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Neurotoxicity and transcriptome changes in embryonic zebrafish induced by halobenzoquinone exposure

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ABSTRACT

Halobenzoquinones (HBQs) are emerging disinfection byproducts (DBPs) with a widespread presence in drinking water that exhibit much higher cytotoxicity than regulated DBPs. However, the developmental neurotoxicity of HBQs has not been studied *in vivo*. In this work, we studied the neurotoxicity of HBQs on zebrafish embryos, after exposure to varying concentrations (0-8 μmol/L) of three HBQs, 2,5-dichloro-1,4-benzoquinone (2,5-DCBQ), 2,6-dichloro-1,4-benzoquinone (2,6-DCBQ), and 2,5-dibromo-1,4-benzoquinone (2,5-DBBQ) for 4 to 120 hr post fertilization (hpf). HBQ exposure significantly decreased the locomotor activity of larvae, accompanied by significant reduction of neurotransmitters (dopamine and γ-aminobutyric acid) and acetylcholinesterase activity. Furthermore, the expression of genes involved in neuronal morphogenesis (*gfap*, α*1-tubulin*, *mbp*, and *syn-2*α) were downregulated by 4.4-, 5.2-, 3.0-, and 4.5-fold in the 5 μmol/L 2,5-DCBQ group and 2.0-, 1.6-, 2.1-, and 2.3-fold in the 5 μmol/L 2,5-DBBQ group, respectively. Transcriptomic analysis revealed that HBQ exposure affected the signaling pathways of neural development. This study demonstrates the significant neurotoxicity of HBQs in embryonic zebrafish and provides molecular evidence for understanding the potential mechanisms of HBQ neurotoxicity.

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Introduction

Drinking water disinfection can effectively prevent waterborne diseases. An unavoidable consequence of disinfection is the formation of disinfection byproducts (DBPs) resulting from reactions between disinfectants and natural organic matter in the source water (Krasner *et al.*, 2006). Human exposure to DBPs occurs mainly via consumption and dermal absorption (e.g., showering and swimming). DBP exposure has

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been linked to a number of adverse human health effects, including risks of developmental defects and bladder cancer (Cantor *et al.*, 2010; Glauner *et al.*, 2005; Gonsioroski *et al.*, 2020; Gonsioroski *et al.*, 2021; Jeong *et al.*, 2015; Liviac *et al.*, 2010; Plewa *et al.*, 2010; Säve-Söderbergh *et al.*, 2020). Several epidemiological investigations have shown a potential association between exposure to the regulated DPBs such as trihalomethanes (THMs) and neural tube defects (Bove *et al.*, 1995; Dodds and King, 2001; Klotz and Pyrch, 1999). *In vivo* studies have shown that dichloroacetic acid (DCAA) and dibromoacetic acid (DBAA) exposure can induce neurotoxic effects in rats (Moser *et al.*, 2004; Moser *et al.*, 1999). *In vitro* studies have demonstrated that some DBPs, such as haloacetic acids (HAAs), use GADPH as a target molecule to induce adverse biological responses (Pals *et al.*, 2011). The impact of alkylated GADPH is linked to the induction of human neurological degenerative diseases (Butterfield et al., 2010). However, the effects of emerging DBPs on neurodevelopment remain unclear.

Halogenated benzoquinones (HBQs), an emerging group of aromatic DBPs, occur widely in chlorinated drinking water (Zhao *et al.*, 2010) and swimming pools (Lou *et al.*, 2019; Wang *et al.*, 2013). *In vitro* studies have shown that the cytotoxicity of HBQs is up to 1000-fold higher than that of the regulated DBPs (Wang *et al.*, 2014). *In vivo* zebrafish tests have shown that the LD_{50} values of several HBQs are up to 500 times higher than those of the regulated DBPs and that HBQs cause zebrafish deformation (Wang *et al.*, 2018). Exposure to 2,6-DCBQ impacts the reproductive development of female zebrafish (Song et al., 2022). HBQ exposure activates reactive oxygen species (ROS), resulting in a variety of oxidative damage (Du *et al.*, 2013; Li *et al.*, 2018; Lou *et al.*, 2019; Wang *et al.*, 2018), such as peroxidation of neurolipids, which results in neurotoxic effects (Al Olayan *et al.*, 2020; Garza-Lombo *et al.*, 2018; Shaw *et al.*, 2020; Wu *et al.*, 2012). Interestingly, recent research has shown that HBQ exposure can induce human neural stem cell cycle arrest at S-phase (Fu et al., 2017). However, the neurotoxicity of HBQs *in vivo* has not been studied.

Zebrafish is a useful *in vivo* model for assessing neurotoxicity (Parng *et al.*, 2007), including variations in neurodevelopment, neurochemistry, and neurobehavior (Eddins *et al.*, 2010). The activity of acetylcholinesterase enzyme (AchE), a widely used biomarker for neurotoxins, can be measured in larvae (Gravato *et al.*, 2021; Shi *et al.*, 2021). The roles of synapsin-IIα (*syn-2α*), myelin basic protein (*mbp*), glial fibrillary acidic protein (*gfap*) and *α1-tubulin* in neuronal morphogenesis have been extensively studied. The locomotion behaviors of zebrafish occur in a predictable sequence (Drapeau *et al.*, 2002). The locomotor activity of zebrafish has been widely used as a sensitive indicator for neurotoxic investigation (Legradi *et al.*, 2015; Selderslaghs *et al.*, 2010), because behavioral maturation is dependent on spontaneous neural activity during development (Drapeau *et al.*, 2002). Previous studies have suggested spontaneous movements to be essential for hatching (Kimmel *et al.*, 1974; Ogungbemi *et al.*, 2019). Therefore, locomotor activity serves as an indicator for the neural development of zebrafish. These established indicators are useful for studying the neurotoxicity of emerging DBPs.

In the present study, we used zebrafish embryos as the *in vivo* model to examine these neurotoxicity indicators after exposure to three HBQs (2,5-DCBQ, 2,5-DBBQ, and 2,6-DCBQ) at varying concentrations from 4 to 120 hr post fertilization (hpf). We analyzed zebrafish locomotor behavior, neurotransmitters, and the expression of related genes in the neurodevelopmental system. We also examined the spatial changes in the expression of the neuro-marker gene *gfap* using whole-mount *in situ* hybridization (WISH). Finally, we carried out transcriptomic analysis to explore the potential molecular mechanisms of neurotoxicity.

1. Materials and methods

1.1. Chemicals and reagents

Standards of 2,5-DCBQ, 2,6-DCBQ, and 2,5-DBBQ were purchased from Tokyo Chemical Industry (TCI; Tokyo, Japan). Dimethyl sulfoxide (DMSO) was obtained from Sigma-Aldrich (St. Louis, MO, USA); it was used to dissolve the three HBQs and as the solvent control in each assay. The protease of *Streptomyces griseus* was purchased from Sigma-Aldrich and was used to remove chorions from embryos in the early stages. N-Phenylthiourea (PTU), SigmaSpin™ Sequencing Reaction Clean-Up, 4% paraformaldehyde (PFA), and methylcellulose were purchased from Sigma-Aldrich and were used for the whole-mount *in situ* hybridization (WISH) assay.

1.2. Zebrafish embryo collection and chemical exposure

Wildtype AB strain zebrafish (three months old, obtained from the Institute of Hydrobiology, Chinese Academy of Sciences) were maintained in 28±0.5 °C aerated water with 0.25%-0.75% (W/V) salinity, and were used to hatch embryos for all exposure experiments. Adult zebrafish, two males and two females in each tank, were separated the night before the collection of fertilized eggs. Thirty embryos were randomly selected, placed in each well of a 6-well plate, and exposed to varying concentrations of HBQs (0-8 μmol/L) ranging from 4 to 120 hpf. The exposure concentrations were chosen based on the LD_{50} of the three HBQs (mean± SD): 2,6-DCBQ 6.6 ± 0.2 μmol/L, 2,5-DCBQ 4.6 ± 0.2 μmol/L, and 2,5-DBBQ 5.6 ± 0.2 μmol/L (Wang et al., 2018). The exposure time was chosen based on the developmental processes of zebrafish embryos, as described in the following sections. In all experiments, three replicate wells were prepared for each exposure group. Both control and exposure groups contained less than 0.007% (V/V) DMSO in 3 mL of water in each well, which did not produce detectable viability difference compared to water alone.

1.3. Measurement of spontaneous movement and hatching rates after exposure to 2,6-DCBQ

Spontaneous movement typically begins between 17-28 hpf, and hatching starts gradually from 48 hpf (Kimmel et al., 1974). We chose 32 hpf as the exposure endpoint of early spontaneous movement experiments. In brief, zebrafish embryos were exposed to varying concentrations of 2,6-DCBQ (0, 2, and 4, and 8 μmol/L) from 4 to 32 hpf (before hatching), and their spontaneous movement was measured by the zebrafish microscopic behavioral analysis system (DanioScope). The

8 μmol/L of 2,6-DCBQ was included in this initial set of experiments to show the effects on hatching because 2 and 4 μmol/L 2,6-DCBQ did not significantly change the hatching rate compared to the control. Three parallel experiments were conducted for each treatment group, and 20 embryos were observed in each set of parallel experiments. At the end of the exposure (32 hpf), the embryos were videotaped using a Leica microscope at a rate of 24 frames per second (fps), and the activity of the zebrafish embryos in the video files was analyzed using DanioScope software. This system can automatically identify embryos, select regions of interest within the embryonic chorion, and automatically measure embryo activity. The metric for analysis was the activity of zebrafish embryos, characterized by the percentage of total time in motion. Studies have shown that spontaneous movement acts as a critical factor in embryonic hatching (Ogungbemi et al., 2019). Hence, the hatching rates were recorded at 72 hpf when more than 90% of embryos hatched under normal conditions.

1.4. *Tracking locomotor behavior under alternating light/dark stimulation*

The effects of HBQs (2,5-DCBQ, 2,6-DCBQ, and 2,5-DBBQ) at the concentrations0, 0.5, 2.5, and 5.0 μmol/L on locomotor behavior of zebrafish larvae were analyzed using Noldus EthoVision® XT video tracking software (version 11.5, Noldus Information Technology, The Netherlands) and Noldus DanioVision® zebrafish tracking hardware system. After exposure for 120 hpf, zebrafish larvae were placed in 24-well plates (one larva per well) at 28°C and acclimated for 10 min before locomotor distance and speed were monitored; then the larvae were kept under light-dark switching stimulation (three cycles of 5 min light/5 min dark). Sixty larvae (each treatment group consisted of three parallel sets of 20 larvae each) were evaluated for locomotor activity based on the video at 25 fps.

1.5. *Determination of neurotransmitters*

The levels of the neurotransmitters dopamine, serotonin, and gamma-aminobutyric acid were determined using ELISA kits. Zebrafish embryos were exposed to varying concentrations (0, 0.5, 2.5, and 5.0 μmol/L) of HBQs (2,5-DCBQ, 2,5-DBBQ, and 2,6-DBCQ) from 4 to 120 hpf. Thirty larvae were randomly taken from each concentration treatment group, placed in a 1.5 mL centrifuge tube, and washed twice with cold PBS buffer; then, 500 μL of PBS and two clean and dry steel beads were added for high-throughput tissue grinding and homogenization (60 Hz, 30 min). The homogenate was centrifuged at 4°C and 3000 r/min for 20 min. The concentrations of the neurotransmitters (ng/mL) and corresponding proteins (mg/mL) in the supernatant were measured to obtain the content of each neurotransmitter (ng/mg prot). The enzymatic activity of acetylcholinesterase (U/mg prot) was determined using the acetylcholinesterase (AchE) assay kit according to the instructions.

1.6. *qRT-PCR and WISH*

Trizol (Invitrogen, Burlington, ON, Canada) was used to extract total RNA from 30 living larvae after their exposure to 0, 0.5, 2.5, and 5.0 μmol/L of HBQs (2,5-DCBQ, 2,5-DBBQ, and 2,6-DBCQ) for 4 hpf to 120 hpf. Genomic DNA was removed using RNase-free DNase I (Qiagen, Hilden, Germany). RNA degradation and contamination were checked using RNase-free agarose gel electrophoresis. RNA purity (on the basis of OD260/280 ratio) was measured using a NanoDrop 2000c (Thermo Scientific, Rockford, IL, USA). The OD260/280 ratios of all RNA samples ranged from 1.8 to 2.0. The GenBank accession numbers (obtained from NCBI) and forward and reverse primer sequences are listed in Appendix A Table S1. The fold-change in gene expression levels was calculated using the $2^{-\Delta\Delta Ct}$ method.

The total RNA extracted from the larvae was converted to cDNA. The PCR-amplified sequence of the *gfap* gene was used as the template for the synthesis of RNA probes. The primer sequences are listed in Appendix A Table S2. The PCR products were transcribed to RNA probes using the *in vitro* transcription reaction that includes primers, T7 RNA polymerase, and promoter sequence with the DNA template. The RNA probes were labeled with digoxigenin-UTP. The antisense RNA probes were used to hybridize with the *gfap* mRNA to detect this gene, and the sense RNA probes were used to detect false positive staining and non-specific staining in the negative control (untreated embryos).

Appendix A Fig. S1 shows the method for zebrafish embryo WISH; the procedures have been described in detail previously (Thisse and Thisse, 2008; Yang et al., 2020). Briefly, on the first day, the embryos were hybridized overnight with the solution containing the probe after rehydration, fixation, proteinase K digestion, and pre-hybridization. On the second day, the embryos were incubated overnight with anti-digoxigenin antibodies conjugated to alkaline phosphatase. On the third day, the chromogenic substrate was added for the enzymatic reaction to generate luminescence. Subsequently, the embryos were observed and imaged every 30 min.

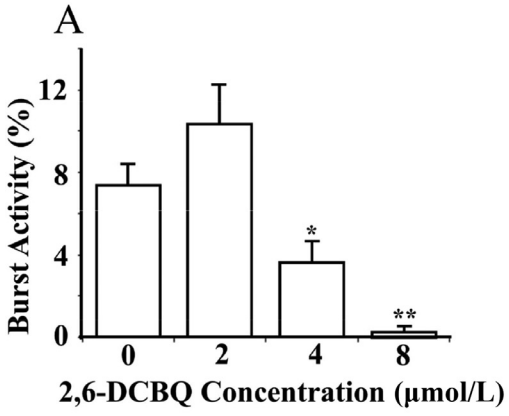
1.7. *Bioinformation analysis of transcriptomics*

The extracted total RNA was sent to Novogene, according to the company's instructions. Sequencing procedures and raw data analysis were performed as described previously (Zhao *et al.*, 2021; Meng *et al.*, 2020). For these differentially expressed genes (DEGs, relative to the control) that were observed after exposure to 2,6-DCBQ (4 and 8 μmol/L), we performed the functional annotation and gene ontology (GO) clustering using DAVID (Database for Annotation, Visualization and Integrated Discovery) Bioinformatics Resources 6.8. The GO terms (Biological Process (BP), Cellular Component (CC), and Molecular Function (MF)) were obtained by analyzing the differentially expressed neural genes.

1.8. *Statistical analysis*

Statistical analysis was performed using GraphPad Prism 7 (GraphPad, La Jolla, CA, USA). Experimental results were expressed as the mean ± standard deviation (SD) or as the mean ± the standard error of the mean (SEM). Statistical comparisons of analyses in which multiple treatment groups were tested (including locomotor activity, neurotransmitter content, and gene expression) were assessed using a one-way

A



B

2,6-DCBQ Concentration (μmol/L)	Hatching rate(72hpf) Mean ± SD %
0	98 ± 4
2	96 ± 2
4	93 ± 3
8	*61 ± 5

Note: * indicates a significant decrease in hatching rate.

Fig. 1 – (A) The percentages of burst activity for zebrafish embryos exposed to 2,6-DCBQ at 36 hpf. Three replicates were performed for each exposure group (20 larvae, 3 replicates). One-way ANOVA with Dunnett's post-test: *$p<0.05$, **$p < 0.01$. (B) Statistical hatching rate of zebrafish larvae exposed to 2,6-DCBQ.

analysis of variance (ANOVA) with a Dunnett's post-test. Differences were considered statistically significant at $p < 0.05$.

2. Results and discussion

2.1. *Effect of HBQs on developmental and spontaneous movement of zebrafish embryos*

To evaluate HBQ neurotoxicity, we first investigated the effects of 2,6-DCBQ on early spontaneous neural activity of zebrafish embryos by measuring the spontaneous motor activity. The exposure concentrations were chosen around the LD_{50} of 2,6-DCBQ (6.6 μmol/L) at 24 hpf reported in our previous study (Wang et al., 2018). Fig. 1A shows that spontaneous motor activity decreased significantly by 49% ($p<0.05$) and 93.3% ($p<0.01$) after exposure to 4 and 8 μmol/L DCBQ at 32 hpf, respectively, and no statistical difference was observed for the 2 μmol/L group, compared to the DMSO control group. The hatching rate was not significantly affected in the exposure groups of 0, 2, and 4 μmol/L 2,6-DCBQ at 72 hpf, whereas it was significantly decreased in the 8 μmol/L 2,6-DCBQ exposure group at 72 hpf (Fig. 1B). The reduction of the spontaneous motor activity of zebrafish after 2,6-DCBQ exposure indicates neurodevelopmental disorders and changes in neurotransmitters as previous studies have reported (Mahabir et al., 2013; Wang et al., 2015; Wu et al., 2016).

2.2. *Effect of HBQ exposure on the movement of larval zebrafish under light/dark stimulation*

Subsequently, we evaluated the effects of HBQs on larvae movements under the stimulation of alternating 5-min light/dark intervals for 30 min at 120 hpf using the zebrafish behavior analyzer. Three structurally similar HBQs were examined and compared in this set of experiments. Figure 2 shows that the motor distance (A1, B1, and C1) and velocity (A2, B2, and C2) significantly decreased after exposure to HBQs. The movement distances are summarized in Appendix A Table S3. Here, we highlight the results that showed significant decrease when compared to the DMSO solvent control group. For the 2,6-DCBQ (0.5, 2.5, and 5 μmol/L) exposure groups compared to the DMSO control group, the decreases in the average movement distances of larvae (Fig. 2-A1) were 23%-39% under dark stimulation for 5-10 min, 13%-47% for 15-20 min, and 7%-47% for 25-30 min, depending on the dose. As shown in Fig. 2-A2, the speed of larvae movements was decreased by 24%-41% for 5-10 min and 11%-41% for 15-20 min, depending on the concentration. Similar effects were observed for 2,5-DCBQ (Fig. 2-B1 and 2-B2) and 2,5-DBBQ (Fig. 2-C1 and 2-C2). For the 2,5-DCBQ (0.5, 2.5, and 5 μmol/L) exposure groups, the decreases in the average movement distances of larvae were 22%-41% for 5-10 min, 39%-49% for 15-20 min, and 25%-36% for 25-30 min, while the speed of larvae movements were decreased by 24%-65%. For the 2,5-DBBQ (0.5, 2.5, and 5 μmol/L) exposure groups, the decreases (%) in the average movement distances of larvae were 35%-65% for 5-10 min, 15%-49% for 15-20 min, and 5%-38% for 25-30 min, and the speed of larvae movement was similarly decreased by 12%-55%. The three HBQs had similar effects on the movement distance and speed of the larvae. The results demonstrated that exposure to concentrations of HBQs as low as 0.5 μmol/L can significantly affect the locomotor performance of larvae. Zebrafish were significantly more active under dark stimulation than light stimulation, supported by the results in Fig. 2. This is consistent with a previous report that larval zebrafish are sensitive to lighting conditions, known as the visual motor response or dark photokinesis (Lau *et al.*, 2011).

2.3. *Effect of HBQ exposure on the levels of neurotransmitters in zebrafish embryos*

The changes in the early spontaneous movement of embryos (Fig. 1) and locomotor performance of larvae (Fig. 2) led us to examine the effects of HBQ exposure on neurotransmitters. Figure 3 and Appendix A Table S4 present the levels of two neurotransmitters, dopamine (DA) and gamma-aminobutyric acid (GABA), and acetylcholinesterase (AchE) after HBQ exposure. Significant decrease in the levels of DA, GABA, and

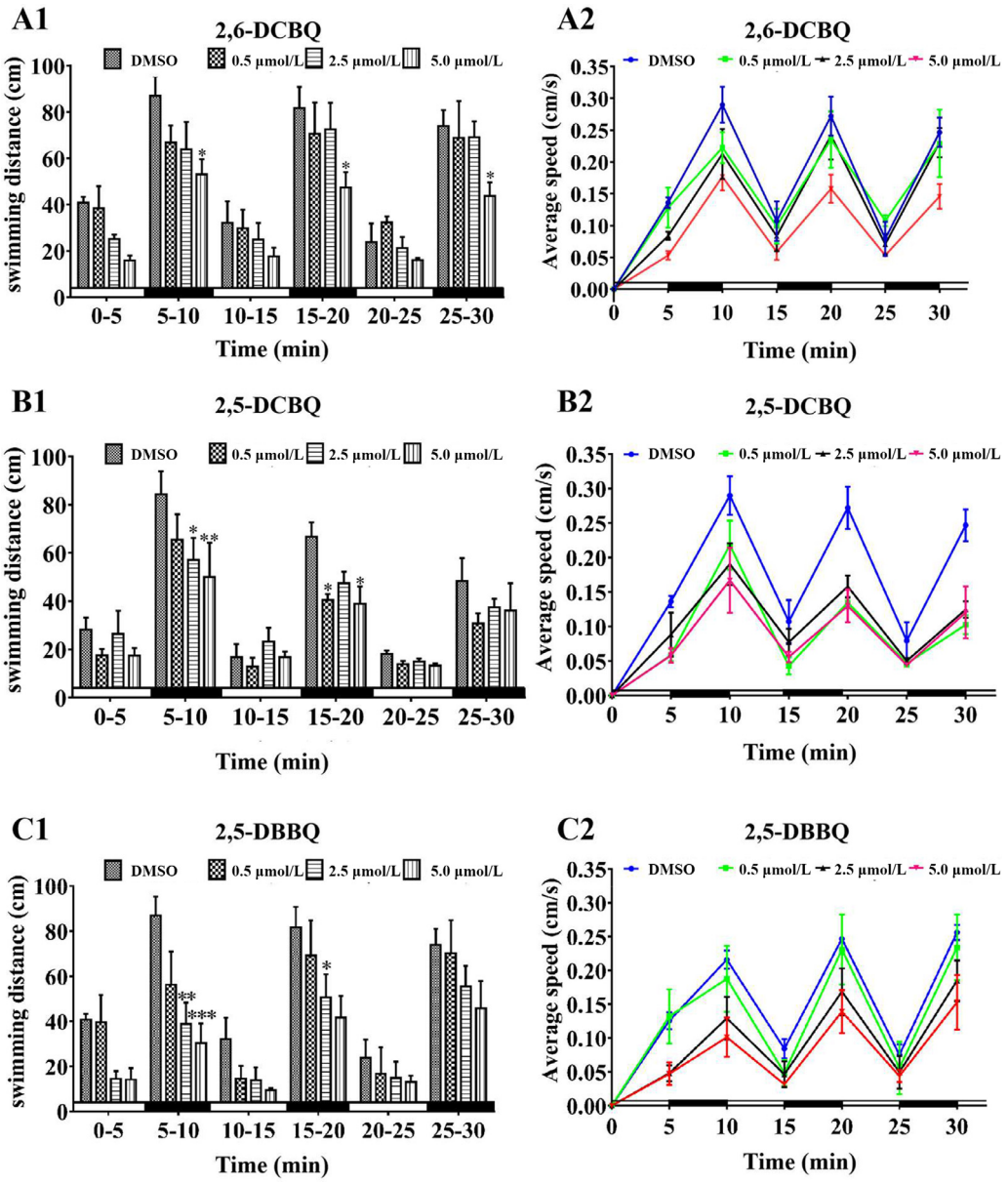


Fig. 2 – (A1- C1) represent the average swimming distance of the embryos exposed to 2,6-DCBQ, 2,5-DCBQ, and 2,5-DBBQ at 120 hpf under the conditions of alternating light/dark stimulation. (A2-C2) represent the average speed of the embryos exposed to 2,6-DCBQ, 2,5-DCBQ, and 2,5-DBBQ at 120 hpf under the conditions of alternating light/dark stimulation. DMSO was the solvent control without HBQ. Error bars represent mean ± SD. Three replicates were performed for each exposed group for 20 larvae per replicate. One-way ANOVA with Dunnett's post-test: *$p<0.05$, **$p<0.01$, ***$p < 0.001$. These measurements are summarized in Appendix A Table S3. The white and black bars on the X-axis indicate light and dark periods, respectively.

AchE activity was detected after exposure to varying concentrations of 2,5-DCBQ and 2,5-DBBQ. The two HBQs have the same structure and the effects of the Cl and Br substations can be compared. In the 0.5, 2.5 and 5 μmol/L 2,5-DCBQ exposure groups, reductions in DA and GABA were 25%-55% and 32%-58%, respectively. In the 2.5 and 5 μmol/L 2,5-DBBQ exposure groups, reductions in DA and GABA were 56%-73% and 41%-51%, respectively. In the 5 μmol/L 2,5-DCBQ and 2,5-DBBQ exposure groups, AchE activity significantly decreased up to 47% and 46%, respectively. Even at 0.5 μmol/L concentration, both 2,5-DCBQ and 2,5-DBBQ inhibited AchE activity. In general, the reduction of GABA and the inhibition of AchE activity was dose-dependent; 2,5-DCBQ and 2,5-DBBQ had similar effects. These results are consistent with previous studies that revealed an association between alterations in neurotransmitters and abnormalities in CNS function (Buckley *et al.*, 2010; Meng *et al.*, 2018). Noticeably, the GABAergic system acts as a switch for zebrafish retinal ganglion cells in response to light,

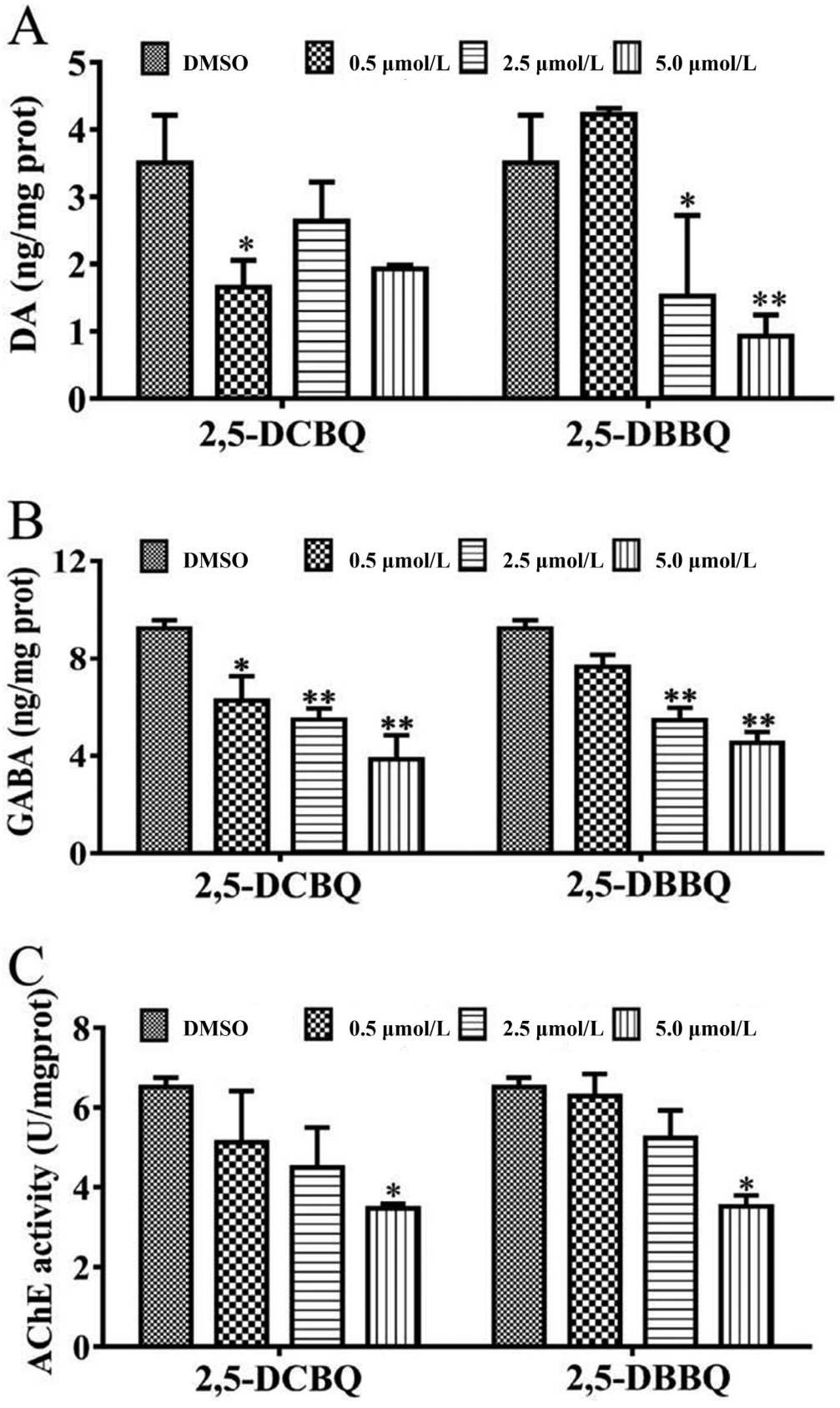


Fig. 3 – Neurotransmitter content and enzyme activity of zebrafish embryos exposed to 2,5-DCBQ and 2,5-DBBQ at 120 hpf: (A) dopamine, (B) gamma-aminobutyric acid, (C) acetylcholinesterase activity. DMSO is the solvent control group. Error bars represent mean ± SEM. Three replicates were performed for each exposure group, and one-way ANOVA with Dunnett's post-test: *$p < 0.05$, ** $p < 0.01$.

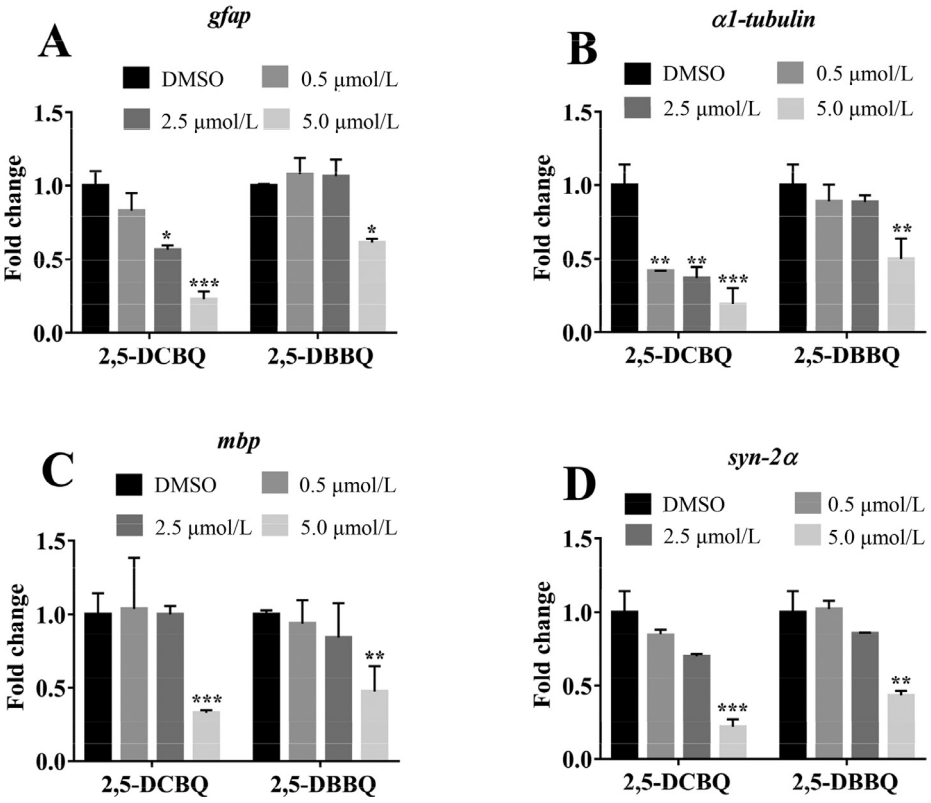


Fig. 4 – Expression profiles of the *gfap* (A), *α1-tubulin* (B), *mbp* (C), and *syn-2α* (D) genes in zebrafish larvae at 120 hpf after exposure to 2,5-DCBQ (0, 0.5, 2.5, and 5 μmol/L) and 2,5-DBBQ (0, 0.5, 2.5, and 5 μmol/L). DMSO was the solvent control group without any HBQ. Error bars indicate the mean±SEM. Three replicate experiments were performed for each exposure group. One-way ANOVA with Dunnett's post-test: *$p<0.05$, ** $p< 0.01$, ***$p < 0.001$.

and disruption of GABAerbic signaling can induce behavior under light-dark transitions (Zhang *et al.*, 2010). These results demonstrated the effects of HBQ exposure on the function of the neurotransmitter system.

2.4. *HBQ effects on the expression of neurodevelopment-related genes in larvae*

To support the neurotoxicity of HBQs with molecular evidence, we subsequently examined the expression of the marker genes of the CNS reported in previous studies (Wang Q et al., 2015; Wang et al., 2015; Wu et al., 2016). Four marker genes involved in neuronal development (*gfap*, *α1-tubulin*, *mbp* and *syn-2α*) were analyzed using qRT-PCR. The *gfap* gene is highly expressed in astrocytes; it is one of the most common markers of mature astrocytes (Sofroniew and Vinters, 2010). The expression of the *α1-tubulin* gene is neural-specific and regeneration-inducible in the neural development of zebrafish (Gulati-Leekha and Goldman, 2006), and it has been used for the screening of developmental neurotoxicity (Fan *et al.*, 2010). The *mbp* gene is expressed in oligodendrocytes; myelin basic protein (MBP) is the main component of the myelin sheath (Brösamle and Halpern, 2002). The *syn-2α* gene is the biomarker of synapse formation and plays a vital role in synaptogenesis and neurotransmitter release (Kao *et al.*, 1998; Nielsen and Jørgensen, 2003). The *mbp* gene plays an important role in neuronal morphogenesis, and is required for axonal myelin formation in the zebrafish CNS.

Figure 4 shows the changes in the expression of the four genes after exposure to HBQs compared to the DMSO group. The transcripts of the two cytoskeleton-related genes (*gfap* and *α1-tubulin*) were significantly downregulated in larvae after exposure to HBQs, compared to the DMSO control group. Figure 4A shows that the expression of the *gfap* gene was downregulated by 1.8- and 4.4-fold in the 2.5 and 5 μmol/L 2,5-DCBQ exposure groups, respectively, and by 2.0-fold in the 5 μmol/L 2,5-DBBQ exposure group. Figure 4B shows that the expression of the *α1-tubulin* gene was downregulated by 2.4-, 2.7-, and 5.2-fold in the 2,5-DCBQ exposure groups (0.5, 2.5, and 5 μmol/L), respectively, and by 1.6-fold in the 5 μmol/L 2,5-DBBQ exposure group. The aberrant expression of the *gfap* and *α1-tubulin* genes suggests that HBQ exposure leads to impaired axon and dendrite extension in zebrafish neuronal cells, which in turn leads to poor neurotransmitter formation and release. In addition, the expression of *mbp* gene was significantly downregulated by 3.0-fold in the 5 μmol/L 2,5-DCBQ exposure group, and by 2.1-fold in the 5 μmol/L 2,5-DBBQ exposure group (Fig. 4C). The expression of *syn-2α* genes was significantly downregulated by 4.5-fold and 2.3-fold in the 5 μmol/L 2,5-DCBQ and 5 μmol/L 2,5-DBBQ exposure groups,

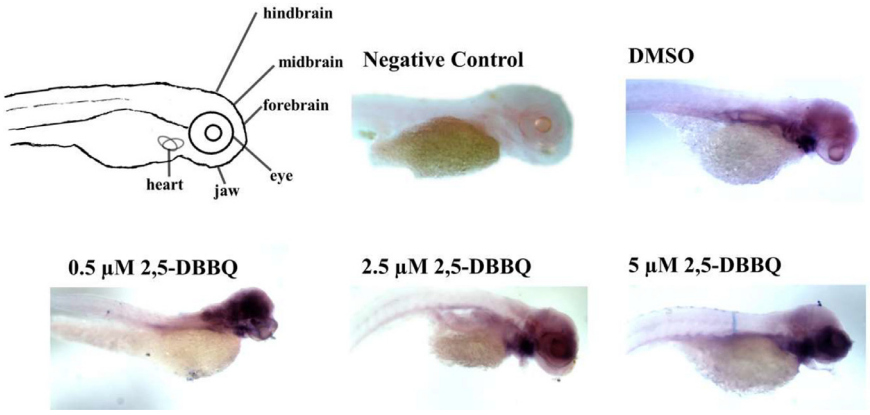


Fig. 5 – The *gfap* gene spatial expression in zebrafish embryos exposed to 2,5-DBBQ (0, 0.5, 2.5, 5 μmol/L) in whole-mount *in situ* hybridization at 96 hpf. The top left is the lateral view anatomy of zebrafish. Negative control (normal embryos without treatment) was stained using the negative probes to exclude nonspecific staining (no false positive) (Appendix A Table S2); DMSO was the solvent control group without any HBQ. The violet black indicates the expression area of the *gfap* gene. Scale bar, 450 μm.

respectively (Fig. 4D). These results (Fig. 4) demonstrated that HBQ exposure induced adverse effects on the development of neuronal systems.

In addition, we visualized the spatial expression of the *gfap* gene by WISH. The glial fibrillary acidic protein (GFAP) is a marker of retinal and astrocytic cells in fish and mammals, mainly present in astrocytes of the CNS (Nielsen and Jørgensen, 2003). As shown in Fig. 5 , the negative control was used to exclude nonspecific staining and to detect the interference of endogenous alkaline phosphatase. No false positive was detected. In the DMSO solvent control group, the *gfap* gene was mainly expressed in the brain. In the exposure groups, the *gfap* gene was expressed in both the brain and the eyes of zebrafish. It is noteworthy that the expression of the *gfap* gene in the eyes was significantly upregulated with increasing exposure concentrations, suggesting that 2,5-DBBQ exposure impacted the neural retina of zebrafish embryos. This result supports the mechanisms of locomotor behavior under dark stimuli, and the lower activity of the high exposure group. The results support that HBQ exposure adversely impacts the visual nerve of zebrafish.

2.5. *Transcriptomic analysis supporting neurotoxicity in zebrafish larvae after exposure to 2,6-DCBQ*

Based on the sequencing results, we analyzed 1407 significantly differentially expressed genes (DEGs, relative to the control) in the 8 μmol/L 2,6-DCBQ exposure group. Then, we performed the Gene Ontology (GO) analysis of these DEGs. GO analysis classified DEGs into three main categories, cellular components (CC), molecular functions (MF), and biological processes (BP). As shown in Table 1, the top ten significantly enriched GO terms describe components, functions, or processes of the oxidation-reduction system and mitochondrial respiratory chain, including oxidoreductase activity, oxidation-reduction process, peroxidase activity, respiratory chain, and mitochondrion. These results are consistent with previous studies on the effects of HBQ-induced oxidative stress (Li et al., 2018; Wang et al., 2018). Therefore, the high-energy swimming behavior is inhibited to a certain extent because of the positive correlation between severe oxidative stress and ATP consumption (Agalakova and Gusev, 2012; Hemalatha and Prince, 2016). To investigate the causes of behavioral changes in zebrafish under light and dark stimuli, we performed an in-depth analysis of DEGs and found a number of signaling pathways related to eye development, such as response to light stimulus, photoreceptor outer segment, detection of light stimulus, retina development, visual system development, photoreceptor activity, phototransduction, and cellular response to light stimulus. One possible reason for ocular tissue lesions due to mitochondrial dysfunction and high energy-dependent function of ocular tissues has been reported (Dorokhov *et al.*, 2015; Liberski *et al.*, 2022). Transcriptomic analysis also showed several DEGs related to the cytochrome c oxidase family and energy metabolism (Appendix A Table S5). Hence, the main reason for the decrease in locomotor activity under dark stimuli caused by HBQ exposure may be attributed to the disruption of the respiratory electron transport chain induced by oxidative stress, which leads to abnormal energy metabolism and ultimately results in decreased swimming activity and impaired visual function.

It is well known that movement behavior and visual perception are both regulated by the nervous system. We observed that 10 DEGs were enriched in both of the two biological processes related to neurogenesis (53 DEGs) and visual system development (26 DEGs) (Tables 1 and 2). Meanwhile, six DEGs were enriched in phototransduction, response to light stimulus, and the photoreceptor activity pathway, which were upregulated (Table 2). All six DEGs belong to the cone opsin 1 family. The zebrafish retina contains five types of photoreceptors (Hamaoka et al., 2002). One study showed that 5 μg/L BDE-209 exposure induced neurobehavioral effects correlated with the upregulation of four cone opsins (Zhang *et al.*, 2020). These results show that opsins are potential targets for the response of optic genes to HBQ exposure. We also found that the GABA-A receptor complex (3 DEGs) and cir-

Table 1 – Details of the top 10 and neural & visual development related GO terms.

Description	Category	DI	GO Term	P-Value	Down	Up
top ten GO terms	Molecular Functions	GO:0016491	oxidoreductase activity	4.26E-11	19	62
	Biological Process	GO:0055114	oxidation-reduction process	5.21E-11	79	24
	Biological Process	GO:0030198	extracellular matrix organization	2.79E-10	4	25
	Biological Process	GO:0097237	cellular response to toxic substance	1.30E-08	14	4
	Molecular Functions	GO:0005518	collagen binding	6.90E-08	1	9
	Molecular Functions	GO:0005504	fatty acid binding	7.19E-08	0	12
	Molecular Functions	GO:0004601	peroxidase activity	1.74E-07	4	9
	Cellular Component	GO:0070469	respiratory chain	3.94E-07	7	30
	Cellular Component	GO:0005739	mitochondrion	5.46E-07	22	64
	Cellular Component	GO:0005581	collagen trimer	6.31E-07	0	16
GO terms related to neural and visual development	Biological Process	GO:0009416	response to light stimulus	0.001803775	5	9
	Cellular Component	GO:0001750	photoreceptor outer segment	0.001812457	0	8
	Biological Process	GO:0042133	neurotransmitter metabolic process	0.002164368	0	7
	Biological Process	GO:0009583	detection of light stimulus	0.004308825	1	7
	Biological Process	GO:0060041	retina development in camera-type eye	0.007386354	9	8
	Biological Process	GO:0150063	visual system development	0.013139595	14	12
	Molecular Functions	GO:0009881	photoreceptor activity	0.016217277	0	7
	Biological Process	GO:0022008	neurogenesis	0.022832969	24	29
	Biological Process	GO:0007602	phototransduction	0.026251224	0	6
	Biological Process	GO:0071482	cellular response to light stimulus	0.045110389	1	6

Table 2 – DEGs were enriched in GO terms related to neurogenesis and visual system development.

GO Term	ID	Regulation	Fold Change	Gene Name	Description
neurogenesis & visual system development	ENSDARG00000098663	up	2.121963734	*fgf19*	fibroblast growth factor 19
	ENSDARG00000100558	up	1.517861336	*slbp*	stem-loop binding protein
	ENSDARG00000044601	up	1.346533386	*rtn4a*	reticulon 4a
	ENSDARG00000025428	down	2.213301514	*socs3a*	suppressor of cytokine signaling 3a
	ENSDARG00000022712	down	1.807843759	*stat3*	signal transducer and activator of transcription 3
	ENSDARG00000027423	down	1.780311882	*igf1ra*	insulin-like growth factor 1a receptor
	ENSDARG00000007377	down	1.678729754	*odc1*	ornithine decarboxylase 1
	ENSDARG00000007377	down	1.678729754	*odc1*	ornithine decarboxylase 1
	ENSDARG00000019743	down	1.612825104	*dctn1a*	dynactin 1a
	ENSDARG00000060323	down	1.32365762	*exoc5*	exocyst complex component 5
phototransduction & response to light stimulus & photoreceptor activity	ENSDARG00000044862	up	4.780296153	*opn1lw1*	opsin 1 (cone pigments), long-wave-sensitive, 1
	ENSDARG00000044861	up	2.975433759	*opn1lw2*	opsin 1 (cone pigments), long-wave-sensitive, 2
	ENSDARG00000097008	up	2.7449883	*opn1mw1*	opsin 1 (cone pigments), medium-wave-sensitive, 1
	ENSDARG00000044280	up	2.249025471	*opn1mw2*	opsin 1 (cone pigments), medium-wave-sensitive, 2
	ENSDARG00000045677	up	2.29278329	*opn1sw1*	opsin 1 (cone pigments), short-wave-sensitive, 1
	ENSDARG00000017274	up	1.957713974	*opn1sw2*	opsin 1 (cone pigments), short-wave-sensitive, 2

Table 3 – Details of the top 10 and neural & visual development related GO terms.

GO Term	DEGs Name	Description	Regulation	Fold Change
GABA-A receptor complex	*zgc:110204*	Gamma-aminobutyric acid (GABA) A receptor, subunit alpha 4	down	1.876481413
	loc562831	gamma-aminobutyric acid receptor subunit pi-like	down	2.515072521
	gabra1	gamma-aminobutyric acid (GABA) A receptor, alpha 1	down	1.814761552
circadian rhythm	*clocka*	clock circadian regulator a	down	1.749133009
	npas2	neuronal PAS domain protein 2	down	1.874986229
	htr7a	5-hydroxytryptamine (serotonin) receptor 7a	down	2.799171731
	nocta	nocturnin a	down	1.995153851
	nfil3-6	nuclear factor, interleukin 3 regulated, member 6	down	1.400886429

cadian rhythm (5 DEGs) pathways were downregulated. As shown in Table 3, the three genes of the GABA-A receptor complex were downregulated, which was consistent with the result of the levels of GABA. Notably, a gene enriched in the biological process associated with circadian rhythm was downregulated by 2.8-fold, namely the 5-hydroxytryptamine (serotonin) receptor 7a (*htr7a*) gene. Several studies have shown that depletion of serotonin leads to a reduction of locomotor behavior in zebrafish (Airhart *et al.*, 2012; Wang *et al.*, 2016), and that serotonin promotes development and regeneration of spinal motor neurons in zebrafish (Barreiro-Iglesias et al., 2015). Thus, in our results, the inhibition of locomotor behavior in zebrafish may be partly caused by the disruption of serotonergic signaling. To date, most studies have attributed the pollutant-induced neurobehavioral changes primarily to abnormal thyroid function (Branchi et al., 2003; Fonnum and Mariussen, 2009). However, the consistent abnormalities in neurobehavioral and visual system development in our findings may provide a new insight into the neurotoxic mechanisms of HBQs.

3. Conclusion

This study demonstrated that HBQ exposure induced impacts on the neurodevelopment of embryonic zebrafish, supported by multiple factors ranging from behavior to the expression of specific genes controlling neurodevelopment. HBQ exposure resulted in abnormal locomotor behavior, reduced levels of neurotransmitters (DA and GABA), and inhibition of AchE enzyme activity. The damage was accompanied by alterations in the expression of genes related to neurodevelopment. Transcriptomic analysis further revealed that HBQ exposure altered the expression of several genes associated with neural and ocular visual development. These findings contribute to our understanding of the developmental toxicity of HBQs.

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Appendix A Supplementary data

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jes.2022.03.042.

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