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Response mechanisms to joint exposure of triclosan and its chlorinated derivatives on zebrafish (*Danio rerio*) behavior

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HIGHLIGHTS

- Joint toxicity of triclosan and its main products (TDTs) to zebrafish behavior.
- TDTs-stress produced larval abnormality and adult anxiety- or autism-like behavior.
- Behavioral effects focus on social interaction, preference, T-maze tests and so on.
- Abnormal behavior from changes in the related gene, biomarker and pathological tissue.
- TDTs produced vascular ablation in head and occurrence of massive apoptosis in brain.

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ABSTRACT

Triclosan (TCS), 2,4,6-trichlorophenol (2,4,6-TCP) and 2,4-dichlorophenol (2,4-DCP) frequently co-exist in real-world aquatic environments; the latter two contaminants contributing to TCS photolytic products or chlorinated derivatives. There is a paucity of information regarding their joint toxicity to aquatic organisms leading us to study their effects on the swimming behavior of zebrafish (*Danio rerio*). Herein, we reported that 0.28 mg/L TDT exposure (mixtures of TCS, 2,4,6-TCP and 2,4-DCP) enhanced 24-hpf embryonic spontaneous movement frequency, 96-hpf larval activity; however, the 0.56 and 1.12 mg/L TDT treatments decreased all of these behavioral endpoints. All adult behavioral tests demonstrated that chronic TDT exposure (0.14 mg/L) led to hyperactivity and restlessness in adult zebrafish. A 0.14 mg/L TD DATE /@ "M/d/yyyy" 11/21/2017T treatment led to anxiety-like behavior in a bottom dwelling test and excessive panic and low hedging capacity in a conditioned place preference test. Social interaction test demonstrated that zebrafish preferred quiet and isolated space in response to TDT stress. Zebrafish memory was significantly decreased in a T-maze experiment. Whole mount *in situ* hybridization of *pax2a* and *bcl2l11* genes revealed that their differential expression in the brain and skeleton were related to the corresponding phenotypic behavioral abnormality. A series of biomarker and estrogen receptor assays demonstrated that TDT acute exposure caused abnormal energy metabolism and neurological diseases. AO staining revealed that TDT exposure produced vascular ablation in the head, as well as the occurrence of massive apoptosis in the brain. TEM observation showed pyknosis of nucleus following TDT exposure. These results allow assessment of mechanisms for zebrafish abnormal behavior in response to TDT exposure, and are useful for early intervention and gene therapy of contaminant-induced diseases.

1. Introduction

Triclosan (TCS), a broad-spectrum antifungal agent, is extensively used in a variety of personal care and industrial products, including hand soap, detergent, deodorant, toothpaste and household goods (Ducey and Sapkota, 2011). As an endocrine disrupting chemical, it has a negative impact on the environment and human health, especially aquatic ecosystems (Alrajab et al., 2015). Environmental behavior studies on TCS demonstrated that after reaction with chlorinated ions, TCS may form many stable

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chlorinated derivatives (Supplementary Fig. 1), among which 2,4,6-trichlorophenol (2,4,6-TCP) and 2,4-dichlorophenol (2,4-DCP) are the most prevalent chlorinated phenolic pollutants in aquatic environments (Canosa et al., 2005; Fiss et al., 2007).

TCP and DCP can be formed through TCS photolysis or chlorination reactions, and they are also widely used in agriculture, industry and household items for purposes such as pesticides, wood preservatives and personal care products. They are frequently detected at ng/L-mg/L level in surface and ground waters, industrial sewage, and drinking water, their maximum concentrations detected in Yellow River up to 0.28 and 0.20 mg/L, respectively (Gao et al., 2008; Zhong et al., 2010). The raw influent TCS ranged from 3000 to 14000 ng/L, whereas the effluent ranged from 161 to 462 ng/L in Red River basin wastewater/sewage treatment plants (Canosa et al., 2005). In surface water of the Taihu Lake, 2,4,6-TCP concentration ranged from 0 to 840 ng/L, 2,4-DCP ranged from 0 to 143 ng/L (Zhong et al., 2010). Therefore, they are listed as priority pollutants by China, USA and European Union, and also as carcinogens by the International Agency for Research on Cancer (Chen et al., 2009).

In aquatic environments, TCS, TCP and DCP are often found to co-exist. Paul and coworkers (2013) reported that Japanese medaka fish exposure to 0.17 mg/L TCS for 9 days resulted in significantly reduced swimming speeds (Paul et al., 2013). TCS may cause severe central and peripheral neurotoxicity along with environmental diseases, such as triggering fibromyalgia, respiratory muscle weakness, myocarditis and rheumatoid diseases. At mg/L levels, TCS can produce 30–50 types of disease symptoms after ingestion for 6–8 months, and can lead to joint and muscular pain disorders after ingestion for 18 months (Larsson et al., 2014).

Sublethal biological effects, especially alterations of swimming behavior, have great importance in evaluation of environmental toxicity and ecological risk for contaminants (Jin et al., 2009). Previous toxicological studies have focused on individual TCS or 2,4-DCP species and therefore few data are available concerning joint compound exposure that is common in real-world environments. Thus, studies examining combined toxicity of TCS and it chlorinated derivatives are of great basic and practical importance in evaluation of TCS toxicities in the environment.

We previously used two toxicity assessment methods, including Toxic Unit, and Mixture Toxicity Index, to evaluate the interactions of TCS, 2,4-DCP and 2,4,6-TCP. All of these assessments demonstrated that compound interactions produced additive toxicological effect (data not shown in this text). To date, few data are available on the toxicity of TCS and its derivatives using zebrafish (*Danio rerio*) as a model organism. Zebrafish have become a valuable model animal as they are highly homologous to humans and easy to maintain, observe and manipulate (Zhang et al., 2016). In addition to their similar brain structure, zebrafish also share similar regulatory processes underlying behavior with human, which makes zebrafish ideal for neuropsychiatric disease studies. Results of this study allow assessment of ecological risk to co-existence of TCS and its two chlorinated derivatives, and further our understanding of nervous system and neurodegenerative diseases, which contribute to health prevention, early intervention and gene therapy of drug-induced diseases.

2. Material and methods

2.1. Ethics statement

This study strictly followed protocols for the care and use of laboratory animals by the Institutional Animal Care and Use Committee (IACUC) at Wenzhou Medical University (Wenzhou, China). All zebrafish surgery was performed on ice to decrease suffering.

2.2. Chemicals reagents

TCS and its two chlorinated products (2,4-DCP and 2,4,6-TCP) were purchased from Sigma-Aldrich (St. Louis, USA): TCS (CAS No. 3380-34-5, 99.9% of purity), 2,4,6-TCP (CAS No. 88-06-2, 98%), and 2,4-DCP (CAS No. 120-83-2, 99%). Chemical structures, transformation relationships and relevant compound information are shown in Supplementary Fig. 1 and Supplementary Table 1. Mixtures of TCS, 2,4-DCP and 2,4,6-TCP used for zebrafish exposure were designated as TDT.

2.3. Zebrafish maintenance and exposure protocols

Wild-type (AB strain) zebrafish (*Danio rerio*) were raised in dechlorinated and filtered water at 28 °C with a 14-h light:10-h dark photoperiod (light on at 8 a.m.). Zebrafish maintenance followed (Westerfield, 2000).

In order to make the TDT-exposure dose to be close to their environmentally monitored concentrations, the maximum toxicity concentration ratio of TCS, 2,4,6-TCP and 2,4-DCP, as well as an orthogonal test with five factors and three levels were determined and evaluated using Latin 3.1 software (Sharetop, Shenzhen, China). The average mortality rate and the optimal ratio among TCS, 2,4,6-TCP and 2,4-DCP ($n = 3$) were computed. Subsequently, LC50 values were determined on the basis of the optimal concentration ratio of TCS, 2,4,6-TCP and 2,4-DCP (TCS:2,4,6-TCP:2,4-DCP = 1:2:4). A series of TDT concentrations (0, 0.14, 0.28, 0.56 mg/L), which were far less than the LC_{50} values (median lethal concentration) of the mixture (2.28 mg/L) was chosen for zebrafish continuous exposure from embryos (6 hpf, hours post-fertilization) to adults (90 dpf). Prior to locomotor behavioral testing, larvae were evaluated using a microscope and any dead or malformed individuals were excluded. The TDT-exposure solutions were renewed daily to maintain stable water quality and TDT concentrations. The schematic illustration on whole experimental procedures is shown in Supplementary Fig. 2.

2.4. Behavioral assessment

2.4.1. Embryonic behavior and larval locomotion

All embryonic and larval locomotion tests were performed in 96-well plates at 27.5 ± 1 °C during the light phase between 9 a.m. and 4 p.m. The embryonic spontaneous movement per minute (times alternating tail coils) was observed for 5 min at 24 hpf, recorded using a microscope camera (SZX16, Olympus, Japan), and automatically analyzed by DanioScope (Noldus IT, Wageningen, Netherlands). For the larval locomotion experiment, larvae for 96 biological replicates were transferred to 96-well plates (one larva for each well, a total of 96 larvae for each treatment including control group), and placed into the DanioVision Observation Chamber (Noldus IT, Wageningen, Netherlands) after exposure to TDT. Video was analyzed using EthoVision XT software (Noldus IT, Wageningen, Netherlands) to track individual movement of zebrafish in each of the 96 wells.

A tapping stimulation test was conducted using 96-hpf zebrafish after a 5-min adaptation, and the tapping intensity was set at 8 levels. Video was collected for 5 min, and analyzed to compute the mean speed, which was used to appraise the sound-evoked startle reaction and adaptability.

After TDT exposure to larvae from 6 to 96 hpf, the autonomous movement and the light-to-dark test were conducted at 120 hpf. For the larval autonomous movement, video was collected for 5 min after a 5-min dark adaptation, and analyzed by software to calculate the mean velocity and distance traveled. These results were used to assess the effects of TDT exposure on early larval locomotion ability.

After a 10-min dark adaptation, the light-to-dark test began with 5 min of 100% light and 5 min complete darkness using two replicates from the light-to-dark cycle test. All locomotor activities during the light-to-dark photoperiod were analyzed, and the velocities were averaged into 1-min time bins.

2.4.2. Adult fish behavioral assessment

Four behavioral tests were performed as follows: (1)bottoming dwelling test (BDT) for general mental status and locomotor activity measurement; (2) conditioned place preference (CPP) test as a classical experimental model for drug psychiatric dependence assessment; (3) social interaction test for social preference judgment; and (4) T-maze test for cognitive ability. All tests were performed at 27.5 ± 1 °C during the light phase between 9 a.m. and 4 p.m., and the test tank was placed in a soundproof box to avoid external interferences. Videos were recorded by a camera placed 0.6 m above the tank and connected to a computer. The automated analyses of traces were performed by Ethovision XT software and all test endpoints were recorded as mean ± SD (standard deviation).

(i)Bottom dwelling test (BDT)

Zebrafish from all groups were individually tested to assess anxiogenic-like effects on their behavior in a 1.5-L trapezoidal tank (15 × 28 × 23 × 7 cm; height × top-length × bottom-length × width; Aquatic Habitats, Apopka, FL, USA) (Kyzar et al., 2012). All tanks were maximally filled with water and divided into equal upper and lower compartments by a line on the outside wall. Following a 5-min habituation period, the locomotor activity was video-recorded for 10 min to determine behavioral parameters.

(ii)Conditioned place preference (CPP)

CPP was assessed by recording zebrafish behavior in the non-preference side to measure the zebrafish guts, which likely engaged mechanisms similar to those of classical conditioning (Wang et al., 2016). The testing tank dimension was 25.5 × 15 × 20 cm (length × width × depth). Differential visual cues divided the test tank into two halves: one half was colored light-brown and the other half colored white with two black spots placed at the bottom of the tank. Depth of water was kept at 2.5 cm from the bottom to reduce stress (Wang et al., 2016). Zebrafish that favored the light-brown side escaped to avoid the white background and black spots; therefore the experimental tank was considered biased. Following a 5-min habituation period, the locomotor activity was video-recorded for 10 min.

(iii)Social interaction test

Zebrafish are social aquatic organisms that prefer swimming in cohorts. The test tank (52 × 26 × 30 cm; length × width × depth) was divided into two compartments, left zone (social zone) and right zone (non-social zone), and zebrafish could freely swim from one zone to the other (Fig. 3B). The social zone was connected to a transparent glass tank, in which 8 adult zebrafish (4 male and 4 female) were placed. Zebrafish in the social zone and transparent glass tank could see each other. In contrast, the inner side of the non-social zone was non-transparent. After a 15-min adaptation period, social interaction behavior was video-recorded for 10 min.

(iv)T-maze experiment

T-maze experiments are often used for studies of zebrafish learning discrimination or location preference behavior (Wang et al., 2016). Food bait was used as a reward in the left side ("b" zone), and tapping as a sound stimulation in the right side ("c" zone) (Fig. 3A). Prior to T-maze testing, zebrafish training was conducted for 7 days (15 min each time; 5 times per day). After training, adult zebrafish were individually placed in the starting zone ("a" zone). The baffle in the "a" zone (red line of "a" zone in Fig. 3A) was quickly taken out, and the locomotor activity was video-recorded for 3 min for subsequent software analysis.

2.5. qRT-PCR analysis and whole-mount in situ hybridization (W-ISH) for neurodevelopmental-related genes

To determine differentially expressed genes of zebrafish at the transcriptional level *in vivo*, qRT-PCR and W-ISH were performed. Total RNA from 96 homogenized zebrafish larvae in each replicate TDT exposure (0, 0.28, 0.56 and 1.12 mg/L) from 6 to 120 hpf was isolated using TRIzol regent with elfa as the endogenous reference (Mccurley and Callard, 2008). Sequences of cDNA probes for the two genes (*pax2a* and *bcl2l11*) were labeled with digoxigenin (DIG) (Supplementary Fig. 3) in W-ISH. The 6-hpf embryos were exposed to TDT at 0, 0.28, 0.56 and 1.12 mg/L, and both control and treatment groups were treated with 0.5% *N*-phenylthiourea (PTU, Aladdin, Shanghai, China). The 72- and 120-hpf larvae were collected for W-ISH (Thisse and Thisse, 2008).

2.6. Acridine orange (AO) staining and cranial vasculature observation

After TDT-exposure to zebrafish from 6 to 72 hpf at a series of concentrations (0, 0.28, 0.56 and 1.12 mg/L), 30 larvae were collected for AO staining according to Zeng and coworkers (Zeng et al., 2014). Apoptotic cells, which appeared as obvious bright spots, were identified with a fluorescence microscope (Leica, Heidelberg, Germany).

Tg (flk1:mCherry) zebrafish (CZ63), purchased from China National Zebrafish Resource Center (Wuhan, China), were used to determine the effects of TDT-exposure on cranial vasculature. Images of Tg (flk1:mCherry) zebrafish after TDT-exposure from 6 to 72 hpf (0, 0.28, 0.56 and 1.12 mg/L) were recorded with a laser scanning confocal microscope (Olympus, Tokyo, Japan).

2.7. Detection of biomarkers, ERα and ERβ

Zebrafish were exposed to varying concentrations of TDT from 6 to 120 hpf. Each group included 200 zebrafish larvae and three biological replicates. Zebrafish were accurately weighed and a 9-fold (w/v) saline or PBS buffer solution added and homogenated in an ice bath to prepare a 10% tissue homogenate. The tissue homogenate was centrifuged at 2500 rpm for 10 min, and the supernatant was collected and diluted for protein analysis. Sample protein concentration was measured using TPierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Shanghai, China). CK activity (U/mg.prot) and MDA concentration (nmol/mg.prot) were determined following manufacturer's instructions for test kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). ERα and ERβ activity (U/mg.prot) were detected by ELISA kits supplied by Shanghai Xianmian Biological Technology Co. (Shanghai, China).

2.8. Histopathological observations for brain and spinal cord tissues

To assess mechanisms responsible for abnormal changes in biomarkers and related molecules, we observed histopathological injuries to the central nervous system (brain and spinal cord) by means of haematoxylin and eosin (H&E) staining; the effects of TDT exposure on ultra-structure were also evaluated by transmission

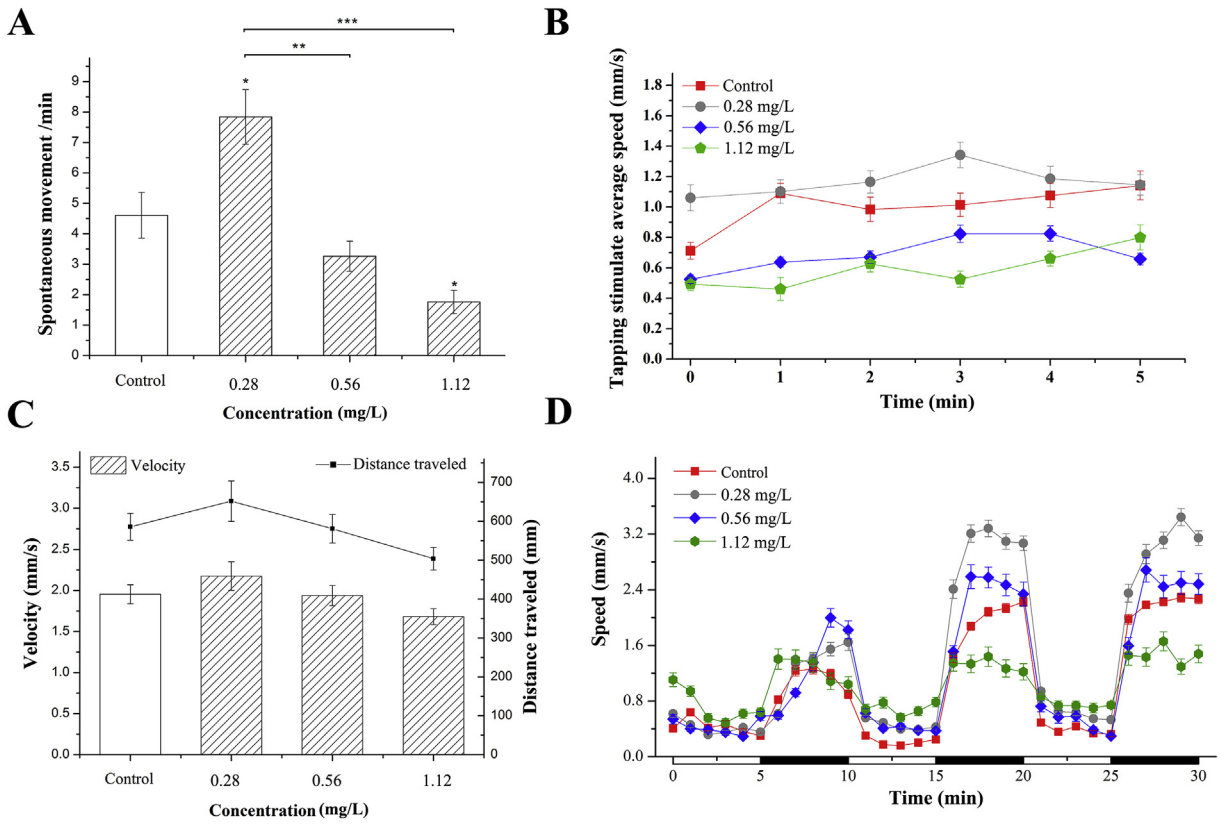

Fig. 1. Behavioral effects of TDT exposure on zebrafish embryos and larvae. Note: (1) A, Zebrafish embryonic spontaneous movements per minute after exposure to TDT from 6 to 24 hpf; (2) B, The swimming speed of zebrafish larvae, exposed to TDT from 6 to 96 hpf, when subjected to a tapping stimulation at 96 hpf; (3) C, The distance traveled and swimming speed of zebrafish larvae at 120 hpf after exposure to TDT from 6 to 96 hpf; (4) D, The swimming speed of zebrafish larvae at 120 hpf after exposure to TDT from 6 to 96 hpf when subjected to a 30-min dark-to-light photoperiod at 120 hpf; (5) "*", "***" and "****" indicate significance levels at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

electron microscopy (TEM). The isolated tissue treatments and H&E staining were conducted using standard protocols. Morphological changes and structural damage in tissues were assessed and photographed using optical microscopy (DM2700 M, Leica, Germany).

To observe ultra-structural changes, dissected tissue was cut into 1 mm^3 blocks, and fixed in glutaraldehyde at 4°Cfor 2 days. Treated tissues were observed using TEM (H7500, HITACHI, Japan).

2.9. Statistical analysis

Experimental data were reported as mean ± SD (standard deviation; the biological and technological replicates for each endpoint are listed in Supplementary Table 2). Post-hoc Tukey tests were used for multiple mean comparisons among different experimental groups. All statistical analyses were conducted with SPSS 18.0 (SPSS, Chicago, USA) using a $^{*}p < 0.05$, $^{**}p < 0.01$, or $^{***}p < 0.001$ significance level, unless otherwise stated.

3. Results

3.1. Effects of TDT on behavior of zebrafish embryos and larvae

By means of acute exposure, we investigated the lethal and teratogenic effects of single-compound TCS, 2,4,6-TCP and 2,4-DCP exposure on zebrafish embryos from 6 to 120 hpf, the EC_{50} (median effective concentration) and LC_{50} values were computed by the Boltzmann equation. In order to determine the maximum toxicity concentration ratio of TCS, 2,4,6-TCP and 2,4-DCP, an orthogonal test with five factors and three levels was conducted to acquire the optimized concentration ratio of 1:2:4 (Supplementary Table 3). According to the combined effects of TDTs on zebrafish hatching, mortality and malformation rates, two methods were used to evaluate the combined toxicity of TDT: Toxic Unit (TU) and Mixture Toxicity Index (MTI), the interactions among TDTs were shown to be partly additive toxicological effect (Cao et al., 2014). The 120-hpf LC_{50} and EC_{50} values for the TDT mixture (TCS+2,4,6-TCP+2,4-DCP) were 2.28 and 1.16 mg/L, respectively (Supplementary Fig. 4). As a result, the selected exposure concentrations (0.14, 0.28, 0.56 and 1.12 mg/L, corresponding to 0.78, 1.54, 3.09, and 6.17 μmol/L) in this study were far less than the LC_{50} values of TDTs.

Embryonic zebrafish are often used to investigate the neural networks that coordinate locomotive behavior. Zebrafish demonstrate robust locomotive behavior early in development and have relatively simple and accessible nervous systems compared to mammalian systems (Mckeown et al., 2009). Spontaneous movements averaged 4.61 (number of zebrafish tail swings per minute) for the 24-hpf embryos in the control group. When exposed to 0.28 mg/L TDT, spontaneous movement frequency was significantly ($p < 0.05$) increased to 7.84 vs 4.61 in the control group. With increasing TDT concentrations (0.56 and 1.12 mg/L), spontaneous movement frequency was prominently inhibited to 3.5 and 1.8, respectively.

Embryonic spontaneous movement is the initial movement of zebrafish when behavior becomes gradually controlled by the central nervous system with the development of the motor nervous system (Jin et al., 2009). Embryonic spontaneous movement (Fig. 1-A) suggested that mixed exposure of TCS, 2,4-DCP and 2,4,6-TCP disrupts the normal development of movement neurons and the implementation of nerve conduct function.

To further investigate the effects of TDT exposure on locomotor

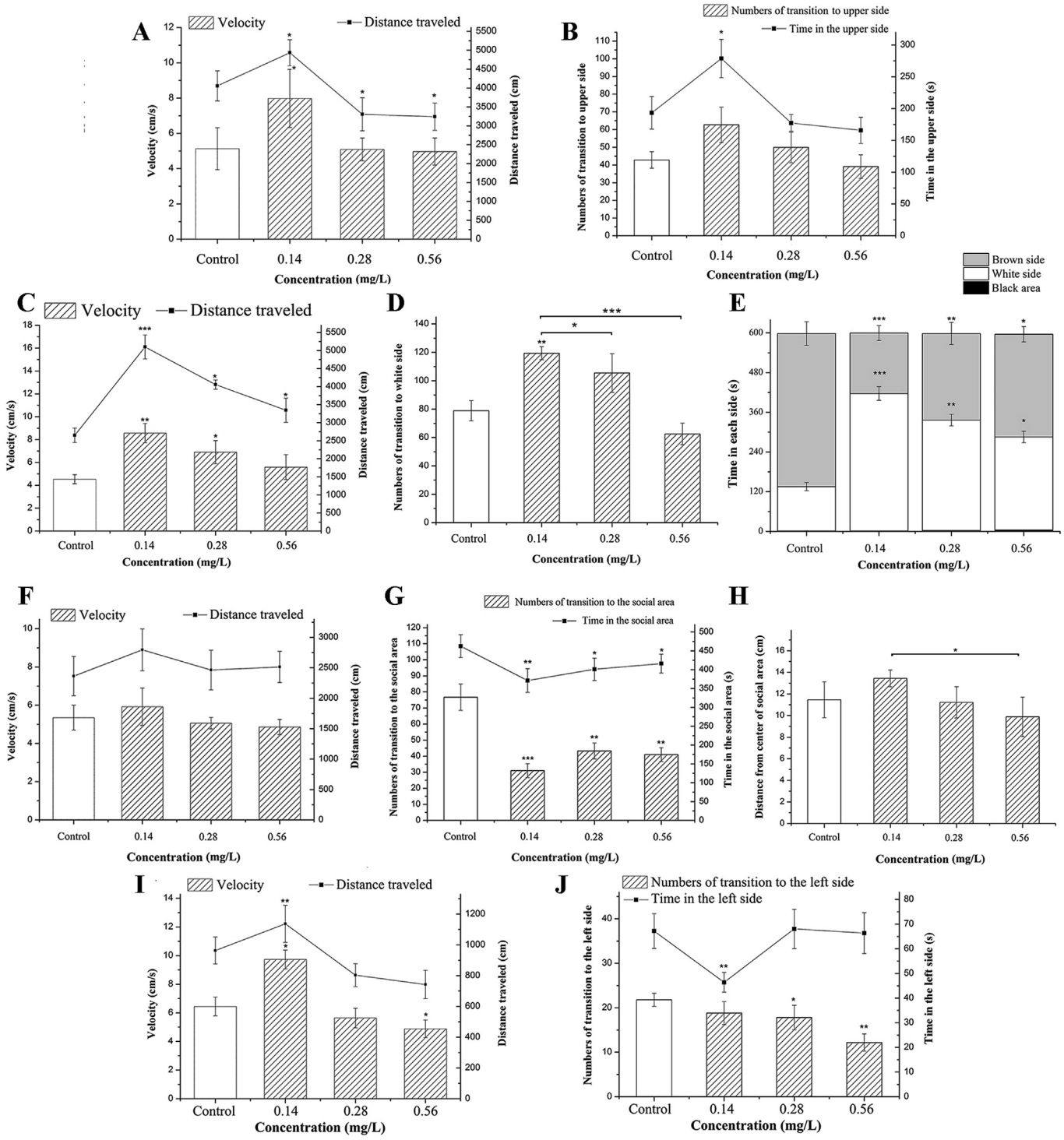

Fig. 2. Effects of TDT exposure on 90-dpf adult zebrafish behavior. Note: (1) A-B, The novel tank test; C-E, CPP test; F–H, The social interaction test; I-J, T-maze test (2) A, Mean velocity and distance traveled; B, Number of transitions to upper portion and time in the upper tank; (3) C, Mean velocity and distance traveled; D, Number of transitions to white side in the CPP test; E, Time in each side in the CPP test; (4) F, Mean velocity and distance traveled in the social interaction test; G, Number of transitions to the social area and time in the social area in the social interaction test; H, Distance from center of social area in the social interaction test; (5) I, Mean velocity and distance traveled; J, Number of transitions to the left side and time in the left side; (6) "*", "**" and "***" indicate significance levels at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

behavior of larval zebrafish, three tests were performed. Larval tapping stimulation tests showed that when larvae at 96 hpf were exposed to 0.28 mg/L TDT, a higher average swim speed was observed compared to the control group. However, average speeds were lower in the 0.56 and 1.12 mg/L TDT-exposure groups compared to the control group (Fig. 1-B). This indicates that low TDT concentrations increased zebrafish fear and restless states, but higher concentrations inhibited sensitivity to sound stimulation suggesting a retardation phenomenon.

In the 120-hpf larvae spontaneous movement test, the contrasting TDT-exposure concentrations and the control group showed similar swimming activities. Although there were some

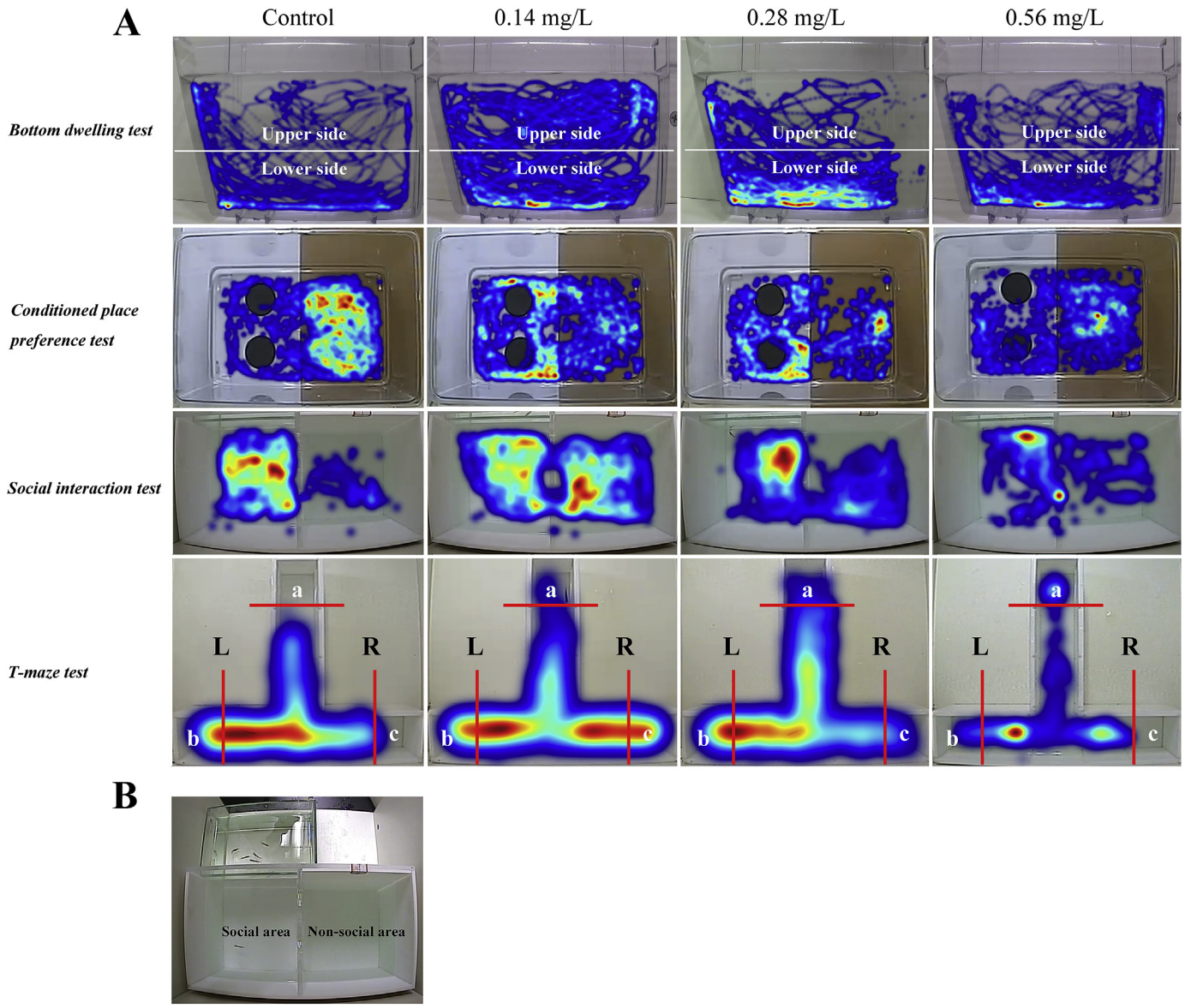

Fig. 3. Heat map of 90-dpf adult zebrafish behavior and the social interaction test experimental setup diagram. Note: (1) A, 'a' arrow, start; 'b' arrow, bait zone (left side); 'c' arrow, stimulating zone (right side); (2) B, The social interaction test experimental setup diagram; (3) blue to green = lower frequency, yellow and red = higher frequency. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

increasing or decreasing trends for the average velocity in different treatments, no significant differences were observed (Fig. 1-C).

The effects of light-to-dark photoperiod on zebrafish average swimming speed are shown in Fig. 1-D. After 8-min light-to-dark stimulation, all TDT-exposure treatments showed higher activity than the control group during the light period, especially for the 1.12 mg/L treatment. During the dark period, the 0.28 and 0.56 mg/L treatments showed prominently higher activity than the control group, but swimming speed was significantly inhibited ($p < 0.05$) in the 1.12 mg/L treatment (Fig. 1-D).

In sum, the series of locomotor behavioral tests revealed that different TDT-exposure concentrations cause abnormal movement including disturbance to movement activity, swim speed of embryos and larvae, sound response and light-to-dark rhythm.

3.2. Chronic TDT exposure effects on behavior of adult zebrafish

Sublethal TDT exposure to zebrafish from 6 hpf (embryonic stage) to 90 dpf (sexually mature stage) resulted in less than 10% mortality and no obvious morphological abnormalities, except for a slight decrease in body weight and length when compared to the control group. There was no prominent difference in sex differentiation between TDT-exposure treatments and the control group. Therefore, the TDT-exposure concentrations were set at 0, 0.14, 0.28 and 0.56 mg/L for investigating the effects of TDT on adult zebrafish behavioral characteristics (Supplementary Table 4).

(i)Bottom dwelling test

The mean swim velocity was highest ($p < 0.05$) in the 0.14 mg/L treatment with an increase of 50–60% compared to the other treatments (Fig. 2A). This phenomenon is clearly reflected in the zebrafish motion heatmap (Fig. 3A). There was a significant decrease in mean swim velocity and distance traveled for the 0.28 and 0.56 mg/L treatments when compared to the 0.14 mg/L treatment ($p < 0.05$). The number of transitions between the upper and

lower tank compartments was highest and longest ($p < 0.05$) for the 0.14 mg/L treatment group among the three TDT treatments, an increase of 10% and 50% compared to the control group (Fig. 2B). Time spent at the bottom of the tank increased for the control, 0.28 and 0.56 mg/L treatments compared to the 0.14 mg/L treatment. The number of transitions to the upper tank position and the time in the upper position were highest and longest ($p < 0.05$) in the 0.14 mg/L treatment, an increase of 46% and 44% compared to the control group. Zebrafish in the control, 0.28 and 0.56 mg/L treatments increased their time at the bottom of the tank compared to the 0.14 mg/L treatment, which indicates a preference for quiet and isolation.

In total, the bottom dwelling test demonstrated that long-term chronic TDT exposure in the 0.14 mg/L treatment led to hyperactivity and restlessness for adult zebrafish; however, with a further increase of TDT exposure concentrations, zebrafish displayed a quiet or depressed state.

(ii)Conditioned place preference (CPP)

To determine the effects of chronic TDT exposure on guts, panic reaction, behavior preference, visual sensitivity and color discrimination capacity, a series of CPP experiments were performed. The distance traveled and the numbers of transitions to the white side increased by ca. 80% and 45%, respectively, in the 0.14 mg/L treatment compared to the control. In contrast, the time spent in the white side was prolonged ($p < 0.05$) by ca. 230% in the 0.14 mg/L treatment group as compared to the control (Fig. 2C–E and 3A). Overall, the 0.14 mg/L TDT exposure led to excessive panic, restlessness and low hedging capacity. Zebrafish in the control group innately preferred the brown area vs. the bright area, and also had a preference to avoid the black dangerous area. However, the three TDT-exposure treatments resulted in a higher staying time in the bright area (white side) compared to the control group. This was especially notable for the 0.14 mg/L treatment. Additionally, higher TDT concentrations led to a higher frequency of zebrafish time spent in the black dangerous area indicating a decreased tendency to avoid or discriminate risk (Fig. 3A).

(iii)Social interaction

Zebrafish, like other fish and humans, are social and cooperative species (Wang et al., 2016). To appraise the risk for the occurrence of autism due to TDT exposure, a series of social parameters were evaluated to express social ability, clustering, psychology and so on. The mean velocity and distance traveled was similar to the changes observed for the bottom dwelling and CPP tests (Fig. 2F–H). In the control group, total swimming time in the social area was significantly higher ($p < 0.01$) than for the background (non-social) area. This indicates that zebrafish preferred social and clustering life vs. autism life. For the 0.14 mg/L treatment, time spent in the social area was decreased, but the time transition to the background side was increased demonstrating a restless state. As TDT concentrations increased to 0.28 and 0.56 mg/L, the time spent in the social area was significantly decreased ($p < 0.01$ or $p < 0.001$) by 15% and 10% as compared with the control (Fig. 2G). Additionally, the distance from the center of the social area was reduced among all TDT treatment groups, demonstrating that zebrafish preferred a quiet and isolated life under TDT stress (Figs. 2H and 3A).

(iv)T-maze experiment

T-maze is often used for appraising capacities of zebrafish memory, recognition and response sensitivity to external stimuli (Gaikwad et al., 2011). For the T-maze test, time in the bait zone (left side in Fig. 3A; "b" zone) was about 67 s in the control group, which demonstrated that zebrafish had a high memory and recognition capacity compare to other groups. As for the starting area, no zebrafish reverse movement was observed in the control group ("a" zone), and almost no movement track was found in the stimulating zone ("c" zone), suggesting that zebrafish had a high capacity for avoiding a perceived dangerous zone. For the 0.14 mg/L treatment, mean velocity and distance traveled were significantly enhanced (Fig. 2I, $p < 0.05$ or $p < 0.01$), but the time in the left side was significantly reduced ($p < 0.01$). In contrast, the 0.28 and 0.56 mg/L treatments had a significant decrease ($p < 0.05$ or $p < 0.01$) in the number of transitions to the left side compared to the control (Fig. 2J). These results demonstrate that TDT exposure severely affects zebrafish memory for the bait or stimulating side, resulting in movement without destination. With more severe TDT stress, some zebrafish were unable to identify direction, and reversely moved toward the starting line ("a" zone) as shown in Fig. 3A heat map. This was especially notable for the 0.56 mg/L treatment where the time spent in the narrow "a" zone was relatively long, suggesting that zebrafish tended to be lonely and autistic.

3.3. qRT-PCR and W-ISH characterization of neurodevelopmental-related genes

To further investigate the molecular mechanisms associated with the induced abnormal behavior, 11 neurodevelopmental-related genes were selected for qRT-PCR analyses on the basis of previous studies (Mihaela et al., 2014). The qRT-PCR results showed three trends for the 11 neurodevelopmental-related genes due to TDT exposure. The genes, *pax6a*, *hoxb1b* and *Foxo3a* were up-regulated with increasing TDT concentrations and were concentration dependent. These genes are involved in development of the nervous system, such as *pax6a* and *hoxb1b* roles as brain marker genes expressed in various parts of the brain. *FoxO3a* is essential for the maintenance of neural development (Peng et al., 2010). Additionally, 7 other genes (*pax2a*,*bcl2l11, fasL, ncam1a, ERα, PSEN1 and app*) were up-regulated in the 0.28 mg/L TDT treatment. However, with a further increase of TDT concentrations (0.56 and 1.12 mg/L), these 7 genes were down-regulated except for *fasL* and *ERα*. The *pax2a* gene participates in the development of the protocerebrum and deutocerebrum border. The *bcl2l11*and*Fas* genes are involved in neural apoptosis (Li et al., 2014) and *ncam1a* plays an important role in the development of the zebrafish nervous system (Langhauser et al., 2012). The *PSEN1* and *app* genes are associated with neuropathological lesions typically observed in Alzheimer's disease and have been identified as definite pathogenic genes for Alzheimer's disease (Takao et al., 2002). Abnormal changes in the above-mentioned genes possibly promoted apoptosis of neuron cells, which was verified by AO staining (Fig. 5C), and inhibited regeneration of new neurons and stem cells. ERα and ERβ are broadly distributed in the brain and closely related to a wide variety of nervous system diseases (Mehra et al., 2005).

Based on qRT-PCR data (Fig. 4A and B), two differentially expressed genes, *pax2a* and *bcl2l11*, were selected for W-ISH to directly observe the expression position and changes in whole larval zebrafish. As shown in Fig. 4C, *pax2a* was mainly expressed in 72-hpf zebrafish brain (protocerebrum, deutocerebrum and tritocerebrum), ear, eye, and anterior spinal cord, and expressed at low levels in pancreas, heart and intestinal epithelia cells. As compared with the control group, expression of *pax2a* significantly increased ($p < 0.01$) mainly in brain, spinal cord and intestinal epithelia of the 72-hpf larval zebrafish in both 0.28 and 1.12 mg/L treatments. When comparing the 1.12 and 0.28 mg/L treatments, a prominently decreased expression was observed for the spinal cord of the 1.12 mg/L treatment (Supplementary Fig. 5A). According to the

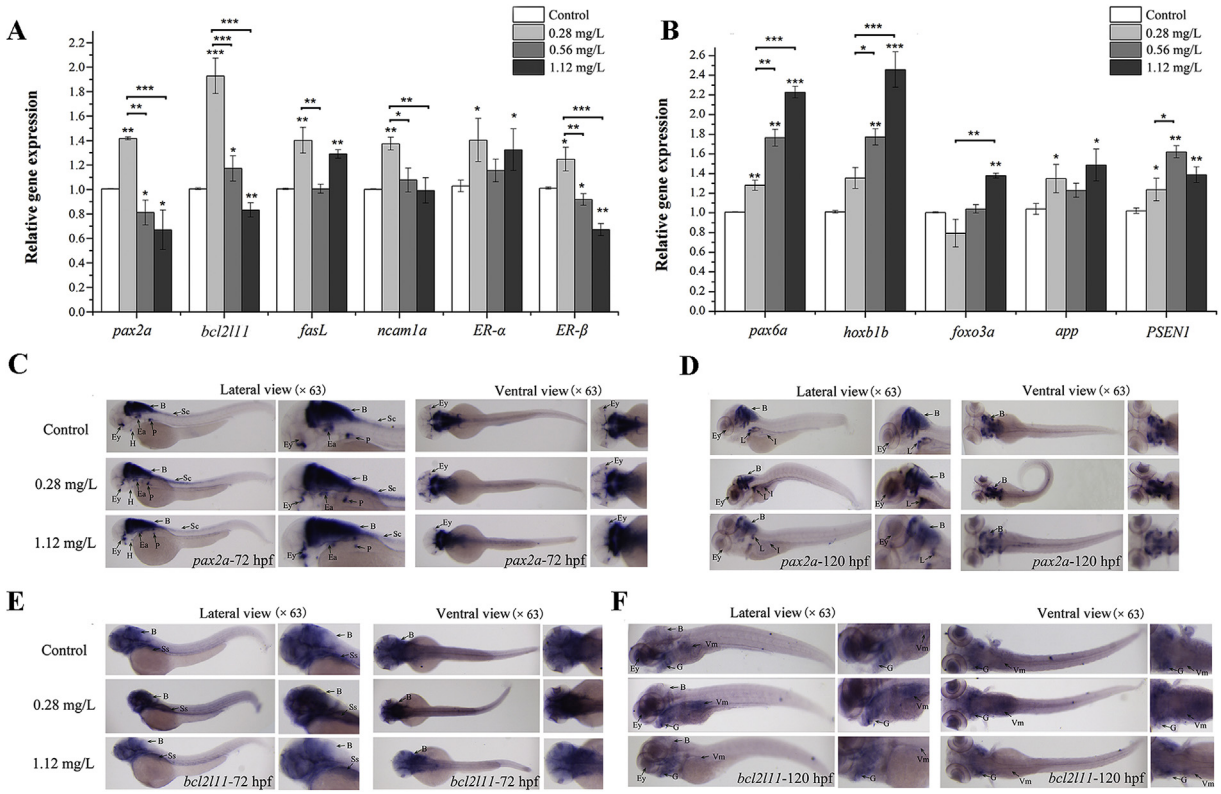

Fig. 4. Differential expression of the neurodevelopmental-related genes by qRT-PCR and W-ISH of *pax2a* and *bcl2l11* genes. Note: (1) A-B, Differential expression of the neurodevelopmental-related genes by qRT-PCR; (2) "*", "**" and "***" indicate significance levels at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively; The significance codes apply to comparisons among the different experimental groups; (3) C, W-ISH of *pax2a* expression in 72-hpf zebrafish larvae; (4) D, W-ISH of *pax2a* expression in 120-hpf zebrafish larvae; (5) F, W-ISH of *bcl2l11* expression in 72-hpf zebrafish larvae; (6) G, W-ISH of *bcl2l11* expression in 120-hpf larvae; (7) Abbreviations in Fig. 4C–F: B, brain; P, pancreas; Sc, spinal cord; H, heart; I, intestinal; L, liver; Ey, eye; Ea, ear; Ss, swimming sac; G, gills; Vm, visceral mass; (8) Fig. 4C–F shows the expression of hybridization signals by lateral and ventral view in control and treatment groups.

ventral view of the 0.28 mg/L treatment, the expression of *pax2a* was enhanced in the eye compared to the control group (Fig. 4C). In contrast, *pax2a* was mainly expressed in brain, liver, pancreas and intestines, but not in spinal cord and eye of the 120-hpf larval zebrafish (Fig. 4D).

The *bcl2l11* gene was mainly expressed in brain, visceral mass and skeleton of 72-hpf larval zebrafish with a significant increase in the 0.28 mg/L treatment and decrease in the 1.12 mg/L treatment compared to the control group (Supplementary Fig. 5B). Its expression was most prominently changed in the swimming bladder and skeleton. As for the 120-hpf larval zebrafish, *bcl2l11* was mainly expressed in the visceral mass and gills, while expressed at low levels in skeleton and protocerebrum (Fig. 4F). The 120-hpf larval zebrafish showed similar gene expression trends as found for the 72-hpf larval zebrafish. Overall, the W-ISH of *pax2a* and *bcl2l11* was in agreement with their corresponding qRT-PCR results. The expressions of both *pax2a* and *bcl2l11* were mainly in the brain, and thus their abnormal expressions under TDT exposure would be expected to lead to behavioral abnormalities.

3.4. Detection of biomarkers, ERα and ERβ

Zebrafish and mammals have high similarity in energy metabolism, and their locomotor behavior is a direct result of nerve conduction, muscle contraction, and energy transfer (Yin et al., 2014). Creatine kinase (CK) catalyzes the reversible transfer of phosphorylases between adenosine diphosphate (ADP) and creatine phosphate, which plays an important role in cell energy metabolism. CK activity was significantly increased ($p < 0.05$) in the 0.28 mg/L treatment, which supports the high swimming velocity and excited state at low-level TDT exposure. These results are consistent with the above-mentioned behavioral tests for both larval and adult zebrafish. The malonaldeyhde (MDA) content in serum and tissue represents the rate and intensity of lipid peroxidation *in vivo*, which indirectly reflects the degree of free radical damage in the body and serves as an important indicator of tissue damage (Huang et al., 2011). MDA concentrations increased with increasing TDT-exposure concentrations (Fig. 5A). When excess MDA accumulates in tissues, severe lipid peroxidation occurs resulting in further damage to organs. No significant changes in ERα were observed for TDT treatments compared to the control group. In contrast, ERβ activity increased significantly ($p < 0.05$) in the 0.28 mg/L treatment, while the 0.56 and 1.12 mg/L treatments were similar to the control group (Fig. 5B).

Overall, the integrated results from qPCR, ELISA and biomarker detection demonstrated that the 0.28 mg/L TDT treatment resulted in abnormal energy metabolism and neurological damage.

3.5. Acridine orange (AO) staining and microscopic observation

The cranial vasculature is essential for the survival and development of the central nervous system and is important in stroke and other brain pathologies (Shima and Mailhos, 2000). Images of Tg (flk1:mCherry) 72-hpf zebrafish using a laser scanning confocal microscope showed prominent cranial vasculature ablation (Fig. 5D). In the control group, the cranial vascular network was clear and integrated, but after TDT exposure became disordered with more branches and mild vascular ablation. In the 1.12 mg/L

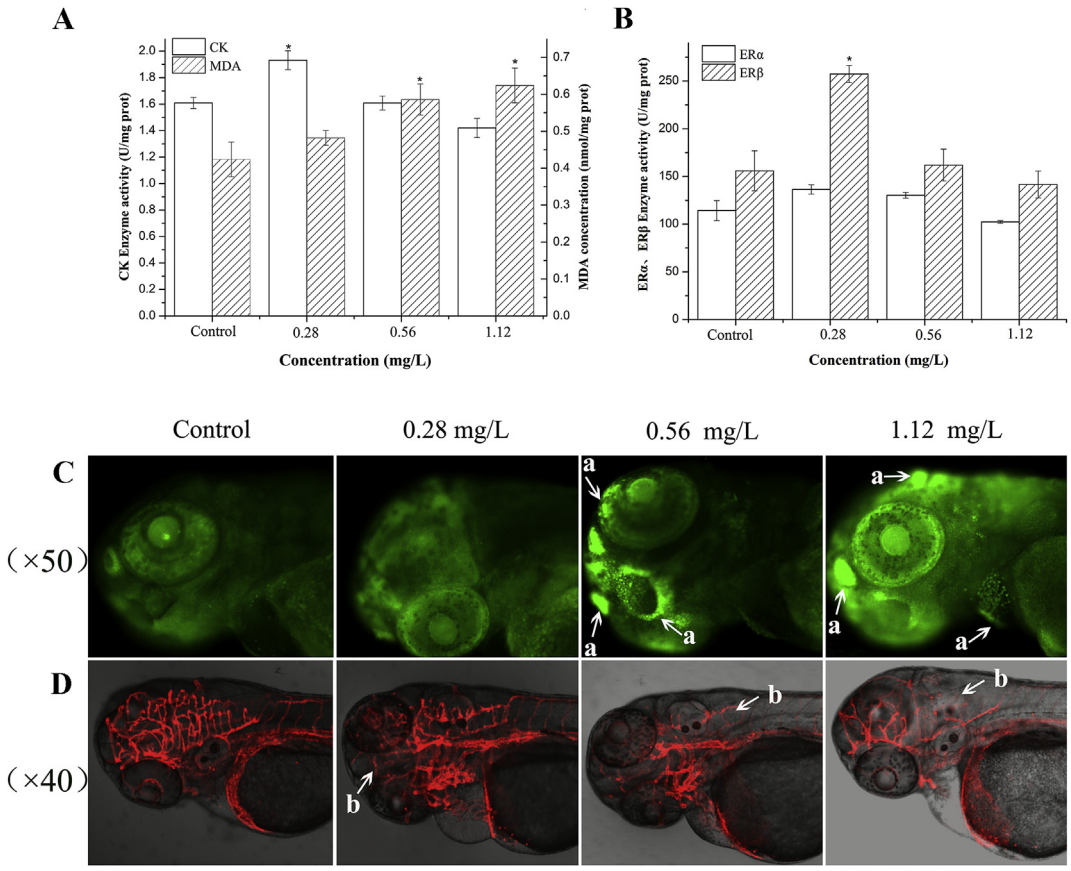

Fig. 5. Detection of biomarkers, ERα and ERβ, apoptosis of zebrafish brain by AO staining and the changes of cranial vasculature after TDT exposure from 6 to 72 hpf. Note: (1) A, CK activity (U/mg.prot) and MDA (nmol/mg.prot) concentration; (2) B, ELISA for ERα and ERβ activity (U/mg.prot); (3) "*" indicates significance at $p < 0.05$ level. (4) C, Lateral view in control and treatment groups after AO staining; 'a' arrow indicates apoptosis signal in the brain; (5) D, Changes in networks of cranial vasculature; 'b' arrow shows the cranial vascular ablation.

treatment, a large area of vascular ablation was observed with almost complete disappearance of vascular branches and a smaller coverage range (Fig. 5D). These results demonstrate that TDT exposure seriously affects the normal vascular network of the head. Blood vessels and nerve fibers are distributed throughout the body in an orderly pattern, often alongside one another. Thus, these results indicate that TDT exposure may affect the development of the zebrafish embryonic nervous system.

To examine the effects of TDT exposure on development, we investigated apoptosis in embryos during their development. In the control group, natural apoptotic phenomenon was observed in the brain. In contrast, TDT-exposure groups displayed apoptotic cells stained with AO primarily in the head that showed a significant concentration dependence ($p < 0.05$) (Fig. 5C). Low TDT concentrations (0.28 mg/L) resulted in apoptosis, while high concentrations (1.12 mg/L) led to widespread apoptosis in larval zebrafish. These results demonstrate that zebrafish brain development was very sensitive to TDT concentration.

3.6. Histopathological analysis of brain and spinal cord tissues

The nervous system contains two main categories or types of cells: neurons and glial cells. Following HE staining of adult zebrafish brain, ventriculomegaly ("b"), decreased number of neurons ("a"), glial cell proliferation and formation of glial scars ("c") were observed (Fig. 6A–D). These observations indicate the occurrence of a neuronal abnormal apoptosis phenomenon in the periglomerular gray zone (PGz) (Fig. 6C–D). In the control group, brain PGz showed oval nucleus in the neurons, evenly distributed chromain, clear and complete nuclear membrane, abundant free ribosome in cytoplasm, and an intact organelle structure with granular endoplasmic reticulum and mitochondria, as well as a tight cell junction (Fig. 6-E and I). For the 0.56 mg/L TDT treatment, the ultra-structure of neurons changed to varying degrees and showed concentration-dependent nuclear pyknosis, smaller nucleus, more heterochromatin, ablation and swelling of the nuclear membrane, reduced free ribosome, ablation of cytoplasm, decreased cell junction, and even intercellular separation and vacuolization. The tissue damage observed by TEM was consistent with that documented by HE staining, reflecting that upon TDT exposure pyknosis and apoptosis of the nucleus possibly resulted in a narrow PGz, abnormal signal transduction and further behavioral and memory disorders.

Following HE staining of the adult zebrafish spinal cord, uniform distribution for glial cells, compact fiber structure and clear nucleus were observed in the control group (Fig. 7A). In contrast, loose and hollow fiber structures were observed in the 0.14–0.56 mg/L treatments (Fig. 7B–D). For the 0.28 and 0.56 mg/L treatments, the HE staining of neuronal nucleus became deeper and glial cells were accumulated (Fig. 7C and D). In comparison, the 0.56 mg/L treatment led to necrosis of the neurons, around which many cavities occurred, and the neurons were surrounded by inflammatory cells, which are common in brain and infectious diseases of the spinal cord such as polio (Fig. 7D).

TEM observations of the control group showed oval astrocyte nucleus, regular distribution of chromatins, clear nuclear

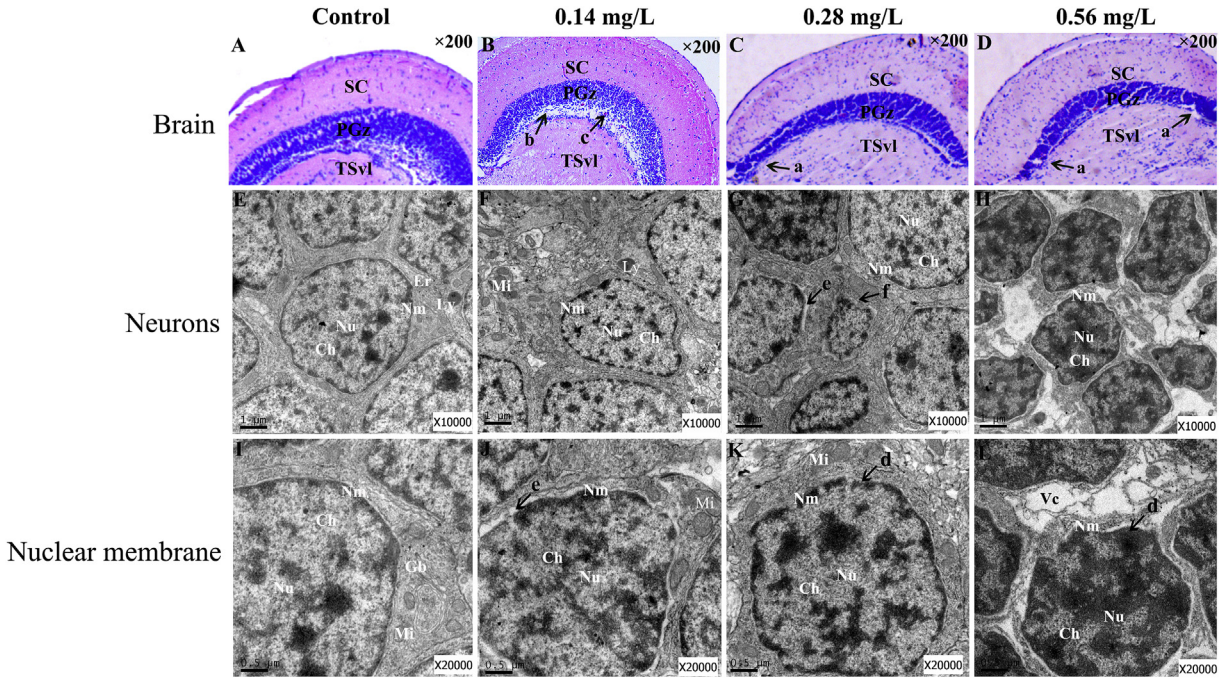

Fig. 6. Histopathological observations on adult zebrafish brain. Note: (1) A, HE dyeing of adult zebrafish brain; (2) A, E and I: Control group, B-D, F-H and J–N: TDT treatment group; (3) Abbreviations in Fig. 7A–D: SC, stratum marginale; PGz, periglomerular gray zone; TSvl, ventrolateral nucleus of semicircular torus; (4) E-N, TEM images of neurons in adult zebrafish periglomerular gray zone (PGz); (5) Nu: nucleolus, Nm: nuclear membrane; Ch, chromatin; Mi, mitochondria; ER, endoplasmic reticulum; Gb, Golgi body; Ly, lysosome; Vc, vacuole; (6) "a arrow" in Fig. 6C and D shows the decreased number of neurons in TDT-exposure treatments; "b arrow" in Fig. 6B shows ventriculomegaly; "c arrow" shows glial cell proliferation and the formation of glial scar; "d arrow" in Fig. 6J and M shows the nuclear membrane shrinkage; "e arrow" in Fig. 6N shows nuclear membrane edema; and "f arrow" in Fig. 6H shows the nuclear membrane dissolved.

membrane, and more mitochondria in astrocytes (Fig. 7D). However, different TDT-exposure levels demonstrated a concentration-dependent pyknosis of nucleus ("e"). For example, the 0.28 mg/L treatment produced swelling of astrocytes (Fig. 7K,"h"), while the 0.56 mg/L treatment resulted in more heterochromatin, organelle damage, loosened cytoplasm and vacuolar degeneration (Fig. 7L, "f"). The neurons in the control group had oval motor neurons in spinal gray matter, regular distribution of chromatin, clear nuclear membrane, and intact organelles (Fig. 7M). In contrast, TDT exposure led to pyknosis of nucleus ("e"), smaller free ribosome, reduced mitochondria and endoplasmic reticulum in the 0.14, 0.28 and 0.56 mg/L treatments (Fig. 7N–P). It was noteworthy that large cavities in cells ("f") and vacuolization resulting from fractured mitochondrial cristae ("g") occurred in the 0.28 and 0.56 mg/L treatments (Fig. 7O and P).

4. Discussion

TCS, 2,4-DCP and 2,4,6-TCP are known endocrine disruptors and they further affect thyroid functions and live-birth index in rats (Chammui, 2017). Previous studies reported the effects of BPA on early neurodevelopment (vom Saal and Hughes, 2005), but there is a paucity of information concerning neurotoxicity to zebrafish embryonic development under TDT exposure. TDT may lead to the abnormal development of the nervous system by means of affecting key molecules of larval zebrafish brain development such as thyroid hormone receptors in plasma and transcription factor receptor (ahr) (Paul et al., 2013). In order to evaluate neurotoxicity to zebrafish larvae and elucidate possible molecular mechanisms on abnormal behavior, we tested a series of behavioral parameters and analyzed the abnormal changes of locomotor- and neurotoxity-related molecules at transcriptional and translational levels. Behavioral parameters and molecular expression changes were determined at three critical stages (24 hpf, embryonic stage; 72 hpf, larval stage; 120 hpf, another important larval window stage). In the light-to-dark photoperiod experiment on 120 hpf larval zebrafish, the vertebrate OFF-retinal and ON-retinal ganglion cells were affected by TDT exposure. Transcription factor pax2a has been reported to have a prominent effect on eye development (Hoyle et al., 2004). In the CPP and social interaction tests, abnormal behavior at low TDT concentrations (0.14 mg/L) possibly resulted from a visual disorder. W-ISH and qPCR results showed that the transcriptional level of *pax2a* was significantly different in the 0.28 mg/L treatment compared to the control group. These results indicate that TDT may have an indirect effect on larval zebrafish brain development.

Because TCS is prevalent in our environment, it raises great concern for pregnant women and infants (Casas et al., 2011). Environmental estrogens have different effects on estrogen and androgen receptors, which are a function of biological species and toxicant concentrations (Zhang et al., 2002). Herein, the 0.28 mg/L treatment led to changes of ERα at the transcriptional level, and also changes in *ERβ* at both transcriptional and translational levels. *ERβ* is involved in the regulation of advanced brain functions such as learning, memory and neurodegenerative diseases. As a result, the *ERβ* gene knock-out seriously affects learning and memory capacities (Liu and Dluzen, 2007). In this investigation, TDT exposure led to abnormal expression of the *ERβ* gene, and further affected zebrafish memory capacity, which was reflected in the T-maze experiment. In behavioral tests, low-level exposure (0.28 mg/L) led to high zebrafish activity, whereas high concentration (1.12 mg/L) contributed to lower activity. Based on biomarker detection, CK increased in the 0.28 mg/L treatment, which indicates that low-level exposure can lead to abnormal energy metabolism and neurological disease. TDT-exposure could decrease the memory capacity of adult zebrafish, which maybe contribute to

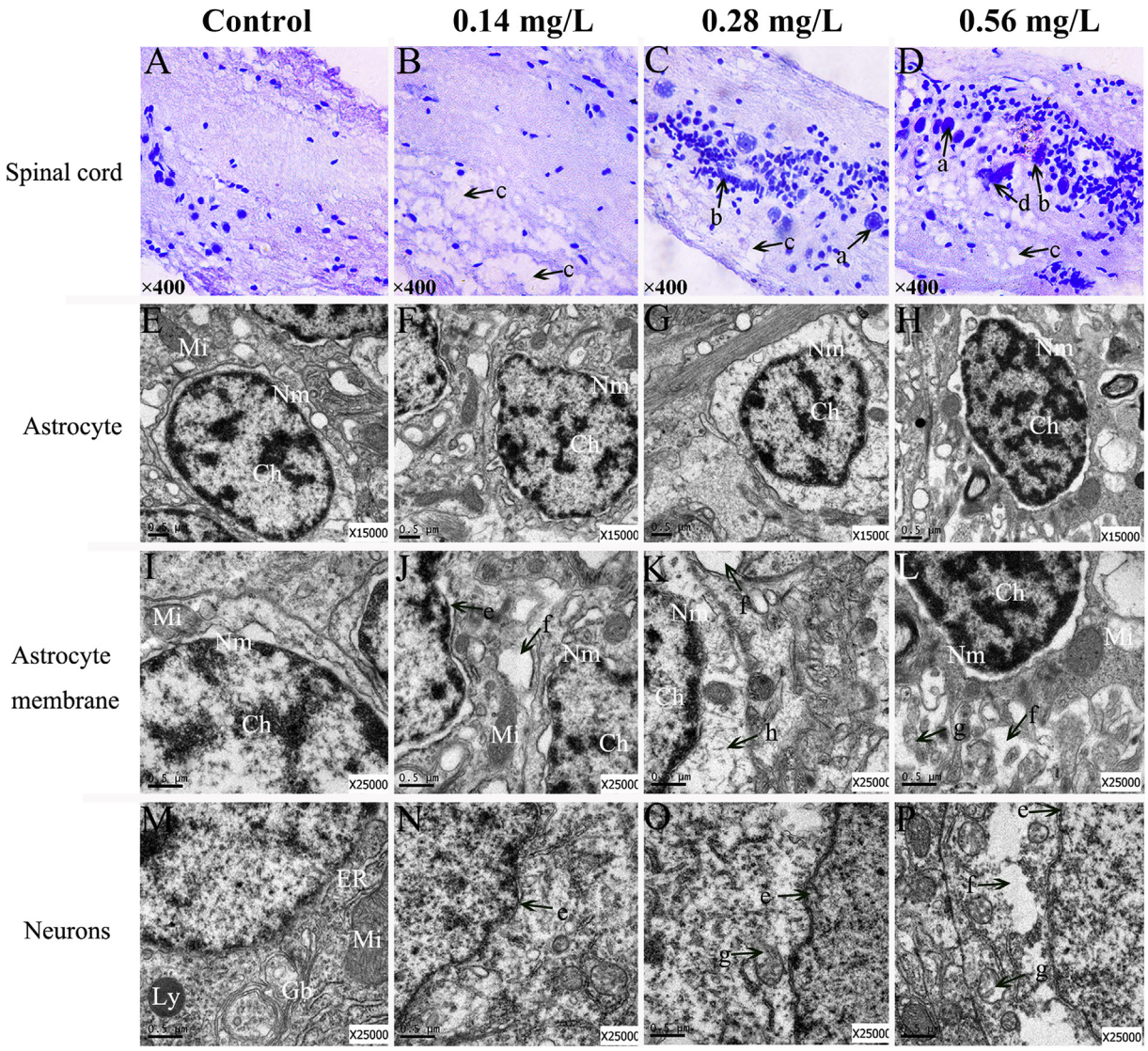

Fig. 7. Histopathological observations on adult zebrafish spinal cord. Note: (1) A-D, HE dyeing of adult zebrafish spinal cord; (2) A, E, I and M: Control group, B-D, F–H, J-N, M–P: TDT treatment groups; (3) E-H, I-L, TEM images of astrocyte and astrocyte membrane in adult zebrafish spinal cord; (4) Nm: nuclear membrane; Ch, chromatin; Mi, mitochondria; ER, endoplasmic reticulum; Gb, Golgi body; Ly, lysosome; (5) "a arrow" in Fig. 7C–D shows the staining of neuronal nucleus became deeper in TDT-exposure treatments; "b arrow" in Fig. 7C–D shows the glial cells were accumulated; "c arrow" in Fig. 7A–C shows loose and hollow fiber structures; "d arrow" in Fig. 7D shows necrosis of the neurons, around which many cavities occurred, and the neurons were surrounded by inflammatory cells; "e arrow" in Fig. 7J–L and N–P shows pyknosis of nucleus; "f arrow" in Fig. 7J–L and P shows vacuolar degeneration; "g arrow" in Fig. 7L,O and P shows fractured mitochondrial cristae.

Alzheimer's disease. Alzheimer's disease is a central nervous system degenerative disease mainly appearing as progressive cognitive and memory impairment; although the specific pathogenesis is not clear, it may be related to estrogen and its receptor (Andreeva et al., 2017). Two established Alzheimer's disease-related genes (*PSEN1* and *app*) were up-regulated in the 0.28 mg/L TDT treatment, which further supports the premise that TDT's neurotoxicity resulted in zebrafish behavioral abnormality in the T-maze experiment.

Previous studies reported a variety of behavioral tests and models such as the open field test, shoaling test, and impoverished housing, among which the light-to-dark test, CPP, T-maze and BDT were the most popular experimental models for zebrafish behavioral studies (Stewart et al., 2012) (Kysil et al., 2017); investigated the comparative analyses of zebrafish anxiety-like behavior using a novel tank test (NTT) and light-dark test (LTD), and found that some NTT and CPP endpoints, and endocrine (cortisol) levels were closely related to anxiety. Our results are consistent with these investigations demonstrating that CPP, BDT, ERα and ERβ were linked with anxiety- or autism-like diseases. Although there are a large number of behavioral endpoints, an integral parameter that reflects the level of zebrafish abnormal behavior is still missing. Therefore, creation of an integral index based on multiple factors and endpoints may be warranted. These findings may help optimize drug screening procedures by choosing more appropriate models and endpoints for testing anxiolytic, anti-autism and Alzheimer's drug treatments.

Locomotive behavior in vertebrates, such as swimming, relies on neural networks in the brain and spinal cord. Histopathological observations demonstrated that TDT exposure might inhibit the development of primary motor neurons such as pyknosis of the nucleolus and cavitation of cytoplasm in the brain and spinal cord. MDA content showed a positive concentration-dependence on TDT, and the high TDT-exposure levels produced severe tissue damage.

We believe that the tissue damage documented in the histopathological observations might be an appropriate metric for evaluating behavioral abnormality. The qRT-PCR results showed that TDT exposure significantly affected the expression of marker genes (*pax2a, pax6a and hoxb1b*) and apoptosis-related genes (*Foxo3a, bcl2l11 and fasL*) in larval zebrafish. These molecular changes might result in disorders of brain and spinal cord tissue, which were further supported by W-ISH and AO staining. However, further investigations are required to develop detailed mechanisms linking TDT exposure to histopathological changes. Overall, the results obtained in this study promote the application of zebrafish as a vertebrate model organism for investigating inborn disorders of metabolism and pharmaceutical treatments, as well as for assessing behavioral abnormalities related to these disorders.

5. Conclusions

In this investigation, a series of experiments were conducted to analyze the effects of TDT exposure on zebrafish swimming behavior, which included bottom dwelling, conditioned place preference, social interaction and T-maze tests. Under joint exposure of TCS, 2,4-DCP and 2,4,6-TCP from embryonic and adult stages (24 hpf-90 dpf), zebrafish showed a series of abnormal behavior such as anxiety-like, hyperactivity, restlessness, excessive panic, autism and memory disorder symptom. Low TDT-exposure (0.28 mg/L) promoted zebrafish swimming activity, but high concentration treatments (0.56 and 1.12 mg/L) inhibited movement behavior. AO staining at 72-hpf zebrafish demonstrated that TDT-exposure produced vascular ablation in the head, as well as the occurrence of massive apoptosis in the brain. Also, HE staining and TEM observation for adult zebrafish showed pyknosis of nucleus. qRT-PCR and W-ISH verified that TDT-exposure significantly affected the expressions of neurodevelopment-related genes (*pax2a, pax6a and hoxb1b*) and apoptosis-related genes (*Foxo3a, bcl2l11 and fasL*). Moreover, the changes of biomarker and estrogen receptor indicated TDT acute exposure led to abnormal energy metabolism and neurological diseases. These observations allow us assessment of ecological risk to co-existence of TCS and its two derivatives, and also further our understanding on the molecular mechanisms for zebrafish abnormal behavior in response to pollutant stress.

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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.2017.11.106.

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