvae has been used as a sensitive biomarker for estrogenic disruption in oviparous organisms (Zhang et al., 2005; Miller et al., 2007; de Vlaming et al., 2007). Expression of VTG is under the control of 17 β -estradiol, which can bind to specific endocrine receptors (ERs) in response to the stimulation of endocrine-disrupting chemicals (Verderame and Limatola, 2010).

In most zebrafish screening studies, either pre-mature or mature life stages have been used (Fenske et al., 2001; Brion et al., 2002). After morphological gonad differentiation stage, ovaries and testes experience progressive growth and maturation after 60 dpf (Segner et al., 2003). Thus, we selected 2-month-old zebrafish in order to detect the developmental or reproductive effects of BPAF in this sensitive windows. In our study, zebrafish were exposed to different concentrations of BPAF (0, 0.05, 0.25, or 1 mg/L) for 28 days. The toxicity of BPAF was evaluated by studying histopathological alterations in the liver and gonads, whole-body hormone levels of testosterone (T), estradiol (E2), and free triiodothyronine (FT3), and expression of the hepatic vitellogenin (VTG) gene.

MATERIALS AND METHODS

Chemicals

BPAF (98% purity) was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). We dissolved BPAF in dimethyl sulfoxide (DMSO) to form stock solutions (1 and 4 mg/L) and stored them away from light. All other chemicals used in this study were analytical grade.

Fish Maintenance and Chemical Exposure

Two-month-old zebrafish were maintained in recirculating aquarium tanks at $28 \pm 0.5^\circ\text{C}$ with a 12:12 light/dark cycle and fed twice daily with fresh *Artemia* nauplii.

The zebrafish were assigned to one of four experimental groups: vehicle control (0.1% DMSO) or 0.05, 0.25, or 1 mg/L BPAF. Each group consisted of duplicate 6 L glass tanks (sexes held separately, $N = 4$ tanks/group, nine males/nine females per tank). The fish were acclimated for 1 week prior to the experiment. After acclimation, we measured the body weight and length of each fish (females: 0.28 ± 0.02 g, 2.98 ± 0.08 cm; males: 0.26 ± 0.02 g, 2.95 ± 0.05 cm). Following this, the fish were exposed to the appropriate dose of BPAF for 28 days. Half of the exposure water in each tank was renewed daily. Both exposure and control groups received 0.1% (v/v) DMSO. Fish were fed with fresh *Artemia* nauplii twice daily. After 28 days, all fish were frozen on ice for the measurement of body weight and length. We used the following formula to calculate the condition factor: $\text{CF} = 100 \times [\text{body weight (g)}/\text{total length}^3 \text{ (cm)}]$.

Histology

The liver, gonads, gill, and intestine were collected for histological examination. The tissues were fixed in paraformaldehyde solution (4%, w/v) for 24 h then dehydrated in ethanol, embedded in paraffin, then sectioned (3 μm), and stained with hematoxylin and eosin. Each section was examined carefully under a light microscope. In females, we counted 100 follicles in each ovarian section and classified their maturation stage following the description in Selman et al. (1993) and Clelland and Peng (2009). We categorized oocyte development into the following four stages: stage I (primary growth stage), stage II (cortical alveolus stage), stage III (vitellogenesis), and stage IV (oocyte maturation). The number of oocytes in each of the four developmental stages in each ovary was expressed as a proportion of the total number of oocytes.

Hormone Measurements

Each fish was homogenized in 1.2 mL phosphate-buffered saline (PBS; 0.01 M PBS, pH 7.2) at 0°C . The homogenate was centrifuged at $13,000 \times g$ for 15 min at 4°C . We col-

lected the supernatant to detect the whole-body levels of free triiodothyronine, estradiol, and testosterone following the methods documented (Zhang et al., 2011).

Quantification of Superoxide Dismutase Activity and Malondialdehyde Content

At the end of the exposure period, we removed the livers from six females/males in each group and immediately stored them at -80°C for analysis. The liver was homogenized in 400 μL physiological saline (0.9%, w/v) and centrifuged at $2800 \times g$ for 15 min at 4°C . The supernatants were used for the measurement of superoxide dismutase (SOD) activity (U/mg protein) and malondialdehyde (MDA) content (nmol/mg protein) following instructions in reagent kits from Nanjing Jian Cheng Bioengineering Institute (Nanjing, China). The protein concentration in the homogenates was measured using the Bradford assay (Bio-Rad, Hercules, CA).

Gene Expression

The homogenized zebrafish livers were prepared for RNA extraction using Trizol reagent (Invitrogen, Carlsbad, CA). We estimated the concentration of total RNA based on the absorbance at 260 nm on a UV-spectrophotometer (BioPhotometer plus, Eppendorf, Germany). RNA quality was assessed based on the ratio of absorbance at 260 nm to that at 280 nm as well as its characteristic bandings on a 1% agarose formaldehyde gel. The synthesis of first-strand complementary DNA (cDNA) was performed with M-MLV reverse transcriptase (Promega, Madison, WI) with 2 μg RNA reverse transcribed in each sample.

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using a SYBR Green PCR kit (Toyobo, Tokyo, Japan). The primers were designed with the assistance of Primer Premier 5.0 (Premier, Palo Alto, CA). The forward and reverse primer sequences for *VTG* were 5'-A GCTGCTGAGAGGCTTGTTA-3' and 5'-GTCCAGGATTTCCCTCAGT-3', respectively. Determination of transcription levels of *VTG* was conducted in duplicate and normalized to β -actin messenger RNA content. The cycle threshold (CT) value was calculated to indirectly assess the relative change in expression levels based on the $2^{-\Delta\Delta\text{CT}}$ method (Livak and Schmittgen, 2001).

Statistical Analysis

All data are expressed as the mean $\pm$ standard error of mean (SEM). SPSS statistical software (Version 13.0) and Sigma Plot 10.0 were used for statistical analysis. The significant differences between control and treated groups were determined using a one-way analysis of variance and Tukey's multiple range test. Differences were statistically significant if $P \leq 0.05$.

TABLE I. Effect of BPAF exposure on growth and condition factor in zebrafish

Zebrafish	BPAF (mg/L)	Length (cm)	Weight (g)	Condition Factor
Female	0	3.20 ± 0.04	0.332 ± 0.011	1.02 ± 0.04
	0.05	3.14 ± 0.03	0.349 ± 0.012	1.13 ± 0.05 ^a
	0.25	3.20 ± 0.03	0.350 ± 0.008	1.07 ± 0.02
	1	3.20 ± 0.03	0.325 ± 0.009	1.02 ± 0.02
Male	0	3.20 ± 0.02	0.287 ± 0.005	0.88 ± 0.02
	0.05	3.18 ± 0.02	0.287 ± 0.006	0.89 ± 0.02
	0.25	3.13 ± 0.02 ^a	0.266 ± 0.005 ^a	0.87 ± 0.01
	1	3.14 ± 0.03 ^a	0.282 ± 0.009	0.91 ± 0.02

The values represent the mean ± SEM of two replicate tanks (nine fish per replicate).

^a*P* < 0.05 indicates a significant difference between exposure groups and the control group.

RESULTS

Growth

There was no significant difference in the body length and weight of females among the BPAF-treated groups. The condition factor was significantly higher in females in the group exposed to 0.05 mg/L than in the controls (Table I). In male fish, BPAF did not cause dramatic changes in condition factor. However, the fish treated with 0.25 mg/L BPAF were shorter and lighter than the control. In addition, exposure to 1 mg/L BPAF made male fish shorter than the control (Table I).

Histological Examination

There was no apparent damage to the liver of male fish that were exposed to lower concentrations (0.05 and 0.25 mg/L) of BPAF relative to the control [Fig. 1(a–c)]. However, the hepatocytes of male fish exposed to 1 mg/L BPAF were swollen and irregularly shaped, in contrast to the compact and polygonal-shaped hepatocytes in the control fish [Fig. 1(d)]. In addition, we noted vacuolization in the liver in the groups exposed to 1 mg/L BPAF. However, BPAF treatment did not result in any hepatic damage in any female fish (figure not shown).

Exposure to BPAF did not cause any obvious alterations in the gills and intestines of both sexes compared with the control (Fig. 1).

In males, we identified germ cells in all stages of spermatogenesis. We observed acellular areas in the testis of fish exposed to 1 mg/L BPAF [Fig. 2(d)], suggesting a reduction of spermatids.

In contrast to the control fish, the majority of cells from BPAF-treated females were in stage I, although a small number of cells were at stage IV [Fig. 2(f–h)]. Exposure to 0.25 and 1 mg/L BPAF resulted in a significantly higher proportion of stage I cells and caused significantly lower proportion of stage IV cells relative to the control fish (Fig. 3), suggesting that BPAF inhibits ovarian maturation.

Sex Hormones and Thyroid Hormone

The sex hormone and thyroid hormone levels in male and female fish are given in Figures 4 and 5, respectively. In male fish, the T levels in the whole-body homogenates were reduced in a dose-dependent manner [Fig. 4(A)]. However, the E2 levels increased with an increase in the concentration of BPAF. This increase was most evident in the fish exposed to 1 mg/L BPAF [Fig. 4(B)]. In female fish, T levels increased following exposure to 0.05 and 0.25 mg/L BPAF but decreased in the 1 mg/L treatment groups. In addition, we observed an increase in E2 levels in the 0.05 and 1 mg/L groups but a slight decrease in the 0.25 mg/L group relative to the control. Furthermore, the ratio of T/E2 in male fish decreased in a dose-dependent manner [Fig. 4(C)]. The total FT3 levels increased in females in all exposure groups, and were significantly higher in the 1 mg/L group compared with the control (Fig. 5).

SOD Activity and MDA Content

There was no difference in SOD activity among the control and treatment groups of both genders after 28 days exposure. Similarly, exposure to BPAF had no impact on liver MDA content in males and females (data not shown).

VTG Gene Expression

In males, exposure to 1 mg/L BPAF caused significant upregulation of the *VTG* gene in the liver. The expression of *VTG* tended to increase in females in all treatments, but the difference was only significant in the 0.25 mg/L treatment group (Fig. 6).

DISCUSSION

BPAF may be a more toxic and more potent endocrine disruptor than BPA. Concern about its potential to harm humans and wildlife requires us to apply multiple animal models to investigate the potential toxicological outcomes.

Fig. 1. Light micrographs of liver, gill, and intestine tissue from male zebrafish. Arrow indicates vacuolization. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Although the endocrine-disrupting effects of BPAF have been documented *in vitro* and *in vivo* (Matsushima et al., 2010; Feng et al., 2012), little is known about the mechanism of its endocrine-disrupting activity on zebrafish. For the first time, we demonstrated that exposure to BPAF significantly altered testosterone levels and *VTG* gene transcription in male zebrafish. In addition, BPAF exposure resulted in acellular areas in the testis or inhibited oocyte maturation. Taken together, these observations suggest that BPAF exhibits estrogen-like activity in zebrafish.

In our study, there were no significant effects on overall fitness of zebrafish in the different treatment groups of both sexes. However, the histological examination revealed damage to the liver of treated male zebrafish, characterized by

hepatocellular swelling and vacuolation. The liver plays an essential role in metabolism, detoxification, and homeostasis (Tao and Peng, 2009) and is the target organ for most bisphenols. The oxidative stress response is of great importance in metabolizing toxicants and protecting hepatocytes in the liver (Bainy et al., 1996). Tyl et al. (2008) noted that BPA exposure led to hepatocyte hypertrophy in adult mice and rats. The toxicological mechanism by which bisphenols affect the liver is complicated and is related to structural features of bisphenols. For example, BPA induces oxidative stress by decreasing antioxidant enzyme activities, resulting in liver abnormalities in rats (Bindhumol et al., 2003). However, our data suggest that the antioxidant enzyme SOD is not significantly affected by BPAF exposure in zebrafish.

Fig. 2. Histological changes in zebrafish testis and ovary after BPAF exposure. The acellular areas were observed in males exposed to 1 mg/L BPAF (asterisk). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

This is in contrast to the results of Bindhumol et al. (2003), likely because of the variation in chemical structure between BPA and BPAF. Interestingly, histological observation of the zebrafish exposed to BPAF revealed hepatocellular vacuolization, primarily in males, suggesting that the hepatic toxicity was gender-specific. The difference in the ability to eliminate and metabolize contaminants between males and females may explain the gender-specific toxicity. It has been reported that several contaminants accumulated more into liver of male zebrafish than females, indicating a better detoxification metabolism of pollutants in females than in males (Tao and Peng, 2009; Wen et al., 2014).

Thyroid hormones are critical for regulating somatic growth and differentiation of many tissues and organs in fish (Power et al., 2001). Disruption of thyroid hormones is known to cause severe impairment, including growth retardation, neurological defects, and metabolism disorders (Yen, 2001; Liu and Chan, 2002; Jugan et al., 2010). Our results showed that BPAF exposure resulted in a dose-dependent increase in FT3 levels in females, suggesting that BPAF can induce thyroid endocrine disruption by disturbing thyroid hormone levels in zebrafish. This is the first observation of thyroid endocrine disruption by BPAF, although several studies have noted similar effects for other bisphenols. For example, BPA is known to affect TH-regulated development *in vivo* (Zoeller et al., 2005; Iwamuro et al., 2006). Recently, Gentilcore et al. (2012) demonstrated that BPA can affect the pituitary–thyroid axis by regulating expression of genes related to thyroid hormones synthesis. Based on the structural resemblance to BPA, we hypothesize that BPAF may induce thyroid-disrupting activity by binding to the thyroid hormone receptor or TH transporter proteins and regulating the expression of genes involved in thyroid hormone synthe-

sis. However, the underlying mechanism by which BPAF causes thyroid endocrine disruption remains unclear and requires further investigation.

In vertebrates, sex steroids such as E2 and T play a role in sex differentiation, sexual maturation, and reproductive success (Chang et al., 2013). Alterations in sex steroid concentrations may directly affect HPG axis, steroid-binding proteins, or aromatase activity (Mills and Chichester, 2005; Hecker et al., 2005). BPAF exposure altered sex hormones in male zebrafish in a dose-dependent manner, resulting in a decrease in T levels but an increase in E2 levels. Our observations are consistent with a previous study, which showed that BPAF caused a dramatic decline in serum testosterone

Fig. 3. The average percentage of follicles present at different stages. Values represent mean $\pm$ SEM (* $P < 0.05$, ** $P < 0.01$, $n = 5$).

Fig. 4. Effects of BPAF exposure on T (A) and E2 (B) levels, and the ratio of T/E2 (C). Values represent the mean $\pm$ SEM of six individual fish from two replicates. Significant differences ($P < 0.05$) between control and exposure groups are represented by *.

in SD male rats by affecting genes and proteins related to the testosterone biosynthesis pathway (Feng et al., 2012). Moreover, we observed a concentration-dependent decrease in the ratio of T/E2 in males after BPAF exposure. In fish, the ratio of T/E2 has been used to detect the imbalance of

Fig. 5. Effect of BPAF exposure on levels of FT3 in adult zebrafish. Values represent the mean $\pm$ SEM of six individual fish from two replicates. Significant differences ($P < 0.05$) between control and exposure groups are represented by *.

sex hormones (Folmar et al., 1996; Orlando et al., 2004). In addition, the T/E2 ratio was proposed as an indicator of aromatase activity, an enzyme that converts T to E2 (Liu et al., 2009). Hence, the decrease in the ratio of T/E2 in our study provides clear evidence that the balance of sex hormones was disrupted by BPAF, and suggests that BPAF also induced the conversion of T to E2.

E2 is thought to disturb the functioning of leydig cells and the production of testosterone. Because of the inhibition of testosterone, the process of spermatogenesis was negatively influenced, subsequently leading to a reduction in mature sperm (Hecker et al., 2005). We found obvious pathologic changes, such as the presence of acellular areas in the testicular tissues of male fish exposed to 1 mg/L BPAF. Our results

Fig. 6. Effect of BPAF exposure on VTG gene expression in the zebrafish liver. The results represent the mean $\pm$ SEM ($n = 6$). A significant difference between treatment and control groups is indicated by * ($P < 0.05$) or ** ($P < 0.01$).

agree with those of Feng et al. (2012), who suggested that the testes may be a primary target organ for BPAF. In female fish, exposure to BPAF inhibited follicular development, characterized by an increase the proportion of stage I follicles and a reduction in the proportion of stage IV follicles following exposure to 0.25 or 1 mg/L BPAF. We attributed this to the direct effects of changes in sex hormone levels.

We measured liver *VTG* gene expression to assess the estrogenic activity of BPAF. Previous studies have shown that *VTG* gene expression can be induced in male fish after exposure to some bisphenols. For example, a 180-day exposure to BPA (10, 200, or 400 µg/L) induced an increase in *VTG* levels in F1 male zebrafish in a concentration-dependent manner (Keiter et al., 2012). In our study, we observed a significant increase in *VTG* gene expression in male fish exposed to the highest BPAF concentration (1 mg/L), accompanied by a concentration-dependent increase in E2 levels. Meanwhile, *VTG* expression levels were upregulated in all female groups that were exposed to BPAF, consistent with the increase in E2 levels. Thus, we conclude that the upregulation of *VTG* gene expression in both sexes is a consequence of increased E2 levels after BPAF exposure. Alteration of *VTG* gene expression confirms the potential for BPAF to exert endocrine-disrupting effects on zebrafish. The underlying mechanism or subsequent outcomes should be evaluated in a future study.

CONCLUSION

Exposure to BPAF disrupted several toxicological end points in zebrafish. In male fish, waterborne exposure to BPAF caused damage to the liver, and resulted in a concentration-dependent decrease in T levels but increase in E2 levels. This led to induction of acellular areas in the testes and significant upregulation of *VTG* gene expression. Likewise, BPAF altered the levels of sex hormones, thereby contributing to retardation of oocyte development and an increase in *VTG* gene expression in female fish. Our results highlight the potential endocrine-disrupting effects of BPAF, suggesting that BPAF can affect the synthesis of sex hormones, and thus disrupt the development of gonads and *VTG* gene expression. Given these effects, we conclude that BPAF is a new EDC that may cause adverse effects to the aquatic environment. Further study is needed to uncover the mechanism by which BPAF exerts its endocrine-disrupting effects and assess its potential hazards to aquatic ecosystems.

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