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Tetrabromobisphenol A caused neurodevelopmental toxicity via disrupting thyroid hormones in zebrafish larvae

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HIGHLIGHTS

- TBBPA altered the thyroid hormones and genes along HPT axis in zebrafish larvae.
- TBBPA induced neurodevelopmental toxicity in zebrafish larvae.
- One mechanism of neurodevelopmental toxicity of TBBPA was via affecting the THs.

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ABSTRACT

Tetrabromobisphenol A (TBBPA), one of the most widely used brominated flame retardants (BFRs), has resulted in worldwide environmental contamination. TBBPA has been reported as a thyroid endocrine disruptor and a potential neurotoxicant. However, the underlying mechanism is still not clear. In this study, zebrafish (*Danio rerio*) embryos (2 h post-fertilization, hpf) were exposed to different concentrations of TBBPA (50, 100, 200 and 400 μg/L) alone or in combination with 3,3′,5-triiodo-*l*-thyronine (T3, 20 μg/L + TBBPA, 200 μg/L). The results confirmed that TBBPA could evoke thyroid disruption by observations of increased T4 contents and decreased T3 contents, accompanied by up-regulated *tshβ*, *tg* mRNA and down-regulated *ttr* and *trβ* mRNA levels in zebafish larvae. TBBPA-induced neurodevelopmental toxicity was also indicated by down-regulated transcription of genes related to central nervous system (CNS) development (e.g., *α1-tubulin*, *mbp* and *shha*), and decreased locomotor activity and average swimming speed. Our results further demonstrated that treatment with T3 could reverse or eliminate TBBPA-induced effects on thyroidal and neurodevelopmental parameters. Given the above, we hypothesize that the observed neurodevelopmental toxicity in the present study could be attributed to the thyroid hormone disruptions by TBBPA.

1. Introduction

Tetrabromobisphenol A (2,2-bis (4-hydroxy-3,5-dibromophenyl) propane; TBBPA), primarily used as a reactive flame retardant in printed circuit boards, is among the highest-production-volume and current-use brominated flame retardants (BFRs) (Lévy-Bimbot et al., 2012). Since many BFRs (i.e. the penta- and octa-congeners of polybrominated diphenyl ethers, PBDEs) have been banned, TBBPA is considered as a substitute and have been registered under the regulation concerning the registration, evaluation, authorization and restriction of chemicals (REACH). So the global production of TBBPA will continue to increase (Liu et al., 2016). The extensive use of TBBPA has caused worldwide pollution. High levels of TBBPA has been reported in surface water (4870 ng/L), sediment (518 ng/g dry wright) and fish species (126.4 ng/g dry weight in kidney) from Chaohu Lake in China, where a high volume of printed wiring boards and electronics components are manufactured (Yang et al., 2012; Liu et al., 2016). Therefore, the potential risks of TBBPA to the ecological environment as well as human beings received extensive concerns.

Due to structural resemblance to thyroid hormone, TBBPA has been suggested to interfere with the thyroid hormonal system. For example, TPPBA suppressed T3-enhanced *Rana rugosa* tadpole tail shortening and resulted in significantly increased serum thyroxine (T4) content in *Platichthys flesus* (Kitamura et al., 2005; Kuiper et al., 2007b). A study using combined transcriptomic and proteomic approaches also depicted an interference of thyroid homeostasis by

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TBBPA (0.75 and 1.5 μM) in adult zebrafish (de Wit et al., 2008). Moreover, TBBPA exposure during the early developmental stages also caused neurodevelopmental deficits in zebrafish, including delayed cranial motor neuron development, inhibited primary motor neuron development and loosed muscle fiber (Chen et al., 2016). But the underlying mechanism is not well expounded. In vertebrates, thyroid hormones (THs) are believed to play an essential role in embryonic development. TBBPA has been suggested to be able to modify T3-mediated up-regulation of gene expression, many of which are related with neurodevelopment, in a neural cell line derive from mouse cerebellum (Guyot et al., 2014). Hence, it would be possible that the observed neurotoxicity of TBBPA were attributed, at least partly, to the alterations of THs levels.

For this purpose, we evaluated the thyroid disrupting potency and neurodevelopmental toxicity in zebrafish embryos/larvae after exposure to a series of concentrations of TBBPA, and aimed to reveal the role of TBBPA induced toxicity to the thyroid hormone system using combined treatments with T3. The results can further improve our understanding of the thyroid disruption and developmental toxicity of TBBPA, and provide more information for their environmental risk assessment.

2. Materials and methods

2.1. Chemicals

TBBPA (CAS: 79-94-7; purity > 97%) and T3 (3,3',5-triiodo-L-thyronine) were purchased from Sigma (St. Louis, MO, USA). Stock solutions of TBBPA (2 mg/mL) and T3 (1 mg/mL) were dissolved in dimethyl sulfoxide (DMSO), respectively, and then stored at −20 °C. TRIzol regent and SYBRGreen PCR kit were purchased from Invitrogen (Carlsbad, CA, USA) and Toyobo (Osaka, Japan), respectively. All other chemicals were of analytical grade.

2.2. Zebrafish maintenance and embryo exposure

Adult wild-type zebrafish (AB strain, ~5 months) were maintained and embryos were exposed at 28 ± 0.5 °C on a 14/10 h light-dark cycle as previously described by Zhu et al. (2014). Exposure experiments were consisted of two parts: (I) single exposure to TBBPA, and (II) co-exposure to TBBPA with T3. For the TBBPA single exposure experiment (part I), normally developed embryos at 2 h post-fertilization (hpf) were randomly distributed into glass beakers (approximately 500 embryos each) containing 600 mL exposure media. The embryos were exposed to different concentrations of TBBPA (50, 100, 200 and 400 μg/L, nominal concentrations). According to OECD guideline for fish embryo toxicity (FET) test, the embryos were immersed in the test solutions at the beginning of blastula stage (~2 hpf), and ended at 144 hpf, by which time they were free-swimming larvae and most organs were completely developed (Chan et al., 2009). The exposure concentrations of TBBPA were selected based on the results from previous study, where the lowest observed effect concentration on THs levels in European flounder (*Platichthys flesus*) was found to be 50 μg/L (Kuiper et al., 2007a), and also our results of preliminary experiment using zebrafish (data not shown). As reported in the Environmental Health Criteria 172 the half-life of TBBPA varies from 6.6 to 80.7 days in different seasons (WHO, 1995). Kuiper et al. (2007a) have verified the waterborne concentrations during the semi-static exposure cycles. The TBBPA levels in aquariums without fish decreased to 71% of the initial levels after 72 h, indicating that TBBPA was relatively stable in water. While those in aquariums with zebrafish dropped to 22 ± 6% of nominal dose after 72 h, indicating bioaccumulation and metabolism in zebrafish (Kuiper et al., 2007a). Therefore, in the present study, the exposure solutions were renewed every day by 50% to maintain the TBBPA levels. The preliminary experiment results also showed that larvae in solvent control group (0.01% DMSO, vol/vol) were not distinguished from those in water control group by any features determined in the present study. Therefore, solvent control group was used as control in the following experiments. For each control and TBBPA exposure groups, there were four replicates, and the final concentration of DMSO was 0.01% (vol/vol). The survival, malformation (eg., axial spinal curvature, pericardial edema and yolk-sac edema) and hatched larvae were recorded during the exposure period. At the end of exposure, 100 zebrafish larvae from one replicate were weighted at the same time, and the total weight was then converted to get the average body weight of each larva. The mean value of four replicates were considered as the average body weight of zebrafish larvae in each group. Then the larvae were sampled randomly for subsequent assays, including measurement of THs contents, genes transcription, acetylcholinesterase (AChE) activity, and behavior.

In part II, zebrafish embryos (2 hpf) were exposed to TBBPA (200 μg/L), T3 (20 μg/L), or TBBPA + T3 (200 μg/L + 20 μg/L) until 144 hpf to investigate whether TBBPA-induced developmental effects were related to the THs levels changes. Solvent control was also included. For each control and treated groups, there were four replicates, and the final concentration of DMSO was 0.01%. T3 was used for exogenous compensation for THs, and 20 μg/L was selected based on our previous studies where no significant effect on survival rate of zebrafish larvae was observed (Wang et al., 2013). For TBBPA, 200 μg/L was selected based on the results from part I where levels of thyroid hormone change dramatically. The exposure protocol and parameters determined were the same as part I.

All tests were conducted according to the guidelines for the care and use of laboratory animals of the National Institute for Food and Drug Control of China.

2.3. RNA isolation and quantitative real-time polymerase chain reaction

Approximately 30 normal zebrafish larvae from each beaker (n = 4 replicates) were randomly sampled for total RNA extraction using Trizol reagent. The quality and purity were examined by 1% agarose-formaldehyde gel electrophoresis with ethidiumbromide staining and 260/280 nm ratios, and concentrations were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). The reverse transcription was performed using the PrimeScript® RT reagent kit (Takara, Dalian, China), and then the relative expression of several genes involved in THs regulation and neurodevelopment were examined by real-time PCR. The selected genes included thyroid-stimulating hormone (*tshβ*), thyroglobulin (*tg*), transthyretin (*ttr*), thyroid hormone receptor (*trβ*), *α1-tubulin*, myelin basic protein (*mbp*), and sonic hedgehog a (*shha*). The selected genes' primer sequences were obtained using the online program Primer 3 (http://frodo.wi.mit.edu/; see Table S1). By using geNorm (http://medgen.ugent.be/genorm), the stability of several housekeeping genes including ribosomal protein l8 (*rpl8*), 18S ribosomal RNA (*18S rRNA*), beta actin (*β-actin*), elongation factor 1 alpha (*ef1a*), and glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) was analyzed. *β-actin* was the most stable gene for TBBPA, or T3, or TBBPA + T3 exposed zebrafish larvae, thus was selected as the reference gene for the transcriptional assay. Q-PCR was carried out on an ABI 7300 system (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) using the SYBR Green PCR kit (Toyobo, Osaka, Japan) and changes in target gene mRNA levels were analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.4. Thyroid hormone extraction and measurements

Whole-body THs extraction and measurement was conducted as previously described by Chen et al. (2012b). Briefly, approximately 200 zebrafish larvae (50 from each beaker) were collected as one sample (n = 3 replicates), and homogenized in ELISA buffer with an Ultra-Turrax T8 Basic Homogenizer (IKA, Staufen, Germany). Samples were intermittently sonicated on ice, centrifugated, then the supernatant was collected and stored at −80 °C for measurement of THs. Zebrafish larvae TH levels were measured using a commercial ELISA kit for fish (USCN Life Science Inc., Wuhan, China). Standard curves for both T4 and T3 ($R^2 > 0.99$) were established using serial dilutions of standard that were provided in the kits. The THs contents were calculated using corresponding standard curve. The intra-assay and inter-assay coefficients of variation were <10% and <12% for T3 and T4. The limit of detection (LOD) for T3 and T4 were 0.1 ng/mL and 1.2 ng/mL, respectively.

2.5. Acetylcholinesterase activity measurement

Approximately 100 normal zebrafish larvae (20–30 from each beaker) were collected as one sample (n = 3 replicates), and homogenized in tris-citrate buffer on ice and centrifuged as previously descried (Chen et al., 2012a). The supernatant was transferred, and the enzyme activity was measured with an AChE assay kit (Nanjing Keygene Biotech, Co, Ltd.). Prior to detection, a gradient dilution of sample were performed to find out the optimized detecting concentration. The results indicated high sensitivity of this kit, and a dilution ratio of 1:49 were employed for detecting AChE activity in the supernatants. Protein concentrations in the same batch of supernatant samples were measured by Bradford method, and were used to normalize enzyme activity. The optical density was recorded at 412 nm, and the AChE enzyme activity was expressed as nanomoles per minute per milligram protein.

2.6. Locomotor activity measurement

The locomotor activity was monitored using a Video-Track system (ViewPoint Life Sciences, Inc., Montreal, Canada) as previously described by Zhu et al. (2016). Briefly, zebrafish larvae were placed into 24-well plates (1 larva per well), and each plate contained all replicates for all control and treated groups. There were 12 plates for both part I and II of this experiment. After 10 min adaption, larval swimming behavior was monitored in reaction to 5 min dark-5 min light-5 min dark transitions. While the dark-light stimulation test reflects changes in general locomotor activity, it is a simple and suitable behavioral assay for juvenile zebrafish (Steenbergen et al., 2011). Frequency of movements, distance traveled and total duration of movements were collected every 30s. A total of 48 larvae per treatment (12 larvae per replicate and four replicates per treatment) were assessed. The data were analyzed using custom Open Office. Org 2.4 software (http://www.openoffice.org).

2.7. Statistical analysis

Data are expressed as the mean ± standard error (SEM). All data were verified for normality and homogeneity of variance using the Kolmogorov–Smirnov test and Levene's test. Differences between the control group and each exposure group were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison using SPSS v16.0 (SPSS, Chicago, IL, USA). $p < .05$ was considered to be statistically significant. The summary of statistics was given in Table S2 and S3.

3. Results

3.1. Developmental toxicity

The developmental parameters are shown in Table S4. For all control and treated groups, the survival rates were over 80%, and the hatching rates were over 90%. The hatching rates, body length and weight were not significantly changed in any group treated with TBBPA, T3 or TBBPA+T3 compared with the control. At 144 hpf, the survival rates were significantly decreased in 400 μg/L TBBPA treated group, while the malformation rates were significantly increased in 200 and 400 μg/L TBBPA treated groups compared with the control. In addition, the survival and malformation rates of zebrafish larvae co-treated with 200 μg/L TBBPA with 20 μg/L T3 were not significantly changed when compared with those treated with 200 μg/L TBBPA alone (Table S4).

3.2. Whole-body content of THs in zebrafish larvae

At 144 hpf, TBBPA exposure caused a dose-dependent increase in whole-body content of T4 and showed significant differences in 200 and 400 μg/L groups when compared to the control (by 90% and 115%, $p < .05$, Fig. 1A). However, exposure to T3 (20 μg/L) significantly decreased T4 contents by 72.9% ($p < .05$; Fig. 1a). Exposure to TBBPA+T3 (200 μg/L + 20 μg/L) also decreased T4 contents. Although this T4 level were not significantly different from the control group ($p > .05$), it was significantly different from the 200 μg/L TBBPA treated group ($p < .05$; Fig. 1a).

In contrast, whole-body contents of T3 in TBBPA treated zebrafish larvae showed dose-dependent decreases with significant differences in the 200 and 400 μg/L TBBPA treated groups when compared to the control (by 34.5% and 47.4%, $p < .05$, Fig. 1B). However, exposure to T3 significantly increased T3 contents by 248% ($p < .05$; Fig. 1b), and exposure to TBBPA + T3 also increased T3 contents, and this T3 level were significantly different from the control group as well as 200 μg/L TBBPA treated group ($p < .05$; Fig. 1b).

3.3. Acetylcholinesterase activity

In zebrafish larvae, exposure to TBBPA caused a dose dependent increase in AchE activity and there was a significant difference between larvae exposed to 400 μg/L when compared to the control (118.4%, $p < .05$, Fig. 2A). While exposure to T3 alone and TBBPA + T3 caused significant decrease of AChE activity to the similar extent, and the resulted AChE activity was significantly different from 200 μg/L TBBPA treated group ($p < .05$; Fig. 2B).

3.4. Gene transcription

The relative expression of selected genes involved in the regulation, transport and synthesis of THs were examined. TBBPA exposure caused significant up-regulation of *tshβ* and *tg* mRNA levels by 1.5-, 1.5-, 1.6-fold and 1.5-, 1.5-, 1.5-fold at 100, 200, and 400 μg/L, respectively ($p < .05$; Fig. 3A). Exposure to T3 alone and TBBPA+T3 caused more significant up-regulation of these two genes by 1.9-, 2.8-fold and 2.1-, 2.9-fold, and the resulted mRNA levels were both significantly different from those of 200 μg/L TBBPA treated group ($p < .05$; Fig. 3B). However, *ttr* and *trβ* mRNA levels were significantly down-regulated by 2.7-, 1.5-fold and 2.2-, 1.5-fold in 200 and 400 μg/L TBBPA treated groups ($p < .05$ Fig. 3A), but remained unchanged in T3 alone or TBBPA+T3 treated groups compared with the control ($p > .05$; Fig. 3B).

In addition, we also evaluated the relative expressions of several genes related to neurodevelopment in zebrafish larvae. The mRNA

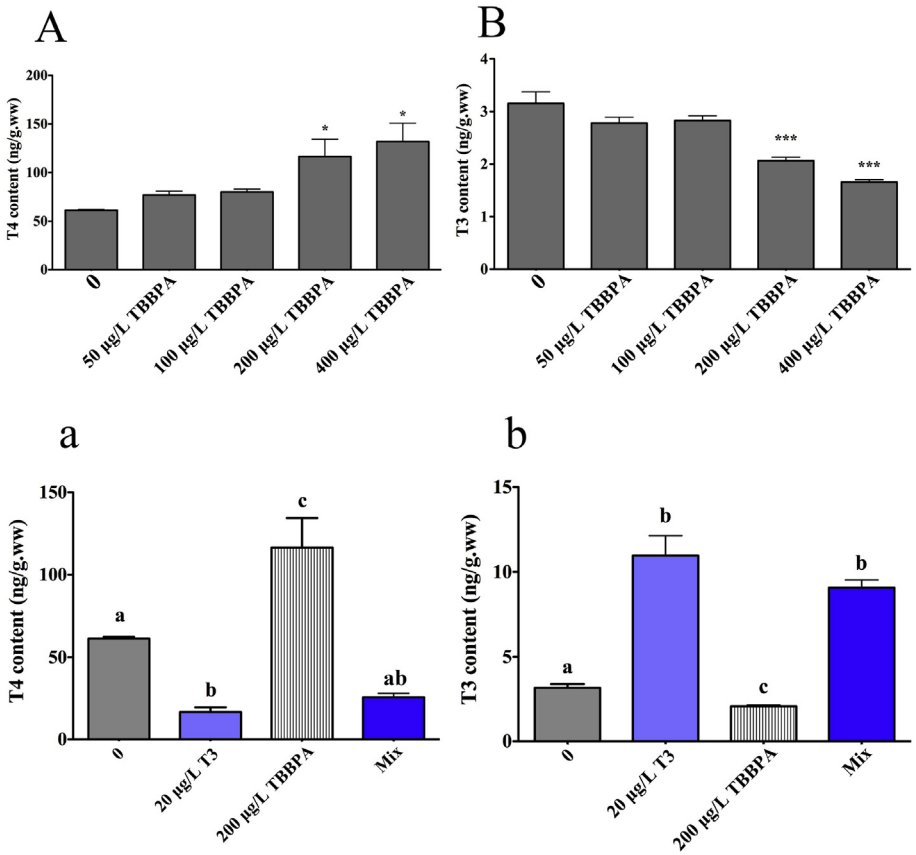

Fig. 1. The effects of TBBPA (0, 50, 100, 200, and 400 μg/L) single exposure or 200 μg/L TBBPA co-exposure with 20 μg/L T3 on whole-body contents of total T4 (A or a) and T3 (B or b) in zebrafish larvae after exposure for 144 hpf. Values are the mean ± SEM (ng/g ww) with three replicate samples and each replicate contained 200 larvae. $^{*}p < .05$ and $^{***}p < .001$ indicate significant differences between exposure groups and the corresponding control group. Different letters denote statistically significant differences among groups (Tukey's test; $p < .05$).

levels of *α1-tubulin*, *mbp*, and *shha* significantly down-regulated by 1.3-, 1.4-, 1.4-fold and 1.5-, 1.8-, 1.7-fold in 200 and 400 μg/L TBBPA treated groups ($p < .05$, Fig. 4A), but remained unchanged in T3 alone or TBBPA+T3 treated groups compared with the control ($p > .05$; Fig. 4B).

3.5. Locomotor behavior

At 144 hpf, zebrafish larvae were subjected to light-dark-light transition stimulation, and the locomotor activity were evaluated and shown in Fig. 5. The average swimming speed of zebrafish larvae was significantly decreased in 200, and 400 μg/L TBBPA treated groups during the first dark period, and also in 100, 200, and 400 μg/L TBBPA treated groups during the light period and the second dark period as compared with the control ($p < .05$; Fig. 5a). However, the average swimming speed of zebrafish larvae in T3 alone or TBBPA+T3 treated groups remained unchanged in any dark or light periods compared with the control (Fig. 5b). The locomotor activities during light-dark-light transition stimulation showed similar changing trends which also decreased in TBBPA treated groups but not changed in T3 alone or TBBPA+T3 treated groups (Fig. 5A and B).

4. Discussion

Our present study showed that embryonic exposure to TBBPA caused thyroid hormone disruptions and neurotoxicity in zebrafish larvae, while co-treatment with T3 could reverse these effects, suggesting that TBBPA-induced neurotoxicity could be attributed to the disruptions of thyroid hormones.

Many studies have reported that THs plays an important role in differentiation of major organs during embryonic development in both lower and higher vertebrates (Oppenheimer et al., 1995). In bony fish, THs are more important in a variety of organs and metabolic processes than any other hormones (Janz, 2000). Previous studies have shown that TBBPA could disturb the thyroid hormones in experimental animals. For example, in European flounder (*Platichthys flesus*), chronic exposure to TBBPA (~50–500 μg/L, norminal concentrations) could result in significant increase of plasma T4 levels, but showed no effects on plasma T3 levels (Kuiper et al., 2007b). In addition, exposure to 0.5 μg/L TBBPA for 28 days could significantly decrease plasma TT4 and TT3 levels in goldfish (*Carassius auratus*) (Qu et al., 2008). The disagreement among these results may be related to the differences in species, life stage of the tested animals, as well as different exposure period and sample types in these studies. While in the present study, we found that in zebrafish larvae whole-body T4 contents were significantly increased and T3 contents were significantly decreased upon embryonic exposure to TBBPA. Our results confirmed that TBBPA could disturb the thyroid hormones *in vivo*.

We then examined the expression of genes involved in the hypothalamic-pituitary-thyroid (HPT) axis. In zebrafish, *tshβ* gene is the major regulator of the thyroidal axis, either positively or negatively controlling the concentrations of circulating THs. A previous study reported that 96-h exposure of zebrafish larvae immediately after hatching to TBBPA at concentrations of

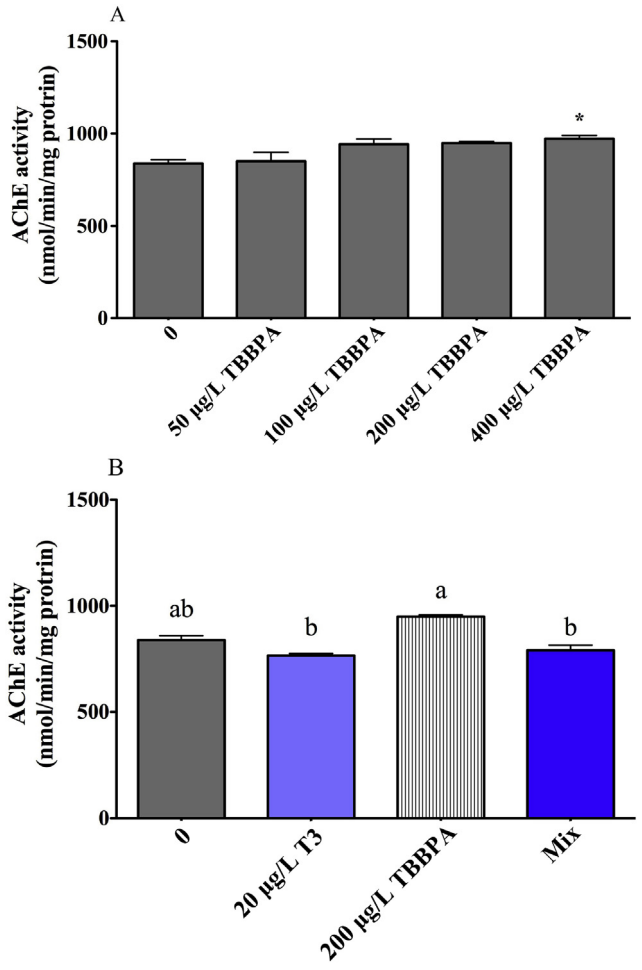

Fig. 2. Acetylcholinesterase (AChE) activity in zebrafish larvae following exposure to various concentrations of (A) TBBPA (0, 50, 100, 200, and 400 μg/L), (B) 200 μg/L TBBPA, 20 μg/L T3, and in combination of them (Mix) at 144 hpf. The values are presented as the mean ± SEM of four relicates. * indicate significant differences between exposure groups and the corresponding control group ($p < .05$). Different letters denote statistically significant differences among groups (Tukey's test; $p < .05$).

530–3950 μg/L caused significant up-regulation of *tshβ* mRNA levels (Chan and Chan, 2012). While embryonic exposure to 100–400 μg/L TBBPA till 120 hpf were reported to result in no change of *tshβ* mRNA levels in zebrafish larvae (Baumann et al., 2016). In the present study, the mRNA levels of *tshβ* were significantly increased in zebrafish larvae upon embryonic exposure to 200 and 400 μg/L TBBPA, which were consist with previous studies. Moreover, the mRNA levels of *tg*, which is the scaffold protein for synthesis of the biologically active THs, were also significantly increased in zebrafish larvae upon embryonic exposure to TBBPA. Hence the elevated mRNA levels of *tshβ* and *tg* indicated that the synthesis of THs were promoted upon TBBPA exposure, and this may explain the elevated T4 contents in zebrafish larvae.

In contrast, we found that the mRNA levels of *ttr* were significantly down-regulated in zebrafish larvae upon embryonic exposure to TBBPA. TTR mainly acts as a "buffering" allowing THs to be released or bound, and also a transporter of THs to specific organization, where T4 were converted to bioactive T3 (Zoeller et al., 2007). Previous studies have demonstrated that TBBPA can compete with T4 for binding to TTR (Hamers et al., 2006). The down-regulated transcription of *ttr* gene may lead to reduced amount of TTR proteins available to bind and transport free T4 to target organs, and this may contribute to the decreased T3 contents. The nuclear receptor TRβ acts as a ligand-mediated transcription factor to stimulate or inhibit expression of target genes (Wang et al., 2014; Chan and Chan, 2012). In previous studies, embryonic exposure to TBBPA did not significantly changed *trβ* mRNA levels in zebrafish larvae (Chan and Chan, 2012; Baumann et al., 2016). However, in the present study, we found that the mRNA levels of *trβ* were significantly down-regulated in zebrafish larvae upon embryonic exposure to 200 and 400 μg/L TBBPA. Hence the down-regulated *ttr* and *trβ* transcription may be related to the decreased T3 contents, which may lead to impairment of THs-regulated biological processes such as embryonic neurodevelopment.

Accordingly, we also examined the transcription of several genes involved in central nervous system (CNS) development, to test whether TBBPA caused neurotoxicity in zebrafish larvae. These genes included *α1-tubulin*, *mbp*, and *shha*. Specifically, the *shha* gene encoded protein is essential for axonal guidance cues in the spinal cord commissural axons and retinal ganglion cell axons (Ishitobi et al., 2007; Kolpak et al., 2005). MBP can modulate the myelin levels in the axons of CNS in developing zebrafish, whereas α1-tubulin encoded an intermediated filament protein that is essential for microtubule cytoskeleton development or axon and dendrite regeneration (Alm et al., 2008). These genes were considered as indicators of impairment in neurodevelopment, and their changes have been reported to be related with neurobehavioral changes in zebrafish upon exposure to BFRs such as BDE-209 and DE-71 (Zhu et al., 2016; Wang et al., 2016). In the present study, the transcription of *α1-tubulin*, *mbp* and *shha* were all down-regulated in zebrafish larvae upon TBBPA exposure, and their changing profiles were positively related to T3 contents.

AChE is necessary for neuronal development in zebrafish embryos (Behra et al., 2002) and changes of its activity has also been implicated as a marker of developmental delay (Yang et al., 2001). In a previous study, exposure to 0.625 and 1.25 mg/L TBBPA caused no significant effects on AChE activity in zebrafish larvae (Usenko et al., 2016). However, in the present study, TBBPA exposure significantly increased AChE activity. The discrepancy observed between these studies may be due to differences in exposure concentration and time. Increased activity of AChE will overhydrolyze ACh toward choline in nerve synapses and neuromuscular junctions and thus decrease the concentration of ACh. As a consequence, decreased ACh concentration could probably lead to hypoactive muscular contraction and behavioral response (Chen et al., 2012a).

The locomotor activity and average swimming speeds of zebrafish larvae were significantly reduced upon exposure to TBBPA. Previously, similar findings on behavioral effects by TBBPA have been reported both in rodents and fish. Nakajima et al. (2009) found that exposure to TBBPA (5 mg/kg body weight) increased horizontal activity in the open-field test as well as lengthened freezing behavior in the contextual fear conditioning paradigm in mice. Zebrafish larvae exposed to 5 μM TBBPA during 8–48 hpf spent significantly more time in the freezing state, and less time in the medium and burst activity state, and their average activity were significantly decreased compared to the control larvae (Chen et al., 2016). Such behavioral changes has been widely used as an indicator for neurotoxicity of environmental pollution (Zhu et al., 2016; Unno et al., 2014; Chou et al., 2010). Thus the above results confirmed that embryonic exposure to TBBPA impaired the neurodevelopment zebrafish larvae and lead to neurobehavioral alterations.

Previous studies have suggested that alterations in THs contents might cause a disorder of the CNS development (Fernie et al., 2005). With this in mind, T3 were used to expose zebrafish embryos alone or in combination with TBBPA to verify whether the observed

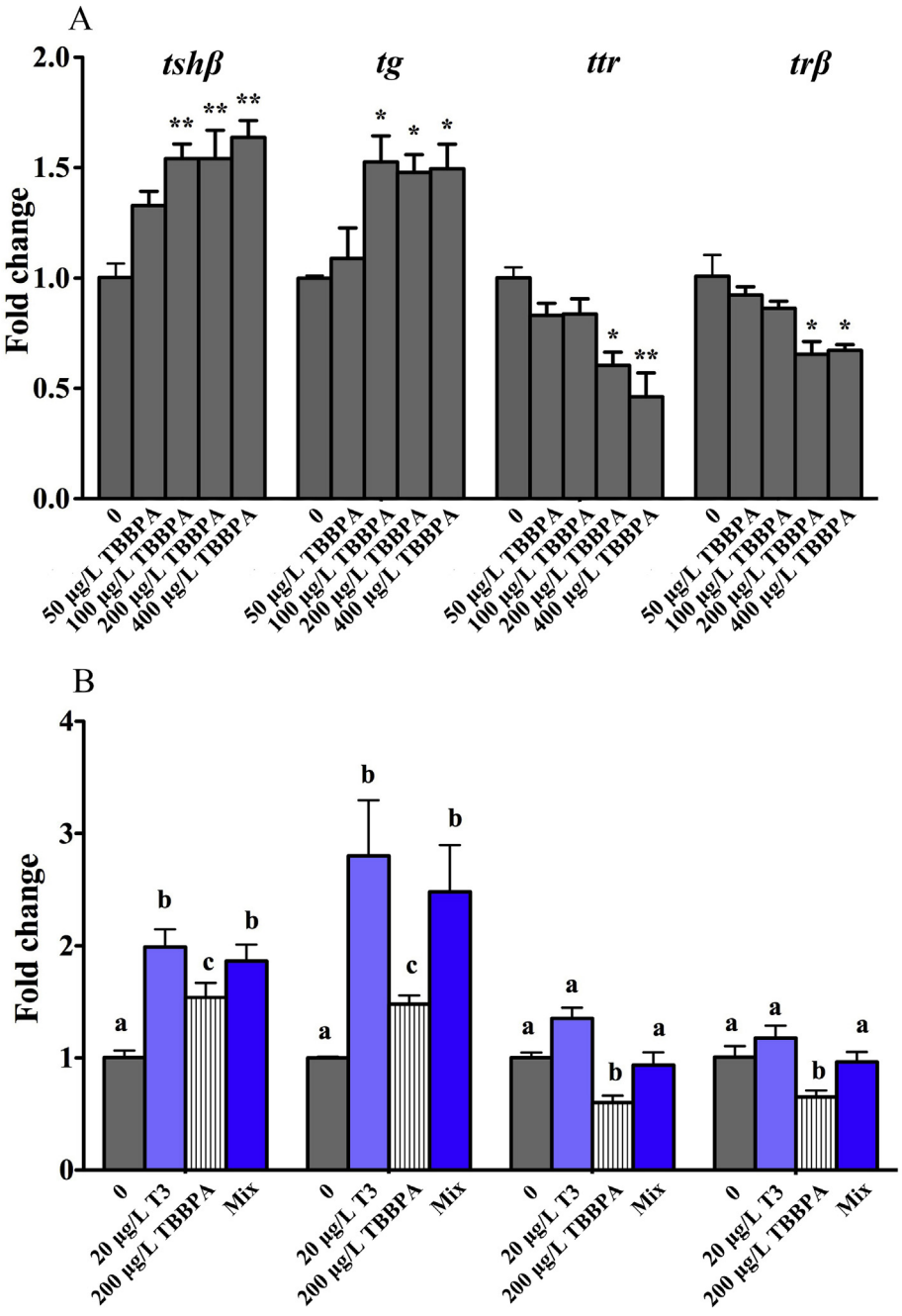

Fig. 3. Thyroid hormone relative mRNA expression levels of *tshβ*, *tg*, *ttr*, and *trβ* in zebrafish larvae exposed to (A) various concentration of TBBPA (0, 50, 100, 200 and 400 μg/L), or (B) 200 μg/L TBBPA, 20 μg/L T3, and in combination of them (Mix) from 2 hpf until 144 hpf. Values are expressed as mean ± SEM of four replicates. $^{*}p < .05$ and $^{**}p < .01$ indicateds significant difference between the exposure groups and control group. Different letters denote statistically significant differences among groups (Tukey's test; $p < .05$).

neurotoxicity in the present study could be attributed to the changed THs contents upon TBBPA exposure. The results showed that T3 treatment resulted in decreased T4 contents and increased T3 contents in zebrafish larvae, which were opposite to those in TBBPA-exposed larvae. While THs contents in larvae treated with TBBPA + T3 showed similar changes with those in T3 treated group, which means that co-treatment with T3 could reverse TBBPA-induced disruptions of THs levels. At transcriptional level, T3 treatment increased mRNA levels of genes involved in THs synthesis including *tshβ* and *tg*, and enhanced TBBPA-promoted transcription of these two genes. T3 treatment did not affect the mRNA levels of *ttr* and *trβ* in zebrafish larvae, but could eliminate TBBPA-induced transcriptional suppression of these two genes. Given the above, T3 co-treatment could reverse or eliminate TBBPA-induced thyroid disruptions in zebrafish larvae.

In the case of neurotoxicity parameters, T3 treatment lead to decreased AChE activity, and co-treatment with T3 reversed TBBPA induced slight increases of AChE activity. T3 showed no significant effects on neither the mRNA levels of *α1-tubulin*, *mbp* and *shha* nor the light-dark stimulated behaviors of zebrafish larvae. But T3 can eliminate TBBPA-induced suppression of these parameters. Hence these results suggested that replenishment of T3 rescued the transcriptional and behavioral changes caused by TBBPA in zebrafish larvae. Therefore, we believed that the observed neurotoxicity

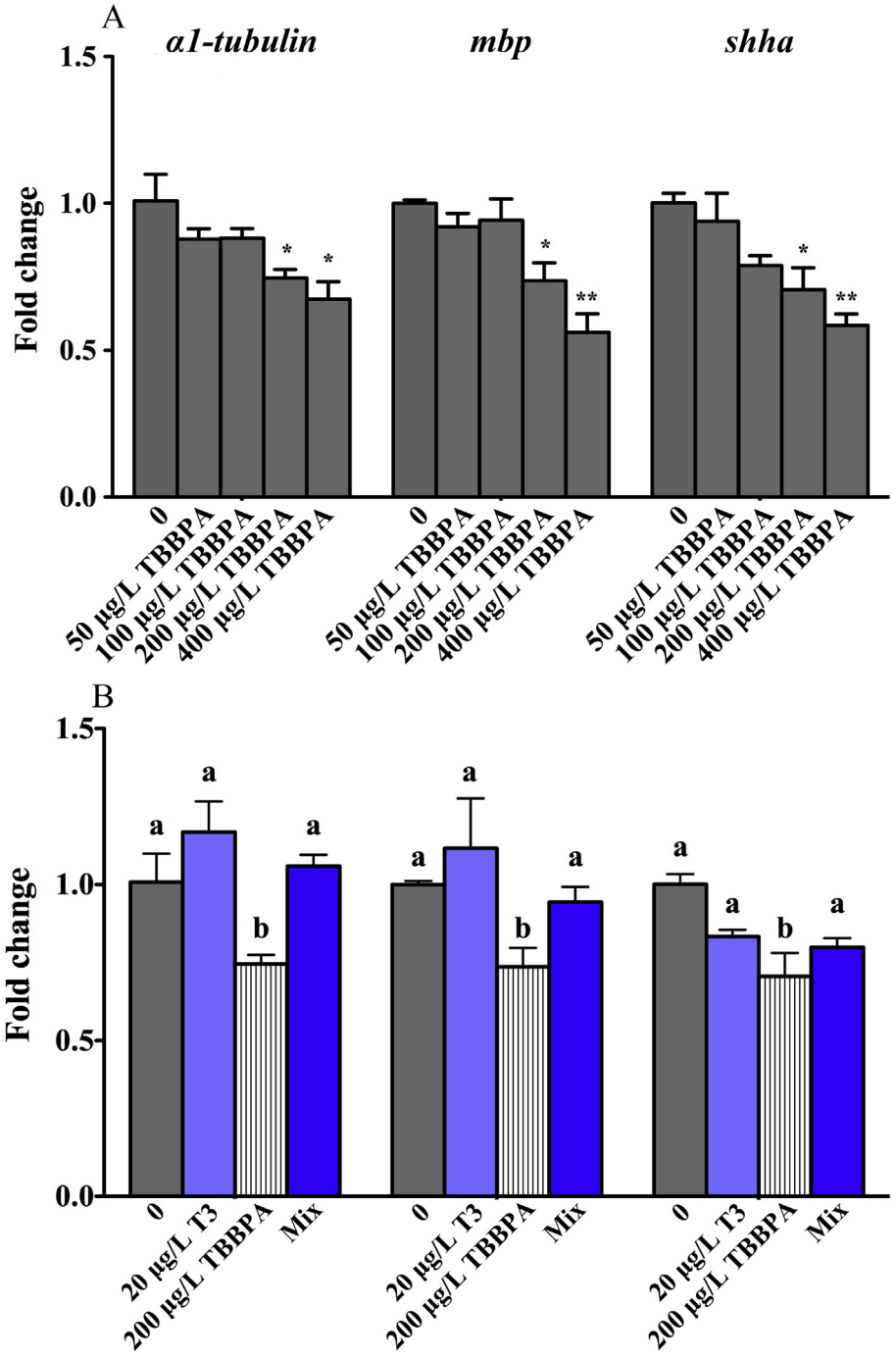

Fig. 4. Central nervous system related mRNA expression levels of *α1-tubulin*, *mbp*, and *shha* in zebrafish larvae exposed to (A) various concentration of TBBPA (0, 50, 100, 200, and 400 μg/L), or (B) 200 μg/L TBBPA, 20 μg/L T3, and in combination of them (Mix) from 2 hpf until 144 hpf. Values are expressed as mean ± SEM of four replicates. $^{*}p < .05$ and $^{**}p < .01$ indicateds significant difference between the exposure groups and control group. Different letters denote statistically significant differences among groups (Tukey's test; $p < .05$).

caused by TBBPA exposure could be attributed to the disruption of THs levels, especially T3 levels.

However, our results also indicated that thyroid endocrine system might be not the only targets of TBBPA. For instance, in this study as well as a previous study by McCormick et al. (2010), significantly increased malformation rates were observed in zebrafish larvae after embryonic exposure to TBBPA at 200 μg/L and higher. However, co-treatment with T3 did not eliminate TBBPA-induced teratogenesis in zebrafish larvae (Table S4). Therefore, other mechanism than thyroid hormone disruptions may be responsible for TBBPA-induced developmental toxicity.

5. Conclusion

In summary, our findings showed that embryonic exposure to TBBPA lead to alterations of thyroid hormone levels and related genes, and also caused neurotoxicity as indicated by altered transcription of genes involved in neurodevelopment, AChE activity and behavioral changes in zebrafish larvae. We also found that these changes could be reversed or eliminated by co-treatment with T3.

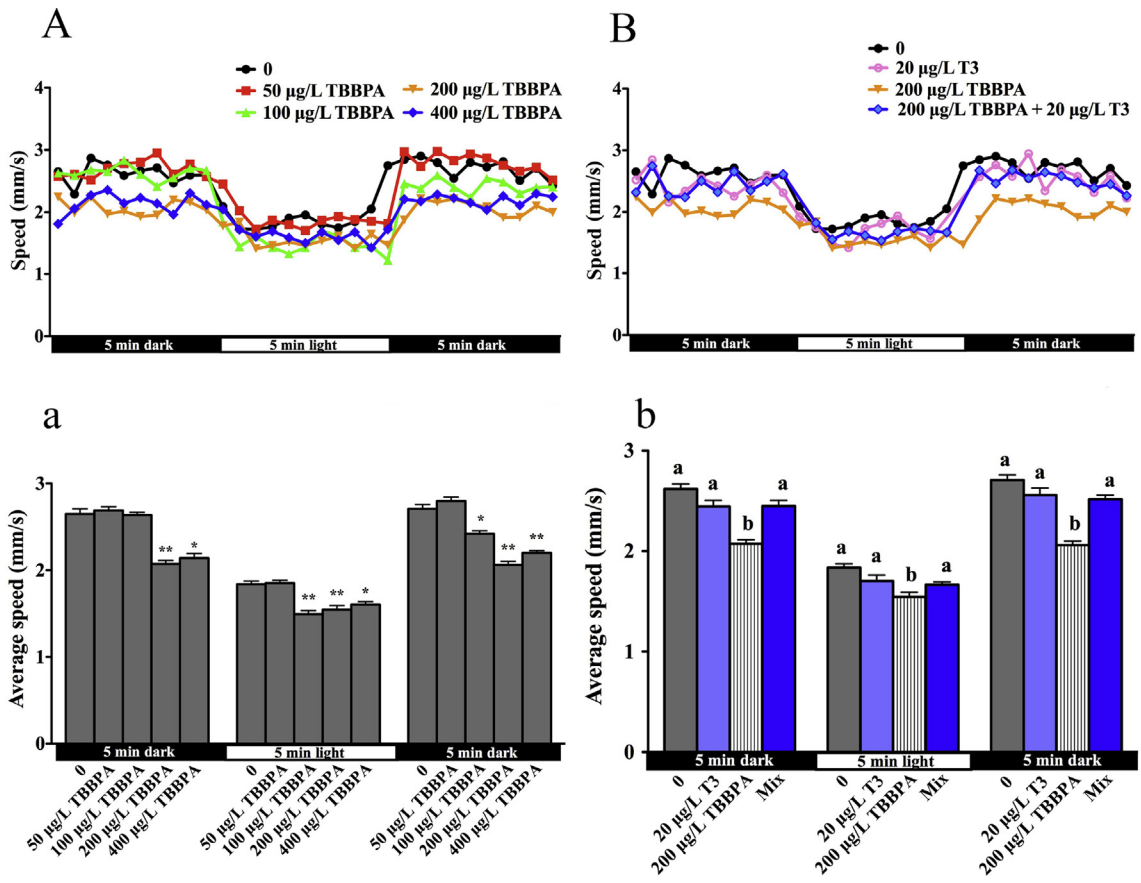

Fig. 5. Locomotor traces and average swimming speed of the zebrafish larvae during the dark-light-dark photoperiod stimulation test after exposure to (A and a) various concentration of TBBPA (0, 50, 100, 200, and 400 μg/L), or (B and b) 200 μg/L TBBPA, 20 μg/L T3, and in combination of them (Mix) from 2 hpf until 144 hpf. Values are expressed as mean ± SEM of four replicates and each replicate contained 12 larvae. $^{*}p < .05$ and $^{**}p < .01$ indicateds significant difference between the exposure groups and control group. Different letters denote statistically significant differences among groups (Tukey's test; $p < .05$).

Therefore, our results demonstrated that the neurodevelopmental toxicity of TBBPA was cause via affecting the regulation of thyroid hormones. Considering the increasing demand for TBBPA and TBBPA-based materials, it is anticipated that TBBPA pollution will concomitantly become more serious in the future. Therefore, the potential influence of TBBPA to wild animals as well as human health is worthy of long-term attention.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.chemosphere.2018.01.080.

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