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Emerging contaminant 1,3,6,8-tetrabromocarbazole induces oxidative damage and apoptosis during the embryonic development of zebrafish (*Danio rerio*)

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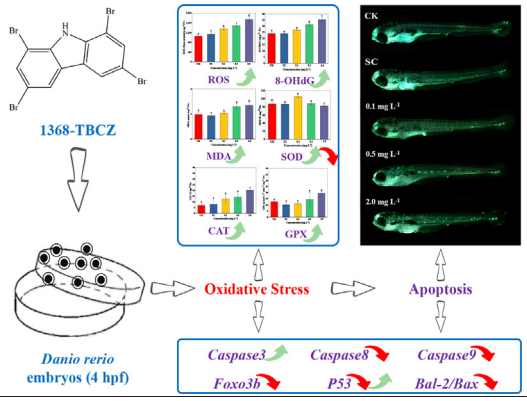
HIGHLIGHTS

- The 96-h LC50 of 1368-TBCZ to the fish embryo is greater than 2.0 mg L^{-1}.
- The hatching and malformation rates of embryos were slight affected by 1368-TBCZ.
- Exposure to 1368-TBCZ inhibited the skeletal and heart development of zebrafish.
- 1368-TBCZ induced oxidative stress, DNA damage and apoptosis in larvae zebrafish.

GRAPHICAL ABSTRACT



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ABSTRACT

Since polyhalogenated carbazoles (PHCs) have been widely detected at high concentrations in multiple environmental media in recent years, the health risk of exposure to these compounds has drawn increasing attention. Most studies have mainly focused on their dioxin-like toxicity, which is induced through the AhR pathway, because PHCs have structures similar to those of polychlorinated dibenzofurans (PCDFs). In addition, most xenobiotic compounds induce oxidative stress in organisms, which is a more common mechanism of toxicity induction. However, there is limited information regarding the oxidative stress and damage induced by PHCs in vivo. The PHC 1,3,6,8-tetrabromocarbazole (1368-TBCZ) is detected at high concentration and frequency. In the present study, the toxic effects (acute toxicity, developmental toxicity, oxidative stress, and apoptosis) induced by 1368-TBCZ at three different concentrations were investigated using zebrafish embryos. It was concluded that the 96 h median lethal concentration (LC_{50}) of 1368-TBCZ for zebrafish embryos was greater than 2.0 mg L^{-1}. The results showed that 1368-TBCZ had little effect on the hatching rate of zebrafish embryos. However, 1368-TBCZ at 0.5 and 2.0 mg L^{-1} inhibited skeletal and cardiac development. It promoted ROS production, CAT enzyme activity, lipid peroxidation, DNA damage, and apoptosis, even at the lowest dose (0.1 mg L^{-1}). In addition, 1368-TBCZ influenced oxidative stress-related gene expression, upregulating the expression of *caspase 3* and *p53* at 2.0 mg L^{-1} and inhibiting the expression of *caspase 9*, *FoxO3b*, and *Bcl-2/Bax*. The present study comprehensively evaluated 1368-TBCZ-induced toxicity in zebrafish, providing valuable data for better evaluation of the potential risks posed by this PHC.

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1. Introduction

Since polyhalogenated carbazoles (PHCs) have been widely detected at high concentrations in multiple environmental media in recent years, they have attracted increasing attention as emerging contaminants (Lin et al., 2016). It has been reported that most PHCs are not target compounds during human synthesis; they are mainly generated as byproducts during the synthesis processes of indigo dyes (Parette et al., 2015) and photoelectric materials (Dumur, 2015; Fang et al., 2016), and the disinfection process of drinking water (G.W. Wang et al., 2019). Moreover, some natural processes (Fromme et al., 2018) including forest fires, bacterial secretion (Ji et al., 2019b) and the biological transformation of carbazoles, are also potential sources of PHCs in the environment.

Multiple sources contribute to the widespread distribution of PHCs in the environment, with concentrations equally to or even higher than those of some conventional contaminants, such as polybrominated diphenyl ethers (PBDEs) (Kuehl et al., 1984; Qiu et al., 2019; Zhu and Hites, 2005). Researchers measured the concentrations of different PHCs in soils and found that they ranged from 13.5 ng g^{-1} to 1167 ng g^{-1} (Mumbo et al., 2015; Reischl et al., 2005; Trobs et al., 2011). Fromme et al. (2018) examined PHCs in dust and air samples, with the median (95%) concentration of the PHCs detected ranged from 1.2 to 13.3 ng g^{-1} in dust samples and 6.1 to 7.7 pg m^{-3} in air samples. However, most PHCs are found in aquatic systems, such as sediment. Wu et al. (2017a) showed that the total concentration of PHCs in the sediments of Lake Tai (China) reached as high as 15.8 ng g^{-1} dry weight (dw), and the median value was 1.54 ng g^{-1} dw. Qiu et al. (2019) studied the distribution of PHCs in surface sediments of Sanmen Bay in the East China Sea and found that PHCs were ubiquitous in sediments, and the concentration of the $\sum$PHCs ranged from 7.7 to 17.5 ng g^{-1} dw (the median value was 11.3 ng g^{-1} dw). Guo et al. (2017) indicated that PHCs amounted to more than 3000 tons in the sediments of the upper reaches of the Great Lakes. Wu et al. (2018) studied the PHCs in the Great Lakes (United States) and reported that the median concentration of PHCs ranged from 54.7 to 154 ng g^{-1} lipid weight (lw) or 6.8 to 28.0 ng g^{-1} wet weight. It has also been reported that PHCs can accumulate in organisms. Wu et al. (2017b) reported that the median concentration of PHCs ranged from 33.7 to 164 ng g^{-1} lw in different aquatic organisms (e.g., bivalves, sport fish, and harbor seal) in the San Francisco bay. The median concentrations of PHCs were 0.1, 0.34, and 0.63 for DDTs, PBDEs, and chlordane, respectively, in the fish of the Great Lakes (Wu et al., 2018).

The widespread distribution and high environmental concentrations of PHCs and the health risk posed by these compounds have also drawn attention, but the toxicology data on PHCs are insufficient to date. Because PHCs have a structure similar to that of dioxin, the first phase of risk studies on PHCs focused on their dioxin-like toxicity, especially the response of dioxin-like toxicity biomarkers such as CYP1A and CYP1B in cell lines in vitro. These studies confirmed that some PHCs can lead to toxicity to organisms by activating the aryl hydrocarbon receptor (AhR), which is considered a classical mechanism for the biological effects of most dioxins (Ji et al., 2019a; Ji et al., 2019b; Tian et al., 2018). Previous studies (Mumbo et al., 2015; Riddell et al., 2015) showed that PHCs have dioxin-like activity in rat hepatocellular carcinoma cells and in Ah-responsive MDA-MB-468 breast cancer cells. Ji et al. (2019a) showed that most of the PHCs measured produced an AhR agonist effect similar to that of dioxin and changed the expression levels AhR downstream genes. The toxicity of 2,3,6,7-tetrachlorocarbazole (2378-TCCZ) and 27-DBCZ to zebrafish development, particularly the heart, were identified (Fang et al., 2016); and showed that these tested PHCs induced significant toxicity in the heart development of zebrafish. Ji et al. (2019b) studied the biotoxicity of 27-DBCZ, and the transcriptome analysis showed that 90 genes in zebrafish showed different expression after exposure to 27-DBCZ, and many of the affected pathways were related to AhR activation, showing some developmental toxicity consistent with AhR activation.

The biological toxicity of compounds is often induced through a variety of pathways. The AhR pathway is a typical way for dioxin-like compounds to cause biological toxicity. In addition, most compounds induce oxidative stress when they enter the organism. Wang et al. (2020) believed that oxidative stress is one of the mechanisms of toxic effects caused by external contamination and that it can change the immune function of an organism, causing damage to cell components and, in severe cases, even leading to apoptosis. Tian et al. (2019) pointed out that oxidative stress is related to developmental toxicity in zebrafish embryogenesis and is one of the causes of abnormal embryonic development. However, there is limited information regarding the effects of 1368-TBCZ on oxidative stress and DNA damage in aquatic organisms.

One of the most abundant PHCs found in the environment (Pan et al., 2019), 1,3,6,8-tetrabromocarbazole (1368-TBCZ, Fig. S1), is a byproduct formed in the manufacturing of the blue dye, 5,5′,7,7′-tetrabromoindigo (Ma et al., 2019; Parette et al., 2015). Previous studies reported that detected concentration of 1368-TBCZ detected in the sediments of different areas ranged from 0.12 ng g^{-1} to 65 ng g^{-1}, which was the most among the three PHCs measured in the Great Lakes (Guo et al., 2014; Wu et al., 2016; Wu et al., 2017a; Wu et al., 2018; Zhu and Hites, 2005). Toxicological studies of 1368-TBCZ have shown that at a high dose (> 50 μM L^{-1}, equal to 24.15 mg L^{-1}), it can cause a slight deformation of the heart and causes conformation and secondary structural changes in human serum albumin (Fang et al., 2016; Yan et al., 2018). Lin et al. (2016) also stated that the carbazole substituted by bromine at 1,3,6,8 may contribute to its higher biotoxicity.

Danio rerio is widely used in aquatic toxicology research due to its advantages (Yang et al., 2018; Zhang et al., 2020). The OECD has proposed the zebrafish embryo toxicity test as a standard test to measure the toxicity of chemicals (OECD, 2013; Strähle et al., 2012).

In the present study, zebrafish embryos were selected to elucidate the effects of 1368-TBCZ on aquatic organisms using the following measures: acute toxicity, developmental toxicity (heart and skeletal development), oxidative stress (indicated by levels of reactive oxygen species (ROS), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) levels), and oxidative damage (indicated by levels of malondialdehyde (MDA) and 8-hydroxydeoxyguanosine (8-OHdG) and apoptosis rate), oxidative damage levels, and expression of apoptosis-related genes (*Bax*, *Bcl-2*, *caspase 3/8/9*, *FoxO3b*, and *p53*). The present study comprehensively evaluated the toxicity of 1368-TBCZ in *Danio rerio*, providing valuable data for understanding the effects of PHCs on aquatic ecosystems to further and better assess the potential risks and regulation of PHCs.

2. Materials and method

2.1. Chemicals

1368-TBCZ ($C_{12}H_5Br_4N$, CAS 55119-09-0, purity >95%) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Dimethyl sulfoxide (DMSO, 99.9% pure, Sigma, Missouri, USA) was used to prepare stock solutions. Kaitong Chemical Reagent Co., Ltd. (Tianjin, China) and Solarbio Science & Technology Co. (Beijing, China) provided the other chemicals (analytical grade). We used kits from Nanjing Jiancheng Bioengineering Research Institute (Jiangsu), Hengyuan Biotechnology Co., Ltd. (Shanghai), Solar Technology Co., Ltd. (Beijing, China) to measure total protein and lipid peroxidation levels, three measures of antioxidant activity changes, 8-OHdG content level, and apoptosis rate. The primers, reagents, and kits for gene expression measurement were purchased from CoWin Biosciences Co., Ltd. (Beijing, China) and Sangon Biotechnology Co., Ltd. (Shanghai, China).

2.2. Fish handling and egg collection

Adult zebrafish (5 months old, *Danio rerio*) were purchased from the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan,

China). Before the experiment, the zebrafish were acclimated for more than two weeks in the laboratory according to the required conditions for environmental adaptation. The fish were maintained in a glass aquarium equipped with a filtration and disinfection system following the conditions depicted in Diekmann et al. (2004). The following details conditions were maintained: chlorine-free and disinfected tap water was maintained at 26 ± 1 °C, pH 7.4–8.1, and DO ≥80%, with a 14 h:10 h light-dark cycle. The zebrafish were fed manually twice a day, with the total weight of feeding less than 3% of the total fish. One-half of the water in the fish tank was replaced every 3 days, and the tank was cleaned regularly.

The light was adjusted to control spawning according to the nature of the fish, and an in-house egg collector was placed in the aquarium to collect eggs. The eggs were collected 1 h after the light was turned on. The collected eggs were washed and identified carefully under a stereoscopic microscope. Washing and identification processes were completed within 2 hour postfertilization (hpf), and normally fertilized eggs were used for the following experiments.

2.3. Lethality and teratogenicity test

The acute toxicity test of 1368-TBCZ on the embryonic development of zebrafish was carried out according to the OECD (2013). 1368-TBCZ was mixed with an incubation solution (the final concentration of each substance in the incubation solution was NaCl, 13.7 mM L^{-1}; KCl 0.54, mM L^{-1}; Na_2HPO_4, 0.025 mM L^{-1}; KH_2PO_4, 0.044 mM L^{-1}; $CaCl_2$, 1.3 mM L^{-1}; $MgSO_4$, 1.0 mM L^{-1}; and $NaHCO_3$, 0.42 mM L^{-1}). The detailed preparation process of preparing the desired concentration of exposure solution is shown in Text S1. DMSO (solvent control, SC) was used as a cosolvent to increase the solubility of 1368-TBCZ, and the final concentration of DMSO at each concentration in the exposure solution was 0.1% (v/v) (Zhang et al., 2020). I It was found through preliminary experiments that when the concentration of 1368-TBCZ was higher than 2.0 mg L^{-1}, its solubility in water was poor, and it precipitated in the incubation solution. The exposure concentrations were set at 0.1, 0.5, and 2.0 mg L^{-1} based on teratogenicity and lethality in the pre-experiment.

The exposure process for the determination of lethality was performed in 24-well plates with 2 mL of exposure solution with 1 egg per well, and three parallel plates were prepared for each group of different concentrations. The environmental conditions were 26 ± 0.5 °C, 14 h:10 h light:dark cycle controlled using an illumination incubator. The exposure solution was not renewed throughout the 96-h exposure. The mortality rate and the malformation rate in the control group were controlled within 10% and 5%, respectively. If either of these data were fell out of range, the experiment was re-run. During the exposure process, zebrafish embryos were observed 72- and 96-hpf, and the number of hatching deaths and malformed fish, were determined and recorded.

Based on the test results, malformation of the heart and spine was the primary abnormal phenotypes, and another exposure experiment was performed to explore the details. Zebrafish embryos were exposed to 0.1, 0.5, and 2.0 mg L^{-1} 1368-TBCZ in a petri dish with 50 mL of exposure solution and 50 eggs per dish. The incubation solution was used as the blank control (CK). The exposure was semistatic, and one-half of the exposure solution was changed every 24 h. Other conditions were the same as those used in the acute toxicity test.

Seventy-two hours postfertilization, 10 larval fish were randomly selected from each group and the heart rate, and body length was measured to determine the effects of 1368-TBCZ on the heart and skeletal development. For the heart rate, the number of heartbeats observed in 20 s was recorded under a stereomicroscope (Phenix Optics Co., Ltd., Jiangxi, China). For the body length measurement, digital images of each fish on a stage micrometer (1 DIV = 0.01 mm) were used first at specific magnification and resolution under the stereomicroscope, and then, the body length was measured using Image J software.

2.4. Oxidative stress and damage tests

The procedure of exposing embryos of zebrafish was the same as that used for the teratogenicity tests. At 37 hpf, 10 larval fish in each group were randomly selected for AO-EB staining, and others were collected and stored in liquid nitrogen and used for ROS, antioxidant enzyme, MDA, 8-OHdG and q-PCR measures (the exposure procedure was repeated many times, until a sufficient number of the larval fish had been collected).

2.4.1. ROS, antioxidant enzymes, MDA, 8-OHdG level determination

For the ROS, antioxidant enzyme, MDA, and 8-OHdG level determination, 3 parallel homogenate supernatants were prepared for each group. Two hundred larval fish were weighed and placed into a 2 mL centrifuge tube, and 0.86% physiological saline was added at weight (g):volume (mL) = 1:9. Then, the mixture was homogenized on ice and centrifuged at 4 °C and 10,000 rpm for 10 min (Eppendorf 5810R, Eppendorf AG, Germany). After centrifugation, the supernatant was obtained to serve as the experimental solution and stored on ice before the measurements were taken.

The supernatant was directly used for the measurements of protein content, enzyme activity, lipid peroxidation and 8-OHdG content levels (Li et al., 2018; Sun et al., 2019). The measurements were carried out using the relevant kits (Nanjing Jiancheng Bioengineering Research Institute, Jiangsu, China) according to the instructions. For the ROS measurement, the supernatant was recentrifuged at 20,000*g* at 4 °C for 20 min (Zhang et al., 2018). The resuspended sediment was used for the ROS content measurement by an ROS assay kit (Beyotime Biotech. Inc., Shanghai, China) following the manual. A fluorescence spectrometer (RF-5301PC, Shimadzu, Japan) was used to record the fluorescence (excitation at 488 nm and emission at 522 nm). The enzyme activity levels of different samples were normalized by protein concentration and then compared.

2.4.2. AO/EB staining

Because the 120 hpf larval zebrafish were almost completely transparent, acridine orange/ethidium bromide (AO/EB) double fluorescence staining was used to determine the DNA damage and apoptosis caused by 1368-TBCZ exposure (Zhang et al., 2020). The staining was conducted using an AO/EB double dyeing kit from Solarbio Science & Technology Co. (Beijing, China) following the kit instructions. After staining, the larval fish were anesthetized using 0.16 mg mL^{-1} tricaine methanesulfonate (MS-222) for 3 min; and then observed and imaged under an inverted fluorescence microscope (AxioVert. A1, Carl Zeiss AG, Oberkochen, Germany) with excitation at 488 nm. Based on the AO/EB staining results, early-stage apoptotic cells could be observed as highlighted green spots, while later stage apoptotic or dead cells could be observed as highlighted orange red spots.

2.4.3. RT-qPCR

The expression of apoptosis-related genes was measured by RT-qPCR. Briefly, total RNA was extracted by the standard TRIzol method, and approximately 100 frozen larvae were used for each sample. The purity and mass concentration were measured using a nucleic acid concentration analyzer (Nanodrop 2000, Thermo Fisher, USA), and an RNA solution of each sample was adjusted to a standard concentration using RNase-free water. Then, inverse transcription was performed using a RevertAid First Strand cDNA synthesis kit following the manufacturer's recommendations. Then, the qPCR experiment was performed using UltraSYBR Mixture (High ROX, CWBIO, China) on a Real-Time PCR instrument (StepOnePlus, Thermo Fisher Scientific Inc., USA). Detailed information on the primers and the PCR protocol can be found in the supplemental materials (Tables S2, S3, and S4). The relative expression of the different genes in zebrafish embryos was calculated according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.5. Statistics

Excel 2016 (Microsoft, WA, USA), Statistic Package for Social Sciences (SPSS, v19, SPSS Inc., USA), and Origin 2019 (OriginLab Corporation, Massachusetts, USA) were used for data analysis and plotting. The statistical discrepancy between different groups was identified by $p < 0.05$ based on one-way ANOVA followed by Duncan's multiple range test ($n \geq 3$).

3. Results and discussion

3.1. Effects of acute and developmental toxicity of 1368-TBCZ on zebrafish

The mortality rates are presented in Text S2. The hatching rates of fish embryos in each 1368-TBCZ control and treatment group 48 and 72 hpf are listed in Table 1. The results showed that 1368-TBCZ slightly enhanced the embryo hatching rates 48 hpf, but there was no significant difference between the 1368-TBCZ-treated group and the SC group, and the embryos at each exposure concentration hatched until 72 hpf.

As listed in Table 2, the malformation rates of the zebrafish embryos in the treatment group at 72 and 96 hpf were slightly higher (ranging from 5.26% to 20.0%) than those of the embryos I n the control group. In addition, the teratogenic effects were more obvious with increasing concentration, indicating a dose-effect relationship between the concentrations of 1368-TBCZ and the malformation rates of zebrafish embryos. The 1368-TBCZ-induced abnormal phenotypes were mainly spinal curvature and pericardium edema, indicating that 1368-TBCZ could affect skeletal and cardiac development.

As shown in Table 3, 0.5 and 2.0 mg L^{-1} 1368-TBCZ obviously reduced the heart rate of the larval fish. Specifically, the heart rates of the larvae in the 0.5 and 2.0 mg L^{-1} treatment groups were 17 beat min^{-1} less than those of the larvae in the control groups. Although no significant difference was found in the body lengths of the larvae in the 1368-TBCZ-treated group and those in the control group, a slight reduction in body length was found for those in the 0.5 and 2.0 mg L^{-1} 1368-TBCZ- treated groups.

Fang et al. (2016) studied the toxicity of PHCs in developing zebrafish embryos. Their results showed that 1368-TBCZ at a high dose (> 50 μM L^{-1}, equal to 24.15 mg L^{-1}) was associated with a slight deformation of the heart. For the 36-DBCZ-treatment group, zebrafish heart malformations and blood occlusion were observed at high doses (> 25 μM L^{-1}, equal to 8.125 mg L^{-1}). The tested PHCs induced malformation at doses higher than 0.1 μM L^{-1} (equal to 0.0325 mg L^{-1}); e.g., cardiac elongation, yolk sac swelling, and pericardial edema were observed. More interestingly, 27-DBCZ was lethal at dose concentrations higher than 10 μM L^{-1} (equal to 3.25 mg L^{-1}). This finding was similar to our results. The authors pointed out that the PHCs were structurally related and that they induced acute toxicity to affecting zebrafish, with heart toxicity being the most notable effect. This phenotype appeared indicate a dioxin-like toxicity; thus, the authors believed that the toxicity of PHCs may be due to *AhR* agonist activity.

Ji et al. (2019b) also reported that the 96-h LC_{50} of 27-DBCZ for *Danio rerio* was 581.9 μg L^{-1}, which slightly differed from our results, possibly due to the use of different types of PHCs. They evaluated the developmental toxicity of 27-DBCZ on zebrafish based on transcriptomics. They indicated that 27-DBCZ had teratogenic effects on zebrafish, with pericardial edema being the most frequently observed malformed phenotype. Treatment of 27-DBCZ at 0.5, 1, and 2 μM·L^{-1} (equal to 0.1625, 0.325, and 0.65 mg L^{-1}) treatment induced an increase of pericardial edema by 1.68-, 2.09-, and 21.81-fold, respectively, that of the control group. Similar to our experimental results, they speculated that the teratogenic effects of 27-DBCZ may be related to the structural similarity of the compound to dioxin. They also pointed out that 27-DBCZ can adversely affect somatic muscle development, resulting in spinal curvature in zebrafish embryos. This finding was also similar to our results. Ji et al. (2019b) measured the gene expression of *AhR1* to evaluate the relationship between heart toxicity and *AhR*. The increased *AhR1* expression indicated that *AhR* activation may contribute to the induced heart toxicity, which is similar to the results of Fang et al. (2016) and our laboratories.

In addition, previous studies clarified that disruption of vascular development was directly related to developmental toxicity (Ji et al., 2019c; Kleinstreuer et al., 2011). Therefore, we infer that 1368-TBCZ may interrupt the vascular development of zebrafish, leading to the developmental toxicity of zebrafish.

Table 1
The hatching rate of fish embryos in each 1,3,6,8-tetrabromocarbazole control and treatment group at 48 and 72 hpf.

Concentration (mg L^{-1})	48 hpf (%)	72 hpf (%)
SC	3.70 ± 6.42	100
0.5	9.08 ± 5.82	100
1.0	7.12 ± 8.01	100
2.0	7.32 ± 2.78	100

SC, solvent control; hpf, hours post-fertilization.

Table 2
Zebrafish malformation rate in each 1,3,6,8-tetrabromocarbazole control and treatment group at 72 and 96 hpf.

Concentration (mg L^{-1})	72 hpf (%)	96 hpf (%)
SC	1.56 ± 5.64	1.56 ± 2.95
0.5	5.26 ± 8.84	7.02 ± 5.89
1.0	6.15 ± 2.95	13.9 ± 2.95
2.0	7.69 ± 2.95	20.0 ± 14.7

SC, solvent control; hpf, hours post-fertilization.

3.2. Oxidative stress and damage

3.2.1. Effects of 1368-TBCZ on oxidative stress and damage

The degree of oxidative stress and damage to zebrafish embryos exposed to 1368-TBCZ is illustrated in Fig. 1. Generally, free radical production and clearance in organisms are in a state of dynamic equilibrium, but when external pollutants cause an increase in ROS content in organisms, this balance is disrupted, causing cell damage (van der Oost et al., 2003; Zhang et al., 2014). Li et al. (2015) pointed out that when cells are in a state of stress, a large number of ROS will be generated through cell metabolism. Lushchak (2011) also indicated that when the body is stimulated by exogenous contaminants, the ROS levels increase significantly. Studies have shown that a mitochondrion is an important organelle for ROS production. Upon exposure to 1368-TBCZ, the ROS content levels (Fig. 1A) in zebrafish of each treatment group were significantly different from those of the zebrafish in the control group. With increasing concentration, the ROS content in the zebrafish of each treatment group gradually increased, showing a significant upward trend and a clear dose-effect relationship.

The abnormal metabolism of ROS free radicals results in excessive production of ROS, which can induce oxidative stress and lipid peroxidation in cells. Excess ROS can be eliminated by the antioxidant defense system, which includes SOD, CAT, and GPX (Chen et al., 2016; van der Oost et al., 2003). SOD and CAT are the first line defenders against

Table 3
Effects of 1,3,6,8-tetrabromocarbazole exposure on body length and heart rate of zebrafish embryo at 72 hpf.

Concentration (mg L^{-1})	Body length (mm)	Heart rate (beat min^{-1})
CK	3.43 ± 0.0767	185 ± 10.7
SC	3.38 ± 0.121	182 ± 14.2
0.1	3.40 ± 0.172	181 ± 6.87
0.5	3.36 ± 0.157	168 ± 11.6*
2.0	3.32 ± 0.163	168 ± 13.8*

CK, control check; SC, solvent control; hpf, hours post-fertilization.
* $p < 0.05$.

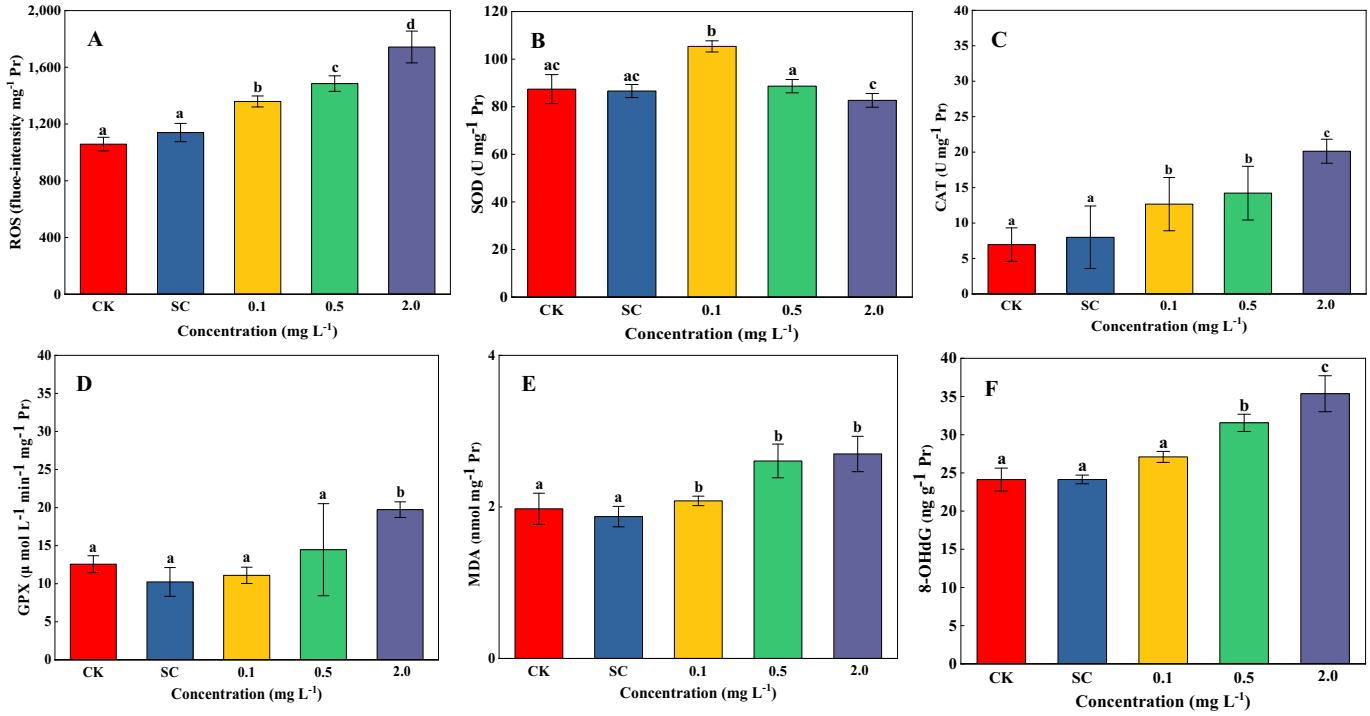

Fig. 1. The degree of oxidative stress and DNA damage to the embryo of zebrafish exposed to three different doses of 1,3,6,8-tetrabromocarbazole (1368-TBCZ, n = 3). The standard error of three samples is represented with an error bar. The lowercase letters above the bars represent significant differences between the groups. CK, control check; SC, solvent control; Pr, protein. (Capital letters A, B, C, D, E and F correspond to the activities of ROS, SOD, CAT, GPX MDA and 8-OHdG, respectively).

excessive ROS in cells. SOD catalyzes O_2^- to generate hydrogen peroxide (H_2O_2) and O_2, and then, CAT catalyzes H_2O_2 to H_2O and O_2. CAT and GPX act together on the H_2O_2 produced and other hydroperoxides, thus reducing the concentration of H_2O_2 in the body and reducing oxidative damage to the organism (Sun et al., 2006; Zhang et al., 2014).

The enzymatic activities of SOD, CAT, and GPX in fish embryos exposed to 1368-TBCZ are illustrated in Fig. 1B, C, and D. We found that low concentrations of 1368-TBCZ promoted increased SOD in zebrafish at a level a significantly different than the level in the control group zebrafish. The SOD content in the zebrafish in treatment groups exposed to medium and high concentrations was not significantly different than that in the control group. In addition, the CAT content in zebrafish of all 1368-TBCZ-treated groups was significantly higher than that in the control group, indicating that 1368-TBCZ can induce an increase in CAT levels in zebrafish. Moreover, the experimental results showed that there was no significant difference in GPX content in the low-medium concentration group and the control group, while it increased significantly in the high concentration treatment group.

According to the theory of ROS (Ali et al., 2018; Mittler, 2002), the reason that the activity of SOD and CAT in the treatment groups was higher than that in the control group may be caused by external factors, which induce an increase in the amount of O_2^- produced in the zebrafish, thereby inducing a significant increase in the biosynthesis of SOD and CAT enzymes. The decrease in SOD content in the high concentration treatment group may increase the degree of stress in the organism, resulting in the rate of excess ROS removal being slower than the ROS production rate (Nascimento et al., 2017; Wang et al., 2006).

Dong et al. (2016) believed that the increase in ROS content can lead to lipid peroxidation. MDA is the end product of peroxidation caused by ROS acting on unsaturated fatty acids and is one of the major by-products of oxidative peroxidation, which can destroy the structure of cell membranes. The MDA content can reflect the extent of intracellular lipid oxidation and cell damage in vivo (Dong et al., 2018; Li et al., 2018; Sun et al., 2019). Similar to the ROS results, the MDA content (Fig. 1E) in the zebrafish in each treatment group was higher after exposure to 1368-TBCZ, and the difference was significantly different than that in the control group. The results from the ROS and MDA indicate that exposure to 1368-TBCZ leads to lipid peroxidation in zebrafish.

Sun et al. (2019) indicated that the degree of DNA damage was related to oxidative stress in organisms. Among various base modification products, 8-OHdG is recognized as a marker of DNA oxidative damage caused by endogenous and exogenous factors and is directly related to an increase in the risk of multiple degenerative diseases and cancer. It is a promising indicator, and the extent of oxidative damage and repair in the body can be evaluated based on the detection of it (Martins et al., 2017).

As illustrated in Fig. 1F, the results showed that there was no significant difference between the low concentration treatment groups (0.1 mg L^{-1}) and the control group, but there were significant differences between the medium and high concentration treatment groups (0.5 mg L^{-1}) and the control group. In the medium and high concentration treatment groups, the 8-OHdG content in the zebrafish was significantly higher than that in the control group, and it increased with the 1368-TBCZ concentration, showing an explicit dose-response relationship. The increased 8-OHdG level indicated that 1368-TBCZ can cause DNA damage in zebrafish.

Apoptosis, an important mechanism of cell death, eliminates damaged cells to maintain the normal ecological balance of organisms (Zhang et al., 2017). Liu et al. (2015) indicated that apoptosis is a fundamental biological phenomenon of cells, and it is an active process by which life is automatically terminated, as determined by genes; therefore, it is often called programmed cell death. Apoptosis can remove abnormal or unuseful cells and plays an important role in the evolution of organisms, the stability of the internal environment, and the development of multiple systems. We used AO/EB double fluorescence staining to determine the apoptosis rate. As shown in Fig. 2, there was no significant apoptosis in the CK or SC treatment groups, and the number of apoptotic cells gradually increased with increasing 1368-TBCZ concentration. This finding indicates that 1368-TBCZ induces apoptosis in zebrafish. In total, DNA damage was affected by 1368-TBCZ in diverse pathways. The mechanism of genotoxicity and cytotoxicity induced by 1368-TBCZ can be elucidated in further studies.

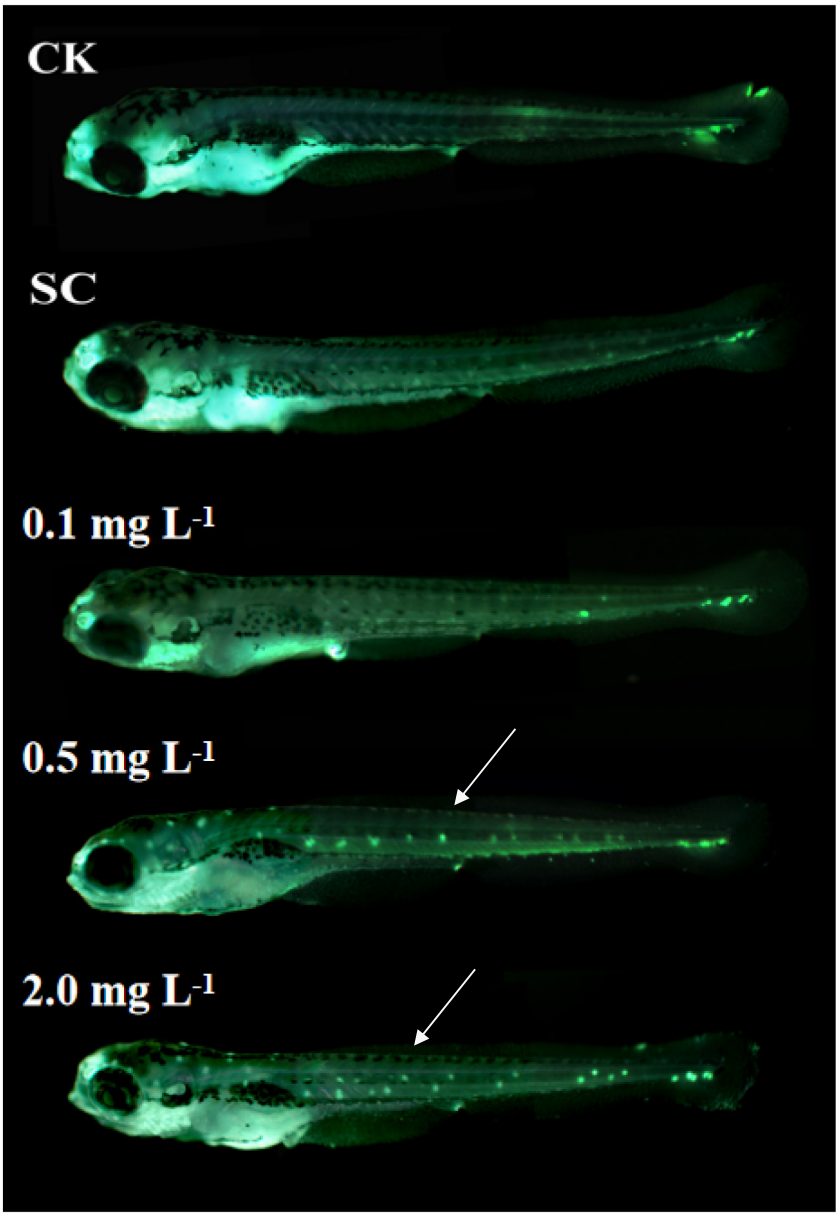

Fig. 2. Apoptosis is affected by 1368-TBCZ exposure of zebrafish larvae 96 hpf. CK, control check; SC, solvent control; hpf, hours postfertilization.

Li et al. (2016) studied the response of antioxidant defense systems in the liver of the freshwater fish *Carassius auratus* exposed to polyfluorinated dibenzo-p-dioxins (PFDDs), whose structure is similar to that of PHCs. Their studies have shown that exposure to PFDDs can inhibit SOD activity and enhance lipid peroxidation in fish. This result is similar to our results, indicating that 1368-TBCZ may have dioxin-like effects. Ji et al. (2019b) performed transcriptional detection of 27-DBCZ on zebrafish developmental toxicity and believed that 27-DBCZ may have induced oxidative damage through a bioconversion pathway, resulting in embryotoxicity. Thus, 1368-TBCZ poses a risk of toxicity to fish embryos, possibly due to dioxin-like effects and through a bioconversion pathway. The details of the mechanism can be elucidated in further studies.

3.2.2. Effects of 1368-TBCZ on gene expression

Liu et al. (2015) pointed out that apoptosis is an active process by which life is automatically terminated by genes, and gene expression can also explain apoptosis in some cases. Therefore, we measured gene expression. The effects of 1368-TBCZ on gene expression in zebrafish embryos are illustrated in Fig. 3. Caspase has an integral role in the process of inducing cells to apoptosis. Studies have found that caspase-activated apoptosis is mainly achieved through two signaling pathways: the death receptor pathway and the mitochondrial pathway (Lin et al., 2018).

The death receptor pathway occurs through activation of a promoter caspase (*caspase 8*, *caspase 9*, etc.), which induces other caspase activities that lead to apoptosis. The mitochondrial pathway, also called the intrinsic pathway, promotes apoptosis by inducing mitochondria to release proapoptotic proteins such as cytochrome *c* (Franco et al., 2009).

Another form of apoptosis induced through the mitochondrial pathway is independent of caspase; that is, apoptosis is mediated by apoptosis-inducing factor (AIF) protein. When the cells are stimulated by internal apoptosis-induction factors, AIF is released from mitochondria, moves to the cytoplasm and eventually enter the nucleus, causing DNA damage and cell death (Norberg et al., 2010).

Bcl-2 was first discovered by Tsujimoto in 1984. In 1988, it was reported that *Bcl-2* can prolong cell survival time, and in 1990, it was confirmed that *Bcl-2* had an anti-apoptosis function (Hardwick and Soane,

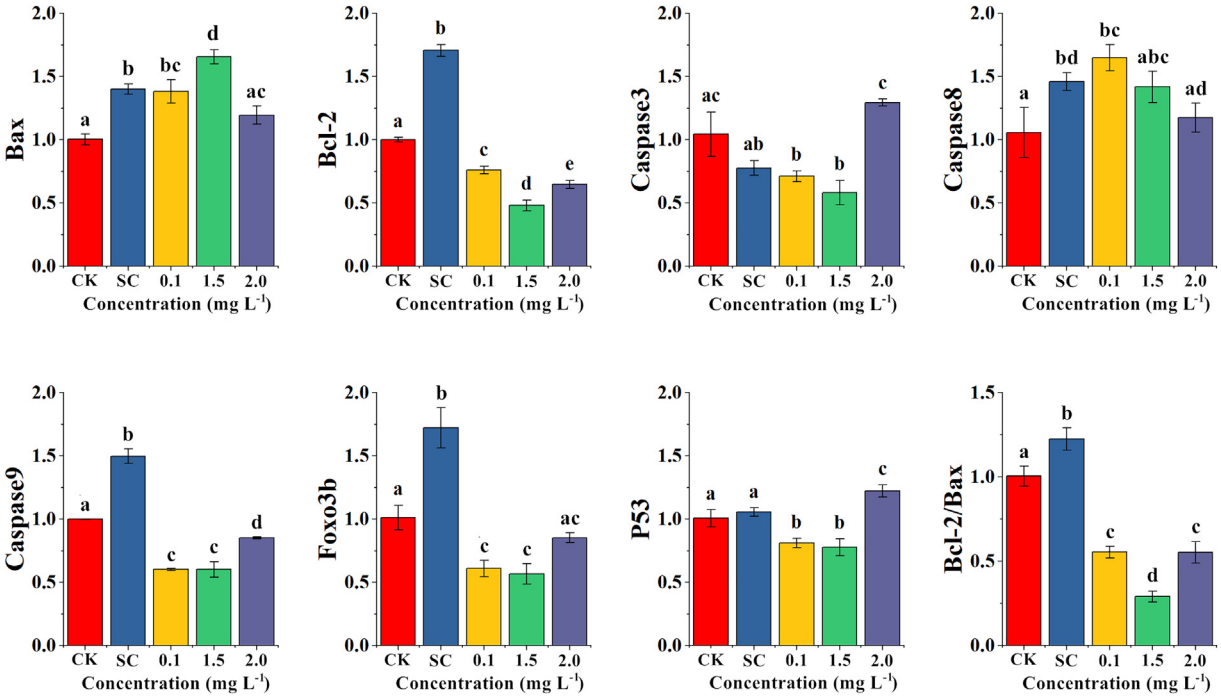

Fig. 3. Effects of 1368-TBCZ on gene expression in zebrafish embryos (n = 3). The standard error of three samples is represented with an error bar. The lowercase letters above the bars represent significant differences between the groups. The ordinate value represents the relative expression of each gene using the CK group as a control. CK, control check; SC, solvent control.

2013). *Bcl-2* and its expression protein can inhibit the apoptosis of a variety of tissue cells; therefore, it is called an apoptosis inhibitory gene. Its expression and regulation are key factors affecting apoptosis (Wensveen et al., 2011). In 1993, Oltvai et al. (1993) first discovered *Bax*, whose main role is to promote cell apoptosis.

Fig. 3 shows that the activity of *caspase 3/8/9* changes to some extent after exposure to 1368-TBCZ. Exposure to low and medium concentrations (0.1 and 0.5 mg L^{-1}) of 1368-TBCZ downregulated *caspase 3* activity, while high concentration exposure (2.0 mg L^{-1}) upregulated *caspase 3* activity. Exposure to 1368-TBCZ upregulates *caspase 8* activity and downregulates *caspase 9* activity. Studies have shown that exposure to harmful substances can change the activity of *caspases*, thereby inducing apoptosis (Sun et al., 2019; G.H. Wang et al., 2019). *Caspase 3* is a key protease in cell apoptosis, and it immediately generates a cascade reaction after activation, making apoptosis inevitable (Seervi and Xue, 2015). In our experiment, the activity of *caspase 3* showed no obvious upward trend, although apoptosis occurred. It may be that another apoptosis pathway was adopted, and cell apoptosis was realized by the releasing of AIF before *caspase 3* was activated.

The fork/winged coil gene, subgroup O (*FoxO*) gene belongs to the forkhead family of transcription factors and has a unique forkhead domain. FoxOs stimulate death receptor ligand expression by inducing the proapoptotic genes of the *Bcl-2* family (Morris et al., 2015). Inactivation of this gene family can lead to the accumulation of ROS in the organism, thereby impairing the normal proliferation of cells (Martins et al., 2016). Studies have shown that, similar to *APOE*, *FoxO3* is a long-lived gene (Brooks-Wilson, 2013). It has also been experimentally shown that *FoxO3* can control the production of cytokines and inhibit the activation and proliferation of NF-κB and T cells, thereby reducing inflammation (Lin et al., 2004).

Fig. 3 indicates that the activity of *FoxO3b* was significantly downregulated after exposure to 1368-TBCZ, and there was a significant difference between the low concentration treatment group and the control group. This result indicated that 1368-TBCZ caused a decrease in *FoxO3b* content in zebrafish, which decreased the immunity of zebrafish and induced apoptosis.

The key to the apoptotic cascade is anti-apoptotic and proapoptotic proteins. *P53* and *Bax* are both anti-apoptotic proteins, while the proapoptotic proteins include *Bcl-2*. Moreover, the tumor suppressor protein *p53* plays an important regulatory role in the process of apoptosis (Schramm et al., 2017).

The *p53* can activate apoptotic signaling pathways and control apoptotic genes such as the those in the *Bcl-2* family (Miguel et al., 2007; Schramm et al., 2017). The results showed that low concentrations of 1368-TBCZ downregulated *p53* activity in the treated group, while *p53* activity was activated in the higher concentration group. According to Félix et al. (2018), *p53* binds to signaling proteins to control lethality and teratogenicity and can activate the apoptosis mechanism by stimulating mitochondria to release cytochrome *c*.

In the treatment groups exposed to 1368-TBCZ, the ratio of *Bcl-2/Bax* decreased significantly, and the differences with the control group were significant, but there was no dose-effect relationship. The ratio of *Bcl-2/Bax* is a key factor that determines the inhibitory effect on apoptosis: upregulation of *Bcl-2/Bax* inhibits apoptosis, and downregulation of the *Bcl-2/Bax* ratio promotes apoptosis (Oltvai et al., 1993); that is, exposure to 1368-TBCZ can induce cell apoptosis in zebrafish.

Although 1368-TBCZ induces low acute toxicity to zebrafish, a low dose of 1368-TBCZ has a significant effect on the induction of oxidative stress and apoptosis. In the future, we should clarify the source of 1368-TBCZ, control the formation of 1368-TBCZ byproducts in the production process, and adopt the elimination process of 1368-TBCZ before it is discharged in the environment to reduce the chance of it entering the ecosystem. This study provides valuable data that can better help people comprehensively evaluate the toxicity of 1368-TBCZ, thereby improving people's awareness of the potential risk of the tested pollutants and the importance of pollution prevention.

4. Conclusions

Taken together, the data show that 1368-TBCZ has slight effects on the hatching rate and teratogenic effects of zebrafish embryos. At a medium (0.5 mg L^{-1}) and high concentrations (2.0 mg L^{-1}) of 1368-TBCZ,

the skeletal and heart development of zebrafish was affected. 1368-TBCZ can promote increases in ROS content, CAT enzyme activity, lipid peroxidation, DNA damage, and apoptosis in zebrafish embryos. Exposure to 1368-TBCZ influences the expression of oxidative stress-related genes, upregulates the expression of *caspase 3* and *p53* at an exposure of 2.0 mg L^{-1}, and has an inhibitory effect on *caspase 9*, *FoxO3b*, and *Bcl-2*/*Bax* expression.

CRediT authorship contribution statement

Jingwen Zhang: Investigation, Writing - original draft. **Cheng Zhang:** Validation, Writing - review & editing. **Zhongkun Du:** Conceptualization, Methodology. **Lusheng Zhu:** Supervision. **Jun Wang:** Software. **Jinhua Wang:** Formal analysis. **Bing Li:** Formal analysis.

Declaration of competing interest

The authors declare that they have no conflict of interest. All the authors have read and approved the manuscript. This work has not been published previously, nor is it being considered by any other peer-reviewed journal.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.140753.

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