Article

Chinese Science Bulletin

Environmental Chemistry

May 2011 Vol.56 No.15: 1548–1555
doi: 10.1007/s11434-011-4471-6

Toxicity of the brominated flame retardant tris-(2,3-dibromopropyl) isocyanurate in zebrafish (*Danio rerio*)

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Received July 28, 2010; accepted September 20, 2010; published online April 13, 2011

Tris-(2,3-dibromopropyl) isocyanurate (TBC) is a heterocyclic brominated flame retardant that was recently detected in the environment in China. TBC is semi-volatile and can accumulate in the lipid of some species, but little is known about its effect on aquatic organisms. We exposed adult zebrafish to 0, 0.25, 1 and 4 mg/L TBC for 28 d and measured the effect on survival, growth, histopathology, hormone levels, enzyme activity, and gene expression. TBC exposure had no effect on survival or growth. We observed significant damage to the liver and gill, including hepatocellular swelling and fatty degeneration in the liver as well as proliferation and edema of epithelial cells in the gills. In addition, exposure to 4 mg/L TBC induced proliferation of goblet cells in the intestine of both sexes, acellular areas in the testis, and thinly scattered vitellogenic granules in vitellogenic oocytes. TBC exposure had no effect on the levels of thyroid hormones, testosterone, estradiol, liver superoxide dismutase activity, malondialdehyde content, and brain cholinesterase activity. By contrast, hepatic vitellogenin and cytochrome *P4501A* gene expression was significantly down-regulated in both male and female zebrafish in response to TBC exposure. Our results suggest that exposure to TBC causes a variety of potential reproductive and endocrine toxic effects.

brominated flame retardants (BFRs), tris-(2,3-dibromopropyl) isocyanurate (TBC), zebrafish, endocrine disrupting effect, vitellogenin

Citation: Zhang X, Li J, Chen M J, et al. Toxicity of the brominated flame retardant tris-(2,3-dibromopropyl) isocyanurate in zebrafish (*Danio rerio*). Chinese Sci Bull, 2011, 56: 1545–1555, doi: 10.1007/s11434-011-4471-6

Brominated flame retardants (BFRs) are added to a variety of commercial products to reduce the risk of fire damage [1]. However, a number of studies have shown that BFRs persist in the environment, can bioaccumulate, and can be transported long distances. Thus, there is reason to be concerned about their potential to cause harm to ecosystems and human health [2,3]. In addition, the concentration of the BFRs has been increasing annually in a range of organisms, including human breast milk [4–6]. This has been addressed to some extent by the ban of polybrominated diphenyl ethers (PBDEs) throughout the industrialized world [7]. However, the levels of other forms of BFRs, including tetrabromobisphenol A (TBBPA), hexabromocyclododecanes (HBCD), and decabromodiphenyl ether (DBDPE) appear to be increasing in the environment [8,9].

Tris-(2,3-dibromopropyl) isocyanurate (TBC) is a heterocyclic hexabrominated flame retardant. TBC is stable and has been widely used in polyolefin, polyphenyl alkenes, unsaturated polyester, synthetic rubber, and fibers [10]. In 2009, TBC was found, for the first time, in the environment in Hunan province, China [11]. High concentrations of TBC were found in river water (2.33–163 ng/L), surface sediments (85.0–6029 ng/g), soils (19.6–672 ng/g), earthworms (9.75–78.8 ng/g), and carp (12.0–646 ng/g) [11]. The annual

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production of TBC is ~500 t in China, which is slightly lower than the production of TBBPA, PBDEs, and HBCDs [12]. Although official production statistics are not available for the past decade, it is thought that the amount of TBC has increased because of the decline in PBDEs production and the rapid industrialization of China. The environmental effects of TBC are of some concern, particularly in light of recent evidence that TBC is persistent and can bioaccumulate [11]. Preliminary *in vitro* toxicological evidence suggests that TBC is not toxic to HepG2 cells [11]. However, nothing is known about the effects of TBC *in vivo*.

Our objective was to evaluate the potential aquatic toxicity of TBC. We exposed adult zebrafish (*Danio rerio*) to a high, medium, and low dose of TBC for 28 d and evaluated the effect on tissue pathology, hormone levels and enzyme activity.

1 Material and methods

1.1 Chemicals

We purchased tris-(2,3-dibromopropyl) isocyanurate (TBC, 97% purity) from Sigma-Aldrich (St Louis, MO, USA). Stock solutions (1000 and 4000 mg/L) were prepared by dissolving TBC in dimethyl sulfoxide (DMSO). All other chemicals were of analytical grade.

1.2 Zebrafish and exposure experiment

We obtained zebrafish (wild-type, AB strain) from the Institute of Hydrobiology, Chinese Academy of Sciences. The fish were reared following the procedures of Westerfield [13]. In brief, the fish were held in fresh water at 26±1.5°C under a 12 h-light: 12 h-dark photoperiod and fed fresh *Artemia* nauplii twice daily.

Prior to the experiment, the adult zebrafish (14 weeks-old) were divided into 4 groups: control (DMSO), low dose TBC (0.25 mg/L), medium dose TBC (1 mg/L), and high dose TBC (4 mg/L). Each group was stocked into triplicate glass tanks (6 L, sexes held separately, N=6 tanks/group, 10 males/10 females per tank) containing 4 L filtered water for 7 d. We measured the body weight and length of each fish (females: 0.35±0.01 g, 2.18±0.02 cm; males: 0.33±0.01 g, 2.19±0.01 cm). Following acclimation, the fish were exposed to the appropriate concentration of TBC for a period of 28 d. The final concentration of DMSO was less than 0.1%. Half of the exposure solution in each tank was replaced daily. At the end of the exposure period, all fish were sacrificed using 0.01% MS-222 and their body weight and length were recorded.

1.3 Histology

We removed the gill, liver, intestine, and gonads from both sexes for histological examination. The tissues were fixed in paraformaldehyde solution (4%, w/v) for 24 h, dehydrated using an ethanol gradient, embedded into paraffin wax, and sectioned at 3 μm. The sections were stained with haematoxylin and eosin (H&E) and examined under a light-microscope. We counted the number of hepatocytes and oocytes within the field of view and noted their developmental stage (cortical alveolus (Coc), vitellogenic oocyte (Voc)).

1.4 Hormone assays

The fish were homogenized in 1.2 mL ice-cold phosphate-buffered saline (0.01 mol/L PBS, pH 7.2) and the whole body homogenate was centrifuged at 13000×g for 15 min at 4°C. The supernatant was removed and the hormones levels were immediately quantified using an Abbott AXSYM immunochemical automated analyzer, using the following kits: AxSYM free triiodothyronine (FT3), AxSYM free thyroxine (FT4), AxSYM Estradiol, and AxSYM Testosterone (Abbott Laboratories, Abbott Park, IL, USA), in accordance with the manufacturer's instructions.

1.5 Quantification of SOD and CHE activity and MDA content

The liver and brain were removed at the end of the exposure period and immediately stored at –80°C until analysis. The liver and brain samples from 5 individuals in each group were homogenized individually in 50 μL of PBS (0.1 mol/L, pH 7.2), then centrifuged at 13000×g for 10 min at 4°C. The supernatant was saved for the measurement of SOD and CHE activity (U/mg protein) and MDA content (nmol/mg) protein using reagent kits from Nanjing Jian Cheng Bioengineering Institute (Nanjing, China). The total protein concentration of each sample was determined using the Bradford assay (Bio-Rad, Hercules, CA, USA).

1.6 RNA extraction and quantitative real-time PCR assay

We prepared total RNA from the homogenized zebrafish liver using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The purified total RNA was measured at 260 and 280 nm using a UV-spectrophotometer (BioPhotometer plus, Eppendorf, Germany). The quality and integrity of the total RNA was verified using the ratio of absorbance at 260/280 nm and by electrophoresis on a 1% agarose formaldehyde gel.

The cDNA was synthesized from 2 μg total RNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer's instructions. We quantified CYP1A and VTG mRNA expression using the following primers: 5′-AGGCGAGAAGGTGTTGGTT-3′ (forward) and 5′-CAGCATTTCTCCTGGCATC-3′ (reverse) for CYP1A and 5′-AGCTGCTGAGAGGCTTGTTA- 3′

(forward) and 5′-GTCCAGGATTTCCCTCAGT-3′ (reverse) for VTG. The primers were designed using Primer Premier 5.0 (Premier, Palo Alto, USA) to amplify a 137 base-pair (bp) fragment of the CYP1A gene and a 95-bp fragment of the VTG gene.

Each PCR reaction mixture (total volume 20 μL) contained 1 μL of cDNA template, 0.1 μmol/L each primer, 7 μL of Milli-Q water, and 10 μL of 2×SYBR QPCR Master Mix (Toyobo, Osaka, JAPAN). The thermal cycling program consisted of a denaturing step (94°C, 5 min) followed by 45 cycles of denaturation (94°C for 20 s), annealing (55°C for 20 s), and extension (72°C for 40 s) in a PTC-200 thermal cycler equipped with a Chr 4 real-time fluorescence detector (MJ Research, Watertown, MA, USA). We measured the gene expression levels in 7 replicates for each treatment group. We used β-actin as an internal standard, using the following primers: 5′-CAACAGAGAGAAGATGACACAGATCA-3′ (forward) and 5′-GTCACACCATCACCAGAGTCCATCAC-3′ (reverse). The cycle threshold (CT) value was obtained using Opticon Monitor 3.0. The target gene expression level was normalized to β-actin. The change in expression levels was analyzed using the $2^{-\Delta\Delta CT}$ method [14].

1.7 Statistical analyses

We used one-way analysis of variance (ANOVA) and Tukey's multiple range tests to compare between the control and treatment groups. A P value of $\leqslant 0.05$ was considered significant. All results are presented as means ± SEM. All analyses were performed in Spss 13.0 and Origin 7.5.

2 Results

2.1 Mortality and growth

There were no differences in mortality among the different groups. The mortality rate was 3.3%, 3.3%, 6.6% and 3.3% in the control, low, medium, and high dose treatment groups, respectively. There was also no difference in body weight (male: 0.41±0.01 g; female: 0.42±0.02 g) and length (male: 2.32±0.03 cm; female: 2.30±0.03 cm) between the control and the high dose treatment group, suggesting that TBC has no effect on fish growth (data not shown).

2.2 Histopathology

TBC exposure caused damage to the liver, gill, and intestine. However, there was no marked difference in the pathological changes between male and female zebrafish.

The hepatic lobules were clearly identified as normal in the control fish (Figure 1(a)). However, the number of hepatocyte nuclei was significantly lower in the low dose treatment group (Figure 1(b)). After 28 d exposure to 4 mg/L TBC, the most significant change was vacuolization (Figure 1(c)).

Exposure to TBC caused damage to the gill primary lamella (Figure 1(e) and (f)), characterized by proliferation and edema of epithelial cells, and amalgamation of secondary gill lamella. The effects of TBC were dose-dependent, with significantly greater damage occurring at the higher concentrations**.** In contrast to the control (Figure 1(g)) group, we observed proliferation of goblet cells in the intestine of fish that were exposed to 4 mg/L TBC (Figure 1(i)).

There were no obvious histological alternations in the testis of the TBC treated groups. We identified all stages of spermatogenetic cells, including spermatogonia, spermatocytes, and spermatids (800×, data not shown). However, we did note the presence of large acellular areas in the testis of fish that were exposed to 4 mg/L TBC, suggesting a decrease in the number of spermatids (Figure 2(c)).

The vitellogenic granules were thinly scattered within the Voc in the high dose group relative to the control. The Voc∶Coc ratio was 1.58, 1.50 and 1.06 in the control and low and high treatment groups, respectively.

2.3 Thyroid hormones and sex steroid levels

TBC had no effect on the growth of zebrafish under the conditions we tested. Similarly, exposure to TBC had no effect on the levels of FT3 and FT4 in both genders (Figure 3(a) and (b)). We also found no difference in the levels of testosterone and estradiol in the whole body homogenates of fish (both sexes) that were exposed to TBC relative to the control group (Figure 3(c) and (d)).

2.4 SOD and CHE activity and MDA content

Exposure to TBC had no effect on SOD activity in the male fish. Conversely, there was an apparent increase in SOD activity in female fish that were exposed to 1 mg/L TBC (Figure 4(a)). However, this effect was not observed in the females exposed to 4 mg/L TBC. TBC exposure had no effect on liver MDA content in both sexes (Figure 4(b)). Similarly, TBC exposure had no effect on CHE activity in the brain of zebrafish (Figure 4(c)).

2.5 *CYP1A* and *VTG* gene expression

Expression of the *CYP1A* gene in the liver was significantly inhibited by TBC exposure (Figure 5(a)). Compared with the control group, the expression of *CYP1A* was 1.26, 3.04 and 5.48 times lower in male fish that were exposed to 0.25, 1, and 4 mg/L TBC, respectively. In the female fish, *CYP1A* expression was significantly down-regulated by 12.71, 9.18, and 6.33 fold, in the low, medium, and high dose TBC treated groups, respectively, compared with control group.

TBC exposure also had a significant effect on *VTG* gene expression in the liver (Figure 5(b)). In the male fish, *VTG*

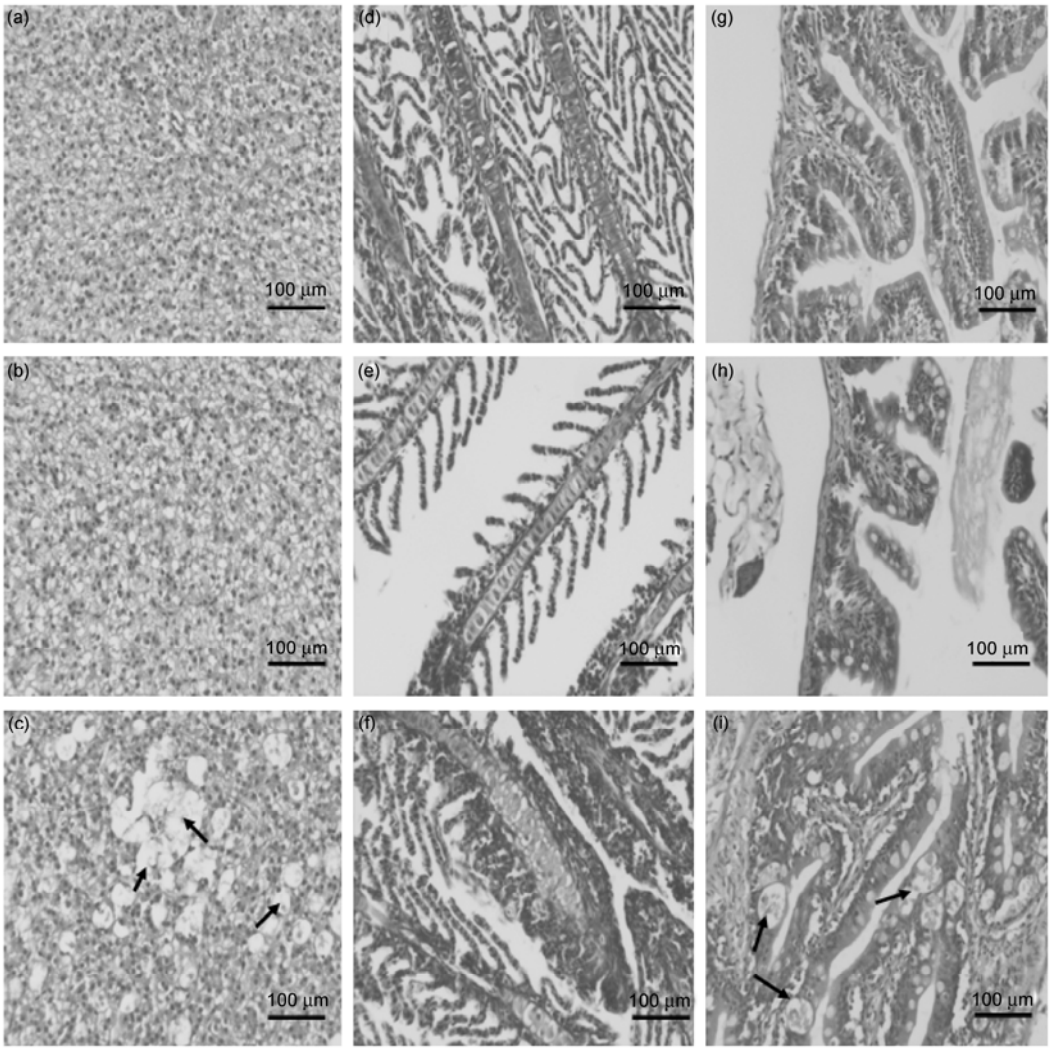

Figure 1 Light micrographs of liver, gill, and intestine from adult zebrafish exposed to TBC. (a) Liver, control; (b) liver, 0.25 mg/L TBC; (c) liver, 4 mg/L TBC treated male, showing vacuolization (arrow); (d) gill, control; (e) gill, 0.25 mg/L TBC; (f) gill, 4 mg/L TBC; (g) intestine, control; (h) intestine, 0.25 mg/L TBC; (i) intestine, 4 mg/L TBC, showing proliferation of goblet cells (arrow).

expression was 1.07, 2.87 and 9.84 fold lower in the fish exposed to 0.25, 1, and 4 mg/L TBC, respectively, though the difference was only significant at the highest dose. Similarly, *VTG* expression was 2.17, 110.13 and 20.36 fold lower in the females exposed to 0.25, 1 and 4 mg/L TBC relative to the controls. The decrease was only significant at the medium and high doses.

3 Discussion

TBC is environmentally persistent and has a tendency to bioaccumulate [11]. Given that the aquatic environment is the ultimate sink for many pollutants, it is important to understand the likely effects of TBC on aquatic organisms. We exposed adult zebrafish to TBC for 28 d, then measured the effect on growth, mortality, hormone levels, and gene expression. To our knowledge these are the first data describing the effects of TBC *in vivo*.

TBC did not appear to have a significant effect on the overall health and somatic fitness of zebrafish in our study. However, TBC is accumulated in lipid-rich organs, such as the brain, liver, intestine, and fat of common carp [11]. Given this, we evaluated the toxicity of TBC in the gill, liver, intestine, and gonads. The gill is the primary gas exchange organ in teleosts and is also a direct target for water-borne pollutants [15]. For example, PBDEs are absorbed through the gill membranes and BDE47 causes apoptosis in the gill epithelial cells of rainbow trout [16,17]. Our results suggest that TBC exposure causes the proliferation of epithelial cells in the gill primary lamella and amalgamation of secondary gill lamella in adult zebrafish. This differs from the effect of PBDEs, likely because of the different mode of accumulation. The liver is also an important target organ for TBC accumulation [11]. Following exposure to 4 mg/L TBC, we noted the presence of large numbers of vacuoles in the liver of both male and female fish. This may have been caused by hepatic cellular swelling and fatty

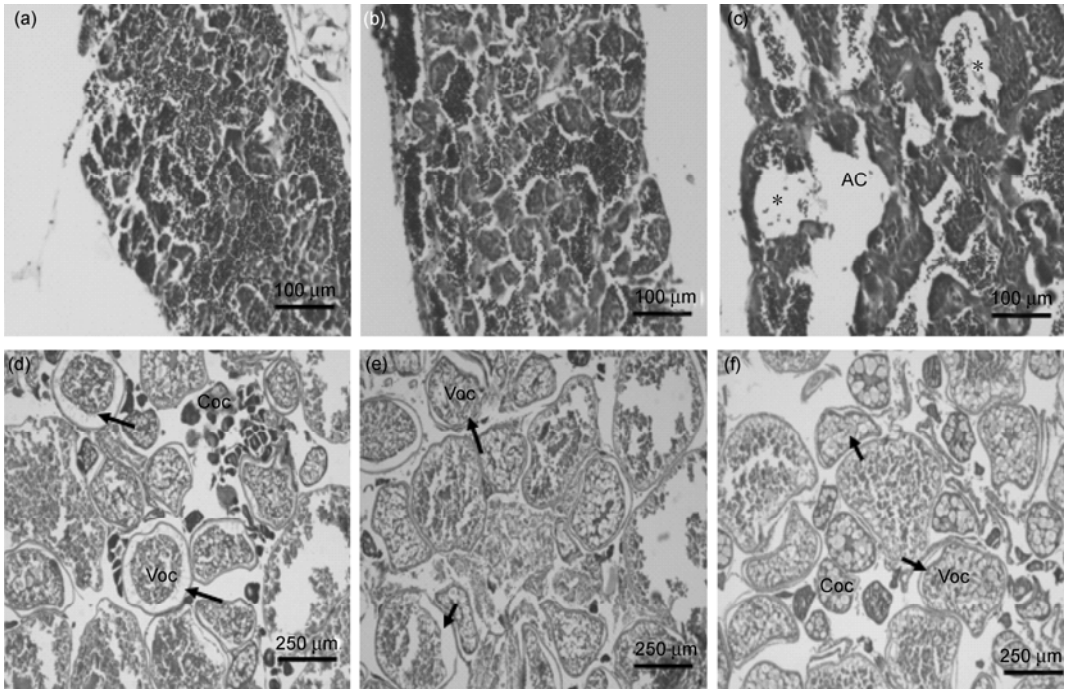

Figure 2 Light micrographs of gonads from adult zebrafish that were exposed to TBC. (a) Control male; (b) 0.25 mg/L TBC treated male; (c) 4 mg/L TBC treated male, showing the acellular areas (AC, asterisk); (d) ovary of control female; (e) ovary of 0.25 mg/L TBC treated female; (f) ovary of 4 mg/L TBC treated female. All arrows point to the vitellogenic oocyte (Voc) stage. Coc=cortical alveolus stage.

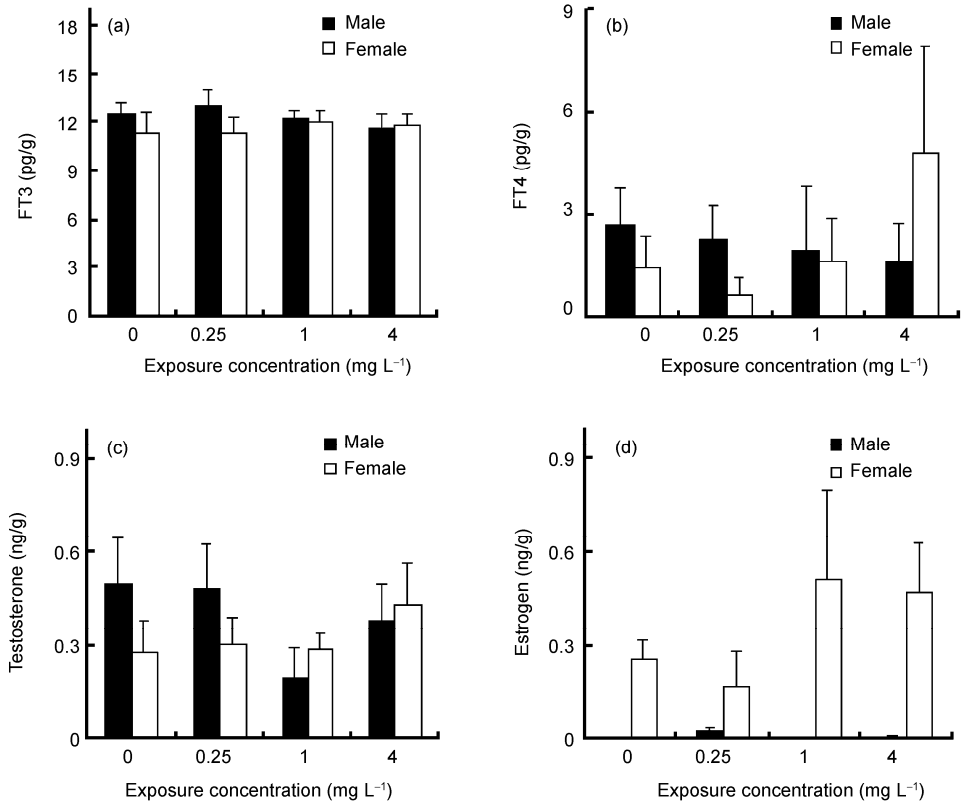

Figure 3 Effect of TBC exposure on levels of thyroid and sex hormones in adult zebrafish. Each bar represents the mean ± SEM (n=5).

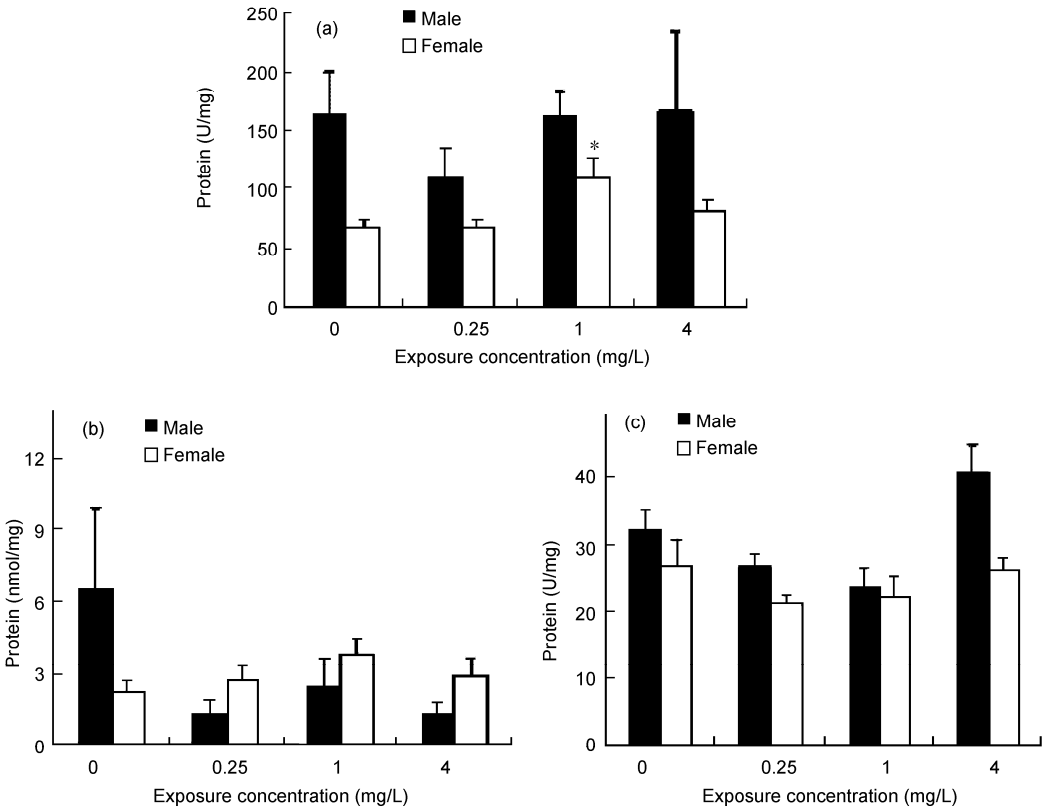

Figure 4 Effects of TBC exposure on SOD and CHE activity and MDA content in adult zebrafish. (a) Liver SOD activity; (b) Liver MDA content; (c) Brain CHE activity. Each bar represents the mean ± SEM (n=5). ∗, Significant difference between treatment and control groups (P<0.05).

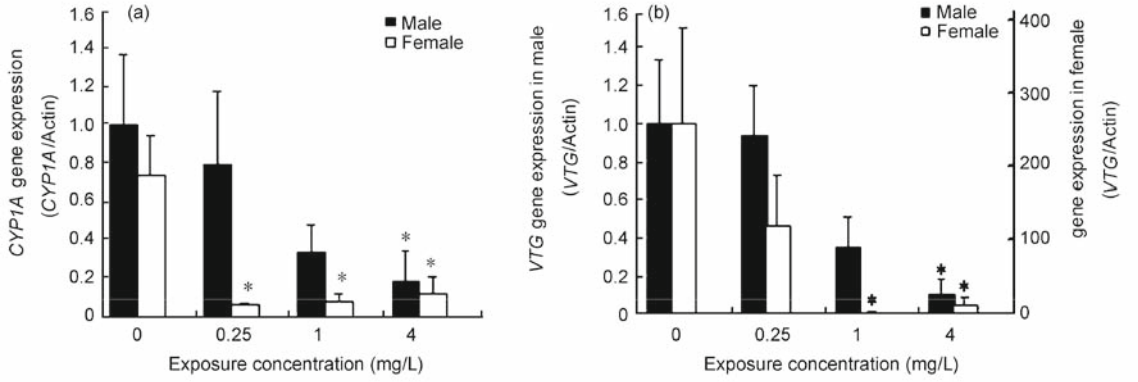

Figure 5 Effect of TBC exposure on *CYP1A* and *VTG* gene expression in the zebrafish liver. (a) *CYP1A* expression; (b) *VTG* expression. Each bar represents the mean ± SEM (n=7). ∗, Significant difference between treatment and control groups (P<0.05).

degeneration. This is consistent with observations of the effect of other BFRs on the liver [18,19]. We hypothesize that steatosis may be caused by: (1) an increase in fatty acid content in the hepatocytes; (2) over-synthesis of triglycercides; and (3) a decrease in apolipoprotein synthesis and inhibition of fat export.

The oxidative stress response plays an essential role in metabolizing toxicants and protecting hepatocytes in the liver [20]. Vertebrates, including teleosts, attempt to reduce the damage from oxidative stress using an antioxidant defense system [21]. Based on histological analysis, TBC exposure caused significant damage to the hepatocytes. However, SOD and MDA were unchanged in the liver, suggesting that there was no increase in free radicals or lipid

peroxidation. Taking together, these observations suggest that zebrafish cannot easily metabolize TBC. This is consistent with previous reports showing that TBC has a tendency to bioaccumulate in certain species, and likely explains the irreversible hepatocyte damage in zebrafish.

Other BFRs, such as PBDE47, inhibit the activity of cholinesterase (CHE) at low doses in teleosts [22]. By contrast, TBC exposure had no effect on CHE activity in zebrafish, suggesting that TBC may be not neurotoxic, at least in this species. This may explain why we did not observe any abnormal behavior in TBC treated fish. However, TBC can pass through the blood-brain barrier and accumulate in the brain [11]. Therefore additional studies are needed to rule out effects on the brain.

Because of the variation in chemical structure, BFRs are absorbed and metabolized via many different pathways [23,24], resulting in a range of toxicity mechanisms. The *CYP1A* family of cytochrome P450s is one of the most sensitive biomarkers of exposure to contaminants and plays a major role in the biotransformation and metabolism of xenobiotics [25]. Compounds such as dioxins and polychlorinated biphenyls induce the expression of *CYP1A* by binding to the aryl hydrocarbon receptor (AHR), causing its translocation to the nucleus and dimerization with the AHR nuclear translocator (ARNT) [26,27]. Because TBC has only recently been detected in the environment, little is known about its toxicity and breakdown in aquatic animals. TBC did not induce the expression of *CYP1A* in the zebrafish liver, suggesting that the breakdown of TBC is not associated with the aryl hydrocarbon receptor signaling pathway. This may explain its tendency to bioaccumulate in certain species [11].

A number of BFRs (e.g. PBDEs, HBCDs and TBBPA) function as endocrine disruptors by affecting thyroid function and altering steroid levels in teleosts [28–30]. However, TBC exposure had no effect on levels of the FT3, FT4 in adult zebrafish, suggesting that TBC does not disrupt thyroid function.

VTG is the precursor of yolk protein vitellogenin in oviparous organisms, including teleosts. VTG is produced in the hepatocytes via a process that is mediated by estrogens. The induction of VTG in male fish plasma or liver tissue is often used as a biomarker for evaluating the estrogenic effects of pollutants [31,32]. Interestingly, TBC exposure inhibited *VTG* gene expression in the liver of adult zebrafish. However, TBC had no effect on levels of estradiol, suggesting that the effect on vitellogenin synthesis was not mediated via the estrogen pathway. Interestingly, the ratio of Voc to Coc was lower in the ovary of TBC exposed fish, suggesting that TBC interrupts the development of oocytes. We speculate that this may have been caused by the reduced expression of VTG in the liver. Furthermore, TBC exposure affected gonad morphology. We noted the occurrence of a large acellular area in the spermary of male gonads and thinly scattered vitellogenic granules in the Voc of female zebrafish, suggesting that TBC may inhibit reproduction. Taken together, our results suggest that TBC may act as an endocrine disrupter because of its effect on VTG production. However, the transportation, metabolism, and biotransformation of TBC involve a series of complex processes. Thus, the mechanisms responsible for the endocrine disrupting effect of TBC remain unclear.

In summary, we demonstrated that subacute exposure to TBC for 28 d had a number of effects in adult zebrafish: (1) TBC is toxic to the liver, gill, intestine, and gonads of adult zebrafish; (2) *CYP1A* gene expression is significantly inhibited by TBC, suggesting that AHR pathway may not be involved in TBC metabolism in zebrafish; and (3) TBC disrupts *VTG* expression, suggesting that TBC is an endocrine disruptor. Our results highlight the need to characterize the toxicity of novel heterocyclic BFRs, such as TBC, as they may present a hazard to aquatic ecosystems.

This work was supported by the National Natural Science Foundation of China (20890112 and 20907017), the National Basic Research Program of China (2009CB421605), the National High Technology R&D Program of China (2007BAC27B01), and the State Key Laboratory of Environmental Chemistry and Ecotoxicology (KF2009-05).

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