보건학석사 학위논문

Thyroid and Sex Hormone Disruption Potentials of HBB (Hexabromobenzene) in Embryo-Larval and Adult Zebrafish (*Danio rerio*)

제브라피쉬(*Danio rerio*) 배아-치어 및 성어를 이용한 HBB(Hexabromobenzene)의 갑상선 및 성호르몬 교란 영향 연구

2023 년 8 월

서울대학교 보건대학원

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이 유 라

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Abstract

Thyroid and Sex Hormone Potentials Disruption of HBB (Hexabromobenzene) in Embryo-Larval and Adult Zebrafish (*Danio rerio*)

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Novel brominated flame retardants (NBFRs) are chemicals that have recently been used as substitutes for conventional brominated flame retardants (BFRs). NBFRs have a different structure from the conventional brominated flame retardants, but have similar physical-chemical properties. NBFRs have been detected in a wide variety of environments, products and biotics. HBB is one of NBFRs with a high log K_{ow} value. HBB has recently been reported to be associated with genes involved in thyroid regulation in aquatic organism. However, despite these potential toxicities, studies on endocrine toxicity by HBB exposure are very limited. The purpose of this study is to determine the mechanism of thyroid toxicity by developmental stage of zebrafish (*Danio rerio*). Following a 7-day exposure to HBB, changes in thyroid hormones and associated gene expression are analyzed in embryo-larval zebrafish. The whole-body thyroid hormones that were measured in larval zebrafish, i.e., total T3 and T4, and free T3 and T4, and

TSH were increased. In addition, central regulatory genes (*crh, trh*), metabolism-related genes (*dio1, dio3a, dio3b*) and hormone synthesis genes (*nis, tg, tpo*) were down-regulated. In adult male fish of 6 months of age, a 21-day exposure to HBB showed the changes in hormone and gene transcription related to HPT (Hypothalamus-Pituitary-Thyroid) axis. In the plasma of adult zebrafish, total T4 and TSH tended to increase. In addition, exposure to HBB lowered E2 (17β-estradiol) and 11-KT (11-ketotestosterone) in the adult fish. The results of this study showed thyroid disrupting responses of HBB in different life stages of zebrafish, and sex hormone disruption in the adult fish. The findings of this study suggest that endocrine toxicities of novel brominated flame retardants that replace conventional brominated flame retardants warrant further investigations.

Keywords : Novel brominated flame retardants (NBFRs), Brominated flame retardants (BFRs), Hexabromobenzene (HBB), Zebrafish, Thyroid hormone, Sex hormone, Endocrine disruption

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1. Introduction

Brominated flame retardants have been used to prevent and delay ignition of consumer materials such as plastics, electricity, electronic circuit devices, televisions, building materials, and fibers (Birnbaum et al., 2004). Polybrominated diphenyl ethers (PBDEs) are among the most frequently used compounds, but have been gradually restricted and phased out worldwide due to the persistence and bioaccumulation, long-distance transport properties, and potential health hazards to the environment and humans (UNEP 2013, UNEP 2018, Wu et al., 2019).

NBFRs (e.g. DBDPE (Decabromodiphenyl ethane), BTBPE (1,2-bis (2,4,6-tribromophenoxy) ethane) and HBB (Hexabromobenzene)) are used as substitutes for PBDEs (Shen et al.,2019, Zuiderveen et al.,2020). NBFRs are utilized and discovered in a range of settings and goods. For instance, 15 NBFRs were detected in car seat foams purchased in China, the United States, and Canada (Wu et al., 2018). The concentration of NBFRs in indoor air in the Spanish office was found to be higher than that of conventional brominated flame retardants (Recheet et al., 2019). Such accumulating use of NBFRs and high trophic magnification potential resulted in widespread occurrences of these compounds. In the coastal waters of the South China Sea in China, 92.7% in 29 species of biota (shell species and fish) were detected for NBFRs. HBB was detected in the tissues of mink whales in the estuary of St. Lawrence, Canada.

NBFRs differ chemically from traditional BFRs, although most of their physico-chemical characteristics (aromatic residues, lipophilic characteristics) are the same (Ezechiáš et al., 2014, Li et al., 2019). Several studies suggest that these shared traits are related to adverse effects of NBFR on organisms of developmental stages including neurodevelopmental disorders (Gan et al., 2016, Gilera et al., 2020). A study (Simond et al., 2019) reported that HBB affects gene regulation involved in the regulation of thyroid and steroid axes.

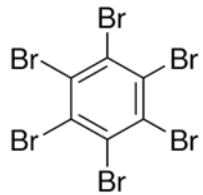
However, there are knowledge gaps for effects of HBB on hypothalamus-pituitary-thyroid (HPT) axis of the endocrine system, particularly in fish. Thyroid hormones are crucial for early development, growth, metabolism, and survival, particularly in the larval stage of fish (Cole et al., 2020). Thyroid hormones have a significant impact on different life phases (Brown et al., 1997, Gilbert et al., 2012, Jabbar et al., 2017). The disruption of thyroid function especially during developmental stages of life may cause various health problems and stunted growth (Busby et al., 2010, Howe et al., 2013 , Lazcano et al., 2023). The use of zebrafish as a model for hormone disruption study has various advantages, including considerable resemblance to the human hormone regulation and a short reproductive cycle (Dooley and Zon, 2000, Ali et al., 2011, Howe et al., 2013, Lazcano et al., 2023). In the present study, we used zebrafish (*Danio rerio*) as an experimental model and investigates endocrine disruption mechanisms, particularly the thyroid control axis, at different stages of life.

2. Materials and Methods

2.1. Reagents

HBB (Hexabromobenzene, CAS No. 87-82-1) purchased from Sigma-Aldrich (St. Louis, MO, USA) (CAT No. 107131-25G). The structure and physicochemical properties of the target material are shown in Table 1. Dimethyl sulfoxide (DMSO, CAS No. 67-68-5) was used as a solvent for all the test chemicals. The concentration of DMSO was set at 0.01% v/v for in vivo studies.

Table 1. Physicochemical properties of HBB in this study

Compound	CAS No	Chemical Structure	Formula	MW(g/mol)[a]	Log K_{ow} [a]	Log K_{oc} [a]	Vapor Pressure (mmHg) at 25°C
HBB (Hexabromobenzene)	87-82-1		C_6H_6	551.5	7.33	5.268	$1.68*10^{-8}$

[a] PubChem

2.2. Zebrafish maintenance and Embryo exposure

2.2.1. Embryo-larval zebrafish exposure

The OECD (Organization for European Economic Cooperation) Test Guideline (No.236 Fish embryo acute toxicity test: Fish embryo acute toxicity test) was followed with a modification of the exposure duration. In-house fish culture chamber conditions (dechlorinated water at 25 1 ℃, 14:10 h light:dark period), the fertilized egg from wild-type zebrafish (*Danio rerio*) is exposed to HBB for 7 days within 3 hours of fertilization.

To measure changes in thyroid-related gene expression, four repeated groups of 30 fertilized eggs randomly placed in 50 mL beakers were exposed to four HBB concentration groups, a control (dechloroinated water), and a solvent control (DMSO 0.01% v/v) for 7 days. To measure thyroid hormone changes, four repeat groups were randomly placed into 300 embryos in 500-mL chamber exposures of fertilized eggs and exposed to three HBB concentration groups and solvent control (DMSO 0.01% v/v) for 7 days. No significant larval mortality was confirmed at the highest exposure concentration (3 mg/L).

Every day, we replaced more than 95% of the medium with fresh medium, after recording survival rate (%) and hatching rate (%) of embryos. To evaluate mRNA expression levels by whole-body, 20–25 larvae per replicate were randomly collected from zebrafish larvae. For thyroid hormone measurement 250 larvae per repeat group were randomly collected in an e-tube (1.7 ml) and stored in a deep freezer until analysis.

2.2.2. Adult zebrafish exposure

Wild-type male zebrafish (6 months old) were obtained from Sin-seong Aquaculture (Korea) and acclimated for more than 7 days in a laboratory environment before the exposure study. The experiment was conducted according to the OECD Test Guideline (No. 230 21-day fish assay : A short-term screening for oestrogenic and androgenic activity, and aromatase inhibition).

For thyroid hormone analysis, a 21-day exposure was performed wit 16 adult fish placed in a replicate, in a 5L glass beaker containing 4L of HBB treatment. Blood was collected from the caudal vein of male zebrafish following 21-day exposure. The plasma was separated by centrifugation, and placed in a tube and stored in a -80℃ freezer. The gonads, liver, thyroid, and brain of each fish were collected for the measurement of expression of the genes involved in the thyroid hormone regulation system.

2.3. Measurement of Thyroid hormone and gene transcription in larval-adult zebrafish

2.3.1. Thyroid hormone extraction and measurement

To measure thyroid hormone, Zebrafish larvae (n = 250) were homogenized using a motor-driven tissue grinder in 250 l of PBS buffer. Then each sample was sonicated for 20 min at 4 ℃ and centrifuged at 13,000g for an additional 20 min at 4 ℃. The supernatant was transferred to e-tube, and the amount of media collected was recorded.

Total T4 (TT4), total T3 (TT3), thyroid-stimulating hormone (TSH), free T3 (fT3), and free T4 (fT4) were measured using an enzyme-linked immunosorbent assay (ELISA) test kit following the protocol of manufacturer with minor modifications. The test kits for thyroid hormones (CAT No. TF E-2100 for Ft3; CAT No. TF E-2200 for Ft4; CAT No. TF E-2300 for T3; CAT No. TF E-2400 for T4) were purchased from LDN (Germany), and the TSH ELISA kit (CAT No. CSB-EQ027261FI) was purchased from Cusabio Technology LLC (Houston, TX, USA). The measurement was conducted using a plate reader (Tecan Infinite 200, Tecan Group Ltd., Mändorf, Switzerland) following the manufacturer's instructions.

2.3.2. RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR) assay

Measurement of transcriptional gene levels was carried out by quantitative real-time PCR (qRT-PCR). Whole-body zebrafish larvae and dissected fish organ samples were homogenized. Total RNA was extracted using an RNeasy mini kit (Qiagen, Valencia, CA, USA) and an RN Plus mini kit (Qiagen, Valencia, CA, USA). After the extraction of total RNA, samples were checked for RNA quality and quantity with Gen5 2.05 (BioTek, Winooski, VT, USA). The complementary DNA was synthesized

from the extracted RNA samples using the Maxima H Minus cDNA Synthesis Kit (ThermoFisher Scientific, Waltham, MA, USA).

To perform quantitative real-time PCR (qRT-PCR), 18 L of premix and 2 L of cDNA sample were added to a 96-well plate. The premix mixture (18 L) contained 10 L of PCR master mix (Power SYBR Green Master Mix, Applied Biosystems by Lift Technologies, Carlsbad, CA, USA), 0.1 L of forward and reverse primer (10 pmol), and 7.8 L of nuclease-free water (Qiagen). Thermal cycling was 95 ℃ for 10 minutes, 40 cycles of amplification at 95 ℃ for 15 seconds, and 60 ℃ for 1 minute. After the final amplification, melting curve analyses were done.

The qRT-PCR was carried out with the QuantStudioTM 3 Real-Time PCR System (Applied Biosystems). The primer sequences of targeted genes in larval and adult zebrafish in this study are listed in Tables 2 and 3, respectively. The relative mRNA expression level of each targeted gene was normalized to the reference gene (*rpl13* for 7 dpf zebrafish) and quantified using the 2Ct method (Livak and Schmittgen, 2001). Using geNorm analysis, the reference gene *rpl13* was selected as the most stable gene in zebrafish among 5 candidate genes, including *rpl13, rpl8, tbp,* and *ef1a*.

Table 2. Primer sequences of larval zebrafish used in this study

Assay	Gene name	Accession No.	Primer Sequence(5'-3')
7 dpf zebrafish	*rpl13*	NM_198143.1	F : TCTGGAGGACTGTAAGAGGTATGC R : TCAGACGCACAATCTTGAGAGCAG
	crhβ	NM_001007379.1	F : CAATTACGCACAGATTCTCCTCG R : GAAGTACTCCTCCCCCAAGC
	trh	NM_001012365.2	F : CATGCTAGAGGACCCCACTG R : GAGCAGCATCAGGTAGCGTT
	trhr	NM_001114688.1	F : AGCATCCAGAAGACAGGTTACA R : CAGAGGGTTGATGGCACTGT
	tshβ	NM_181494.2	F : GCAGATCCTCACTTCACCTACC R : GCACAGGTTTGGAGCATCTCA
	nis	NM_001145763.2	F : TGGTTGGTGTGGTGGTCAGTTA R : GCATCGCAGGGCTTTTGTT
	tg	NM_001329865.1	F : ACAATCCACTGGGTGTGTGTT R : GAGAGCAAAAGACCTGCCCT
	tpo	NM_021467270.1	F : TGATATCTCCTTCACGCCGC R : TCTGTATGGGGAAGCAGGGA
	mct8	NM_001258230.1	F : GTTCGGGAAGATCGGAGACC R : AACACGGCACACTGAGGAAT
	ttr	NM_001005598.2	F : CGGGTGGAGTTTGACACTTT R : GCTCAGAAGGAGAGCCAGT
	hhex	NM_130934.1	F : ACCAGCCTGATCCCGTCTTA R : GACCACAGCAGAGGCTTACC
	nkx1.2a	NM_131589.1	F : AGGACGGTAAACCGTGTCAG R : CACCATGCTGCTCGTGTACT
	dio1	NM_001324404.1	F : GTTCAAACAGCTTGTCAAGGACTTC R : AGCAAGCCTCTCCTCCAAGTT
	dio2	NM_212789.4	F : CTGCCTGTTTTTCGTGCTGT R : GCAGCTTCGCCCAATTTCA
	dio3a	NM_001256003.1	F : CACGGACACAAGCTGGACTA R : GATGTAGATCAGCAGCGCGT
	dio3b	NM_001177935.3	F : TGCTCCTGACCGCCCTTCAT R : ACACCACCGTCGCGTCCTTT
	ugt1ab	NM_213422.2	F : ACGCAGAGCTGTTAGGTCAC R : TCCTTGGTTAGTGGAGCCCT

sult1st5	NM_001199903.1	F : AGCAGTTCCAGGCGTCAGAGG R : GAACCATCTGCTGCGGTCATCTC

Table 3. Primer sequence related to the HPT axis of male zebrafish used in this study

Assay	Gene name	Accession No.	Primer Sequence(5'-3')
Male zebrafish (Thyroid)	*rpl13*	NM_131263.1	F : GCTGAAGGAATACCGCACCA G : TCCAGTAAGCTGTGTTGCCAT
	tshr	NM_001145763.2	F : ATGGCACAAAGCTGGACTCA G : CAAGGAGCATTGGCCCACTA
	nis	NM_001089391.1	F : CCACTGAAGATCGGCAGAAT G : CAGCCAAGCCCATAGAACA
	tg	NM_001329865.1	F : ACAATCCACTGGGTGTGTGTT G : GAGAGCAAAAGACCTGCCCT
	tpo	XM_021467270.1	F : TGATATCTCCTTCACGCCGC G : TCTGTATGGGGAAGCAGGGA
	dio1	NM_001324404.1	F : GTTCAAACAGCTTGTCAAGGACT G : AGCAAGCCTCTCCTCCAAGTT
	dio2	NM_212789.4	F : TGGATGCCTACAAACAGGTGA G : GTCTTACCGCTGATGCTCC
	dio3a	NM_001256003.1	F : GCGCGTACGGAGCTTACTTC G : AGCTCGGAGATGCGGAATCC
	nkx2.1a	NM_131589.1	F : AGGACGGTAAACCGTGTCAG G : CACCATGCTGCTCGTGTACT

Assay	Gene name	Accession No.	Primer Sequence(5'-3')
Male zebrafish (Brain)	*rpl13a*	NM_198143.1	F : GCTGAAGGAATACCGCACCA G : TCCAGTAAGCTGTGTTGCCAT
	trh	NM_001012365.2	F : CATGCTAGAGGACCCCACTG G : GAGCAGCATCAGGTAGCGTT
	crhβ	NM_001007379.1	F : CAATTACGCACAGATTCTCCTCG G : GAAGTACTCCTCCCCCAAGC
	trhr	NM_001114688.1	F : CAGTGCCATCAACCCTCTGA G : GGCAGCGCGGAACTTCT
	mct8	NM_001258230.1	F : CTTCGGATGTCGGAAAACGG G : CCCAGAGTCGTGGCGAAG

	tshβ	NM_181494.2	F : GCAGATCCTCACTTCACCTACC G : GCACAGGTTTGGAGCATCTCA

Assay	Gene name	Accession No.	Primer Sequence(5'-3')
Male zebrafish (Liver)	*rpl13*	NM_131263.1	F : GCTGAAGGAATACCGCACCA G : TCCAGTAAGCTGTGTTGCCAT
	ttr	NM_001005598.2	F : CGGGTGGAGTTTGACACTTT G : GCTCAGAAGGAGAGCCAGTG
	dio1	NM_001324404.1	F : GTTCAAACAGCTTGTCAAGGACT G : AGCAAGCCTCTCCTCCAAGTT
	dio2	NM_212789.4	F : TGGATGCCTACAAACAGGTGA G : GTCTTACCGCTGATGCTCC
	dio3a	NM_001256003.1	F : GCGCGTACGGAGCTTACTTC G : AGCTCGGAGATGCGGAATCC
	ugt1ab	NM_213422.2	F : GCCAGCTTTGATGAACTTGCC G : AACTCCTCCAGTTCCTTGGTT
	sult1st5	NM_001199903.1	F : GAAAGAGGACCCTGCTCGTG G : TTTGCCATGGGGTTTTCTCG
	mct8	NM_001258230.1	F : CTTCGGATGTCGGAAAACGG G : CCCAGAGTCGTGGCGAAG

2.3. Measurement of sex hormone and gene transcription in adult hormone

2.3.1. Sex hormone extraction and measurement

In zebrafish plasma, sex steroid hormones were measured by enzyme-linked immunosorbent assay (ELISA) using commercially available kits. Hormones were extracted from 5 µL of fish plasma. The samples were diluted with 400 µL of ultrapure water. 2 mL of diethyl ether were added in the diluted samples and then centrifuged at 2000 g for 10 minutes. The upper layer was collected, extracted twice with diethyl ether. After the evaporation of diethyl ether, the dried sample was reconstituted with 300 µL of EIA buffer for 120 µL for the zebrafish using ELISA kit (Ji et al., 2010).

2.3.2. RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR) assay

Measurement of transcriptional gene levels was carried out by quantitative real-time PCR (qRT-PCR). Isolated fish organ samples were homogenized, and total RNA was extracted using an RNeasy mini kit (Qiagen, Valencia, CA, USA). Total RNA samples were checked for RNA quality and quantity with Gen5 2.05 (BioTek, Winooski, VT, USA). The complementary DNA was synthesized from the extracted RNA samples using an iScriptTM cDNA synthesis kit (BioRad, Hercules, CA, USA). To perform quantitative real-time PCR (qRT-PCR), 18 µL of premix and 2 µL of cDNA sample was added in a 96 well plate. The premix mixture (18 µL) contained 10 µL of PCR master mix (Power SYBR Green Master Mix, Applied Biosystems by Lift Technologies, Carlsbad, CA, USA), 0.1 µL of forward and reverse primer (10 pmol) and 7.8 µL of nuclease-free water (Qiagen). Thermal cycling was 95◦C for 10 minutes, 40 cycles of amplification at 95 °C for 15 seconds and 60 °C for 1 minute. After the final amplification, melting curve analyses were done. The qRT-PCR was carried out with QuantStudio^{TM} 3 Real-Time PCR System (Applied Biosystems).

The primer sequences of targeted genes zebrafish in this study are listed in Table 4 respectively. The primer sequences in this study were used according to a previous study by Ji et al. (2013). The relative mRNA expression level of each targeted gene was normalized to the reference gene (*rpl13a* for male zebrafish brain; *rpl13* for male zebrafish gonad and liver) and quantified using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Using geNorm analysis, the reference genes, i.e., *rpl13a* and *rpl13* were selected as the most stable gene in zebrafish among 5 candidate genes including *rpl13, rpl13a, β-actin, tbp*, and *elf1a*.

Table 4. Primer sequence related to the HPG axis of male zebrafish used in this study.

Assay	Gene name	Accession No.	Primer Sequence(5'-3')
Male zebrafish (Gonad)	*rpl13*	NM_131263.1	F : GCTGAAGGAATACCGCACCA G : TCCAGTAAGCTGTGTTGCCAT
	star	NM_131663.1	F : TTTCTGGCTGGGATGTCCAC G : GGGTCCATTCTCAGCCCTTAC
	hsd3b2	NM_212797.1	F : TGTTATTGAGGGGGATATCCG G : GCAGGAGCCGTGTAGCTTTAA
	hsd17	NM_205584.2	F :ACGCAGCCTGTATGACCAATA G : TGATGTACTGAGAGCCGTCCA
	cyp11	NM_152953.2	F : GAGGGGTGGACTCGGTTACTT G : GCAATACGAGCGGCTGAGAT
	cyp17	NM_212806.3	F : GGGAGGCCACGGACTGTTA G : CCATGTGGAACTGTAGTCAGCAA
	cyp19	NM_131154.3	F : AGATGTCGAGTTAAAGATCCTGCA G : CGACCGGGTGAAAACGTAGA
	fshr	NM_001001812.1	F : GCTGCTCTTGAATTTCTATTTGATCTAC G : GGTCAATCCGAGGAAAGCATT
	lhr	NM_205625.1	F : TGTGGAGTGTGTTTCGAGTGT G : GCTCTGGGCGATTTCTATTCT

Table 4. (Continued)

Assay	Gene name	Accession No.	Primer Sequence(5'-3')
Male zebrafish (Brain)	*rpl13a*	NM_198143.1	F : GCTGAAGGAATACCGCACCA G : TCCAGTAAGCTGTGTTGCCAT
	gnrh2	NM_181439.4	F : GGTCTCACGGCTGGTATCCT G : TGCCTCGCAGAGCTTCACT
	gnrh3	NM_182887.2	F : CACAACAGCAACAAAGGTGATTC G : CCAGATGCCCAGCAGGTAAT
	gnrhr1	NM_001144980.1	F : GCTCGCCTCTCCACAGTTAT G : GCATCTGGCGGTTGATTTCC
	gnrhr2	NM_001144979.1	F : TGGACCATGAGTGTCGTGTTG G : GCACTGGACAAACTGCTTTGG
	gnrhr4	NM_001098193.1	F : TGGCCTGGGCGATGAGT G : TCGAGTGGTACACTGAGTGAAATTG
	cyp19a1b	NM_131642.2	F : GTCGTTACTTCCAGCCATTCG G : GCAATGTGCTTCCCAACACA
	era	NM_152959.1	F : GGTCCAGTGTGGTGTCCTCT G : CACACGACCAGACTCCGTAA
	er2b	NM_174862.3	F : TTCACCCCTGACCTCAAGCT G : TCCATGATGCCTTCAACACAA
	ar	NM_001083123.1	F : TCTGGGTTGGAGGTCCTACAA G : GGTCTGGAGCGAAGTACAGCAT
	fshβ	NM_205624.1	F : TTGTTCTGGCGCTGCTGTTGC G : TTCTGGGTGTGCTGTGCCAT
	lhβ	NM_205622.2	F : AATGCCTGGTGTTTCAGACC G : AGTATGCGGGGAAATCCTCT

Assay	Gene name	Accession No.	Primer Sequence(5'-3')
Male zebrafish (Liver)	*rpl13a*	NM_198143.1	F : GCTGAAGGAATACCGCACCA G : TCCAGTAAGCTGTGTTGCCAT
	vtg1	NM_001044897.3	F : AGCTGCTGAGAGGCTTGTTA G : GTCCAGGATTTCCCTCAGT
	vtg2	NM_001044913.1	F : GGCTCAGTTCAAGGACAAGC G : TTGCAAAGCAACCACAAGAG

shbg	NM_001014292.2	F : CTGCCGTTCTTCCTGCTGCTGATT G : GCCGACACCAATGACCAGACACTC

2.5. Statistical analysis

The normality of the distribution was assessed using Shapiro-Wilk's test, and the homogeneity of variance was evaluated using Levene's test. To compare the differences among the treatments, a one-way analysis of variance (ANOVA) was performed, followed by Dunnett's or Dunnett's T3 test as a post-hoc analysis. In cases where the data did not exhibit a normal distribution, a nonparametric Kruskal-Wallis test was used. Spearman's rank correlation test was employed to examine the linear trend between the concentration and response. The results are reported as values with the standard deviation of the mean (SD), and a p-value less than 0.05 was considered statistically significant. The statistical analysis was conducted using IBM SPSS Statistics 26 (SPSS Inc., Chicago, IL, USA).

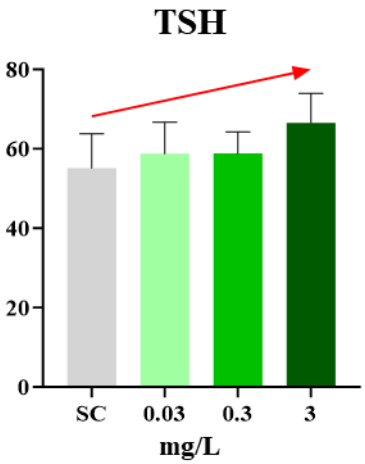
3. Results

3.1. HBB's Effects on Embryo-Larval Zebrafish

3.1.1. Thyroid Hormone and TSH changes

When Following exposure to HBB for 7 days, TT3, TT4, fT3, and fT4 of the larval fish increased in a concentration-dependent manner (Figure 1). Furthermore, all thyroid hormones exhibited a significantly higher pattern in the highest concentration group compared to SC (DMSO 0.01% v/v); TSH showed a dose-dependently increasing trend. TSH was detected at the highest concentration in the highest concentration group, however statistically significance was not observed.

(A)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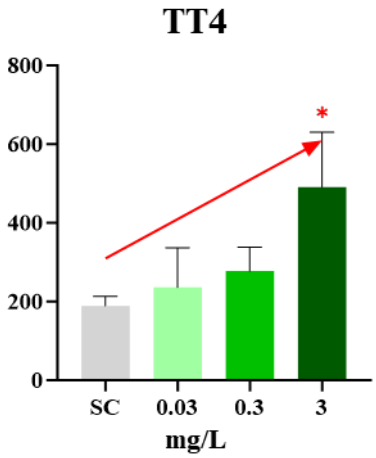
60
40
20
0
SC
0.03
0.3
3
mg/L

(B)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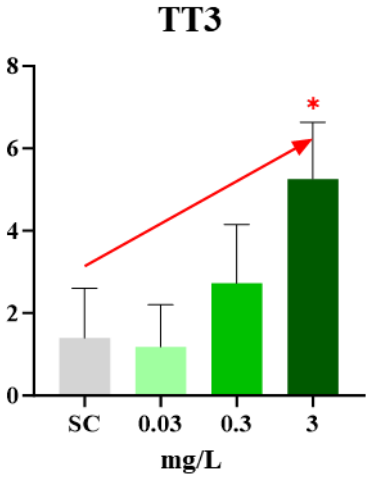
600
400
200
0
*
SC
0.03
0.3
3
mg/L

(C)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6
4
2
0
*
SC
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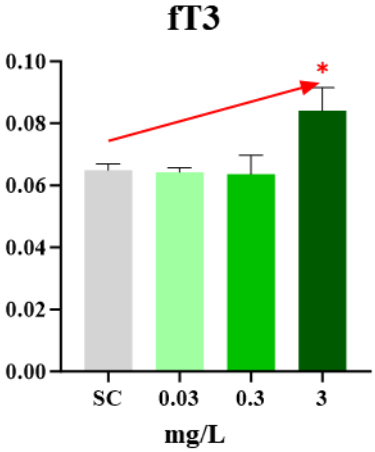
0.3
3
mg/L

(D)


fT3
0.10
0.08
0.06
0.04
0.02
0.00
*
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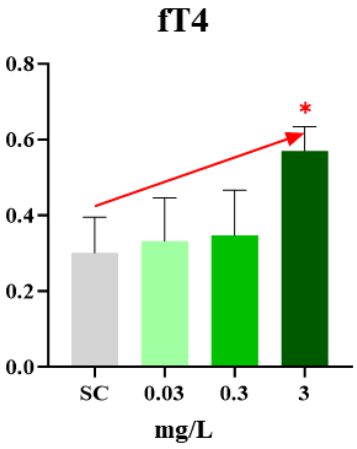
0.03
0.3
3
mg/L

(E)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0.6
0.4
0.2
0.0
*
SC
0.03
0.3
3
mg/L

Figure 1. Thyroid hormone changes in zebrafish larvae exposed to HBB (0.03, 0.3, 3 mg/L; n=4). (A) Thyroid-stimulating hormone (TSH), (B) Total 3 (TT3), (C) Total T4 (TT4), (D) Free T3 (fT3), and (E) Free T4 (fT4) mean concentrations after 7-day exposure are shown as mean ± standard deviation. One-way ANOVA followed by Dunnett's post-hoc test was used for comparison among concentrations. *$P<0.05$.

3.1.2. Gene expression changes

In zebrafish larvae, changes in thyroid hormone-related gene expression have been observed. Changes in thyroid hormone-associated gene expression were found in embryo-larval zebrafish. *crhβ* showed a significantly increased pattern compared to SC, and showed a significant increase at 0.3 mg/L. At the highest concentration (3 mg/L), *dio1* was significantly downregulated, while *dio2* gene was significantly up-regulated in a dose-dependent manner. The transcriptional level of *dio2* gene was significantly increased when compared between 0.3 mg/L and SC. The *dio3a* gene showed a significant increase compared to SC at the highest concentration (3 mg/L), and *dio3b* was slightly upregulated but not significantly. The *tg* showed a significant down-regulating pattern following exposure up to 3 mg/L, while *tpo* showed a non-significant downward pattern up to 3 mg/L. The *hhex* was exhibiting significant down-regulation up to 0.3 mg/L. In *nkx2.1a*, it was significantly lower at 0.3 mg/L compared to SC, but rose again at 3 mg/L. The *ttr* gene showed the lowest transcription at 0.03 mg/L. The *sult1st5* gene showed a concentration-dependent down-regulation.

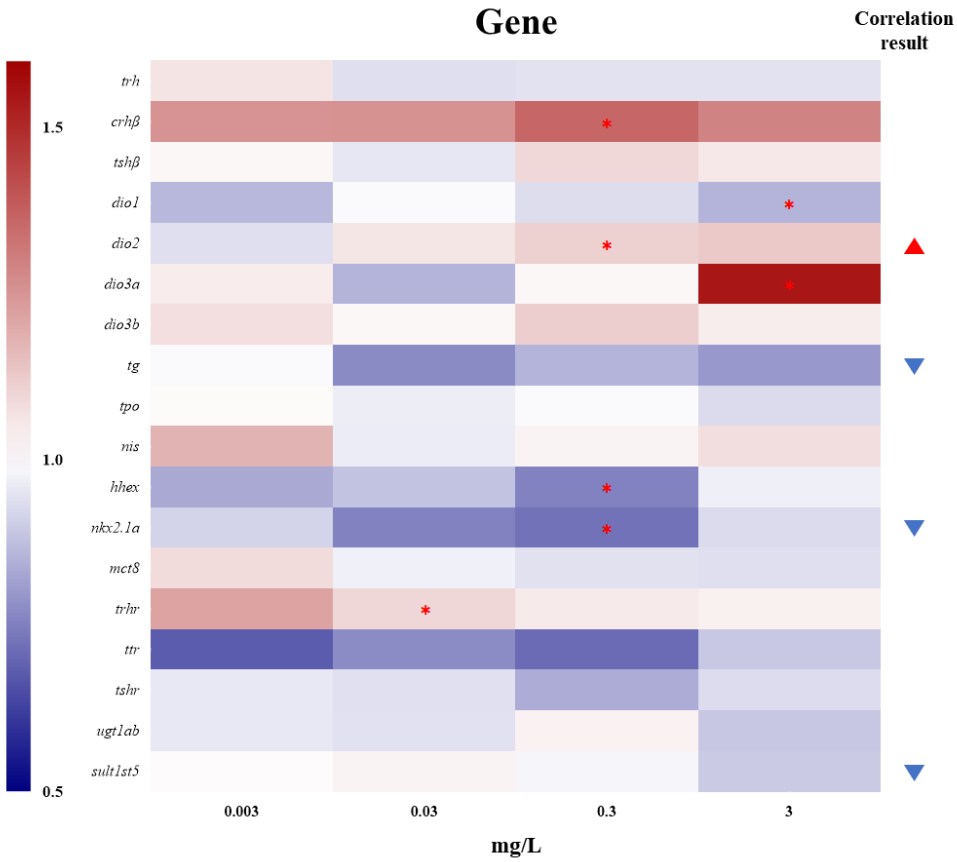


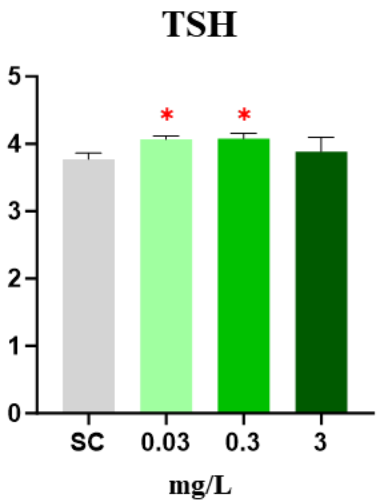
Figure 2. Thyroid hormone-related genes' mean concentrations changed after being exposed to HBB (0.03, 0.3, 3 mg/L; n=4) for 7-days in zebrafish larvae. One-way ANOVA followed by Dunnett's post-hoc test was used for comparison among concentrations. *$P<0.05$. The triangle on the right shows the results for the correlation analysis (blue: down; red: up).

3.2. Effects of HBB on Adult zebrafish

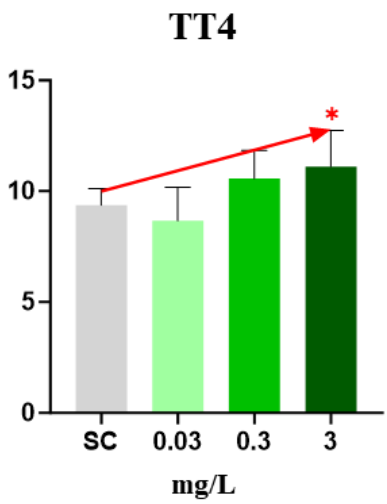
3.2.1. Thyroid Hormone and TSH changes

Thyroid hormone concentrations observed in adult zebrafish after exposure are shown in Fig. 3. When HBB was exposed to 6-month-old adult fish for 21 days, TT3 decreased at the highest concentration (3 mg/L), while TT4 increased in a dose-dependent manner. Also, at the highest concentration (3 mg/L), TT4 showed a significant increase. TSH was significantly increased at 0.03 and 0.3 mg/L but decreased in the highest concentration group.

(A)


TSH
5
4
3
2
1
0
*
*
SC
0.03
0.3
3
mg/L

(B)


TT4
15
10
5
0
*
SC
0.03
0.3
3
mg/L

(C)

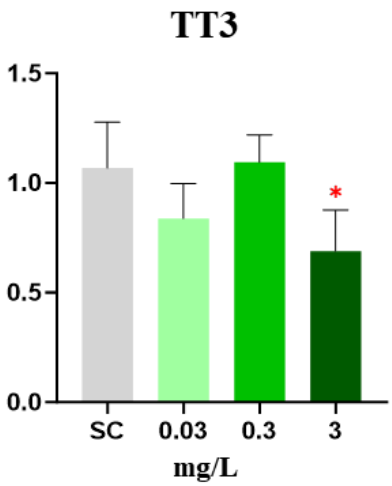
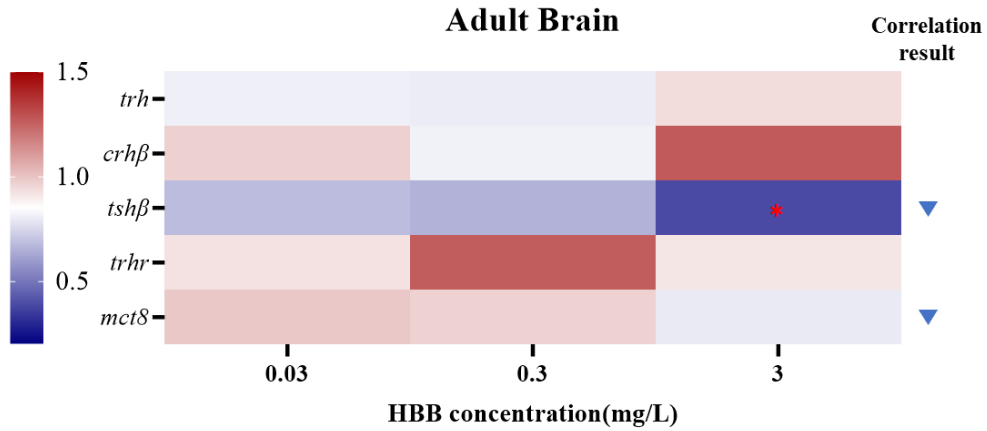

TT3
1.5
1.0
0.5
0.0
*
SC
0.03
0.3
3
mg/L

Figure 3. Thyroid hormone changes in zebrafish adult exposed to HBB (0.03, 0.3, 3 mg/L; n=4). (A) Thyroid-stimulating hormone (TSH), (B) Total T4 (TT4), (C) Total T3 (TT3) mean concentrations after 21-day exposure are shown as mean ± standard deviation. One-way ANOVA followed by Dunnett's post-hoc test was used for comparison among concentrations. *$P<0.05$.

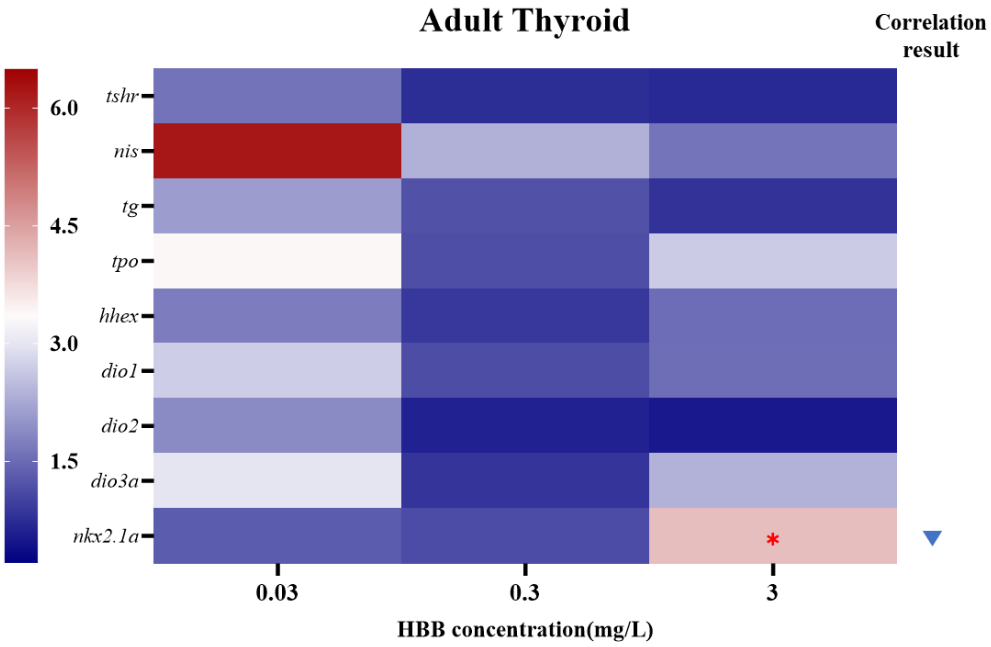
3.3.2. HPT axis gene levels in male zebrafish

After 21-day exposure to HBB, the expression levels of genes related to the HPT axis in male zebrafish were changed (Fig. 4). *tshβ* and *mct8* were significantly decreased in zebrafish brains after HBB exposure in a dose-dependent manner. Compared with SC, *dio2* was down-regulated in all exposure concentrations in the liver, but not significantly. Compared with SC, *mct8* tended to down-regulate in a concentration-dependent manner. In contrast, *dio3a* was up-regulated, but not significantly. The genes related to thyroid hormone synthesis and development, e.g., *nis*, *tg*, and *tpo*, were up-regulated following exposure, while linear dose response relationships were not observed. Furthermore, *dio1*, *dio2*, and *dio3a*, which are related to TH metabolism, showed significant up-regulations compared to SC at the lowest concentration (0.03 mg/L). The transcription of *nkx2.1a* was significantly increased in a dose-dependent response, with the highest concentration (3 mg/L) being significantly increased.

(A)


Adult Brain
Correlation result
1.5
1.0
0.5
trh
crhβ
tshβ
trhr
mct8
*
0.03
0.3
3
HBB concentration(mg/L)

(B)


Adult Thyroid
Correlation result
6.0
4.5
3.0
1.5
tshr
nis
tg
tpo
hhex
dio1
dio2
dio3a
nkx2.1a
*
0.03
0.3
3
HBB concentration(mg/L)

(C)

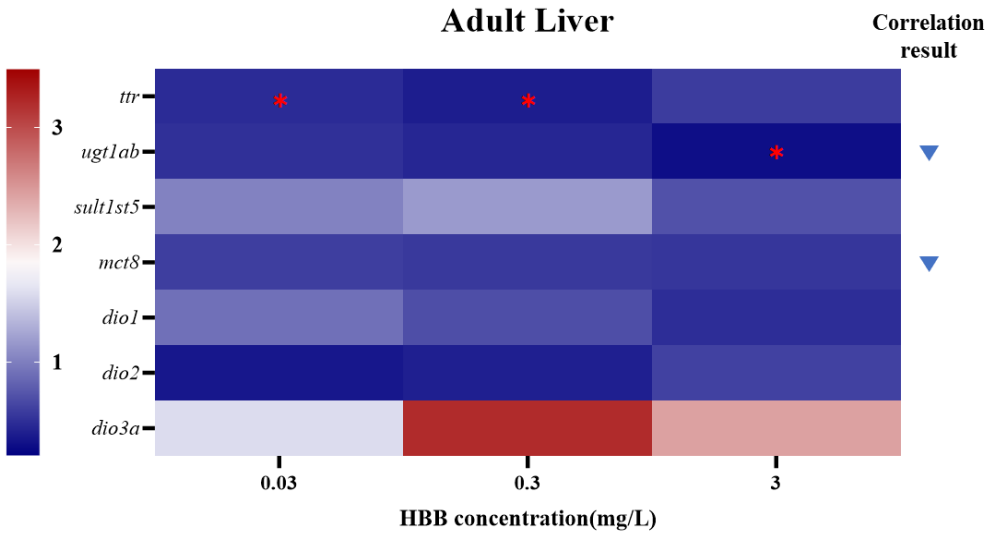
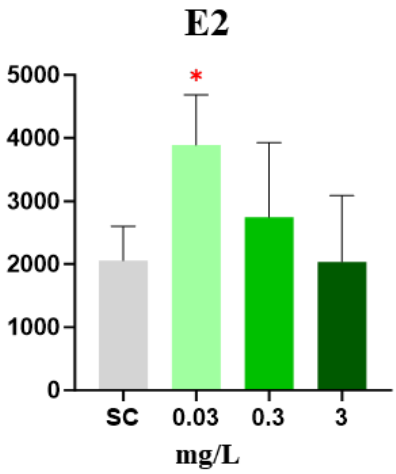


Figure 4. Effects of HBB (0.03, 0.3, and 3 mg/L) and SC (0.01% DMSO v/v) exposure on the levels of genes associated to the HPT axis in male zebrafish following 21-day exposure period are shown in (A) Brain (B) Thyroid (C) Liver. One-way ANOVA followed by Dunnett's post-hoc test was used for comparison among concentrations. *$P<0.05$.

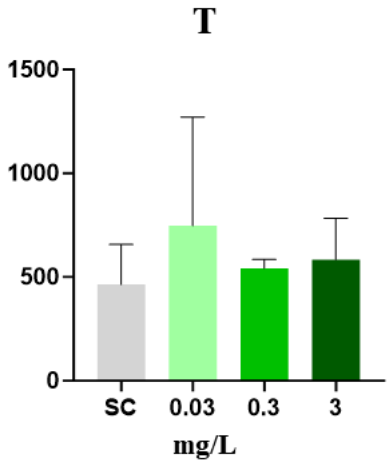
3.2.3. Sex Hormone changes

In the adult male zebrafish following 21-day exposure to HBB, 17β-estradiol (E2) and 11-ketotestoterone (11-KT) showed a significant increase, but only at 0.03 mg/L (Fig. 5). Testosterone (T) was not significantly changed after the exposure.

(A)


E2
5000
4000
3000
2000
1000
0
*
SC
0.03
0.3
3
mg/L

(B)


T
1500
1000
500
0
SC
0.03
0.3
3
mg/L

(C)

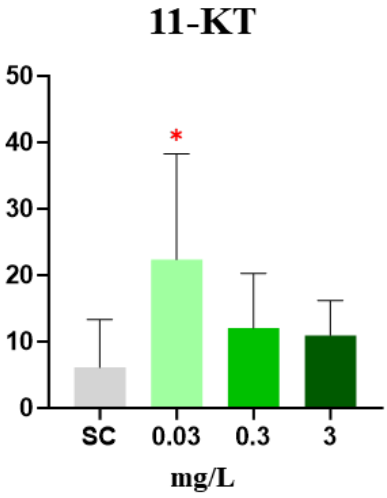
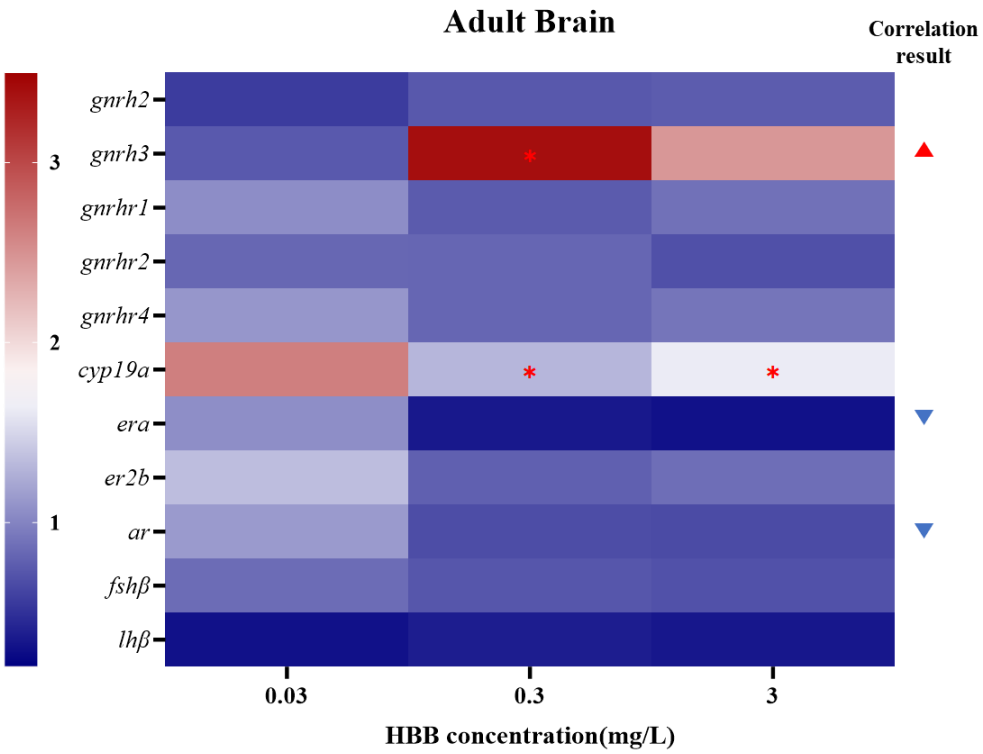

11-KT
50
40
30
20
10
0
*
SC
0.03
0.3
3
mg/L

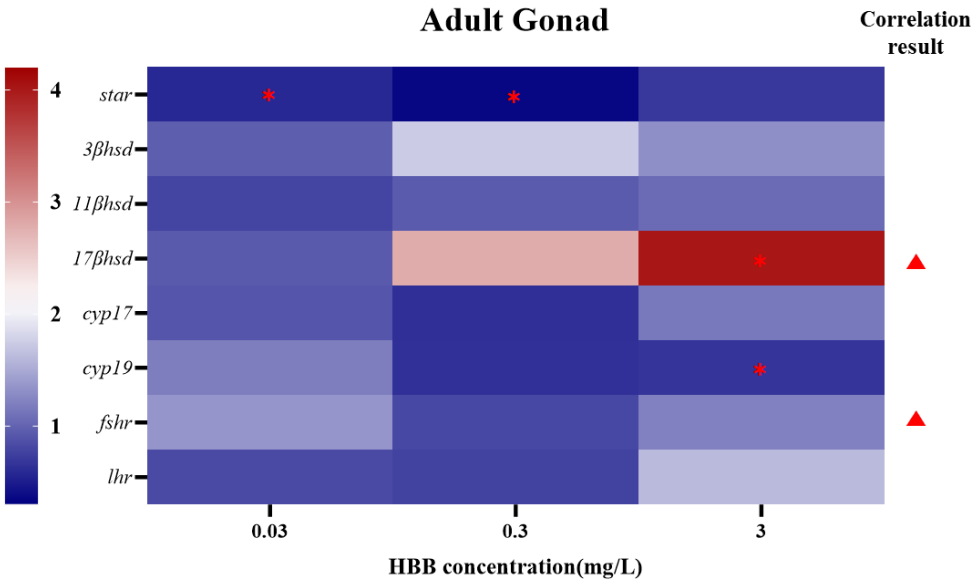
Figure 5. Thyroid hormone changes in zebrafish adult exposed to HBB (0.03, 0.3, 3 mg/L; n=4). (A) 17β-estradiol (E2), (B) Testosterone (T), (C) 11-ketotestosterone (11-KT) mean concentrations after 21-day exposure are shown as mean ± standard deviation. One-way ANOVA followed by Dunnett's post-hoc test was used for comparison among concentrations. *P 0.05.

3.2.3. HPG axis gene levels in male zebrafish

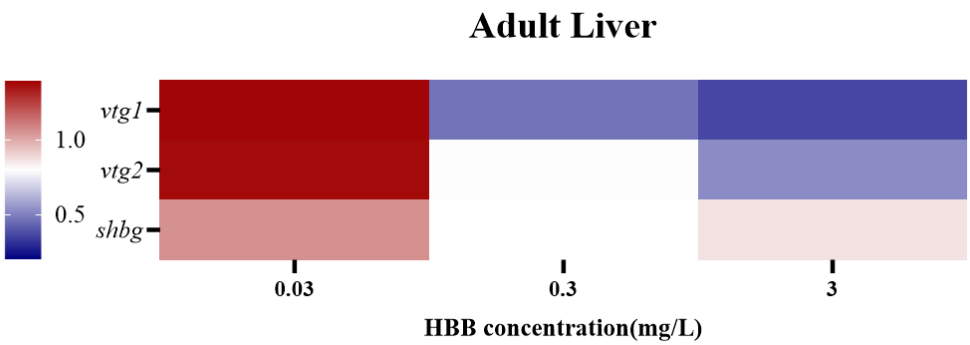
In adult male zebrafish, 21-day exposure to HBB caused changes of several genes related to the HPG axis (Fig. 6). In brain, the concentration-dependent changes of *gnrh3* and *cyp19a* were observed. In gonad, *star* was significantly lower at 0.03, 0.3 mg/L compared than SC. Compared with SC, *17βhsd, fshr* was increased in all concentration.


Adult Brain
Correlation result
gnrh2
gnrh3
gnrhr1
gnrhr2
gnrhr4
cyp19a
era
er2b
ar
fshβ
lhβ
3
2
1
0.03
0.3
3
HBB concentration(mg/L)

(A)


Adult Gonad
Correlation result
star
3βhsd
11βhsd
17βhsd
cyp17
cyp19
fshr
lhr
4
3
2
1
0.03
0.3
3
HBB concentration(mg/L)

(B)



(C)

Figure 6. Effects of HBB (0.03, 0.3, and 3 mg/L) and SC (0.01% DMSO v/v) exposure on the levels of genes associated to the HPG axis in male zebrafish following 21-day exposure period are shown in (A) Brain (B) Gonad (C) Liver. One-way ANOVA followed by Dunnett's post-hoc test was used for comparison among concentrations. *$P<0.05$.

4. Discussion

The general decreases of thyroid hormones in larval zebrafish after exposure to HBB for 7 days indicate that HBB is a thyroid hormone disruptor during the early life stage of zebrafish. The thyroid endocrine system (HPT axis) is responsible for releasing and stimulating hormones for the production and release of thyroid hormones (THs), including tri-iodothyronine (T3) and thyroxine (T4), which play a crucial role in regulating TH dynamics by coordinating their synthesis, secretion, transport, and metabolism (Carr and Patio et al., 2011; Heiko et al., 2011; Sun et al., 2015; Lazcano et al., 2012).

HBB exposure did not cause developmental toxicity (e.g., hatchability, malformations, survival) in larval-embryo/adult zebrafish. These results are consistent with previous studies showing no developmental toxicity in another new brominated flame retardant, DBDPE (Wang et al., 2019). This is consistent with another study showing that BDE-209, a conventional brominated flame retardant, did not affect zebrafish development (Chen et al., 2012).

Thyroid hormone changes in adult male zebrafish, along with sex hormone changes following 21-day exposure to HBB (Figs. 3 and 5) show adverse effects of HBB on both thyroid and sex hormone balances. Our observation also outlines potential link between thyroid and sex hormone regulation: Thyroid hormone (TH), together with follicle-stimulating hormone, are reported to influence androgen biosynthesis by directly and indirectly regulating the expression and activity of steroidogenic enzymes (Liu et al., 2011; Cortes et al., 2014). A recent study (Flood et al., 2013) implicated the promoters of several enzymes and receptors involved in both androgens and thyroid hormones.

Endocrine disruptors may have different effects depending on their life stages. Adult organisms are generally considered to be less sensitive, while reports indicating otherwise are also available

(Dickerson et al., 2007; Zoeller et al., 2012). Therefore, it's critical to comprehend how endocrine disruptors affect endocrine function at various life stages.

4.1. Effects of HBB on embryo-larval zebrafish (*Danio rerio*) in terms of altering thyroid hormone

TT3 and TT4 levels increased after HBB was exposed to embryo-larval zebrafish for 7 days in this study. This result is in line with research showing that DBDPE, a different new brominated flame retardant, raises levels of TT3 and TT4 in the body (Wang et al., 2019). Chen et al. (2012) reported that exposure to BDE-209, a conventional flame retardant, showed a decrease in TT3 and an increase in TT4 in larval zebrafish, which are opposite to the current observations on HBB.

Up-regulations of several thyroid regulating genes such as *crhβ* and *tshβ* genes support the observed increases of thyroid hormones (Figure 2). The corticotropin-releasing hormone (crh) is secreted by hypothalamic neurons and binds to receptors on the anterior pituitary gland. The crh is considered as a regulator of HPT axis, and could be influenced by exposure to thyroid disrupting chemicals in fish (Yu et al., 2013). The *tshβ* gene is a functional element contributing to the stimulation of thyroid stimulating hormone. While statistical significance was not detected, the up-regulating trend of *tshβ* gene coincided with increased TSH levels observed in the current study (Figures 1 and 2). Previous research has demonstrated that PFOS exposure causes comparable changes in hormone and gene levels in zebrafish larvae (Shi et al., 2009). For this reason, a down-regulation of *tshβ* gene together with increased T3 levels has been interpreted as a compensatory transcriptional effort.

The proteins encoded by TH deiodinase genes (*dio1, dio2, dio3*) are important enzymes responsible for bioconversion of thyroid hormones in vertebrates. The *dio2* gene encodes an enzyme that

facilitates the outer ring-deiodination pathway by converting T4 to T3, while *dio1* specifically mediates iodine retrieval and TH degradation (Liu et al., 2019). In a previous study, exposure to PFOS and permethrin increased THs and *dio2* expression (Tu et al., 2016).

Moreover, *dio3* functions as an enzyme that inactivates T3 to T2 (Liu et al., 2019). In this study, *dio2* and *dio3a* levels were both significantly elevated in a concentration-dependent manner. The *dio1* gene however demonstrated a significant decrease. Taken together, these changes in *dio* genes support elevated T3 bioconversion by up-regulated *dio2* gene, but also compensatory efforts to inactivate T3 by up-regulation of *dio3* gene.

Down-regulation of *ugt1ab* and *sult1st5* genes also support increased TH levels. According to Jugan et al. (2010) and Yu et al. (2013), UGT and sulfotransferases are significant players in metabolism and elimination of THs that primarily bind to TH and play an elimination role through glucuronization or sulfation.

The exposure to HBB decreased transcription levels of genes involved in thyroid synthesis, *tg*, *hhex*, and *nkx2.1a*, and small but insignificant transcription levels of *tpo*. Thyroid follicular cell precursors in mammals are developed by the transcription factors nkx2.1a and hhex (Elsalini et al., 2003). The *tpo* promotes the iodination of tyrosyl residue in tg and its binding to iodothyronine, and tg is a protein precursor of TH involved in TH synthesis (Tu et al., 2016). The thyroid electronic factors *nkx2.1a* and *hhex* regulate the expression of *tg* and *tpo*, which are involved in thyroid TH synthesis. Reduced gene levels of *nkx2.1a* and *hhex* decrease the expression of *tg* and *tpo*, respectively. (Chen et al., 2012). In contrast to the current observations, DE-71 exposure in zebrafish larvae (Yu et al., 2010) caused the opposite directions of transcriptional changes in *tg* and *nkx2.1a* genes, and TT4, suggesting that the mode of thyroid disruption of HBB is different from that of DE-71 .

Several new brominated flame retardants (NBFRs) have also been demonstrated to disrupt TH homeostasis, leading to developmental toxicity. In addition, rats exposed to BTBPE, an alternative to octa-BDE, had significantly lower levels of free and total T4 (Weiet al., 2022). Chen et al. (2019) have shown a positive correlation between the concentrations of TT3, TT4, and DBDPE in serum of adult humans, suggesting that chronic human exposure to DBDPE disturbs the homeostasis of thyroid hormones.

4.2. Effects of HBB on male adult zebrafish (*Danio rerio)* in terms of altering thyroid hormone

Adult male zebrafish showed reduced TT3 levels and increased TT4 levels following a 21-day exposure to HBB, which was different from those observed in larval zebrafish. TSH was significantly increased at the concentrations of 0.03 and 0.3 mg/L. This thyroid surge effect suggests that elevated T4 is reduced by a negative feedback mechanism.

The observed increase of TT4 may be explained by up-regulation of the genes of TH synthesis, e.g., *tpo*, *nis*, and *hhex*. These transcriptional changes shown in adult male fish were different from those observed in the larval fish, suggesting that the modes of thyroid hormone disruption may vary by life stage of zebrafish. Down-regulation of *dio2* gene supports the observation of decreased T3, while T4 levels were increased. Similar finding was reported for triphenyltin chloride, e.g., an increase in total T4 levels and a decrease in total T3 levels, indicating that *dio2* is the primary determinant of plasma T3 content (Bianco and Kim, 2006; Wu et al., 2020). The down-regulation of *dio1*, *dio2*, *ugt1ab*, and *sult1st5* genes may support increasing levels of T4, by reduced bioconversion of T4 or elimination. Moreover, up-regulation of *dio3a* gene supports increased metabolic inactivation of T3, supporting the observation of decreased T3 in the adult fish (Figure 3).

In the brain, transcription of *tshβ* was significantly decreased in a dose-dependent manner, which was different from slightly increased TSH levels measured in the fish plasma (Figure 3). Considering relatively transient nature of the transcriptional change, the observed changes in *tshβ* gene may be seen as a secondary response to TSH increase.

4.3. Effects of HBB on male adult zebrafish (*Danio rerio)* in terms of altering sex hormone

Nonliner but clear increases of E2 and 11-KT observed following exposure to HBB (Figure 5) show sex hormone disruption potential of this chemical, along with TH disruption. Sex horones are important because these hormones are responsible for sexual differentiation, sexual maturation, and reproduction (Pradhan and Olsson, 2015). Male zebrafish use 11-KT as their main androgen because, unlike T, it does not transform into E2 in the brain (Fuzzen et al., 2011; Zhang et al., 2020). Additionally, unlike mammals, teleosts use 11-KT rather than T as a major androgen (Borg et al., 1994). Increase of E2 is supported by up-regulation of *vtg* gene in liver (Figure 6). Male fish normally do not express the *vtg*, but environmental estrogens or estrogen mimics can cause male fish to express the *vtg* gene at levels that are detectable (Chen et al., 2015; Chen et al., 2019). Up-regulation of the sex hormone-binding globulin (*shbg*) gene also supports increased levels of sex hormones, because shbg protects steroids from rapid metabolism and degradation, interfering with the availability of androgen in the male reproductive system (Saxena et al., 2014; Miguel et al., 2004).

After exposure to HBB, adult male zebrafish exhibited the down-regulation of several steroidogenic genes (*cyp17, cyp19*, and *star*) in their gonads, although the extent of up-regulation was not great. These observations contradict the observed increases of E2 and 11-KT, and may be interpreted as compensatory transcriptional

efforts against increased sex hormones, while further experimental evidences are warranted. While only male zebrafish were used for measurement in this study, the present observations show that exposure to HBB caused changes in sex hormones of the male adult zebrafish along with transcriptional changes of major sex hormone regulating genes.

5. Conclusion

HBB is among the novel brominated flame retardants with increasing environmental health concern. Our observations show that HBB exposure may cause thyroid hormone disruptions in both larval and adult stages of zebrafish and affect sexual hormones in adult male zebrafish. HBB exposure, whether short-term or long-term, may have significant negative effects on the thyroid endocrine system and potentially early development of freshwater vetebrates. Further investigations are warranted to understand consequences of hormonal disruption in zebrafish.

6. References

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국문 초록

제브라피쉬(*Danio rerio*) 배아-치어 및 성어를 이용한 HBB(Hexabromobenzene)의 성 및 갑상선 호르몬 교란 영향 연구

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Novel brominated flame retardants(NBFRs)는 기존의 브롬화난연제(BFRs)의 대체제로서 최근에 사용되고 있는 화학물질이다. NBFRs 는 기존의 브롬화 난연제와 화학 구조는 다르지만 대부분의 물리-화학적 특성이 유사하다. NBFRs 은 다양한 환경 및 제품에서 검출되어지고 있으며, 환경에 노출됨에 따라 생물상에서도 종종 검출되는 보고들이 증가하고 있다. NBFRs 중 HBB 는 높은 log K_{ow} 값을 가진 물질로 최근 한 수생 생물 내에서 갑상선 조절에 관여하는 유전자와 상관관계를 보인다는 연구가 보고되었다. 그러나 이러한 독성 가능성이 있음에도 HBB 의 노출에 의한 내분비계 독성 연구는 매우 부족한 실정이다. 따라서 본 연구에서는 HBB 에 의해 수서 생물인 zebrafish(*Danio rerio*)의 발달 단계에 따라 갑상선 호르몬 및 성 호르몬 교란을 확인하고 그 기전을 밝히고자 한다. Embryo-larval zebrafish 노출 실험의 경우, 7 일 동안 HBB 을 노출한 후 갑상선 호르몬 및 관련 유전자 발현 변화를 분석한다. Whole larvae 의 갑상선 호르몬을 측정한 결과, total T3(TT3), total(TT4)의 함량이 증가하였으며, free T3(fT3), free T4(fT4)의 함량과 thyroid-stimulating hormone(TSH)의 함량도 증가하는 결과를 보인다. 또한,

갑상선 중추 조절 유전자(crh, trh), deiodination 관련 유전자(dio1, dio3a, dio3b), 합성 관련 유전자(nig, tg, tpo)가 모두 감소되는 결과를 보인다. Adult zebrafish 노출 실험의 경우, 약 6 개월 연령의 수컷 adult zebrafish 을 사용하여 21 일 동안 HBB 을 노출 시킨 후 갑상선 호르몬 및 관련 adult zebrafish 의 각 장기 별 유전자 발현 변화를 분석한다. Adult zebrafish 의 plasma 에서의 갑상선 호르몬 분석 결과, TT3 는 감소하는 경향이 관찰되었으며, TT4 는 증가하는 경향이 보인다. 또한, HBB 의 노출에 의해 E2(17β-estradiol)와 11-KT(11-ketotestosterone)가 감소하는 경향을 보였다. 본 연구 결과 zebrafish 의 다양한 life-stage 에서 갑상선 교란 반응이 관찰되었다. 따라서 기존의 브롬화 난연제를 대체하는 새로운 브롬화 난연제에 대한 추가적인 독성 연구가 수행되어야 함을 시사한다.

Keywords: **새로운 브롬화 난연제(NBFRs), 브롬화 난연제(BFRs), 헥사브로모벤젠(HBB), 제브라피쉬, 갑상선 호르몬 교란, 성 호르몬 교란, 내분비 교란**

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