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Research Paper

The comparative toxicities of BPA, BPB, BPS, BPF, and BPAF on the reproductive neuroendocrine system of zebrafish embryos and its mechanisms

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ABSTRACT

Bisphenol A (BPA) is a well-known endocrine disruptor that has elicited great concern because of its potential toxic effects in organisms. In this study, the effects of BPA and several BPA structural analogs, including BPB, BPS, BPF, and BPAF, on the reproductive neuroendocrine system were evaluated during zebrafish embryonic and larval development. Our results showed that the numbers of gonadotropin-releasing hormone 3 neurons in zebrafish embryos increased after 100 μg/L BPA analog treatment, and exposure to BPA or its analogs at 1 or 100 μg/L increased the expression of reproductive neuroendocrine-related genes and the levels of typical hormones such as LH, FSH, E2, and GH. Moreover, the effects were associated with increases in the activities of *erα*, *erβ*, and *cyp19a* genes. The respective estrogen receptors (ER) and aromatase (AROM) antagonists significantly attenuated the stimulation of *lhβ*, *fshβ*, LH, and FSH expression, thereby proving that BPA analogs affect the reproductive neuroendocrine system via ERs and AROM pathway. Furthermore, we observed that the reproductive neuroendocrine toxicity of BPAF was more similar to that of BPA. This was the first study to comparatively explore the reproductive neuroendocrine toxicities of bisphenols in aquatic organism.

1. Introduction

Bisphenol A (BPA), a well-known endocrine-disrupting chemical, has been frequently used in the production of polycarbonate polymers, epoxy resins, and thermal papers, and the high production volumes and disposal of these products has led to the widespread dispersal of BPA in the environment (Abril et al., 2020). In fact, BPA has also been detected in human serum, urine, umbilical cord blood, amniotic fluid, and placental tissue (Macczak et al., 2017). BPA is thought to be associated with many human diseases such as diabetes, obesity, reproductive disorders, cardiovascular disease, birth defects, and breast cancer (Zhang et al., 2018). Given these adverse effects on human health, some countries, including Canada (2009), United States (2010), European Union (2011), and China (2011), banned the use of BPA in products intended for infants (Pouokam et al., 2014).

Bisphenol B (BPB), bisphenol S (BPS), bisphenol F (BPF), and bisphenol AF (BPAF) are most four commonly BPA analogs, and have been used as alternatives to produce BPA-free products (Pang et al., 2019). BPB is used for the production of phenolic resins, and shows high estrogenic effect to organisms (Cunha and Fernandes, 2011), while BPS is mainly used in epoxy glue, plastic products, and thermal paper (Liao et al., 2012). In addition, BPF is usually applied in industrial

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applications, including lining materials, flooring materials, coatings, and pharmaceuticals, particularly for the products needing increased thickness and durability (Qiu et al., 2018). BPAF is a cross-linker in fluoroelastomers, electronics and optical fibers, and widespread used in plastic production, such as polycarbonate copolymers, food contact polymers, and electronic materials (Li et al., 2020a). The largely use of BPB, BPS, BPF, and BPAF leading to the widely detection of these BPA analogs in different environmental matrices. In a review, by Chen et al. (2016) reported that these BPA analogs were widely detected in different environmental media, food, and human body fluids and possessed potential estrogen-like activity, similar to BPA. For instance, these four BPA analogs were frequently reported the occurrence in river, seawater, and sediment (Huang et al., 2020; Yan et al., 2017; Zhao et al., 2019), also, these chemicals were widely detected in the serum of pregnant women from different provinces of China or the breast milk of Chinese women (Jin et al., 2020; Li et al., 2020b), as well as the urine of American citizens at different ages (Mendy et al., 2020).

Generally, bisphenols could accumulate by bony fishes, a recent study have detected the occurrence of BPA and its analogs in muscle and liver of fish from ocean, and deemed that this may have the potential threat to the human food safety (Barboza et al., 2020). Moreover, several previous studies confirmed that BPB, BPS, BPF, and BPAF exert similar toxicities as BPA (Cano-Nicolau et al., 2016; Chen et al., 2016). For example, Zhang et al. (2018) demonstrated that BPS and BPF, like BPA, could potentially interfere with the thyroid hormone (TH) signaling pathway, and Mokra et al. (2017) found that BPAF exhibited higher potential toxicity than BPA in human blood cells. However, the information about the potential toxicity mechanisms of these BPA analogs on bony fish is still limited.

Gonadotropin-releasing hormone (GnRH), a hypothalamic decapeptide critical to the hypothalamic–pituitary–gonadal (HPG) axis, stimulates the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which regulate normal reproductive and gonadal functions in vertebrates, from the anterior pituitary (Eytan et al., 2010). The neuropeptide kisspeptin (Kiss) has been recognized to play essential roles in regulating GnRH neuron biology (Arianna et al., 2011). One study reported that 17β-estradiol (E2) could induce the release of GnRH and LH in the presence of estrogen receptor alpha (ERα) and could control the development of kisspeptin neurons (Dubois et al., 2015). Similarly, BPA could also impair the kisspeptin neuronal maturation of female mice to interrupt the neuroendocrine system (Ruiz-Pino et al., 2019). Typically, BPA is thought to disrupt the reproductive neuroendocrine system (Qiu et al., 2015) potentially by acting along the HPG axis (Diamanti-Kandarakis et al., 2009). In addition, BPA can induce the expression of *ers* (*erα*, *erβ1*, or *erβ2*) and cytochrome P450 family 19 (*cyp19*, *cyp19a1* or *cyp19a2*) genes through its actions on estrogen receptors (ERs) and the aromatase (AROM) pathway (Chung et al., 2011). ERs play essential roles in the development of the female reproductive tract by regulating the mRNA levels of reproductive neuroendocrine-related genes and hormone levels (Pangas and Rajkovic, 2015). Specifically, during fish development, *cyp19* expression relies on ER expression in the developing brain, suggesting that brain AROM is regulated by ERs (Mouriec et al., 2009); here, the presence or deficiency of aromatase also affects sexual differentiation (Pangas and Rajkovic, 2015). Thus, ERs and the AROM pathway play important roles in the development of the reproductive neuroendocrine systems of higher organisms. However, the mechanisms underlying the toxic effects of BPA analogs, such as BPB, BPS, BPF, and BPAF, on the reproductive neuroendocrine systems of aquatic organisms and the magnitudes of the toxicities of these chemicals still require further clarification.

The zebrafish (*Danio rerio*) is considered as an excellent model organism for exploring developmental toxicity because changes are easily visualized in the early developmental stages (Li et al., 2017). In this study, we used zebrafish embryos to determine the effects of exposure to BPA and BPA analogs, including BPB, BPS, BPF, and BPAF, at 1 and 100 μg/L on the reproductive neuroendocrine system. The effects of BPA analogs on the hatching rate, body length, and motor behavior of embryos or larvae, as well as changes in the expression of reproductive neuroendocrine-related genes [e.g., *kiss1*, *kiss2*, *gnrh3*, FSH beta (*fshβ*), LH beta (*lhβ*), atrial natriuretic peptide (*anp*), renin (*ren*), parathyroid hormone 1 (*pth1*), GH (*gh*), prolactin (*prl*)] and the levels of various hormones (e.g., LH, FSH, E2, and GH), were investigated. Furthermore, the roles of ERs and the AROM pathway, which are potentially affected by the exposure of the reproductive neuroendocrine system to BPA and its analogs, were explored. Our results demonstrated the adverse effects of the BPA alternatives, BPB, BPS, BPF, and BPAF on the reproductive neuroendocrine systems of zebrafish embryos and larvae and further confirmed that these BPA alternatives could induce the similar toxicity effects with that of BPA.

2. Materials and methods

2.1. Chemicals

Analytical-grade BPA [99%, CAS number 80-05-7, 2,2-bis(4-hydroxyphenyl)propane], BPB [99%, CAS number 77-40-7, 2,2-bis(4-hydroxyphenyl)butane], BPS [98%, CAS number 80-09-1, bis(4-hydroxyphenyl)sulfone], BPF [99%, CAS number 620-92-8, bis(4-hydroxyphenyl)methane], and BPAF [99%, CAS number 1478-61-1, 2,2-bis(4-hydroxyphenyl)hexafluoropropane] were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were separately dissolved in dimethyl sulfoxide (DMSO) to obtain 10 g/L stock solutions. Fresh stock solutions were made every week and stored at 4 ℃. All other chemicals used were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Fish maintenance

Wild-type adult zebrafish were maintained in a flow-through aquarium system at 28 ± 0.5 ℃ under a 14:10-h light–dark photoperiod. Fertilized embryos were collected within 1 h after spawning and were maintained in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, and 0.3 mM $MgSO_4$) at 28 ℃. Meanwhile, transgenic adult zebrafish in which the GnRH3 promoter drives the expression of a bright variant of green fluorescent protein [Emerald green fluorescent protein (EMD)] were maintained using the same method. All the experiments were performed in sexually undifferentiated embryos and larvae. All the procedures performed in this study were approved by the Animal Care and Use Committee of Southern University of Science and Technology (JY2019067).

2.3. Experimental design

Zebrafish embryos at 2 h post-fertilization (hpf) were randomly distributed in Petri dishes (100 embryos per dish). Embryos at 2 hpf were exposed to 50 mL of a test solution comprising BPA or an analog at an environmentally relevant concentration of 1 or 100 μg/L in E3 medium (Crain et al., 2007; Muhamad et al., 2016), a blank control (E3 medium alone), or a solvent control (0.005% DMSO in E3 medium). In addition, the ER antagonist ICI 182,780 (ICI, 1 μM) or the AROM inhibitor fadrozole hydrochloride (FAD, 1 μM) was used in a co-exposure experiment with bisphenols to determine which signaling pathways mediate the effects of these bisphenols on the expression of reproductive neuroendocrine-related genes and hormones (Kinch et al., 2015). All treatment groups were created in triplicate (n = 3), and the test solutions were replaced every 12 h. Zebrafish larvae were collected after bisphenols exposure at 120 hpf when the zebrafish fully completed development from embryo to larvae (Nishimura et al., 2016), and immediately immersed in liquid nitrogen, and stored at − 80 ℃ until further experimentation.

2.4. Hatching rate, body length, and motor behavior

The hatching rate, defined as the percentage of hatched embryos among all surviving embryos, was recorded at 48 hpf. The body lengths of five randomly selected larvae from each sample were measured at 120 hpf under a microscope. In addition, the motor behaviors of larvae at 120 hpf were evaluated from 0 to 10 min, and the mean values of the movement distances were determined using DanioVision (Noldus, Wageningen, the Netherlands).

2.5. Confocal microscopy

The embryos (2 hpf) from transgenic adults were exposed to 100 μg/L BPA or its analogs to identify changes in GnRH3 neurons during early embryonic development. At 25 hpf, seven embryos were randomly selected from each treatment group (n = 7), and the forebrain populations of GnRH3 neurons were visualized by confocal microscopy. After the image analysis, the numbers of GnRH3-EMD neurons in the terminal nerve (TN) and hypothalamus (HYPO) were counted in a treatment-blind manner. Detailed information about the images obtained was analyzed as described in our previous study (Qiu et al., 2015).

2.6. RNA isolation, cDNA synthesis, and quantitative real-time polymerase chain reaction (qRT-PCR)

The Quick-RNA™ MiniPrep kit (Zymo Research, USA) was used to extract total RNA from the homogenates of a sample of 20 zebrafish larvae per each sample. The High-Capacity RNA-to-cDNA™ Kit (Life Technologies, USA) was used to synthesize cDNA from 1 μg of the total RNA in a final volume of 20 μL. The SYBR® Green PCR Master Mix kit (Life Technologies, USA) and Mx3000P qPCR System (Agilent Technologies, USA) were used to perform qRT-PCR. The reaction mixture comprised 1 μL of cDNA, 10 μL of 2×SYBR Green Master Mix, and the appropriate forward and reverse primers in a final volume of 20 μL. The PCR conditions were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Fluorescent signals were measured at the annealing/extension step, and each sample from each group was analyzed in duplicate for each tested gene. Melting curve analyses were performed to validate the specificities of the PCR amplicons. The gene encoding ribosomal protein L13A (*rpl13a*) was used as the internal reference gene for each tested sample, based on a previous study (Qiu et al., 2015). A Ct-based relative quantitative expression value of each gene with an efficiency correction normalized to *rpl13a* was calculated using the $2^{-\Delta\Delta Ct}$ method. The primers specific for *rpl13a* and target genes are listed in Table S1.

2.7. Biochemical assays

Zebrafish were sampled from the control and BPA analog treatment groups to determine the changes in hormone levels at 120 hpf. After homogenizing the zebrafish in ice-cold phosphate-buffered saline (pH = 7.0), the homogenates were centrifuged at 12,000 ×*g* for 20 min at 4 °C, and the supernatant of each sample was used for analysis. LH, FSH, E2, and GH levels were measured using a commercial Fish ELISA kit (Nanjing Jiancheng Bioengineering Institute, China). Antibodies specific for each fish hormone were precoated onto a 96-well microplate, and a standard curve was constructed in parallel when each sample was tested. Each sample was tested in triplicate.

2.8. Statistical analysis

Data are shown as the means ± standard errors of the means. In all experiments, intergroup differences were assessed using a one-way analysis of variance (ANOVA), followed by the least significant difference (LSD) test depending on the experimental design. The level of statistical significance was set at $p < 0.05$, which is indicated by * or # in the figures. For the tested parameters, no significant difference was observed between the blank control group (E3 medium without DMSO) and the solvent control group (E3 medium with DMSO), and therefore the blank control group was set as the control group. All statistical analyses were performed using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). The principal component analysis (PCA) was performed using Origin 9.0 (OriginLab, Northampton, MA, USA) with the plug-in. All figures were obtained using Origin 9.0.

3. Results

3.1. Hatching rate, body length, and motor behavior

The embryos in control and BPA or BPA analogs treatment groups were all hatched at 72 hpf, however, 100 μg/L BPA, BPS and BPF significantly accelerated the hatching rate of zebrafish embryos at 48 hpf, as compared with the control (Fig. 1A). The body lengths of zebrafish larvae at 120 hpf were significantly reduced in all 100 μg/L BPA analog treatment groups (Fig. 1B). To determine whether exposure to BPA analogs altered the development of motor behavior, the movement distance of larvae at 120 hpf was measured from 0 to 10 min. As shown in Fig. 1C, exposure to 100 μg/L of BPA, BPB, and BPAF significantly decreased the movement distance of the zebrafish larvae relative to the control treatment. Taken together, our results suggest that exposure to 100 μg/L of most BPA analogs could increase the hatching rate of zebrafish embryos, while reducing the body length and inhibit the motor behavior of zebrafish larvae.

3.2. Effects of BPA analogs on the numbers of GnRH3 neurons in the TN and HYPO

We further explored the effects of BPA and its analogs on the development of GnRH3 neurons in zebrafish embryos. As shown in Fig. 2A, the numbers of GnRH3 neurons in the TN and HYPO were increased after exposure to BPA and its analogs (Fig. 2B–C). Specifically, the numbers of GnRH3 neurons in the TN increased significantly only in BPA-, BPB-, and BPAF-exposed zebrafish embryos (Fig. 2B), whereas those in the HYPO significantly increased in all BPA analog treatment groups (Fig. 2C).

3.3. Effects of BPA analogs on the expression of reproductive neuroendocrine-related genes

To evaluate the effects of exposure to BPA analogs on zebrafish larvae, the expression levels of several reproductive neuroendocrine-related genes were analyzed. As shown in Fig. 3A, when compared with the control treatment, 5 days of exposure to 100 μg/L of BPA, BPB, BPS, and BPAF significantly increased the expression of *kiss1*. Fig. 3B suggests that the expression of *kiss2* significantly increased only in the 1 μg/L BPF and 100 μg/L BPS treatment groups. Fig. 3C–D indicates that the expression of *gnrh3* and *fshβ* increased notably after exposure to 100 μg/L of BPA and BPAF, respectively. Fig. 3E shows that the expression of *lhβ* significantly increased after exposure to 1 μg/L of BPS and BPF and 100 μg/L of BPA, BPS, BPF, and BPAF.

We further analyzed changes in the expression of five endocrine-related genes, namely *anp*, *ren*, *prl*, *gh*, and *pth1*. As shown in Fig. 4, the expression of almost all genes increased in all BPA analog treatment groups relative to the control group. Specifically, Fig. 4A shows that the expression of *anp* increased notably after exposure to 1 μg/L of BPS and BPF and 100 μg/L of all tested BPA analogs. Similarly, the expression of *ren* significantly increased after exposure to 1 μg/L of BPF and BPAF and 100 μg/L of all tested BPA analogs (Fig. 4B), and the expression of *pth1* increased notably after exposure to 1 μg/L of BPAF and 100 μg/L of all tested BPA analogs (Fig. 4C). The expression of *gh* significantly increased after exposure to 1 μg/L of BPF and BPAF and 100 μg/L of all tested BPA

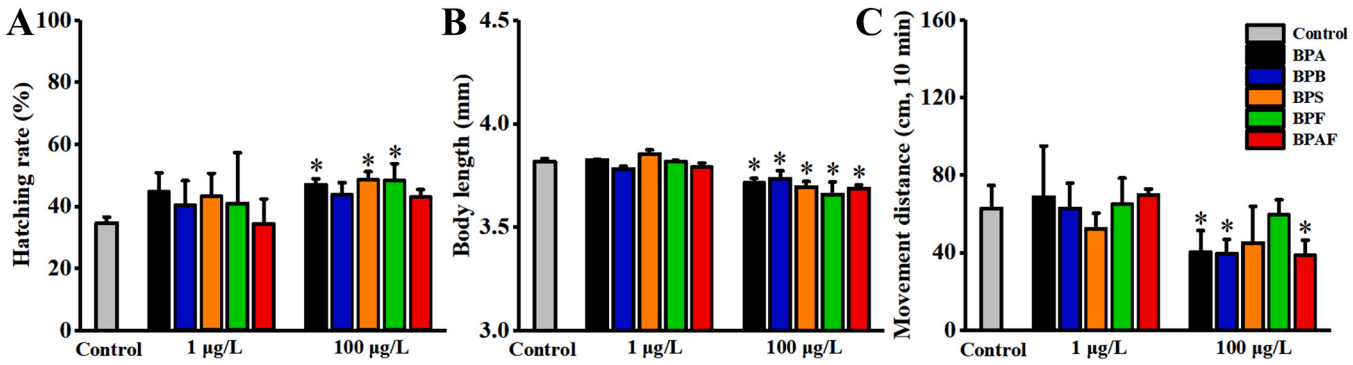

Fig. 1. Effects of BPA, BPB, BPS, BPF, and BPAF (1 and 100 μg/L) on the hatching rates of zebrafish embryos at 48 hpf (A), body lengths of zebrafish larvae at 120 hpf (B), and mean values of the movement distances (cm) of zebrafish larvae at 120 hpf from 0 to 10 min (C). Data are shown as the means ± standard errors of the means relative to the control group (n = 3). * represents a significant difference compared with control group (LSD test, ANOVA, $p < 0.05$).

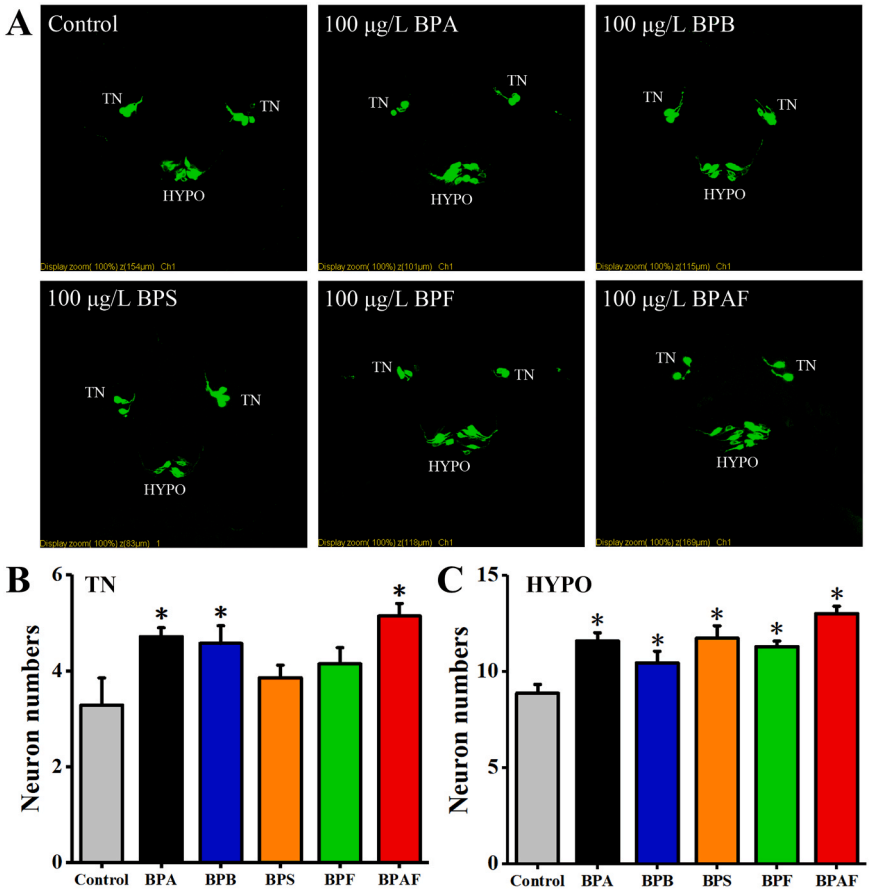

Fig. 2. The numbers of GnRH3 neurons in zebrafish embryos (viewed at 25 hpf) treated with 100 μg/L BPA or its analogs. (A) Typical image of the forebrain populations of GnRH3 neurons in each group. (B) and (C) The numbers of GnRH3 neurons in the terminal nerve (TN, B) and the hypothalamus (HYPO, C) of embryos in each group (n = 7). * represents a significant difference compared with the control group (LSD test, ANOVA, $p < 0.05$).

analogs except BPF (Fig. 4D), whereas that of *prl* increased notably after exposure to 1 μg/L of BPS and 100 μg/L of BPA and BPAF (Fig. 4E).

3.4. Effects of BPA analogs on hormone levels

As shown in Fig. 5, the levels of endocrine hormones, including LH, FSH, E2, and GH, in zebrafish larvae were increased following exposure to BPA analogs. Compared with the control group, the 100 μg/L BPA, BPB, BPF, and BPAF treatment groups exhibited significantly increased LH levels (Fig. 5A). The FSH levels increased significantly after exposure to 1 μg/L of BPAF and 100 μg/L of BPA and BPS (Fig. 5B). In addition, the E2 levels increased notably after exposure to 1 μg/L of BPS and BPAF and 100 μg/L of BPA, BPS, and BPAF (Fig. 5C). Similarly, the GH levels significantly increased after exposure to 100 μg/L of BPA, BPF, and BPAF (Fig. 5D).

3.5. Effects of BPA analogs on ERs and CYP19

We also investigated the mRNA levels of ERs, which are known to ultimately affect reproductive functions. As shown in Fig. 6, the mRNA level of *erα* significantly increased in response to 1 μg/L of BPA and BPAF and 100 μg/L of all BPA analogs relative to the control treatment

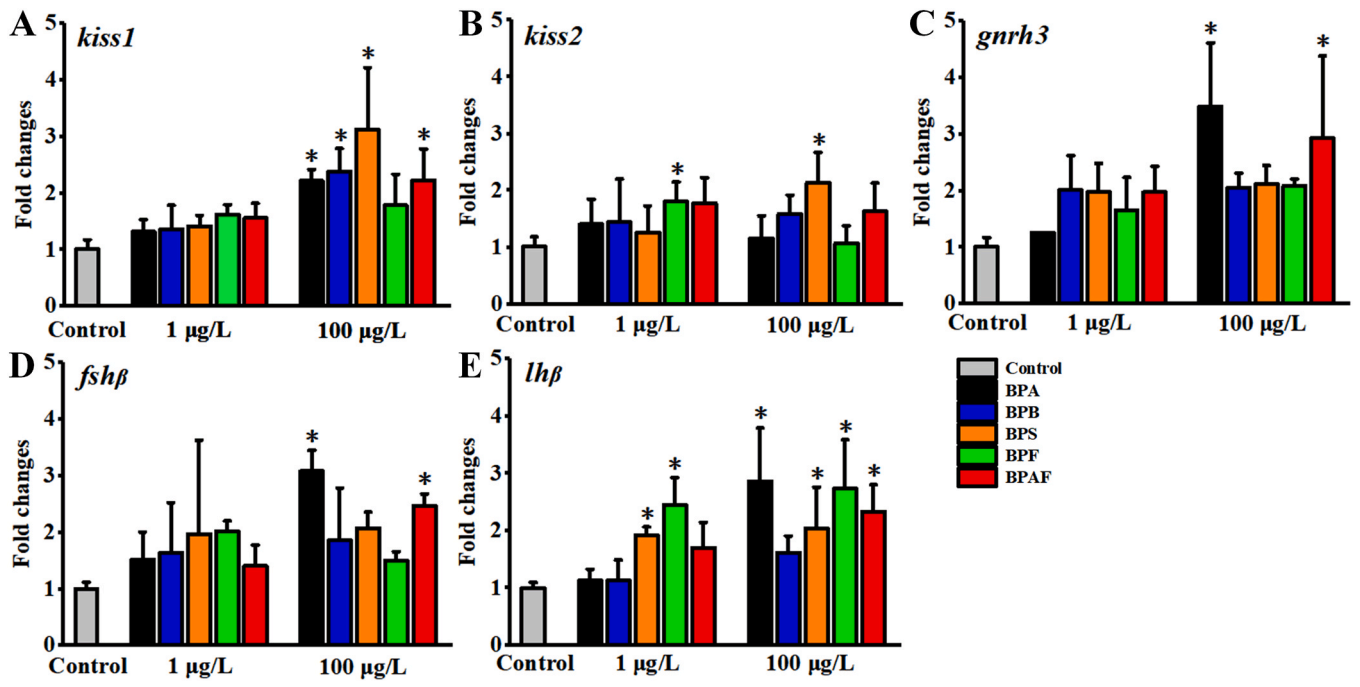

Fig. 3. The effects of BPA and its analogs on the expression of reproductive neuroendocrine-related genes, including *kiss1* (A), *kiss2* (B), *gnrh3* (C), *lhβ* (D), and *fshβ* (E), at 120 hpf. * represents a significant difference compared with the control group (LSD test, ANOVA, $p < 0.05$).

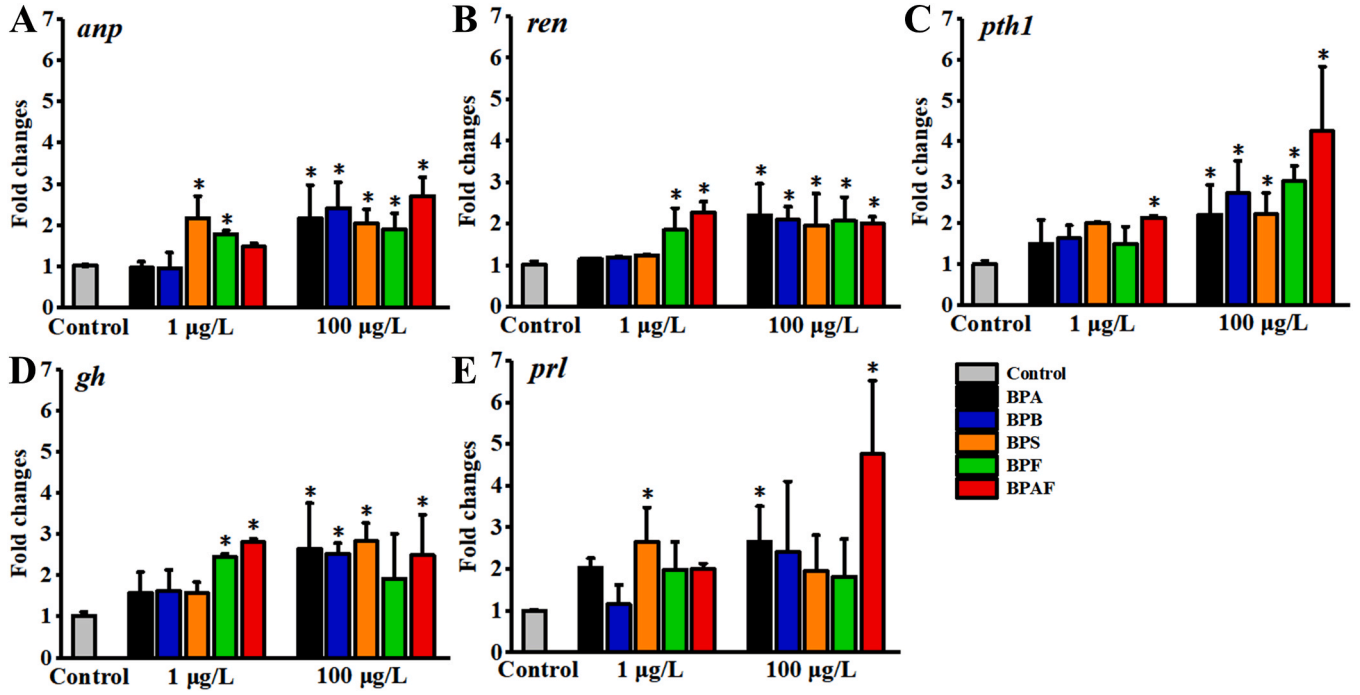

Fig. 4. The effects of BPA and its analogs on the expression of reproductive neuroendocrine-related genes, including *anp* (A), *ren* (B), *pth1* (C), *gh* (D), and *prl* (E), at 120 hpf. * represents a significant difference compared with the control group (LSD test, ANOVA, $p < 0.05$).

(Fig. 6A), whereas that of *erβ1* significantly increased in response to 1 μg/L of BPA, BPB, BPS, and BPF and 100 μg/L of BPB, BPS, and BPAF (Fig. 6B). In addition, the mRNA level of *erβ2* significantly increased after exposure to 1 μg/L of BPF and 100 μg/L of BPB, BPS, and BPAF (Fig. 6C).

The expression of aromatic hydrocarbon receptors was also evaluated. As shown in Fig. 6D–E, the expression of *cyp19a1* significantly increased after exposure to 1 μg/L of BPAF and 100 μg/L of BPA, BPS, and BPAF, whereas that of *cyp19a2* increased notably after exposure to 1 μg/L of BPB, BPF, and BPAF and 100 μg/L of all BPA analogs.

3.6. Effects of the ER antagonist ICI and the AROM inhibitor FAD

To further investigate the possible involvement of ERs and the AROM pathway, we used the ER antagonist ICI (1 μM) and the AROM inhibitor FAD (1 μM) in co-exposure experiments with 100 μg/L BPA analogs. We

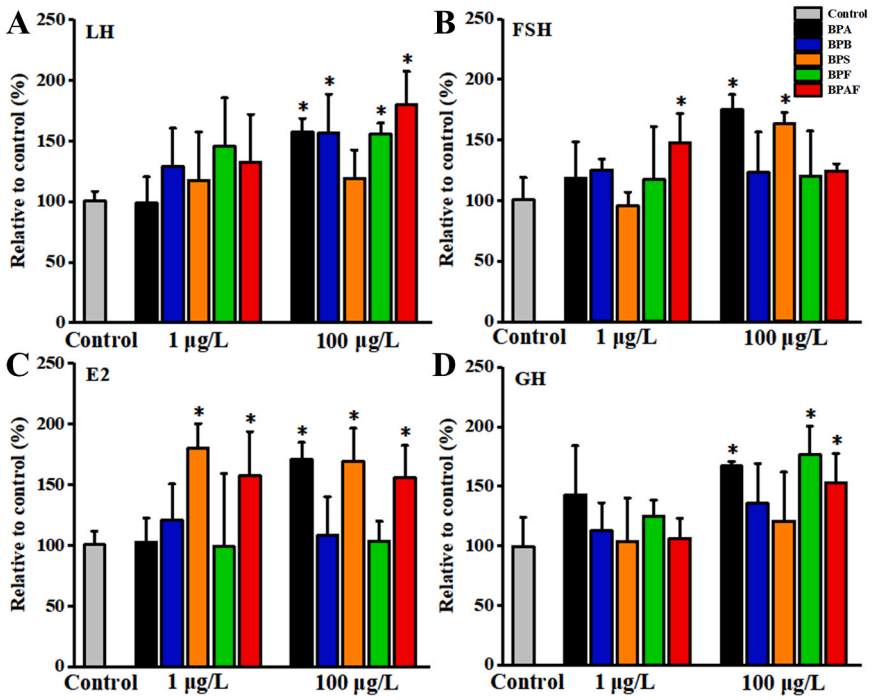

Fig. 5. The effects of BPA and its analogs on the levels of hormones, including LH (A), FSH (B), E2 (C), and GH (D), at 120 hpf. * represents a significant difference compared with the control group (LSD test, ANOVA, $p < 0.05$).

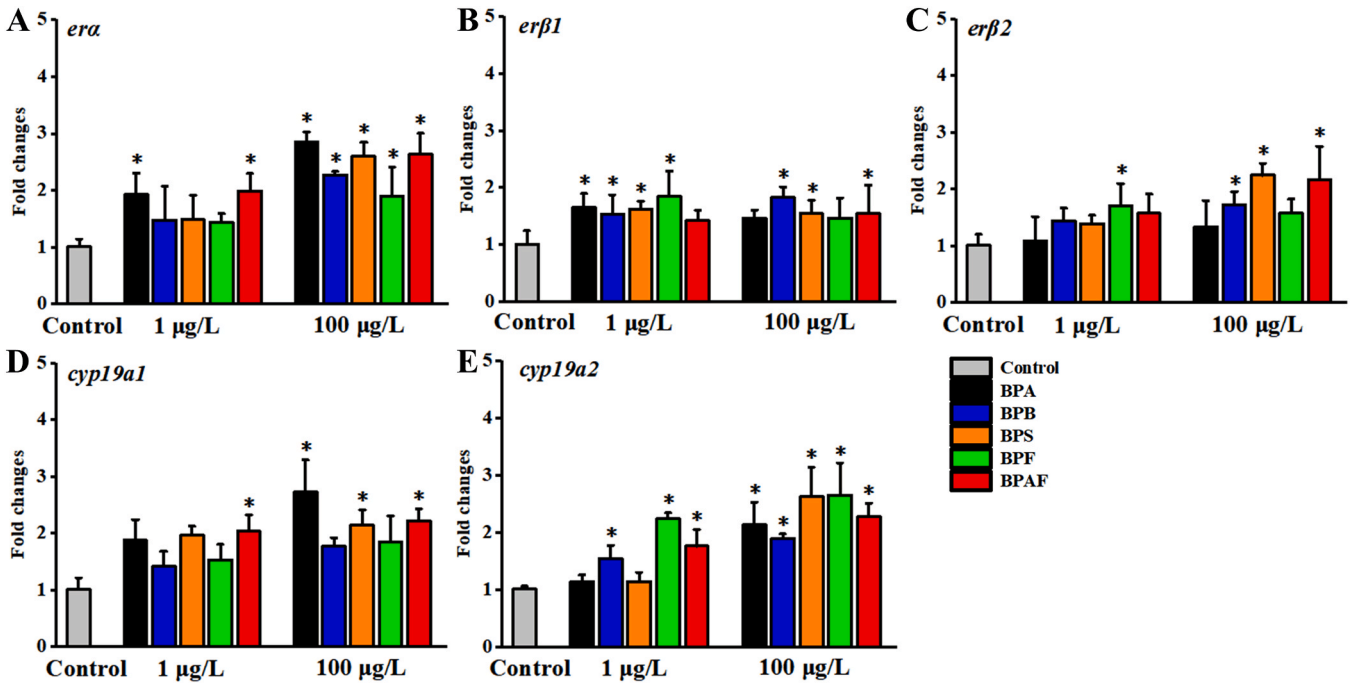

Fig. 6. The effects of BPA and its analogs on the expression of genes encoding ERs, including *erα* (A), *erβ1* (B) and *erβ2* (C), and CYP19 family members, including *cyp19a1* (D) and *cyp19a2* (E), at 120 hpf. * represents a significant difference compared with the control group (LSD test, ANOVA, $p < 0.05$).

found that the survival rates of zebrafish were not significantly altered in this receptor inhibition experiment. However, as shown in Fig. 7, both ICI and FAD attenuated the stimulatory actions of BPA analogs on reproductive neuroendocrine-related gene expression and endocrine hormone levels. Specifically, the expression of *lhβ* significantly decreased after co-exposure to BPA or BPF with ICI and after co-exposure to BPA, BPB, or BPF with FAD, compared with the expression after exposure to the corresponding BPA analogs alone (Fig. 7A–B). Similarly, the expression of *fshβ* also decreased notably after co-exposure to BPA, BPF, or BPAF with ICI or to all BPA analogs with FAD relative to the expression after exposure to the corresponding BPA analogs alone (Fig. 7C–D). The LH levels were significantly decreased in response to BPA or BPAF co-exposure with ICI relative to the levels in response to BPA or BPAF exposure alone (Fig. 7E). In addition, the LH level significantly decreased in response to BPA co-exposure with FAD (Fig. 7F). Meanwhile, the FSH level significantly decreased in response

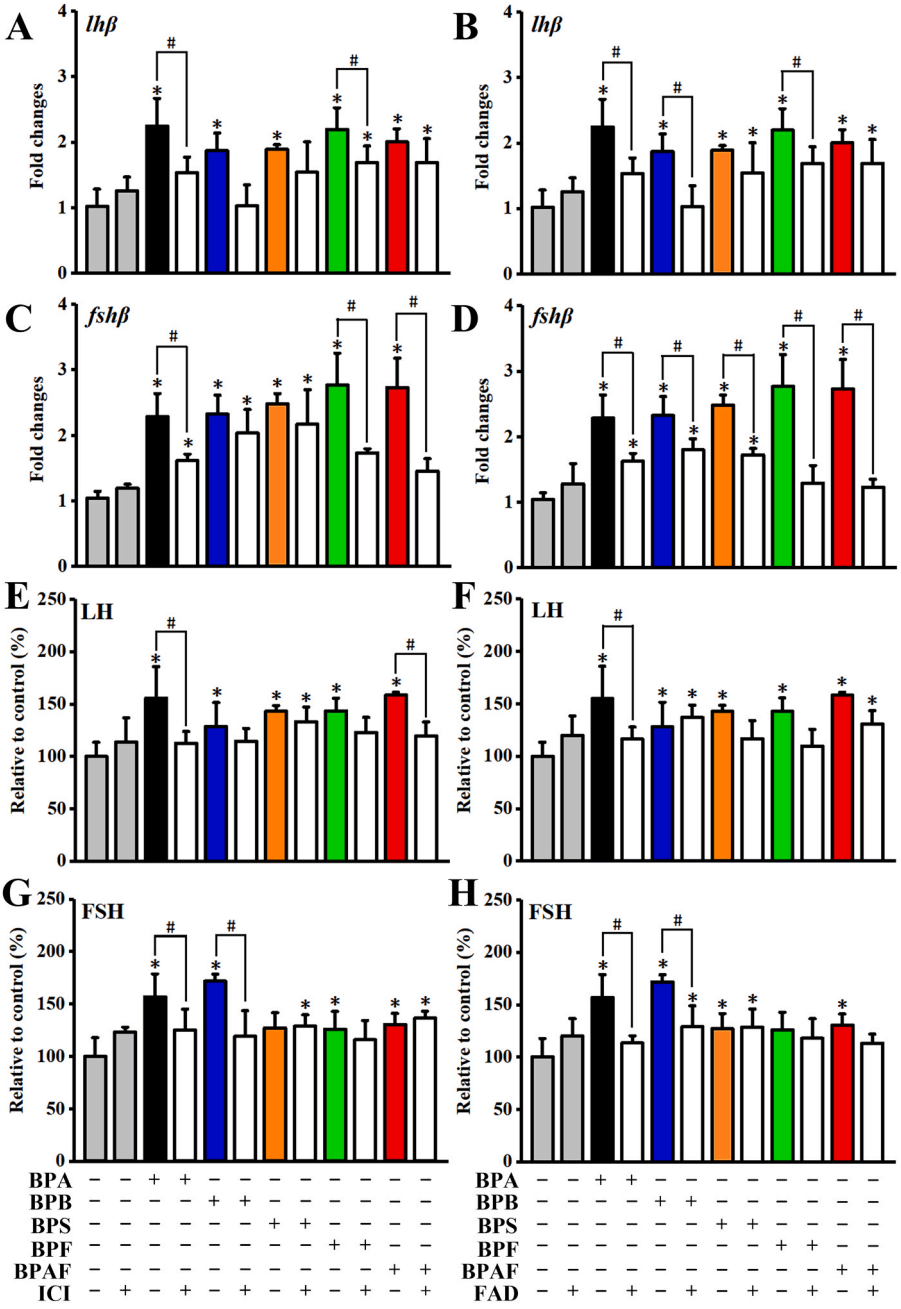

Fig. 7. Changes in the expression of *lhβ* and *fshβ* gene expression and LH and FSH hormone levels following co-exposure to BPA or its analogs with the ER antagonist ICI or the AROM inhibitor FAD. * represents a significant difference compared with the control group, and # represents a significant difference between the two compared groups (LSD test, ANOVA, $p < 0.05$).

to BPA or BPB co-exposure with ICI or FAD relative to BPA or BPB alone (Fig. 7G–H).

4. Discussion

This study investigated the effects of BPA, BPB, BPS, BPF, and BPAF on the reproductive neuroendocrine system in the zebrafish embryo, as well as the underlying mechanism. Consistent with our previous studies on zebrafish larvae or adults (Qiu et al., 2015, 2019), the BPA analogs induced the expression of some reproductive neuroendocrine-related genes and hormones in this study. Meanwhile, we found that the abundance of some indexes in lower concentration were higher than that of higher concentration, including *kiss2*, *fshβ*, *anp*, *ren*, *gh*, *prl*, *FSH*, *E2*, and *erβ1*, with no significant difference. We suggested the phenomenon was a dynamic response process of several parameters upon BPA and BPA analogs exposure. In accordance with the findings from our previous research on BPA (Qiu et al., 2015, 2018), exposure to 100 μg/L of BPA, BPS, and BPF significantly promoted the hatching rates of zebrafish larvae at 48 hpf, indicating that the BPA analogs could accelerate the hatching rate of zebrafish embryos at early stage, which was also similar with previous report that BPA could accelerate the hatching rate of medaka eggs (Ramakrishnan and Wayne, 2008). In addition, consistent with the previous study by Xiao et al. (2019), exposure to a high concentration (100 μg/L) of a BPA analogs could significantly reduce the body lengths of zebrafish larvae at 120 hpf. This observation might be related to the ability of high concentrations of BPA analogs to disrupt thyroid hormone signaling in vertebrates (Heimeier et al., 2009), thereby adversely affecting postembryonic development of

zebrafish larvae. Meanwhile, our results also showed that exposure to a high concentration (100 μg/L) of BPA, BPB, or BPAF could significantly reduce the movement distances of zebrafish larvae. This can be explained by the significant axial muscle damage induced by exposure to these BPA analogs (Wang et al., 2013), which likely contributed to the observed deficits in the swimming activity of zebrafish larvae. In addition, the dysfunction of neuroendocrine systems by these chemicals may also induce the abnormal of swimming activity (Salahinejad et al., 2020).

The structures of BPA and BPA analogs are similar, and therefore the study of the comparative toxicities of different bisphenols is meaningful as an evaluation of the underlying adverse effects of BPA-free products containing BPA alternatives. In this study, we explored the toxicities of five bisphenols, including BPA, BPB, BPS, BPF, and BPAF, on zebrafish larvae. We found that the expression of reproductive neuroendocrine-related genes (*kiss1*, *kiss2*, *gnrh3*, *fshβ*, *lhβ*, *anp*, *ren*, *pth1*, *gh*, and *prl*) increased in various treatment groups, indicating that BPA analogs could disrupt the GnRH system during zebrafish development (Imamura et al., 2020). In addition, the levels of the gonadotropins LH and FSH, two important hormones in the HPG axis, as well as of E2 and GH, were increased in BPA-exposed zebrafish larvae. LH, FSH, and E2 are known to play important roles in sexual development (Wang et al., 2016) and in regulating the reproductive functions of organisms (Radovick et al., 2012). BPA could also affect ovarian maturation of female goldfish with the mediation of HPG axis (Wang et al., 2019). Consistently, our results revealed that BPA treatment could ultimately interfere with the development and reproductive function of the gonads in zebrafish. Therefore, our observation of changes in the expression of reproductive neuroendocrine-related genes, together with the changes in endocrine hormone levels, suggest that all the tested BPA analogs had obvious effects on the GnRH system and HPG axis in zebrafish larvae (Ji et al., 2013; Shi et al., 2015). Notably, 100 μg/L BPA and BPAF exposure significantly induced the expression of all reproductive neuroendocrine-related genes except *kiss2* (Fig. 8A), which was consistent with the finding of our previous study that *kiss1*, rather than *kiss2*, is the key gene involved in the stimulation of GnRH3-expressing neuron development in zebrafish embryos or larvae (Qiu et al., 2015).

GnRH neurons are located in the brains of organisms, where they release GnRH into the pituitary and induce the production of fertility-related hormones (Cortes-Campos et al., 2015). As the main controllers of the reproductive axis, GnRH neurons also control the sexual differentiation of organisms (Clarkson et al., 2014). In our study, the numbers of GnRH3 neurons in the TN and HYPO of zebrafish embryos increased significantly after treatment with 100 μg/L BPA, BPB, or BPAF, whereas the numbers increased significantly only in the HYPO and slightly in the TN after exposure to BPS and BPF, and the embryos in BPAF-exposed group formed the most numbers of GnRH3 neurons. This was consistent with the findings of our previous study (Qiu et al., 2015) and our present analysis of the *gnrh3* mRNA levels. A previous study found that estrogen may activate kisspeptin neurons and then increase the production of GnRH neurons (Herbison and Clarkson, 2008). Accordingly, we found that treatment with BPA and BPA analogs induced the expression of *kiss* and *gnrh3* and increased the numbers of GnRH neurons in zebrafish embryos, suggesting that the early development of the zebrafish reproductive system was significantly affected. This finding is consistent with that of a previous study on the effect of BPA in rats (Adewale et al., 2011).

Interestingly, exposure to BPAF, which is more similar to BPA, altered the expression of more types of genes and hormones than the other three BPA analogs, viz. BPB, BPS, and BPF (Fig. 8A), suggesting that the toxicities of these three BPA analogs on zebrafish larvae differ from those of BPA and BPAF. Meanwhile, our PCA results showed that the clustering of change profiles of detected endpoints related to BPAF was closer to that of BPA with no obvious distinction than was that of other BPA analogs (Fig. 8B–E). Mu et al. (2018) revealed that among BPAF, BPA, BPF, and BPS, BPAF exhibits the highest estrogenic activity

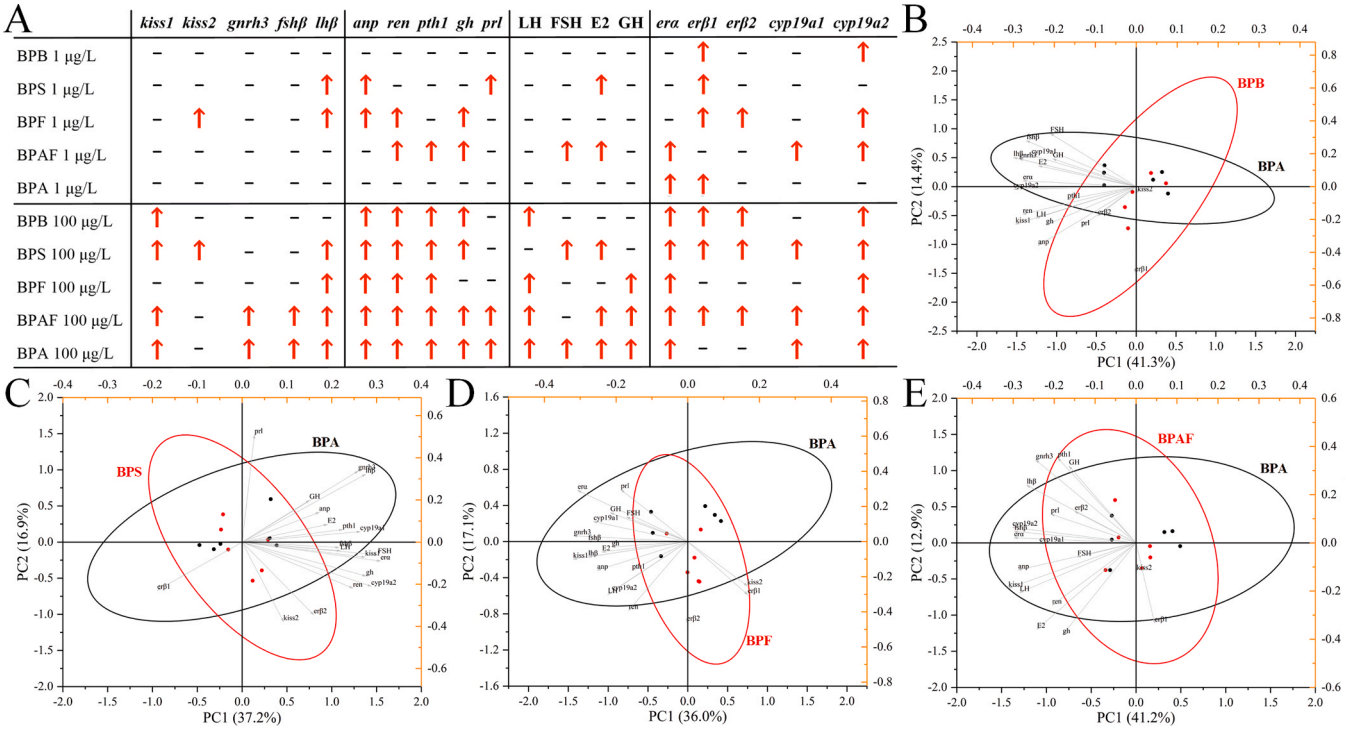

Fig. 8. Comparisons of changes in the expression of all evaluated genes and hormones in zebrafish larvae exposed to BPA and its analogs. (A) The changes in all indexes after exposure to 1 μg/L or 100 μg/L of BPA, BPB, BPS, BPF, and BPAF; the arrows and short bars indicate the upregulation and non-change of the index in each group, respectively. (B–E) The PCA results of all indexes in comparisons of the BPA treatment group with the BPB (B), BPS (C), BPF (D), and BPAF (E) treatment groups.

and toxicity to zebrafish, with an ER binding ability much higher than that of BPA (Matsushima et al., 2010). Rochester and Bolden (2015) reviewed the adverse effects of BPS and BPF on reproductive endpoints and determined that these BPA analogs had a hormonal potency slightly lower than that of BPA, while the effects of BPS on the reproductive system were similar to those of BPA (Kinch et al., 2015). Like BPA, BPA analogs (BPB, BPF, and BPS) could induce oxidative stress and disrupt the rat reproductive systems, with consequent effects on the ovaries, testes, and spermatogenesis (Ijaz et al., 2020; Ullah et al., 2018), whereas BPB and BPS could induce neurotoxicity in the hippocampal neuronal cells of mice (Pang et al., 2019). Taken together, the results of these previous studies and our study indicate that all BPA analogs, including BPB, BPS, BPF, and BPAF, exert reproductive neuroendocrine toxicity in zebrafish larvae, while BPAF, which is more similar to BPA, could change the expression of more reproductive neuroendocrine indexes than other BPA analogs (Moreman et al., 2017; Mu et al., 2018).

As typical estrogen-mimicking chemicals, BPA and its analogs adversely impact estrogen signaling by interacting with two types of ERs (ERα and ERβ). Accordingly, we found that the expression of *erα* and *erβ1* was induced after exposure to BPA and all its tested analogs, whereas that of *erβ2* increased in response to all BPA analogs but not BPA. This could be attributable to the mechanistic differences between BPA and its analogs, as demonstrated by a report by Patisaul et al. (2006) demonstrating that BPA acts mainly through an ERα-mediated pathway. Thus, our results, which were consistent with the results of a study by Le Fol et al. (2017), indicated that BPA selectively activated the ERα alone and that BPS or BPF was more likely to activate both ERα and ERβ. In the research on the function of BPAF in breast cancer cells, Okazaki et al. (2017) suggested that at a low concentration, BPAF could function as an agonist to activate ERα but not ERβ. Similarly, we found that BPAF induced the expression of both *erα* and *erβ* in zebrafish larvae at 100 μg/L, but only *erα* at 1 μg/L. This finding suggests that BPAF exerts its estrogenic effect via ERα when administered at a low concentration, but acts through both ERα and ERβ when administered at a high concentration. In addition, evidence has shown that kisspeptin neurons express ERs (Radovick et al., 2012), and the activation of ERs by bisphenols could lead to the secretion of Kiss1, which would consequently affect GnRH neuronal activity by modulating the E2 level (Pielecka-Fortuna et al., 2008). ERs are also required for the production of LH (Wintermantel et al., 2006), and mediate the impairment of reproductive system of zebrafish induced by BPA (Song et al., 2020). Thus, ERs are essential mediators in the reproductive neuroendocrine toxicity exerted by bisphenols.

Meanwhile, we found that BPA and its analogs could upregulate the expression of the AROM-related genes *cyp19a1* and *cyp19a2*. Aromatase (CYP19) is a crucial enzyme in the regulation of zebrafish sex differentiation at early developmental stages (Fenske and Segner, 2004), and high expression of *cyp19a* triggers the development of fish into females. Perinatal exposure to BPA could increase the intraprostatic levels of aromatase in prostate of offspring rats (Castro et al., 2018), which will affect the development of reproductive organs, while in female zebrafish, BPA could also induce reproductive toxicity by affect the function of HPG axis and change the expression level of *cyp19b* (Molina et al., 2018). Thus, the induction of *cyp19a1* or *cyp19a2* expression in zebrafish larvae by exposure to BPA and BPA analogs in our study may have promoted ovarian development, consistent with the previously reported effects of BPS (Ji et al., 2013). In particular, we found that unlike other BPA analogs, BPB and BPF exerted their effects mainly through the *cyp19a2*-mediated pathway, rather than the *cyp19a1*-mediated pathway, as no significant increase was observed in *cyp19a1* expression. However, Yang et al. (2018) reported the involvement of *cyp19a1* in development and sexual differentiation after more than 20 days of BPF treatment. This difference in the results between the study by (Yang et al., 2018) and our study may be due to the difference in the exposure time, which was only 5 days in our study.

ICI and FAD, the inhibitors of ER and AROM, respectively (Meeuwen et al., 2007), could significantly attenuate the stimulatory actions of BPA and its analogs on the expression of the tested genes (*lhβ* and *fshβ*) and gonadotropins (LH and FSH). According to our results, ICI and FAD could separately attenuate increases in the expression of *lhβ*, *fshβ*, LH, and FSH in BPA-exposed groups. Our previous study showed that BPA, when co-administered with ICI or FAD, could interact with the ER to mediate immune responses in fish (Yang et al., 2015). Kinch et al. (2015) suggested that ER and AROM signaling play important roles in BPA-induced precocious puberty in zebrafish. Therefore, BPA participates in the disruption of the reproductive neuroendocrine system of zebrafish larvae by activating ER and AROM signaling. Meanwhile, we found that co-exposure with ICI or FAD could also relieve the inductive effects of BPA analogs on some indexes, consistent with the findings of previous studies on the estrogenic effects of bisphenols (Boucher et al., 2016; Li et al., 2015). These findings suggest the presence of a complementary interaction between ER- and AROM-activated pathways in response to BPA and its analogs in zebrafish embryos, in accordance with the results of previous studies (Moreman et al., 2017).

5. Conclusions

In conclusion, our study found that exposure to BPA and its analogs decreases the body length and affects the motor behavior of zebrafish larvae. These chemicals increased the expression of reproductive neuroendocrine-related genes, including *kiss1*, *kiss2*, *gnrh3*, *fshβ*, *lhβ*, *anp*, *ren*, *pth1*, *gh*, and *prl*, and contents of hormones such as LH, FSH, E2, and GH. Moreover, BPA and its analogs increased the expression of *erα*, *erβ*, and *cyp19a*, and the respective ER and AROM antagonists, ICI and FAD, significantly attenuated the stimulation of *lhβ* and *fshβ* expression and the increases in LH and FSH levels, proving that BPA and its analogs affect the reproductive neuroendocrine system via cross-talk between the ER and AROM pathways. Thus, the use of BPB, BPS, BPF, and BPAF as alternatives to BPA should be reconsidered in the future, as the effects of these analogs on the reproductive neuroendocrine system are similar to those of BPA and thus pose potential health risks to humans.

CRediT authorship contribution statement

Wenhui Qiu: Methodology, Conceptualization, Software, Writing - review & editing. **Shuai Liu:** Methodology, Software, Data curation, Writing - original draft. **Honghong Chen:** Investigation, Data Curation. **Shusheng Luo:** Methodology, Formal analysis. **Ying Xiong:** Methodology, Software. **Xuejing Wang:** Methodology, Software. **Bentuo Xu:** Validation, Visualization, Supervision, Writing - review & editing. **Chunmiao Zheng:** Writing - review & editing, Supervision, Funding acquisition. **Ke-Jian Wang:** Conceptualization, Funding acquisition, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2020.124303.

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