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Concentration-dependent effects of 17β-estradiol and bisphenol A on lipid deposition, inflammation and antioxidant response in male zebrafish (*Danio rerio*)

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HIGHLIGHTS

- E2 and BPA damaged gonad structure and induced feminization in male fish.
- E2 and BPA promoted lipid deposition mainly in relatively low concentrations.
- Inflammation was enhanced by high concentrations of E2 and BPA.
- BPA caused more severe lipid peroxidation and gonad damage to zebrafish than E2.
- The toxicity of environmental estrogens to zebrafish was affected by concentrations.

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ABSTRACT

Environmental estrogenic compounds are important pollutants, which are widely distributed in natural water bodies. They produce various adverse effects on fish, but their concentration-dependent toxicities in fish metabolism and health are not fully understood. This study investigated the effects of 17β-estradiol (E2) and bisphenol A (BPA) at low and high concentrations on lipid deposition, inflammation and antioxidant response in male zebrafish. We measured fish growth parameters, gonad development, lipid contents and the activities of inflammatory and antioxidant enzymes, as well as their mRNA expressions. All E2 and BPA concentrations used increased body weight, damaged gonad structure and induced feminization in male zebrafish. The exposure of zebrafish to E2 and BPA promoted lipid accumulation by increasing total fat, liver triglycerides and free fatty acid contents, and also upregulated lipogenic genes expression, although they decreased total cholesterol content. Notably, zebrafish exposed to low concentrations of E2 (200 ng/L) and BPA (100 μg/L) had higher lipid synthesis and deposition compared to high concentrations (2000 ng/L and 2000 μg/L, respectively). However, the high concentrations of E2 and BPA increased inflammation and antioxidant response. Furthermore, BPA caused greater damage to fish gonad development and more severe lipid peroxidation compared to E2. Overall, the results suggest that the toxic effects of E2 and BPA on zebrafish are concentration-dependent such that, the relative low concentrations used induced lipid deposition, whereas the high ones caused adverse effects on inflammation and antioxidant response.

1. Introduction

Estrogens such as estrone (E1), 17β-estradiol (E2) and estriol (E3) are compounds naturally synthesized in vertebrates, whereby they play important roles in endocrine and reproductive systems (Yin et al., 2003; Eskicioglu and Hamid, 2012). Synthetic estrogens such as bisphenol A (BPA) and nonyl phenol (NP) are the artificial

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estrogen-mimicking chemicals, which are widely used in medicine and manufacturing industries (Adeel et al., 2016). Of these, BPA is the typical organic synthetic compound with estrogenic effects, which is produced as a key monomer of polycarbonate plastics and epoxy resins (Genuis et al., 2012). It is extensively used in the production of rubber, plastics and circuitry (Chamorro-Garcia et al., 2012). Therefore, BPA together with other environmental estrogenic compounds can easily spread in natural water bodies through discharge of wastewater from humans, livestock and industrial processes (Shrestha et al., 2012). Accordingly, E2 concentration in effluents has been reported to range from 48 to 141 ng/L in Israel and higher levels up to 313 ng/L were detected in Salt River Project water and Huron River water (Kramer et al., 1998; Yoon et al., 2003). The BPA concentration in rivers has been approximated as 21 μg/L in The Netherlands (Crain et al., 2007) and 24538.14 μg/L in Linhe River in China (Lu, 2012), while it was reported as 17200 μg/L in landfill leachates in Japan (Crain et al., 2007). Therefore, BPA and E2 are considered as important pollutants to the aquatic environments. Contrary to drinking water, effluent and landfills, the concentrations of estrogenic compounds in the bodies of aquatic animals are expected to be higher due to bioconcentration and biomagnification (Curieux-Belfond et al., 2005).

Aquatic animals, such as fish are directly exposed to environmental estrogenic compounds and show greater adverse impacts than terrestrial animals. Previous studies have shown that environmental estrogenic compounds weakened maleness and induced the production of oogonia and vitellogenin (VTG) in fish (Knoebl et al., 2006; Kidd et al., 2007). Estrogenic compounds also act as endocrine-disrupting chemicals (EDCs) in vertebrates (Cock and Bor, 2014). For example, estrogenic compounds have been reported to regulate growth and development, energy metabolism, immunity and inflammation in mammals (Wang et al., 2013; Monteiro et al., 2014). Essentially, estrogens regulate energy metabolism mainly by modulating lipid deposition. For example, fat distribution and the prevalence of obesity diseases in animals display sex bias and estrogen treatment after ovariectomy in mice suppressed lipid deposition in liver (Shi and Liu, 2013; Zhu et al., 2013). Estrogens also play significant roles in inflammation and antioxidant capacity as a response to injury and infection (Monteiro et al., 2014). Similar to lipid metabolism, estrogens cause different effects between sexes in inflammatory diseases, such as bowel, cardiovascular, diabetic and autoimmune diseases (Reckelhoff, 2006; Linares et al., 2013).

Previous studies on EDCs such as environmental estrogens (Ruggeri et al., 2008), polychlorinated biphenys (PCBs) (Sun et al., 2018) and antibiotics (Limbu et al., 2018) have revealed that their existence in natural water bodies affect fish health by disrupting energy metabolism. The distorted energy metabolism by estrogenic compounds in fish has been related to their ability to promote lipid accumulation, especially in the liver, which affect lipid metabolism (Cakmak et al., 2006; Santangeli et al., 2018). Notably, the dysregulation of lipid metabolism and immunity response affect general fish health. For instance, excessive fat deposition induces lipotoxicity or fish fatty liver disease (Du, 2014), whereas oxidative stress and inflammation increases mortality in fish (Adeyemi et al., 2014). Therefore, we hypothesized that estrogenic compounds in water may be responsible for various fish abnormalities such as disruptions of metabolism and immunity response, based on their potential endocrine-disrupting effects in vertebrates (Cakmak et al., 2006; Xu et al., 2013). Furthermore, we assumed that different concentrations of environmental pollutants may exert distinct effects on fish similar to other contaminants. For instance, lower concentrations of antibiotics caused more severe toxicological effects on immunity compared to higher concentrations (Limbu et al., 2018). Nevertheless, studies attempting to investigate critically the concentration-dependent effects of estrogenic compounds on fish health are very limited.

The present study examined the effects of low and high concentrations of E2 and BPA on the health of male zebrafish. We selected E2 and BPA because they are widely spread in natural waters. Specifically, we assessed the effects of E2 and BPA on lipid deposition, inflammatory and antioxidant responses, hepatic health and gonadal development in male zebrafish. In the present study, we used 200 ng/L E2 and 100 μg/L BPA to the reflect real environmental concentrations and 2000 ng/L E2 and 2000 μg/L BPA to investigate the potential effects of high exposure to E2 and BPA on aquatic animals. These concentrations were chosen based on the wide range of estrogenic compounds in the natural environments (from few nanograms to thousand micrograms per litre).

2. Materials and methods

The experimental protocols and animal handling in the present study were carried out strictly under the Guidance of the Care and Use of Laboratory Animals in China. This research was approved by the Committee on the Ethics of Animal Experiments of East China Normal University.

2.1. Chemicals and diet preparations

The 17-Beta-Estradiol (E2, CAS: 50-28-2), 2,2-Bis (4-Hydroxyphenol) Propane (BPA, CAS: 80-05-7) and dimethyl sulfoxide (DMSO, CAS: 67-68-5) were purchased from the Adamas-beta (Shanghai, China). The stock solution for each compound was prepared by dissolving 1 g of E2 and 100 g of BPA in 1 L of DMSO. The final DMSO concentrations in the water was 0.005 ml/L. All stock solutions were stored in 20 mL glass amber vials with PTFE-lined solid lids supplied from ANPEL (Shanghai, China).

We prepared a purified diet based on the detailed formulations given in supplementary Table S.1. The purified diet was necessary in order to avoid extra environmental estrogens sources. The exact concentrations of E2 and BPA in the diet were detected as 4.72 ± 0.40 ng/g and 6.71 ± 0.58 ng/g dry weight, respectively.

2.2. Fish and estrogens exposure

About 400 five-month-old AB male zebrafish strain (0.319 ± 0.011 g each) were purchased from the Chinese National Zebrafish Resource Center (Wuhan, China). Before the formal experiments, the fish were acclimated in fresh-dechlorinated water and maintained at required water quality parameters by using compressed air pumps for two weeks at a 12 h light to dark photoperiod. During the acclimatization period, fish were fed on a commercial zebrafish diet (Shandong, China). The water temperature, pH, dissolved oxygen and ammonia nitrogen were kept at 27 ± 1 °C, 7.69 ± 0.13, 5.6 ± 1.3 mg L^{-1} and below 0.02 mg L^{-1}, respectively.

After acclimation, fish were randomly divided into five treatments and exposed to control (fresh-dechlorinated water), E2 (200 and 2000 ng/L) and BPA (100 and 2000 μg/L) hereafter referred to as control, E200, E2000, B100 and B2000 treatments, respectively. Each treatment had 78 fish separated into three replicates (26 fish per replicate, 10 L glass tank). All the fish were fed twice at 9:00 and 19:00 h at 4% of their body weight daily for six weeks. In order to keep relatively stable exposure concentrations, one third of culture water by volume in each glass tank was replaced with fresh-dechlorinated water containing the respective estrogen concentrations every 24 h. All the culture water was replaced by using newly prepared estrogens-water every seven days. During the trial, the real concentrations of E2 and BPA in the culture water were measured by using ultra-high-performance liquid chromatography equipped with tandem mass spectrometry (UHPLC-MS/MS) technology. The E2 contents in the water of control, E200 and E2000 treatments were 0.0006 ± 0.0001, 0.1718 ± 0.0064 and 1.8514 ± 0.0491 μg/L, respectively. The BPA contents in the water of control, B100 and B2000 treatments were 0.109 ± 0.005, 77.452 ± 3.724 and 1920.573 ± 28.576 μg/L, respectively. The detailed method for estrogens detection is presented in the supplementary methods. The weights of fish in each tank were recorded biweekly and the daily feeding amounts were adjusted accordingly.

2.3. Oxygen consumption rate assay

At the end of the exposure, all fish were fasted for overnight. A sample of 45 fish from each treatment (15 fish per replicate) were randomly sampled for oxygen consumption rate (OCR) assay by using the Strathkelvin Instruments 782 Oxygen Meter system (North Lanarkshire, Scotland, UK). The oxygen consumption rate was calculated as: OCR = (fish oxygen consumption, mg/h)/(fish weight, g).

2.4. Estimation of growth performance, condition factor and organ indices

After the oxygen consumption rate assay, all the fish were euthanized by using MS-222 (tricaine methanesulfonate, Sigma-Aldrich, St Louis, MO, USA) and sacrificed for dissection and sampling of organ tissues. Several indexes were carried out to reveal zebrafish growth, including final body weight (FBW), condition factor (CF), viscerosomatic index (VSI) and gonadosomatic index (GSI) based on formulae given below:

$$CF = 100 \times (fish\ weight, g) \Big/ (fish\ legth, cm)^3$$

$$VSI = 100 \times (viscera\ weight)/(fish\ weight)$$

$$GSI = 100 \times (testis\ weight)/(fish\ weight)$$

2.5. Lipid content, enzymes activities and histological assays

A sample of 12 fish from each treatment (four fish per replicate) were collected for total fat content analysis. Total fat content was measured by using the classical methanol-chloroform method (Bligh and Dyer, 1959). The liver, viscera (including intestines, liver, spleen, heart and abdominal fat tissue) and muscle of 12 fish from each treatment (four fish per replicate) were sampled for tissue triglycerides (TG), free fatty acids (FFA) and total cholesterol (T-CHO) assays. The TG, FFA and T-CHO in tissues were determined by using specific commercial kits (Jiancheng Biotech Co., China) according to the manufacturer's guidance.

Another sample of 12 fish liver from each treatment (four fish per replicate) were homogenized in ice-cold phosphate buffer solution (PBS) for determination of immunity and inflammation enzymes activities. The activities of acid phosphatase (ACP), alkaline phosphatase (AKP), total superoxide dismutase (T-SOD), total antioxidant capacity (T-AOC), catalase (CAT) and malondialdehyde (MDA) content in liver were detected by using specific commercial kits (Jiancheng Biotech Co., China) according to the manufacturer's instructions.

Six fish livers and testes from each treatment were fixed in 4% paraformaldehyde for more than 24 h, then embedded into paraffin as described previously (Betancor et al., 2015). Sections of 6 μm thickness were stained by using hematoxylin-eosin (HE) staining and subsequently observed under a microscope (Nikon, Eclipse, TS100). To quantify the proportions of spermatic area occupied by different spermatogonial cell types and hepatic area occupied by lipid vacuoles, the images were analyzed quantitatively by using the ImageJ freeware (National Institutes of Health, Bethesda, MD, USA, http://rsbweb.nih.gov/ij). The areas occupied by spermatids as well as spermatogonia and spermatocytes were expressed as the percentage of the selected total gonad area.

2.6. Total RNA isolation, quantitative real-time PCR (qPCR) and transcriptomic assays

A sample of 12 fish livers from each treatment (four fish per replicate) were randomly sampled for total RNA isolation, cDNA synthesis and quantitative real-time polymerase chain reaction (qPCR). The qPCR was performed as described in our previous study (Limbu et al., 2018). The detailed procedures are presented in the supplementary methods. Elongation factor 1 (*ef1α*) and *β-actin* were used as reference genes due to their stability expression in liver samples from different treatments. All primers sequences used for qPCR genes analysis are listed in supplementary Table S.2. The relative mRNA expression of target genes was calculated as the $2^{-\Delta\Delta Ct}$ method, thereof, $\Delta Ct = Ct\ target - (Ct\ ef1\alpha + Ct\ \beta\text{-}actin)/2$. The qPCR efficiency was between 99% and 102% and the correlation coefficient was above 0.98 for each gene.

A total of 15 hepatic RNAs from each treatment (five hepatic RNAs samples were pooled for each replicate) were prepared after exposure for the RNA-seq transcriptome library. The mRNA isolation, quality control measures of invalid reads and unigenes assembly for further gene analysis were performed as described previously by Lu et al. (2019). The paired-end RNA-seq sequencing library was sequenced by using the Illumina HiSeq 4000 (Illumina, Inc., San Diego, CA, USA; 2 × 150 bp read length) after quantification by using TBS380. The differentially expressed genes (DEGs) between two different samples for each estrogenic compound were calculated based on the fragments per kilobase of exon per million mapped reads (FRKM) method. Functional-enrichment analyses including Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were used for identifying the DEGs enriched in GO terms and signal pathways at Bonferroni-corrected *P*-value ≤ 0.05 compared with the whole transcriptome background. To confirm the accuracy of transcriptomic data, we chose the E2000 treatment as the representative for validation. The validation was performed by using qPCR and detailed results are shown in the Supplementary Fig. S1.

2.7. Statistical analyses

Results are presented as means ± standard error of means (SEM). One-way analysis of variance (ANOVA) was used to compare the differences in measured parameters among control, E200, E2000, B100 and B2000 treatments followed by Duncan's multiple range test. Moreover, two-way ANOVA was used to determine the interactive effects between estrogenic compounds and exposure levels. Significant differences were judged at probability levels of $P \leq 0.05$ for all analyzes. All statistical analyses were conducted by using SPSS 23 statistical software (IBM, Armonk, NY, USA).

3. Results

3.1. The exposure of zebrafish to E2 and BPA changed growth, impaired testis morphology, inhibited spermatid development and induced feminization tendency

The FBW of the fish exposed to E2 and BPA was significantly higher than control (Fig. 1A). The fish OCR, which represents the body metabolic rate, was notably higher in the BPA treatments compared with the control (Fig. 1B). The fish exposed to the low concentrations of estrogenic compounds increased the OCR compared to the high ones (Fig. 1B). The CF of the fish, which represents the ratio of body mass to body length, increased after exposure to E2 and BPA as compared to the control (Fig. 1C). The CF of fish exposed to B100 was significantly lower than fish exposed to B2000 (Fig. 1C). Likewise, the VSI was remarkably enhanced in the fish exposed to E2 and BPA than control (Fig. 1D). The estrogenic compounds and exposure levels interacted to affect CF (Table S.5).

The GSI and gonadal histopathological biopsies were used to diagnose gonad development and detect gonadal damage caused by the estrogenic compounds. The GSI for zebrafish exposed to E2 and BPA was markedly lower than control (Fig. 2A). At the same time, testis biopsies for the fish exposed to E2 and BPA showed smaller and fewer lumps of spermatids than control (Fig. 2B–G). In contrast, the regions of spermatogonia and spermatocytes were macroscopically larger in fish exposed to E2 and BPA than control (Fig. 2H). Moreover, the mRNA expression of several genes such as estrogen receptor 1 (*esr1*; Fig. 2I), vitellogenin 1 (*vtg1*; Fig. 2J) and vitellogenin 2 (*vtg2*; Fig. 2K) were dramatically upregulated in fish exposed to high concentrations of E2 and BPA than control. The sperm-associated antigen 1 (*spag1*; Fig. 2L) and cytochrome P450 1B1 (*cyp1b1*; Fig. 2M) genes, which are involved in blocking fertilization and xenobiotic metabolism, respectively, were upregulated in the fish exposed to BPA than control. Additionally, the mRNA expression of *cyp1c* gene, which is involved in xenobiotic metabolism, increased in fish after exposure to E2 and BPA than control (Fig. 2N). Moreover, the estrogenic compounds and exposure levels interacted to affect spermatid contents and the expression of *vtg1*, *vtg2* and *cyp1b1* genes (Table S.5). These results highlight that exposure to E2 and BPA increased growth performance, impaired testis morphology, inhibited spermatid development and induced feminization tendency in male zebrafish.

3.2. The exposure of zebrafish to E2 and BPA altered functional pathways

Transcriptomic analysis is a suitable method to determine the systemic effects of estrogenic compounds in fish functional pathways. In the present study, the proportions of the main functional pathways in the liver differed significantly among the fish exposed to estrogenic compounds and control (Fig. 3). Among the changed pathways, fish exposed to the low concentrations of E2 and BPA had higher proportions of lipid metabolism pathways than the high concentrations (23% vs 9% in the E2 treatments, and 33% vs 8% in the BPA treatments) (Fig. 3A). However, exposure of fish to the high concentrations of E2 and BPA increased the pathway proportions related to immune system and infectious diseases than those in the low concentrations (29% + 19% vs 6% + 6% in the E2 treatments, and 26% + 11% vs none in the BPA treatments) (Fig. 3A). We then selected the significantly changed genes involved in lipid metabolism and immunity pathways for comparison (Fig. 3B and C). The expression of the genes related to lipid synthesis, such as fatty acid synthase (*fas*), sterol regulatory element binding transcription factor 1 (*srebp-1c*), diacylglycerol O-acyltransferase 2 (*dgat2*), acetyl-CoA carboxylase (*acc*) and peroxisome proliferator-activated receptor gamma (*ppar*γ), were upregulated in fish exposed to the low concentrations compared to the high concentrations, especially in the E2 treatment (Fig. 3B). Nevertheless, exposure of fish to the high concentrations of E2 and BPA upregulated the genes related to lipid transport, including microsomal triglyceride transfer protein (*mtp*), long-chain fatty acid transport protein (*fatp*), apolipoprotein E (*apoE*), cluster of differentiation 36 (*cd36*) and apolipoprotein A

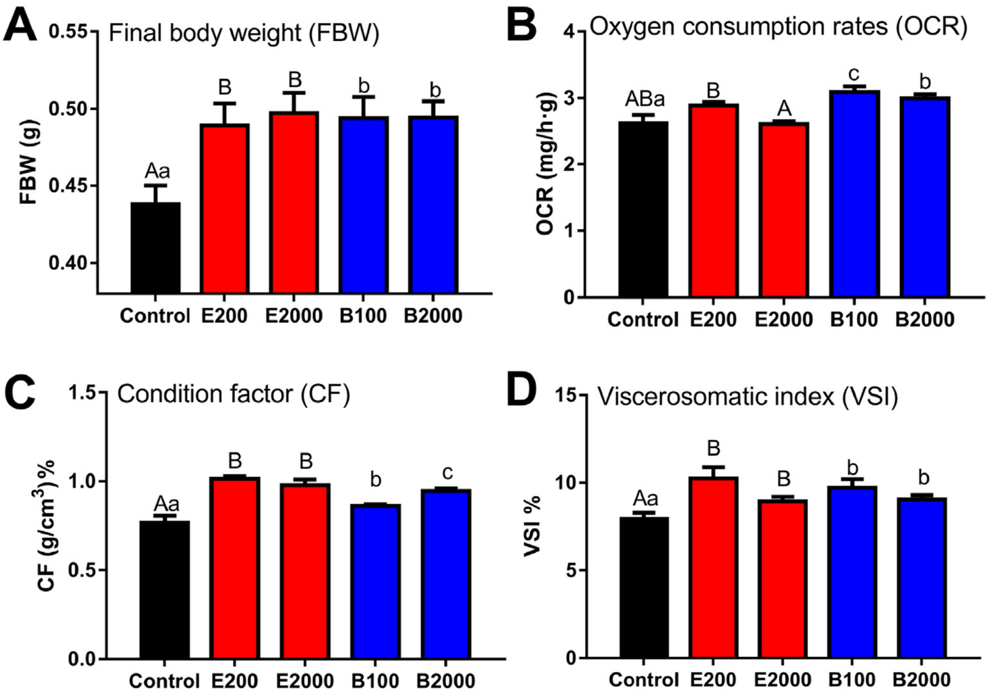

Fig. 1. The growth parameters of zebrafish in response to E2 and BPA exposure.

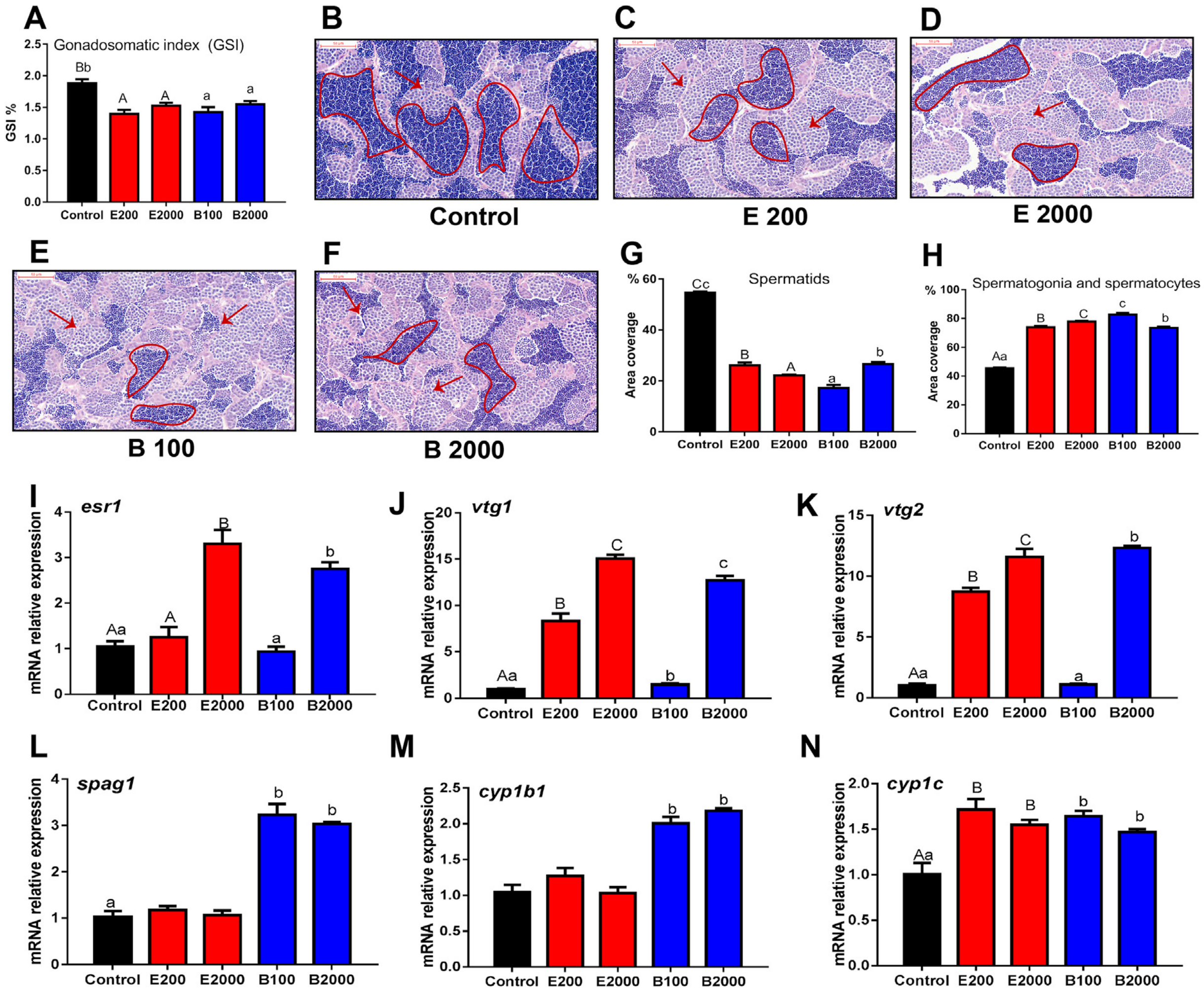

Fig. 2. The effects of E2 and BPA on male zebrafish testis development.

(*apoA*) compared to the low concentrations (Fig. 3B).

Concurrently, exposure of fish to the high concentrations of E2 and BPA upregulated the pro-inflammatory cytokines, including nuclear factor kappa-light-chain-enhancer of activated B cells (*nf-kb*), interleukin-17 receptor (*il-17r*), chemokine ligand 20 (*ccl20*) and interleukin-1 beta (*il-1β*) than the low concentrations (Fig. 3C). Similarly, glutathione S-transferase (*gst*) and glutathione peroxidase (*gpx*), which are indicators of oxidative stress, were also elevated in the fish exposed to high concentrations of E2 and BPA than the low concentrations (Fig. 3C). Moreover, fish exposed to high concentrations of E2 and BPA showed higher expression of growth arrest and DNA-damage-inducible beta (*gadd45β*) gene, which is associated with DNA damage in tissues (Fig. 3C). These findings suggest that the estrogenic compounds altered functional pathways in the male zebrafish in which, the low concentrations of E2 and BPA exposure mainly disturbed lipid metabolism pathways, while high concentrations exposure triggered inflammatory response and oxidative stress.

3.3. The exposure of zebrafish to E2 and BPA changed lipid deposition

Liver plays a crucial role in lipid metabolism, thus the lipid content, lipid distribution and mRNA expression of genes were performed in fish liver to investigate the effects on lipid deposition after exposure to E2 and BPA. Total fat content was notably higher in zebrafish exposed to E2 and BPA compared to control (Fig. 4A). Similarly, the TG content was significantly elevated in the liver (Fig. 4B) and viscera (Fig. 4C) of the zebrafish exposed to E2 and BPA than control. Moreover, liver TG content was higher in fish exposed to the low concentrations of E200 and B100 than the high concentrations (Fig. 4B). The histological analysis indicated that, the quantity and volume of vacuoles in hepatocytes of the zebrafish exposed to E2 and BPA were more numerous and larger than those in the control (Fig. 4G). Moreover, severe fat accumulation was observed in the fish exposed to the low concentrations of E200 and B100 than the high concentrations (Fig. 4G), consistent with the results on TG content in the liver. Furthermore, FFA content in the liver was significantly enhanced in all fish exposed to E2 and BPA than control (Fig. 4E). However, the TG content in zebrafish muscle was comparable among all groups (Fig. 4D). Conversely, the T-CHO content in zebrafish liver exposed to BPA was lower than control (Fig. 4F).

The mRNA expression of genes related to lipid synthesis, catabolism and lipid transport were further measured in the liver. The expression of lipid catabolism-related enzymes such as carnitine palmitoyl transferase 1a (*cpt1a*), hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit beta (*had*) and peroxisome proliferator-activated receptor α (*pparα*) were generally comparable among all treatments, except *cpt1a*, which was

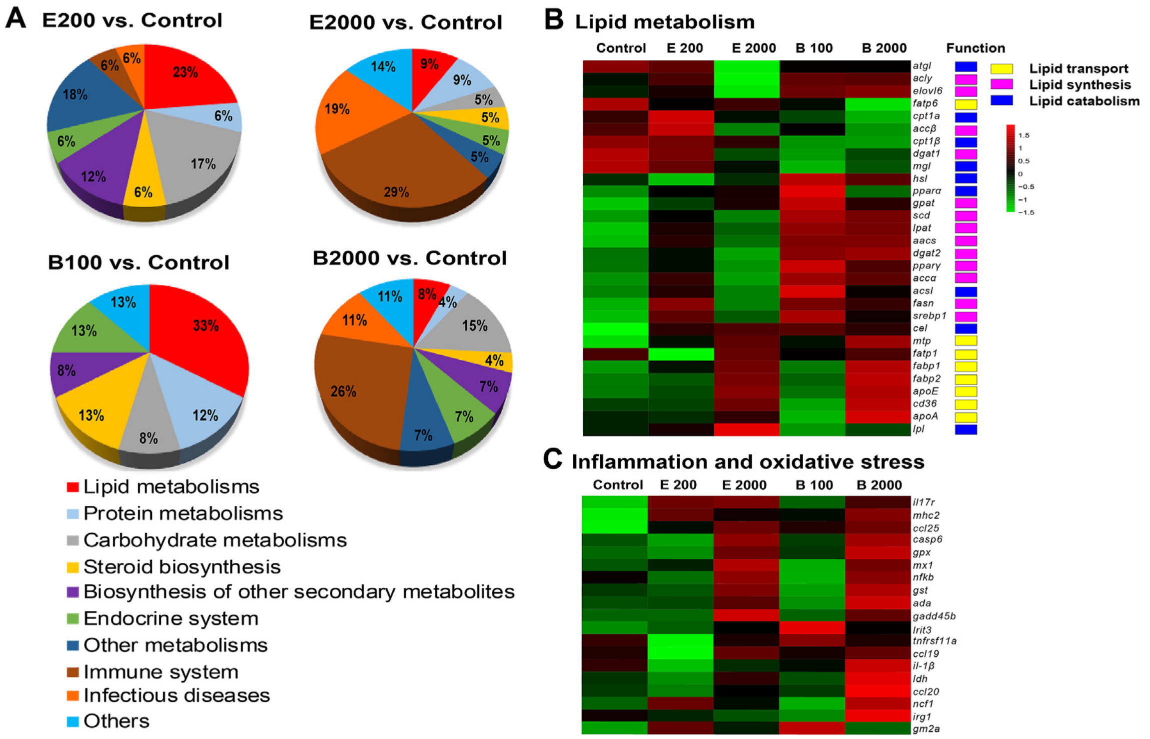

Fig. 3. The altered signaling pathways and gene heatmaps as response to E2 and BPA exposure in fish liver via transcriptomic analysis.

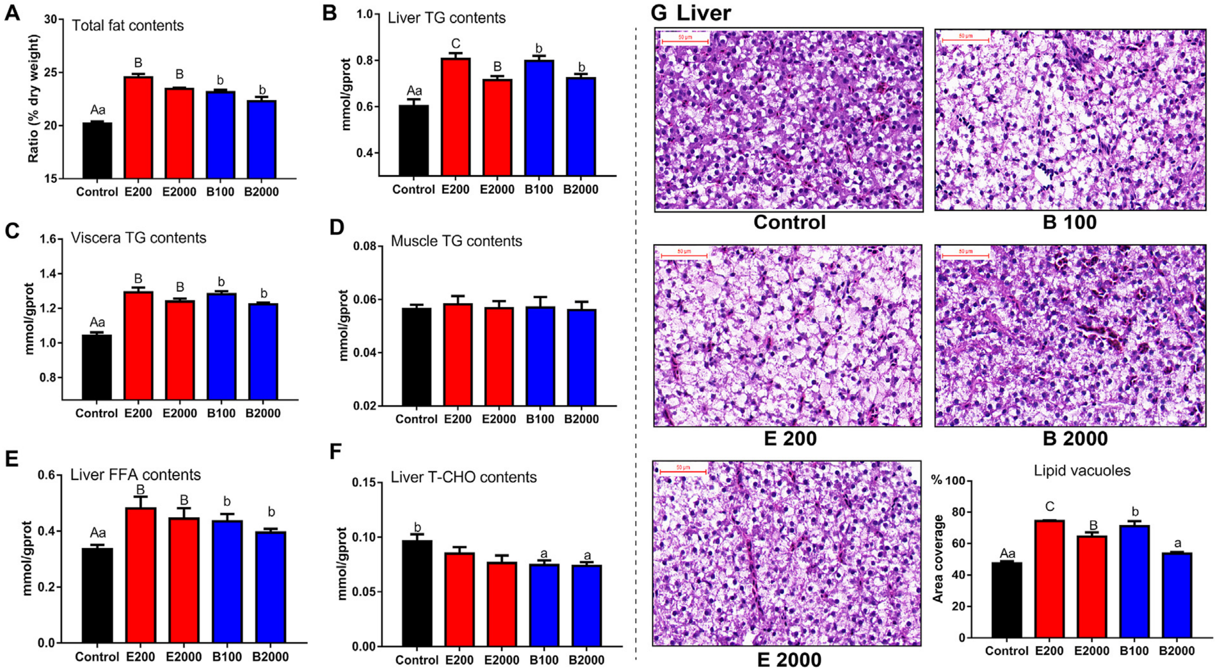

Fig. 4. Fish lipid profiles and contents are presented as response to E2 and BPA exposure.

higher in fish exposed to E200 than E2000 (Fig. 5A). The estrogenic compounds and exposure levels interacted to affect the expression of *cpt1a* and *had* genes (Table S.5). The mRNA expression of lipogenesis genes such as *fas* and *dgat2*, especially *srebp-1c* were significantly upregulated after exposure of zebrafish to the low concentrations of E2 and BPA, compared to high concentrations and control (Fig. 5B). Moreover, the mRNA expression of genes related to lipid transport such as *fatp1*, *cd36* and fatty-acid-binding protein 1a (*fabp1a*) were enhanced after exposure of zebrafish to the high concentrations compared to the low concentrations and control (Fig. 5C), consistent with transcriptomic data (Fig. 3B). In general, these data reveal that E2 and BPA induced fish lipid deposition and

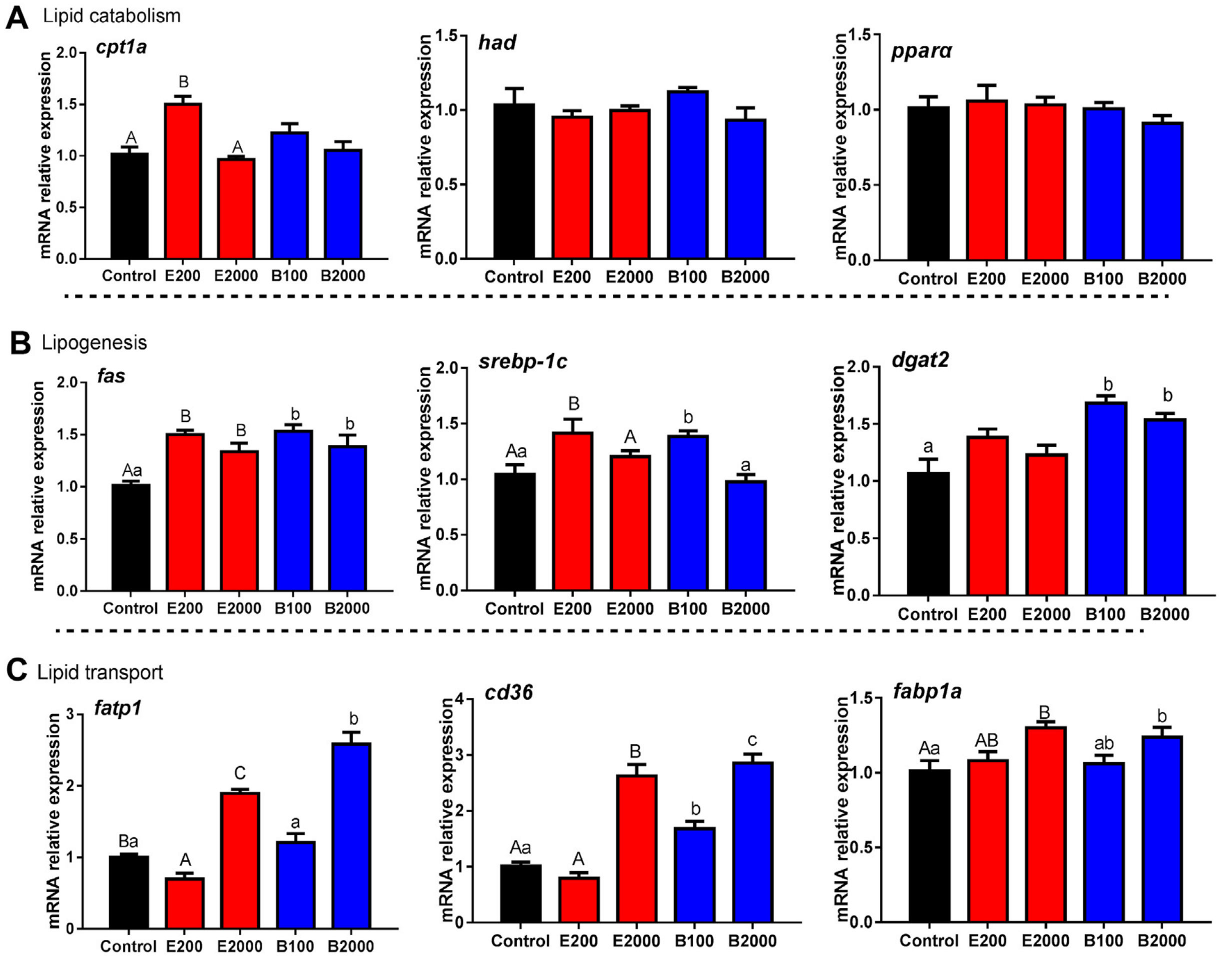

Fig. 5. Effects of E2 and BPA on liver mRNA expressions related to the lipid metabolism.

altered lipid profiles by increasing TG and FFA contents, but decreased T-CHO content. Furthermore, the exposure of fish to the low concentrations of E2 and BPA enhanced lipogenesis, while the high concentrations elevated lipid transport.

3.4. *The exposure of zebrafish to E2 and BPA affected inflammatory response and oxidative stress*

The results of transcriptomic assay showed induced pro-inflammatory response, immunity and oxidative stress in the liver. Therefore, the enzyme activities and mRNA expression of genes related to inflammation and oxidative stress were measured in the zebrafish liver. The ACP and AKP activities were significantly higher in fish exposed to high concentrations of E2 and BPA than in the low concentrations and the control (Fig. 6A and B). In addition, the T-SOD activity was also elevated in fish exposed to E2000 and B2000 compared to the control and the low concentration treatments (Fig. 6C), similar to the T-AOC (Fig. 6D) and CAT (Fig. 6E) activities. However, BPA treatments elevated malondialdehyde (MDA) concentration than control and the E2 treatments (Fig. 6F).

The gene assays in the liver indicated higher expression of immunity related genes such as lysozyme (*lyz*), *acp*, *akp* and Toll-like receptor (*tlr*) in fish exposed to high concentrations of E2 and BPA than those in the low concentrations and control (Fig. 7). Similarly, antioxidant genes such as *sod* and glutathione peroxidase (*gpx*) and inflammatory genes including interleukin 1 (*il-1*), interleukin 6 (*il-6*) and *nf-κb* were also upregulated in zebrafish exposed to high concentrations of E2 and BPA than those in the low concentrations and control of zebrafish. The estrogenic compounds and exposure levels interacted to affect *acp* gene expression (Table S.5). These results confirm that the high concentrations of E2 and BPA induced inflammation and antioxidant capacity in zebrafish, a response which might help fish to resist oxidative stress. Moreover, BPA as the synthetic estrogen, exhibited greater toxicity in lipid peroxidation via dramatically elevating MDA concentration, than the natural estrogen E2.

4. Discussion

4.1. *The exposure of zebrafish to E2 and BPA disturbed growth and feminized male fish*

We determined the growth parameters and biopsied the testis of zebrafish in order to investigate the influences of exposure to E2 and BPA on general body function. Exposure of zebrafish to both E2 and BPA notably increased the body weight, VSI and CF of the fish. Increased body growth might be caused by fat deposition in viscera, similar to results obtained in juvenile Japanese eels fed on diets supplemented with 25 and 50 mg/kg of E2 (Hiroaki et al., 1993). Nevertheless, exposure to estrogenic compounds have been reported to reduce growth in fish such as medaka embryos exposed to 200 μg/L BPA for 24 h (Ramakrishnan and Wayne, 2008).

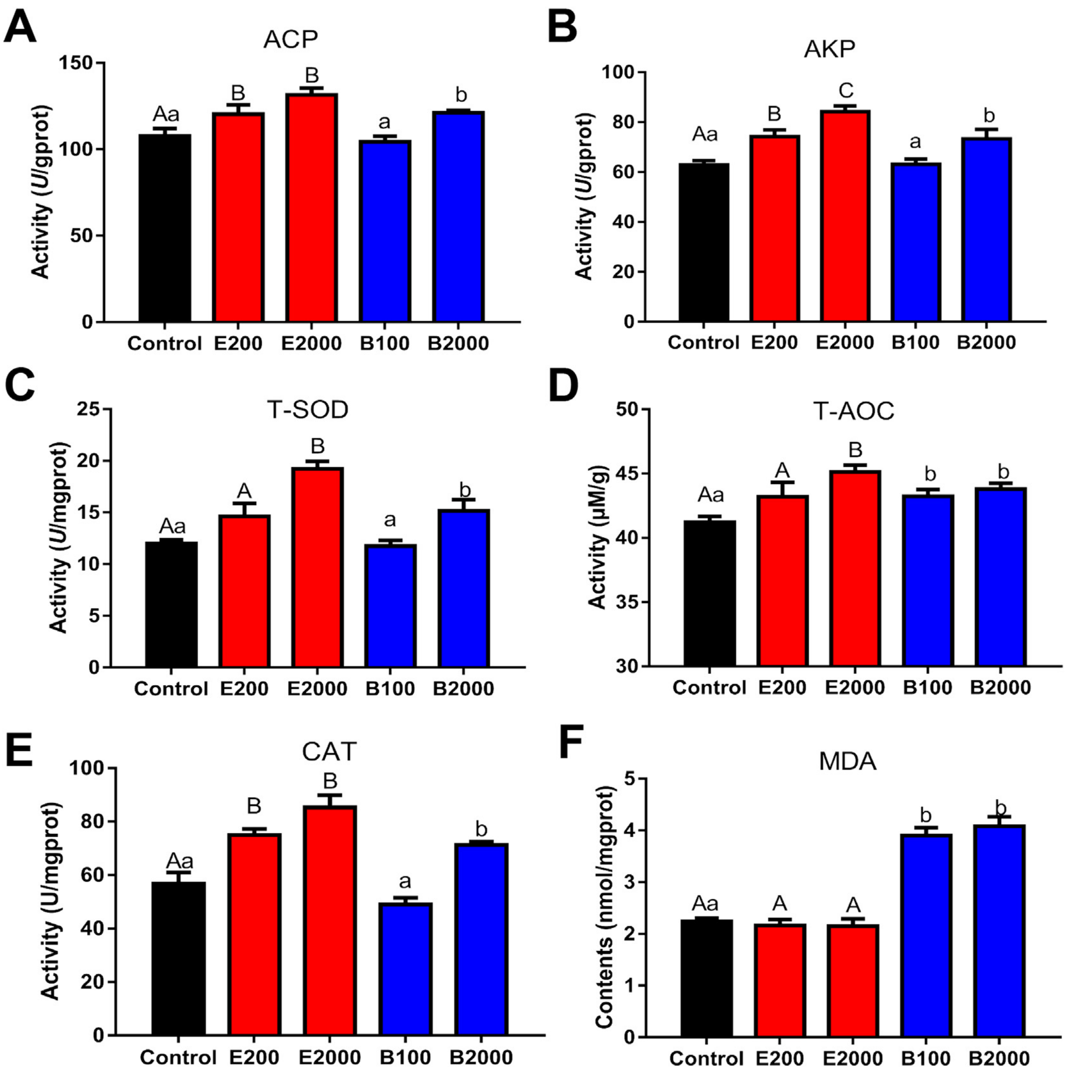

Fig. 6. The antioxidant and immune defense in liver of zebrafish exposed to E2 and BPA.

Similarly, fathead minnow (*Pimephales promelas*) exposed to EE2 at high concentrations (64 and 16 ng/L) for 305 days had inhibited growth and reduced hatching rate compared to low concentrations (0.2, 1 and 4 ng/L) (Länge et al., 2010). These inconsistent results may be caused by variations in toxicity of estrogenic compounds, exposure concentrations and periods, and fish species and their developmental stages.

We relate the increased growth rate, CF and VSI in fish exposed to estrogenic compounds to the upregulation of lipogenesis. This is because, all the fish exposed to estrogenic compounds had higher growth rate, CF and VSI, and at the same time, they had higher body fat, liver and visceral TG contents, especially in the E200 and B100 treatments. In fact, several studies have demonstrated that, the body weight and CF are positively correlated to the mRNA upregulation of genes related to lipogenesis such as *srebp* and *fas* (Loftus et al., 2000; Huang et al., 2010). Conversely, body weight and CF are inversely correlated to mRNA upregulation genes related to lipid catabolism such as *pparα* and *cpt1* (Harrington et al., 2007; Adeogun et al., 2016). In the present study, we only found increased expression of *cpt1* gene in zebrafish exposed to E200, but the mRNA expression of *pparα* gene was statistically similar among all groups (Fig. 5). However, we found significantly increased mRNA expression of *fas* gene in all zebrafish exposed to estrogenic compounds and *srebp-1c* gene in fish exposed to E200 and B100. Therefore, it is reasonable to suggest that, the higher growth performance in zebrafish exposed to estrogenic compounds resulted from the upregulation of lipogenesis.

In the present study, E2 and BPA significantly altered zebrafish testis size and sperm amount, consistent to altered mating behaviors, induced gonad deformation and delayed sexual maturation by environmental estrogens in male fish obtained from previous studies (Scholz and Klüver, 2009; Rose et al., 2013). These results suggest that estrogenic compounds damage testicular structure and sperm development. The expression of genes related to estrogen metabolism, including *vtgs* and *esr*, were upregulated in fish exposed to the high concentrations of E2 and BPA. The VTG is the precursor of egg yolk protein, providing nutrition for embryonic development and growth; thus its induction in male fish has been regarded as an estrogenic effect of environmental pollutants (Matozzo et al., 2008). In the present study, the observed upregulation of *vtgs* and *esr* suggest that, the estrogenic compounds feminized the male zebrafish, especially after exposure to the high concentrations. Indeed, in the natural environment, wild fish live longer in the water containing a variety of environmental estrogens

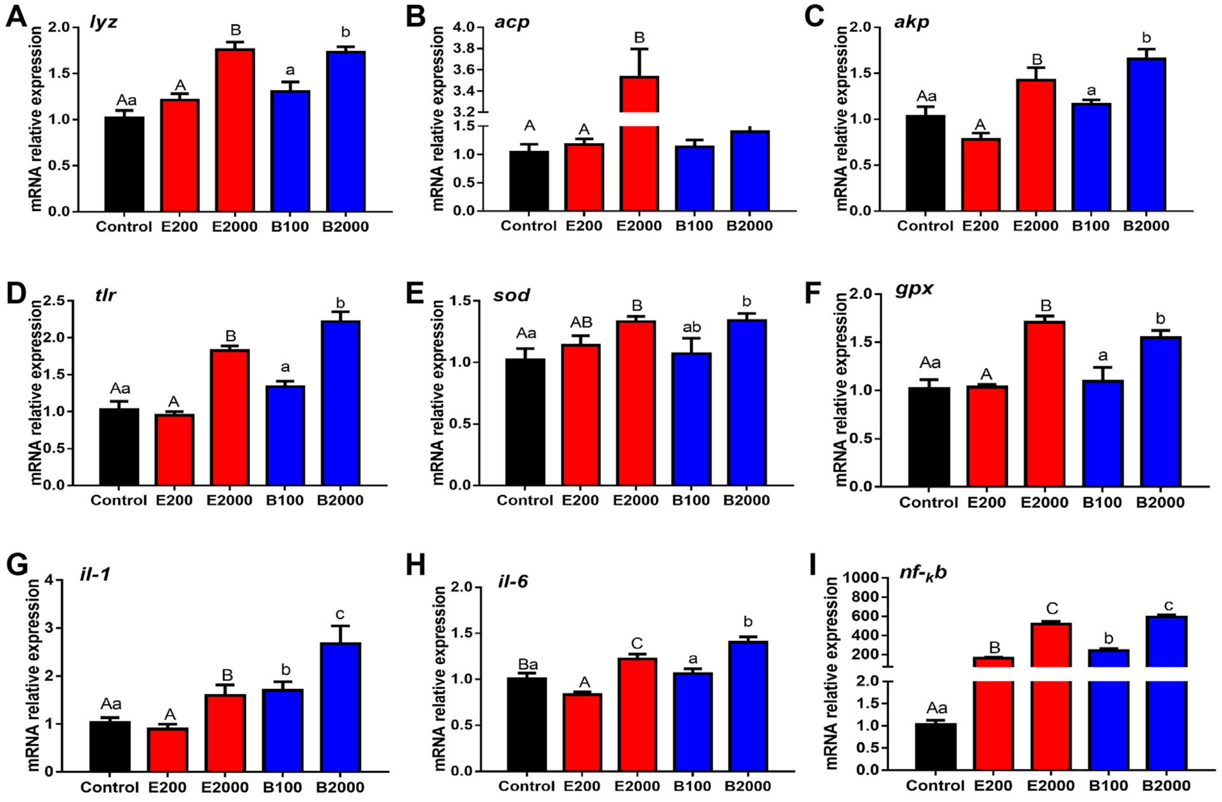

Fig. 7. Effects of E2 and BPA on liver mRNA expressions of immune defense (A–C), antioxidant response (D–F) and inflammation (G–I).

than the period tested in the present study. Therefore, considerable feminization might be occurring more often among wild fish as has been reported by several studies (Sumpter, 1995; Yan et al., 2012). In fact, male flounder (*Platichthys flesus*) and viviparous blenny (*Zoarces viviparus*) showed ovotestis and the sex ratio of juvenile fish was biased towards females in UK coastal waters (Matthiessen et al., 2002). Similarly, Kang et al. (2002) reported that medaka (*Oryzias latipes*) exposed to E2 at a concentration of 463 ng/L for 21 days, displayed a dramatic decrease in GSI and lower fecundity compared to relatively lower concentration of 29.3 ng/L. Moreover, the authors also stated that, male fish exposed to high concentration induced productions of *vtg* in the liver and ova in the testis, compared to lower concentration (Kang et al., 2002). These findings highlight that relatively high concentrations of estrogenic compounds might trigger feminization in male fish. Of note, the estrogenic compounds and exposure levels interacted to induce male fish feminization (Table S5). This result indicates that fish feminization is more sensitive to environmental estrogenic compounds and their concentrations than other physiological processes. Altogether, these findings show that, environmental estrogenic compounds indeed have the potential to disturb fish growth, damage gonadal development and functions. More importantly, concentration-dependent effects of environmental estrogenic compounds induce male fish feminization.

4.2. Concentration-dependent effects of E2 and BPA on lipid deposition

In the present study, we found that E2 and BPA dramatically altered lipid accumulation and metabolic pathways in male zebrafish (Figs. 3, 4A and 5). Moreover, the expression of genes related to lipid synthesis were upregulated in fish exposed to E2 and BPA (Figs. 3B and 5B). These results are similar to increased lipid deposition in fish exposed to environmental estrogens such as increased TG content in gilthead sea bream after BPA exposure (Forner-Piquer et al., 2018) and increased the hepatic lipid content in rainbow trout following E2 exposure (Cakmak et al., 2006). However, the results obtained in fish are contrary to those obtained in mammals. For instance, E2 exposure exerted anti-obesity effects such a decrease in lipid deposition in mice liver fed on a high-fat diet or after ovariectomy (Bryzgalova et al., 2008; Zhu et al., 2013). Furthermore, E2 also indicated anti-obesity effects mediated through estrogen receptor (ESR) by suppressing or activating the expression of genes related to lipid synthesis or catabolism, such as *ppar*γ and *ppar*α, resulting in reduced body fat (Wang and Kigore, 2002; Yepuru et al., 2010). We propose future studies to compare the different mechanisms of estrogenic compounds on lipid metabolism in mammals and fish.

In the present study, the low concentrations of E2 and BPA elevated the lipid deposition and metabolism pathways by increasing lipid synthesis (Figs. 3–5). These results are due to the different mechanisms of estrogen actions. The BPA acts as the typical estrogen by mimicking and binding to ESR, similar to E2 (Hiroi et al., 1999). In general, the classic mechanism of estrogen action is that, two complexes of E2-ESR constitute a dimer, which then binds to the estrogen response elements (EREs) located in target genes thereby regulating physiological processes (Nilsson et al., 2001). However, evidence of ERE independent genomic action has also been reported, whereby E2-ESR acts as a single complex and then activates the target genes without directly binding to EREs (Katzenellenbogen et al., 2000; Björnström and Sjöberg, 2005). Therefore, it is possible that the concentration-dependent effects of estrogenic compounds are due to different mechanisms of estrogen actions.

Interestingly, exposure of zebrafish to the high concentrations of E2 and BPA led to remarkable upregulation of genes regulating lipid transport, including *mtp*, *fatp1*, *apoE*, *apoA* and *cd36* (Fig. 3B). Simultaneously, the expression of genes related to pro-inflammation also increased in zebrafish exposed to the high concentrations (Fig. 3C). These results signify modulation of inflammation after exposure to high concentrations of E2 and BPA. Several studies have shown that, acute inflammation response is manifested by an increase in transport proteins and apolipoproteins, such as high density lipoproteins (HDLs) associated with the proteins APOE, APOB, APOA-IV and APOA-V (Khovidhunkit et al., 2004; David et al., 2010). Moreover, APOD action not only distributes lipids, but also protects the animal from oxidative stress and inflammation in the brain (David et al., 2010). Furthermore, the inflammation-induced proteins serum amyloid A (SAA) and APOJ are also apolipoproteins, which not only transport lipids, but also help to modulate inflammation (Jordan-Starck et al., 1994; Beer et al., 1995). Consequently, high concentrations of estrogenic compounds increased lipid transport such as apolipoproteins, which participated in inflammation process. Together, these findings suggest that environmental estrogenic compounds increase lipid deposition and alter lipid profiles, and the low concentrations of estrogenic compounds notably elevate lipid synthesis and deposition.

4.3. Concentration-dependent effects of E2 and BPA on inflammation and antioxidant response

Inflammation is a protective response, which modulates the activities of inflammatory mediators (Martins et al., 2016). However, overexpression of inflammatory cytokines have been linked to tissue damage and chronic inflammation induces diabetes, cancer and cardiovascular diseases (Haffner, 2006; Benneriah and Karin, 2011). In the present study, pro-inflammation was significantly observed in the E2 and BPA in fish exposed to high concentrations compared to low concentrations (Figs. 3C and Fig. 7G–I), similar to results obtained by Jin et al. (2010) and Xu et al. (2013) in zebrafish exposed to relatively high concentrations of E2 and BPA. Furthermore, E2 and BPA dramatically elevated *nf-kb* expression (Fig. 7I). The *nf-kb* is the main regulator of various pro-inflammatory genes and sustained activation lead to chronic inflammation and oxidative stress (Steffan et al., 2006). Evidently, the activities and expression of genes involved in antioxidant enzymes were also stimulated by E2 and BPA (Figs. 6 and Fig. 7A–F), similar to increased SOD, GST and CAT activities in adult fish after exposure to BPA (Wu et al., 2011; Kaya and Kaptaner, 2016). Conversely, several studies reported that BPA exposed to zebrafish embryos for 168 h post-fertilization inhibited antioxidant parameters (Minghong et al., 2011) and E2 plays a protective role by inhibiting pro-inflammatory cytokines (Ghisletti et al., 2005; Pelekanou et al., 2016). The dysregulation of immune defenses stimulated by estrogenic compounds lead to either immune attenuation or immune hyper-reactivity, inducing immunodeficiency or autoimmune disease, respectively (Kuo et al., 2012). Therefore, the available data on the immunomodulatory effects of estrogenic compounds are still confusing and inconsistent. Such results are attributed to multiple factors, such as variations in exposure periods, estrogen concentrations and expression levels of *esr* (Straub, 2007).

We attribute the differences in immune response in the present study to ESR mechanism on estrogen actions (Rogers et al., 2013). Previous studies have indicated that, both ESRα and ESRβ are distributed in various tissues among fish tissues (Kovats, 2015) including reproductive tissues, T cells, monocytes and macrophages (Mao et al., 2005; Suzuki et al., 2007; Gulshan et al., 2010). Therefore, it is possible that the exposure to low and high concentrations of estrogenic compounds caused different distribution of E2 and BPA among fish tissues, thus induced distinct immune responses, an observation which requires further studies. The results of the present study indicate that the high concentrations of environmental estrogenic compounds damage fish tissue and induce inflammation through the NF-kB pathway, which further stimulates antioxidant capacity as a response. Furthermore, the concentration-dependent effects of environmental estrogenic compounds are not only based on the mechanisms of estrogen action, but also are likely to be caused by the distributional differences in ESRs in the immune response tissues and reproductive cells.

4.4. The differences in tissue injury and metabolism between natural and synthetic estrogens on zebrafish

Although natural and synthetic estrogens belong to environmental estrogenic compounds, they have diverse effects on animals owing to their different origins and properties (Lange et al., 2012). In the present study, exposure of zebrafish to BPA, markedly upregulated the expression of *spag1* and *cyp1b1* genes compared to E2 (Fig. 2L, M). This suggests that synthetic estrogens may cause serious damage to fish gonad development than natural ones, similar to findings reported by Piferrer and Donaldson (1992) who indicated that natural and synthetic estrogens influence differently gonad and sex differentiation in fish. These variations are related to the differences in the ability of natural and several synthetic estrogens to activate the ESRs transcriptions (Caroline et al., 2014). Indeed, BPA exhibited both agonist and antagonist activity for ESRα *in vitro* and acted as an agonist for ESRβ with a lower affinity than E2 (Kuiper et al., 1998; Hiroi et al., 1999). Moreover, BPA can also bind to other hormone receptors, such as thyroid hormone receptor (Zoeller et al., 2005). Therefore, exposure of fish to natural and synthetic estrogens caused diverse gonadal development and damages due to their differences in capacity to activate ESRs and other receptors.

The exposure of zebrafish to BPA increased dramatically the MDA concentration in liver compared to E2, indicating that BPA induces severe lipid peroxidation and oxidative stress in fish (Fig. 6F). These results might be attributed to short half-lives of natural estrogens and their readily elimination rate compared to synthetic ones (Yin et al., 2003). Natural estrogens such as E2 has been reported to degrade quickly in aquifer materials with a half-life of 2 days (Yin et al., 2003). However, the half-life for BPA degradation ranged from 2 to 3 days in river water under aerobic conditions, but the degradation may be extended under anaerobic conditions (Kang and Kondo, 2002). Furthermore, Ying et al. (2003) reported the half-life of synthetic estrogens such as EE2 was around 81 days in aquifer materials. Moreover, synthetic estrogens easily accumulate and are hardly eliminated in fish tissues (Ferreiraleach and Hill, 2001). Therefore, the properties of BPA as a synthetic estrogen lead to the observed severe disruptions of gonad development and tissue oxidative stress in the male zebrafish. Exposure to BPA increased the OCR of the zebrafish than E2, suggesting that the synthetic estrogen caused higher metabolic rates (Fig. 1B). An elevated OCR possibly helped to speed the rate of degradation and detoxification response of the xenobiotic. These findings show that synthetic estrogens can cause diverse toxicological and oxidative effects in fish tissues, which stimulate metabolic rates compared to natural ones.

5. Conclusion

In conclusion, this study demonstrated that the two environmental estrogenic compounds used increased body growth, but damaged gonadal structure and disrupted the general health of male zebrafish. Moreover, both estrogenic compounds also altered the fish lipid profiles and induced lipid deposition by elevating lipid synthesis, particularly after exposure to the low concentrations. Nevertheless, the high concentrations of E2 and BPA induced inflammation response and antioxidant capacity of the fish. Overall, for the first time, this study highlights that the comprehensive effects of estrogenic compounds on fish lipid deposition, inflammation and the antioxidant response are influenced differently by exposure concentrations. Our work provides a foundation for evaluating the toxicological effects of environmental estrogenic compounds in aquatic ecology and aquaculture.

Author contributions

Sheng-Xiang Sun and Zhen-Yu Du conceived and designed this study; Sheng-Xiang Sun, Yun-Ni Zhang, Dong-Liang Lu and Wei-Li Wang performed experiments; Sheng-Xiang Sun and Zhen-Yu Du analyzed the data and wrote the manuscript; Li-Qiao Chen, Mei-Ling Zhang and Samwel Mchele Limbu read and corrected the manuscript. All authors read and approved the final manuscript for submission.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2019.124422.

Final body weight (A), oxygen consumption rates (B), condition factor (C), and viscerosomatic index (D) are presented. The values are presented as mean ± standard error of mean (SEM). Significant differences were determined by one-way ANOVA ($P \leq 0.05$). Capital letters (A-C) above columns mean significantly different among treatments of the control, E200 and E2000. Lowercase letters (a-c) above columns mean significantly different among treatments of the control, B100 and B2000.

Testis weight (A), histopathological findings (B—F), quantification of the spermatic area covered by spermatids, spermatogonia and spermatocytes in percentage (G and H), and mRNA expressions related to feminization and gonad damage (I—N) are presented. Testis biopsies were stained by the hematoxylin-eosin (HE). Scopes inclosed by the red lines in the testis biopsies represents the spermatids, and the red arrows indicate the spermatogonia and spermatocytes. The values are presented as mean ± standard error of mean (SEM). Significant differences were determined by one-way ANOVA ($P \leq 0.05$). Capital letters (A-C) above columns mean significantly different among treatments of the control, E200 and E2000. Lowercase letters (a-c) above columns mean significantly different among treatments of the control, B100 and B2000. Scale bar represents 50 μm. Abbreviations: *esr1*, estrogen receptor 1; *vtg1*, vitellogenin 1; *spag1*, sperm-associated antigen 1; *cyp1b1*, cytochrome P450 1B1.

Altered pathways (A), heatmaps of lipid metabolism (B) and inflammation and oxidative stress (C) in liver from each treatment are presented. The percentages on the pie chart represent the proportion of each different pathway to total ones. Red and green colors in the heatmaps represent induced and repressed genes, respectively. Moreover, the purple, blue and yellow of function section in lipid metabolism represent the genes related to lipid synthesis, catabolism and transport, respectively. Detail data for heatmaps were specified in Tables S.3 and 4.

Fish total fat content (A), triglyceride (TG) content from liver, viscera and muscle (B-D), and liver free fatty acid (FFA) and total cholesterol (T-CHO) content (E and F) in each treatment are observed. Histological slides of liver and quantification of the hepatic area covered by lipid vacuoles in percentage (G). The histological slides were stained by HE to show the tissue structure and fat accumulation. The values are presented as mean ± standard error of mean (SEM). Significant differences were determined by one-way ANOVA ($P \leq 0.05$). Capital letters (A-C) above columns mean significantly different among treatments of the control, E200 and E2000. Lowercase letters (a-c) above columns mean significantly different among treatments of the control, B100 and B2000. Different letters signify significant difference among treatments. Scale bar represents 50 μm.

Liver mRNA expressions of lipid catabolism (A), lipogenesis (B) and lipid transport genes (C). The values are presented as mean ± standard error of mean (SEM). Significant differences were determined by one-way ANOVA ($P \leq 0.05$). Capital letters (A-C) above columns mean significantly different among treatments of the control, E200 and E2000. Lowercase letters (a-c) above columns mean significantly different among treatments of the control, B100 and B2000. Different letters signify significant difference among treatments. Abbreviations: *had*, Hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit beta.

Enzyme activity of acid phosphatase (ACP) and alkaline phosphatase (AKP) in response to E2 and BPA exposure (A and B). Antioxidant response enzyme activities of total superoxide dismutase (T-SOD), total antioxidation capacity (T-AOC), catalase (CAT), and malondialdehyde (MDA) concentrations are presented (C—F). The values are presented as mean ± standard error of mean (SEM). Significant differences were determined by one-way ANOVA ($P \leq 0.05$). Capital letters (A-C) above columns mean significantly different among treatments of the control, E200 and E2000. Lowercase letters (a-c) above columns mean significantly different among treatments of the control, B100 and B2000. Different letters signify significant difference among treatments.

The values are presented as mean ± standard error of mean (SEM). Significant differences were determined by one-way ANOVA ($P \leq 0.05$). Capital letters (A-C) above columns mean significantly different among treatments of the control, E200 and E2000. Lowercase letters (a-c) above columns mean significantly different among treatments of the control, B100 and B2000. Different letters signify significant difference among treatments. Abbreviations: *lyz*, lysozyme; *tlr*, toll-like receptor.

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