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Sex hormones disruption potentials of novel brominated flame retardants and related mechanisms in male zebrafish (*Danio rerio*) and in H295R and MVLN cell lines

수컷 제브라피쉬(*Danio rerio*)와
H295R, MVLN 세포주를 이용한
신종 브롬계난연제(NBFRs)의 성호르몬 교란 영향 연구

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Abstract

Sex hormones disruption potentials of novel brominated flame retardants and related mechanisms in male zebrafish (*Danio rerio*) and in H295R and MVLN cell lines

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Decabromo diphenyl ethane (DBDPE) and 1,2-bis(2,4,6 tribromo-phenoxy) ethane (BTBPE) are novel brominated flame retardants (NBFRs) and have been widely detected in various environment and biota. Their effects on sex endocrine disruption by DBDPE and BTBPE have been reported in fish, however, the knowledge on sex hormones disruption was generally focused on very

limited scope, e.g., vitellogenin induction.

This study used male zebrafish (*Danio rerio*) and two cell lines to investigate the sex hormone disruption potential of DBDPE and BTBPE and its related mechanism. First, male zebrafish was exposed to DBDPE and BTBPE for 14 days to investigate alteration of sex hormones including 17β-estradiol (E2), and 11-ketotestosterone (11-KT), and transcriptional changes of related genes. Both chemicals were also tested by human adrenocortical carcinoma cells (H295R) for alteration of sex hormones and the genes related to steroidogenesis, and human breast carcinoma cells (MVLN) to observe binding affinity with estrogen receptor.

Following male zebrafish for 14 days exposure, E2 levels for DBDPE treatment were significantly increased and E2 and E2/11-KT ratio for BTBPE treatment showed an increasing trend. Following BTBPE exposure, transcription of *era* and *erβ* genes in the liver, showed increasing trends, but not following exposure to DBDPE. In H295R cells, although a few exceptions were observed, both DBDPE and BTBPE showed similar patterns of the change compared with male zebrafish results. In MVLN cells, neither DBDPE nor BTBPE showed affinity for estrogen receptors.

The result of the present study showed that DBDPE and BTBPE have sex hormone disruption potentials at the male zebrafish and cells toward estrogenicity, but by different mechanisms. Since regulation of sex hormones plays crucial roles in sexual development such as gonad gametogenesis and reproduction of humans and animals, further investigations including long-term exposure are

needed to understand their ecotoxicological consequences and more detailed mechanisms.

Key words: novel brominated flame retardants, DBDPE, BTBPE, H295R cell line, MVLN cell line, zebrafish, sex hormones, endocrine disruption

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1. Introduction

Flame retardants are used in various products such as construction materials. plastics, electronic equipment, furniture, and other materials to avoid the spread of fire. About two hundreds compounds are currently used as flame retardants, among them include 75 brominated flame retardants (Covaci et al., 2011). Polybrominated diphenyl ethers (PBDEs) like penta-, octa-, and deca-BDE have been used in great quantity in many products that are frequently used in homes and offices. Endocrine disruption potentials of PBDEs have been reported. BDE-209, one of the most widely used PBDEs, showed a negative association with testosterone levels in male humans (Den Hond et al., 2015). Besides, BDE-209 showed a significant negative association with estradiol in female offspring (Kim et al., 2009), and affected vitellogenin production and steroidogenesis on zebrafish (*Danio rerio*) larvae (Chen et al., 2016). Octa-BDE showed a positive association with testosterone and luteinizing hormone on male humans (Johnson., 2010).

Under the Stockholm Convention, the United Nations Environmental Programme (UNEP) has listed penta- and octa-BDE as the Persistent Organic Pollutants (POPs) because of the persistence, bioaccumulation and adverse health effects (Ashton et al., 2009; Stockholm Convention, www.chm.pops.int). In addition, deca-BDE has been prohibited and phased out in some European countries, the United States (Hess, 2009). After such regulation, the usage of novel brominated flame retardants (NBFRs) like decabromo diphenyl ethane (DBDPE) and 1,2-bis(2,4,6 tribromophenoxy) ethane (BTBPE) has been globally on the rise (Ali et al., 2011), and the volumes were estimated at 180,000 tons/ year in 2011 (Covaci et al., 2011).

DBDPE is one of the alternative flame retardants substituting BDE-209 that has been mentioned by the Stockholm Convention as an additive polymeric materials such as acrylonitrile butadiene styrene (ABS) and in textiles like polyester and cotton (WHO, 1997; Arias, 2001; Kierkegaard et al., 2004; Covaci et al., 2011). Asian countries like China and Japan have reduced the use of BDE-209, and moved to DBDPE (Xiao, 2006; Watanabe and Sakai, 2003; Covaci et al., 2011). Several European countries have imported DBDPE around 3000 tons in 2001 (WHO, 1997, Arias, 2001). BTBPE, which has been also suggested by Stockholm Convention, is intended to replace octaBDE in production of ABS, coatings, and thermoplastics (WHO, 1997). BTBPE has been approximated that about 16,710 tons are in production and usage in 2001 (Verreault et al., 2007).

Frequent use of DBDPE and BTBPE has resulted in their worldwide occurrences in the environment (Table 1). DBDPE and BTBPE have been detected in Norwegian wastewater at levels up to 5.1 ng/L and 1 ng/L, respectively (Nyholm et al., 2013), and in sewage sludge of China at levels up to 1995 ng/g and 1.66 ng/g, respectively (Shi et al., 2009). Both chemicals have been also reported in sediments around the world, at levels up to 1714 ng/g of DBDPE and 57.76 ng/g of BTBPE in China (Guerra et al., 2010; La Guardia et al., 2012; Wu et al., 2010; Zhu et al., 2018). In other environmental media, both compounds have been increasingly detected (Table 1). Higher levels of DBDPE and BTBPE have been reported in ambient air of China (Up to 3578 pg/m^3 and 67.4 pg/m^3) and house dust from UK (Up to 3400 ng/g and 1900 ng/g) (Shi et al., 2009; Stuart et al., 2008).

Some studies have found that DBDPE and BTBPE had a potential

for biomagnification and long-range transport, similar to PBDEs (Law et al., 2006, Wu et al., 2011, Vorkamp and Rigét, 2014; Jin et al., 2016; Guo et al., 2017). DBDPE is persistent in the environment, i.e., no degradation for even 112 days in the sunlit condition (Kajiwara et al., 2007). BTBPE was showed a depuration half-life of 54.1 days in juvenile rainbow trout (Tomy et al., 2007a). Thus, both chemicals can persist also in an organism like fish, birds, and even humans (Table 2). Human may be exposed to these chemicals through food-web magnification (Covaci et al., 2011) and can be at a potential risk (Peijnenburg et al., 1995; Allchin et al., 1999; Rahman et al., 2001).

Similar to PBDEs, DBDPE and BTBPE exposure have been reported to possess sex hormones disruption potentials in recent studies (Table 2). Exposure to DBDPE has resulted in reproductive toxicity in zebrafish embryo and larvae, e.g., delayed hatching time and observed higher mortality (Nakari et al., 2010). BTBPE was reported to increase vitellogenin synthesis in female rainbow trout and fathead minnow (Giraudo et al., 2017; de Jourdan et al., 2011). In addition, DBDPE was founded to increase vitellogenin in hepatocyte of male rainbow trout and Brown trout (Nakari et al., 2009).

Sex hormones play crucial roles in gonad gametogenesis and reproduction of animals. Therefore, disruption in sex hormones can impact on reproductive function and health (Den Hond et al., 2015; Harrison et al., 1997). However, the information on sex hormones disruption by DBDPE and BTBPE was scarce, and the mechanisms of sex hormones disruption were generally focused on transcription of *vtg* gene and its translation.

In this study, zebrafish and two cell lines of H295R (human adrenocortical carcinoma cell) and MVLN (human breast carcinoma

cell) cells were chosen and used for investigation of sex hormone disruption and related mechanisms. Zebrafish has been used as one of the suitable models for screening endocrine disrupting chemicals because of cost-effectiveness, rapid life stage, and physiological similarity with other vertebrates (He et al., 2014). Male zebrafish was chosen to focus on estrogenic effect of the study chemicals. H295R cell line has been suggested by OECD as the test model to observe potential alteration in steroidogenesis pathway (OECD, 2011). Besides, MVLN cell line has been employed to measure binding affinity to estrogen receptor (ER) (Demirpence et al., 1993; Freyberger et al., 2005; Liu et al., 2012; Lee et al., 2019).

The aim of this study was to evaluate the effects on sex hormones synthesis and the potential related mechanism of DBDPE and BTBPE. This study can help not only to enhance understanding of the sex hormones disruption and the related mechanism of DBDPE and BTBPE but also to provide information that can be used for their environmental and human risk assessments for the regulation of NBFRs.

Table 1. Detected concentrations of DBDPE and BTBPE in environmental media worldwide.

Matrix	Compound	Country	Location	Sampling time	Concentration range	Reference
Water	DBDPE	China	Chengdu	2016	ND	Hou et al., 2019
		Norway	Drammen, Lillehammer, Tromsø	2009	ND-5.1 ng/L [a]	Nyholm et al., 2013
	BTBPE	China	Guangdong	2006	0.02 ng/L [a]	Wu et al., 2010
		China	Chengdu	2016	ND-0.24 ng/L	Hou et al., 2019
		Norway	Drammen, Lillehammer, Tromsø	2009	ND-1 ng/L [a]	Nyholm et al., 2013
Sewage sludge	DBDPE	China	-	2005	39-140 ng/g	Ricklund et al., 2008
		China	Guangdong	2007	266-1995 ng/g	Shi et al., 2009
		China	Harbin	2011-2016	255.8 ng/g	Li et al., 2018
		Germany	-	2004	70-220 ng/g	Ricklund et al., 2008
		Norway	Drammen, Lillehammer, Tromsø	2009	1.9-6.3 ng/g [a]	Nyholm et al., 2013
		Canada	-	2004	ND-65 ng/g	Ricklund et al., 2008

Table 1. (Continued)

		USA	-	1999-2000	1.4-160 ng/g	Ricklund et al., 2008
	BTBPE	China	Guangdong	2007	0.31-1.66 ng/g	Shi et al., 2009
		China	Harbin	2011-2016	19.8 ng/g	Li et al., 2018
		Norway	Drammen, Lillehammer, Tromsø	2009	0.7-1.4 ng/g [a]	Nyholm et al., 2013
Sediment	DBDPE	China	Guangdong	2006	1796 ng/g [a]	Wu et al., 2010
		China	Guangzhou, Macau, Hongkong	2013	1.52-1714 ng/g	Zhu et al., 2018
		Spain	Llobregat, Anoia	2005-2006	4.8-23 ng/g	Guerra et al., 2010
	BTBPE	China	Guangdong	2006	4554 ng/g [a]	Wu et al., 2010
		China	Guangzhou, Macau, Hongkong	2013	ND-57.76 ng/g	Zhu et al., 2018
		USA	North Carolina	2009	77-2000 ng/g	La Guardia et al., 2012
Soil	DBDPE	China	Guangdong	2006-2007	17.6-35.8 ng/g	Shi et al., 2009
		China	Shouguang	2014	560 ng/g [a]	Li et al., 2016

Table 1. (Continued)

	.....	Australia	Melbourne	2014	< 5.00-295 ng/g	McGrath et al., 2017
	BTBPE	China	Guangdong	2006-2007	0.02-0.11 ng/g	Shi et al., 2009
		China	Tianjin	2011	0.250-5.90 ng/g	Hong et al., 2016
		Australia	Melbourne	2014	< 0.02-11.4 ng/g	McGrath et al., 2017
Ambient air	DBDPE	China	Guangdong	2007	402-3578 pg/m^3	Shi et al., 2009
	.....	USA	Cleveland, Eagle Harbor and three others	2005-2011	1.2-5.2 pg/m^3 [a]	Ma et al., 2013
	BTBPE	China	Guangdong	2007	3.83-67.4 pg/m^3	Shi et al., 2009
		USA	Chicago, Michigan and three others	2002-2003	2.8-70 pg/m^3	Hoh et al., 2005
		USA	Cleveland, Eagle Harbor and three others	2005-2011	0.4-1.8 pg/m^3 [a]	Ma et al., 2013
House dust	DBDPE	Belgium	Antwerp	2008	55-2126 ng/g	Ali et al., 2011
		Sweden	Örebro, Stockholm, Norrköping	-	ND-121 ng/g	Karlsson et al., 2007

Table 1. (Continued)

	UK	Basingstoke, Hampshire and two others	2006-2007	ND-3400 ng/g	Stuart et al., 2008
	UK	West Midlands	2007-2008	< 20-2467 ng/g	Ali et al., 2011
BTBPE	Belgium	Antwerp	2008	0.5-1019 ng/g	Ali et al., 2011
	Sweden	Örebro, Stockholm, Norrköping	-	2.52-8.15 ng/g	Karlsson et al., 2007
	UK	Basingstoke, Hampshire and two others	2006-2007	ND-1900 ng/g	Stuart et al., 2008
	UK	West Midlands	2007-2008	< 0.5-1741 ng/g	Ali et al., 2011

[a] Average concentrations.

Table 2. Detected concentrations of DBDPE and BTBPE in biota worldwide.

Matrix	Compound	Country	Location	Sampling time	Concentration range	Reference
Fish	DBDPE	China	Guangdong	2006	ND-0.338 μg/g lipid	Wu et al., 2010
		Canada	Winnipeg	2000-2002	ND-2,710 μg/g lipid	Law et al., 2006
		USA	Great lake basin	2010	2×10^{-8}-9.1×10^{-7} μg/g lipid	Guo et al., 2017
	BTBPE	China	Guangdong	2006	< 0.000012-0.00015 μg/g lipid	Shi et al., 2009
		China	Guangdong	2006	0.00171-0.518 μg/g lipid	Wu et al., 2010
		Canada	Winnipeg	2000-2002	ND-840 μg/g lipid	Law et al., 2006
		USA	Great lake basin	2010	5×10^{-8}-3.9×10^{-7} μg/g lipid	Guo et al., 2017
Plankton	DBDPE	Canada	Winnipeg	2000-2002	ND-1,510 μg/g lipid	Law et al., 2006
	BTBPE	Canada	Winnipeg	2000-2002	ND-3,720 μg/g lipid	Law et al., 2006

Table 2. (Continued)

Bird	DBDPE	China	Guangdong	2006	0.0096-0.124 μg/g lipid	Shi et al., 2009
		Korea	Paju, Gyungsangbuk-do and four others	2010-2011	< LOQ-0.35 μg/g lipid	Jin et al., 2016
	BTBPE	China	Guangdong	2006	0.00007-0.00241 μg/g lipid	Shi et al., 2009
		Korea	Paju, Gyungsangbuk-do and four others	2010-2011	< LOQ-3.002 μg/g lipid	Jin et al., 2016
Human blood	DBDPE	China	Guangzhou	2014	30.9-65 ng/g	Qiao et al., 2018
		China	Wenling	2015	4.2-128 ng/g	Liang et al., 2016
		China	Shandong	2016-2017	236-54,400 ng/g	Chen et al., 2019
		China	Shandong	2016-2017	3.15-3,740 ng/g	Chen et al., 2019
		Sweden	Uppsala	1996-2015	< 0.006-0.012 ng/g	Gyllenhammar et al., 2016
	BTBPE	Sweden	Uppsala	1996-2015	< 0.00013-0.032 ng/g	Gyllenhammar et al., 2016

Table 3. Sex hormone and reproductive effects in fish by exposure to DBDPE and BTBPE

Assay	Compound	Species		Concentrations	Results	Reference
In vitro assay	DBDPE	Hepatocyte	Rainbow trout [b]	0, 6.3, 12.5, 25, 50 μg/L	Vitellogenin ↑ as low as 6.3 μg/L and ↓ at higher concentrations	Nakari et al., 2010
			Brown trout [b]	0, 6.3, 12.5, 25, 50 μg/L	Vitellogenin ↑ as low as 6.3 μg/L and ↓ at higher concentrations	Nakari et al., 2010
In vivo assay	DBDPE	Vertabrate	Zebrafish embryo	0, 12.5, 25 μg/L	Hatching ↓ at 25 μg/L Mortality ↑at 12.5 and 25 μg/L	Nakari et al., 2010
			Zebrafish larvae	0, 12.5, 25 μg/L	Mortality ↑ at 12.5 and 25 μg/L	Nakari et al., 2010
	BTBPE		Fathead minnow [a] [b]	0, 0.5 μg/g (Injection in sediment)	Vitellogenin ↑ at 0.5 μg/g (trend), E2. 11-KT no change	de Jourdan et al., 2011
			Rainbow trout [a]	0, 50 μg/g (Feeding)	*vtg* gene ↑at 50 μg/g (trend)	Giraudo et al., 2017

[a] Female

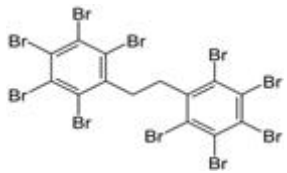
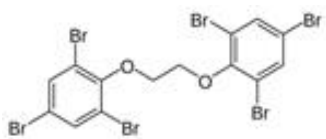
[b] Male

2. Materials and Methods

2.1. Chemicals

DBDPE (Decabromo diphenyl ethane, CAS no. 84852-53-9, purity: >96%) was purchased from Tokyo Chemical Industry (Japan). BTBPE (1,2-bis(2,4,6 tribromophenoxy) ethane, CAS no. 37853-59-1, purity: 98%) was purchased from Toronto Research Chemicals (Canada). The physicochemical properties of DBDPE and BTBPE were described in Table 4. For both *in vivo* and *in vitro* studies, 0.1% (v/v) dimethyl sulfoxide (DMSO) was used as solvent.

Table 4. Physicochemical properties of DBDPE and BTBPE

Compound	Decabromo diphenyl ethane	1,2-bis(2,4,6 tribromophenoxy) ethane
Synonyms	DBDPE	BTBPE
CAS No.	84852-53-9	37853-59-1
Structure		
Usage	Additive, textiles	ABS, thermoplastics, coating
Molecular formulate	$C_{14}H_4Br_{10}$	$C_{14}H8Br_6O_2$
Molecular weight (g/mol)	971.22	687.64
$LogK_{OW}$	13.64 [a]	8.31 [b]
Water solubility at 25°C (mg/L)	1.61×10^{-12} [a]	2.23×10^{-4} [a]

[a] US EPA 2012

[b] EFSA 2012

2.2. Zebrafish culture and exposure

Adult wild-type male zebrafish (about six months old) were obtained from a commercial supplier (Greenfish, Seoul, Korea) and were acclimated in aerated dechlorinated tap water for 7 days before the experiment. For DBDPE and BTBPE exposure, 16 male zebrafish were separated into four replicates, and the replicates with four male fish with 3 L beaker were used. DBDPE or BTBPE, were exposed to the fish, at five concentrations including dechlorinated tap water, and solvent water. The exposure concentration (DBDPE; 0.3, 3, and 30 μM, BTBPE: 0.1, 1, and 10 μM) were chosen based on the preliminary range finding tests (7 days exposure). The maximum concentration which can be soluble in 0.1% (v/v) DMSO, which did not show mortality was determined as the highest experimental concentration. The exposure media (> 80%) were renewed every day.

Following OECD test guideline (No, 204 fish prolonged toxicity test: 14 days), the experiments were carried out for 14 days (OECD, 1984). The experiments conducted in 26 ± 1 °C under a photoperiod of 14:10 h light: dark, and freshly hatched *Artemia nauplii* were used the feed twice a day. For checking the water quality, dissolved oxygen, pH, conductivity, and temperature with old and new exposure media were measured every day. After the 14 days of exposure, each fish in replicate pooled for analysis of transcriptional and hormone change. Each fish dissected out the liver and a heparinized glass capillary tube used to collect blood samples from caudal vein. After centrifugation (8000 rpm for 10 min at 4 °C), plasma was collected from the supernatant. all collected samples were stored at - 80 °C

until genes and sex hormones measurement. Four of the technical replicates and one of the biological replicates were conducted.

2.3. H295R and MVLN cell culture and exposure

H295R cell line was used to measure sex hormones concentration a nd gene related to steroidogenesis pathway (Hecker et al., 2011). H295R cells were maintained at 37 ℃ with 5% CO_2. For H295R culture medium, a mixture of Dulbecco's modified Eagle's medium/Ham's F-12 nutrient mixture (Sigma D206, Sigma Aldrich) with 1% ITS + Premix (BD Biosciences, San Jose, CA, USA), 2.5% Nu-Serum (BD Biosciences) and 1.2 g/L Na_2CO_3 (Sigma Aldrich) was used. The medium was renewed on alternate days, and the cell subculture was conducted when cell confluence was over 80%. or within 7 days. For H295R exposure, cells were seeded at 3.0×10^5 cells/mL density in 24-well plates and incubated for 24 hours using culture medium. Based on the result of WST-1 cell proliferation assay, various concentrations (DBDPE; 0.003, 0.01, and 0.03 μM, BTBPE: 0.1, 0.3, and 1 μM) that showed over 80% cell survival compared to solvent control was dosed. Forskolin (10 μM) was treated to the cells as a positive control. each treatment in three replicates was exposed for another 48 hours. After the exposure, each chemical medium was collected for sex hormones measurement, and the harvested cell was homogenized for gene analysis. Three times of technical replicates and biological replicates were conducted for statistical analysis.

Following the previous studies (Snyder., 2001; Preuss., 2006), MVLN cell line was used to measure binding affinity to ER. MVLN Cell maintained at 37 ℃ with 5% CO_2. For MVLN culture medium, a mixture of hormone-free DMEM/F12 nutrient mixture, 1 mM sodium

pyruvate, and 1 mg/L insulin (Sigma Aldrich) added with 10% dextran-charcoal stripped fetal bovine serum (Hyclone, Logan, UT, USA) was used. The medium was renewed on alternate days, and cell subculture was conducted when cell confluence was over 80% or within five days. Since some compounds in serum such as endogenous hormones may interfere with a baseline of luciferase activity, the culture medium should be changed by charcoal-stripped medium 24 hours before cell plating. Cells were seeded 1.25×10^5 cells/mL density in 96-well plates and incubated for 24 hours. Based on the result of WST-1 cell proliferation assay, various concentrations in three replicates (DBDPE; 0.0003, 0.001, 0.003, 0.01, and 0.03 μM, BTBPE: 0.01, 0.03, 0.1, 0.3, and 1 μM) that showed over 80% cell survival compared to solvent control was dosed for another 72 hours. After the exposure, luciferase activity was analyzed using Steady^{Glo} - Luciferase Assay System (Promega Corp., Madison, WI, USA). The maximum response of 17β-estradiol (E2) was set to 100% and the relative light unit (RLU) for each sample well was converted to maximum induction of luciferase activity (% E2 max Luc). Three times of the technical replicates and the biological replicates were conducted for statistical analysis.

2.4. Measuement of sex hormones and gene transcription in H295R cell and zebrafish

In both plasma of male zebrafish and H295R medium, sex hormones were measured by commercial kits using enzyme-linked immunosorbent assay (ELISA) (Cayman Chemical; 17β-estradiol [Cat No. 501890]). For H295R cell assays for DBDPE, ELISA kit of E2 was used (Cayman Chemical; 17β-estradiol [Cat No. 582251]). In zebrafish, 11-ketotestosterone (11-KT) was measured by the ELISA kit (Cayman Chemical; 11-ketotestosterone [Cat No. 582751]). In H295R cell line, testosterone (T) was measured using a different ELISA kit (Cayman Chemical; testosterone [Cat No. 582701]). Before the measurement, fish plasma and H295R medium were extracted. Ultrapure water (400μl) added to 5 μl of plasma and 500μl of H295R medium. Plasma and the medium sample were extracted with 2ml and 2.5ml diethyl ether twice at 2100 rpm for 10 minutes, respectively. After diethyl ether was evaporated, the dried sample with the plasma and the medium were diluted in 120 μl and 300 μl ELISA buffer respectively for ELISA assay (Ji et al., 2010).

Dissected fish organ samples and harvested H295R cells were collected and homogenized. After the extraction of total RNA using RNeasy mini kit (Qiagen, Valencia, CA, USA), the samples were checked for RNA quality and quantity with a 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). Quantitative real-time PCR (qRT-PCR) was conducted with 20 μl reaction mix containing 18 μl pre-mix with

10 μl of Light cycler® 480 SYBR Green I master mix (Roche Diagnostics ltd., Lewes, UK), 1.8 μL of forward, reverse primer (10 pmol), and 4.4 μl nanopure water and 2 μl cDNA templates. Following $2^{-\Delta\Delta Ct}$ methods (Livak and Schmittgen et al., 2001), the threshold cycle (Ct) value was converted to the relative transcription level of the target gene. As a housekeeping gene, *β-actin* and *rpl8* were employed as a reference gene and the relative expression level of mRNA of each target gene was normalized with that of the reference gene. The primer sequences of targeted genes of zebrafish and H295R cells used in the study were chosen based on our previous study (Liu et al., 2012; Bhuiyan et al., 2019), and the information is listed (Table 5).

Table 5. Primer sequences of H295R cell lines, zebrafish used in the study

Assay	Gene name	Accession No.	Description	Sequence (5'-3')
In vitro H295R	*β-actin*	NM_001101	Forward	CACTCTTCCAGCCTTCCTTCC
			Reverse	AGGTCTTTGCGGATGTCCAC
	star	NM_000349	Forward	GTCCCACCCTGCCTCTGAAG
			Reverse	CATACTCTAAACACGAACCCCACC
	3βhsd	NM_000198	Forward	TGCCAGTCTTCATCTACACCAG
			Reverse	TTCCAGAGGCTCTTCTTCGTG
	cyp17	NM_000102	Forward	AGCCGCACACCAACTATCAG
			Reverse	TCACCGATGCTGGAGTCAAC
	cyp19	NM_000103	Forward	AGGTGCTATTGGTCATCTGCTC
			Reverse	TGGTGGAATCGGGTCTTTATGG
In vivo zebrafish	*rpl8*	NM_200713	Forward	TTGTTGGTGTTGTTGCTGGT
			Reverse	GGATGCTCAACAGGGTTCAT
	vtg[a]		Forward	AAGACCCCTGTCGTTCCAATC
			Reverse	AAACTCGTACTGCAGGGATCC
	era	NM_152959	Forward	CAGACTGCGCAAGTGTTATGAAG
			Reverse	CGCCCTCCGCGATCTT
	erβ	NM_174862	Forward	TTCACCCCTGACCTCAAGCT
			Reverse	TCCATGATGCCTTCAACACAA

[a] Sohn et al., 2016, This primer sequences were designed using Primer 3 online software ver.4.0.0 (http://primer3.ut.ee/)

2.5. Statistical analysis

Shapiro-wilk's test and Levene's test were used for the data normality and homogeneity of variances, respectively. If necessary, log-transformation was employed. For group differences among all treatments, One-way analysis of variance (ANOVA) with Dunnett's t-test was used. linear regression was performed for trend analysis. Differences with $p < 0.05$ were considered statistically significant in all analyses. All data were shown as mean ± standard deviation. IBM SPSS 23.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for data analysis

3. Results

3.1. Male zebrafish 14 days exposure test

3.1.1. Effects on plasma sex hormones in male zebrafish

The effects of DBDPE and BTBPE on 17β-estradiol (E2), 11-ketotestosterone (11-KT), and E2/11-KT ratio in male zebrafish were shown (Figure 1). DBDPE significantly increased E2 concentration (Figure 1A). Following DBDPE exposure, while E2 levels significantly increased at 3 and 30 μM, 11-KT concentrations and E2/11-KT ratio were not significantly altered. Following exposure to BTBPE, increasing trends of E2 and E2/11-KT ratio were observed, but no change of 11-KT concentrations was shown.

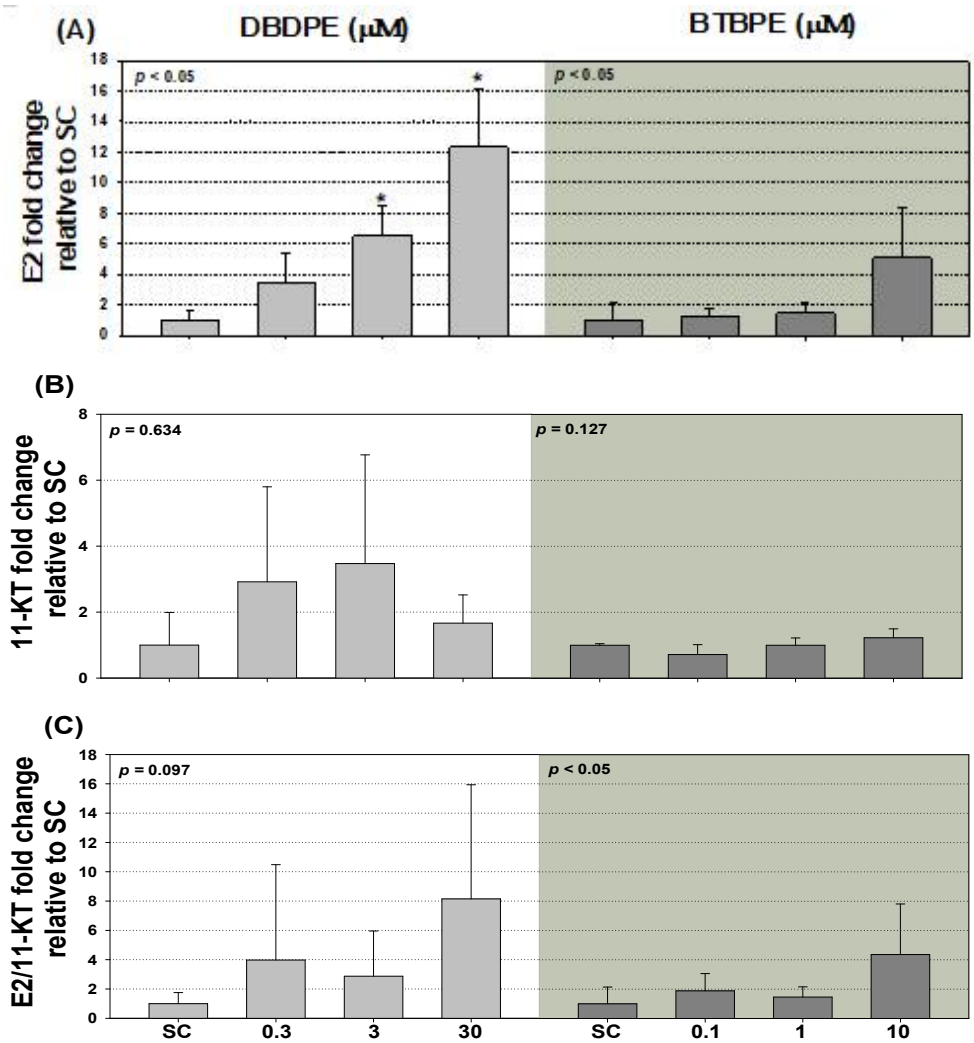


Figure 1. Effects on plasma sex hormone (A) 17β-estradiol (E2) concentration, (B) 11-ketotestosterone (11-KT) concentration, and (C) E2/11-KT ratio in male zebrafish after exposure to DBDPE (0.3-30 μM) and BTBPE (0.1-10 μM) for 14d. The results are shown as mean ± SD of four replicates (n=4). Asterisk (*) presents a significant difference from solvent control (SC, treated with 0.1% DMSO, $p < 0.05$). The p-value for trend was observed based on linear regression analysis.

3.1.2. Effects on transcriptional changes in male zebrafish

After exposure to BTBPE, several gene expressions related to vitellogenin synthesis showed increasing trends in male fish (Figure. 2). Estrogen receptor alpha and beta genes (*era* and *erβ*) showed increasing trends in BTBPE exposure (Figure 2B and C), but not *vtg* gene. None of the gene transcriptions was changed by DBDPE exposure.

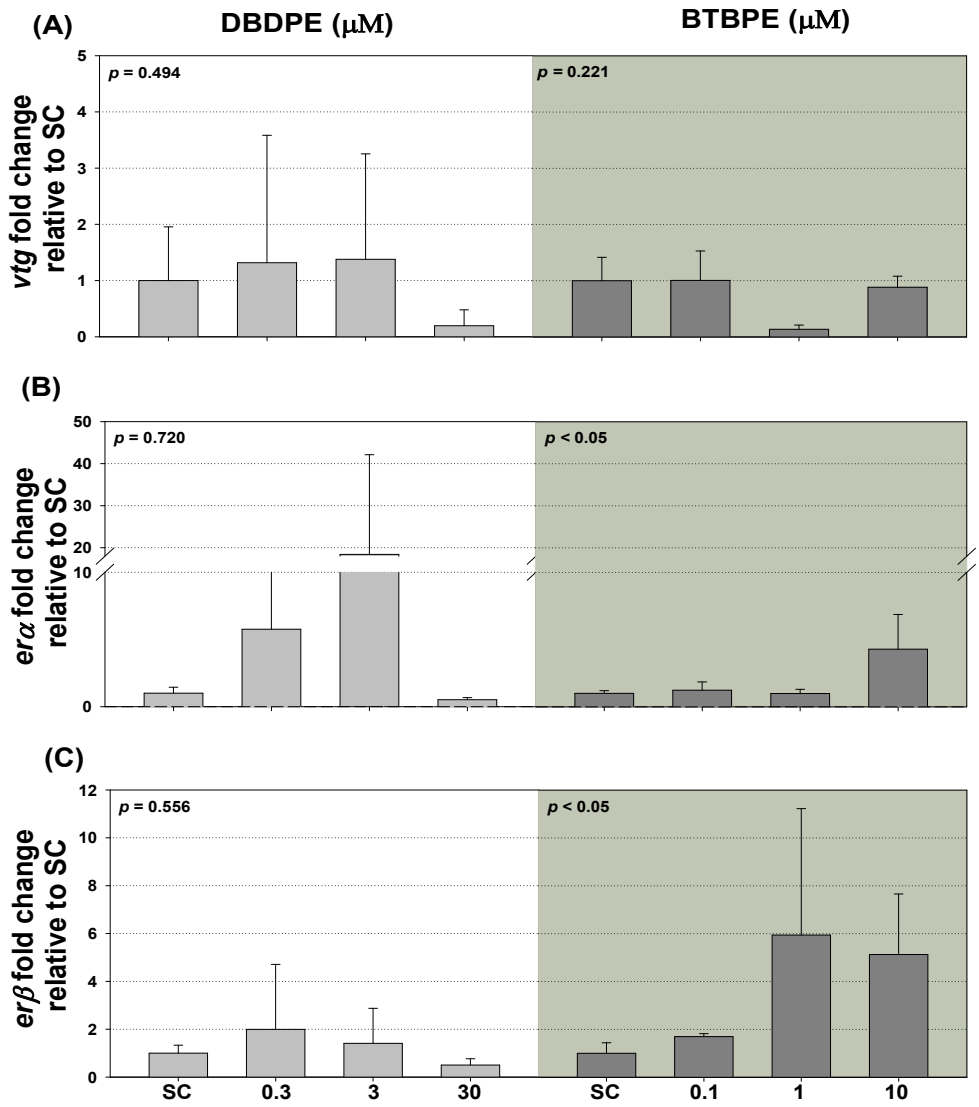


Figure 2. Effects on (A) *vtg*, (B) *era*, (C) *erβ* gene expression in male zebrafish liver after exposure to DBDPE (0.3-30 μM) and BTBPE (0.1-10 μM) for 14 days. The results are shown as mean ± SD of four replicates (n=4). Asterisk (*) presents significant difference from solvent control (SC, treated with 0.1% DMSO, $p < 0.05$). The p-value for trend were observed based on linear regression analysis.

3.2. H295R and MVLN cell line assays

3.2.1. Cytotoxicity of two NBFRs

Based on WST-1 assay, DBDPE showed cytotoxicity in H295R cells, e.g., <80% cell viability at 0.1 μM and greater. BTBPE did not show cytotoxicity even at 1.5 μM in H295R cells (Figure 3A). The doses with no cytotoxicity were used for the H295R assay. WST-1 assay also was employed to MVLN cells before measuring luciferase assay in MVLN cells (Figure 3B). Since DBDPE and BTBPE did not show any cytotoxicity at all concentrations, the same exposure doses in H295R and MVLN cell lines were used.

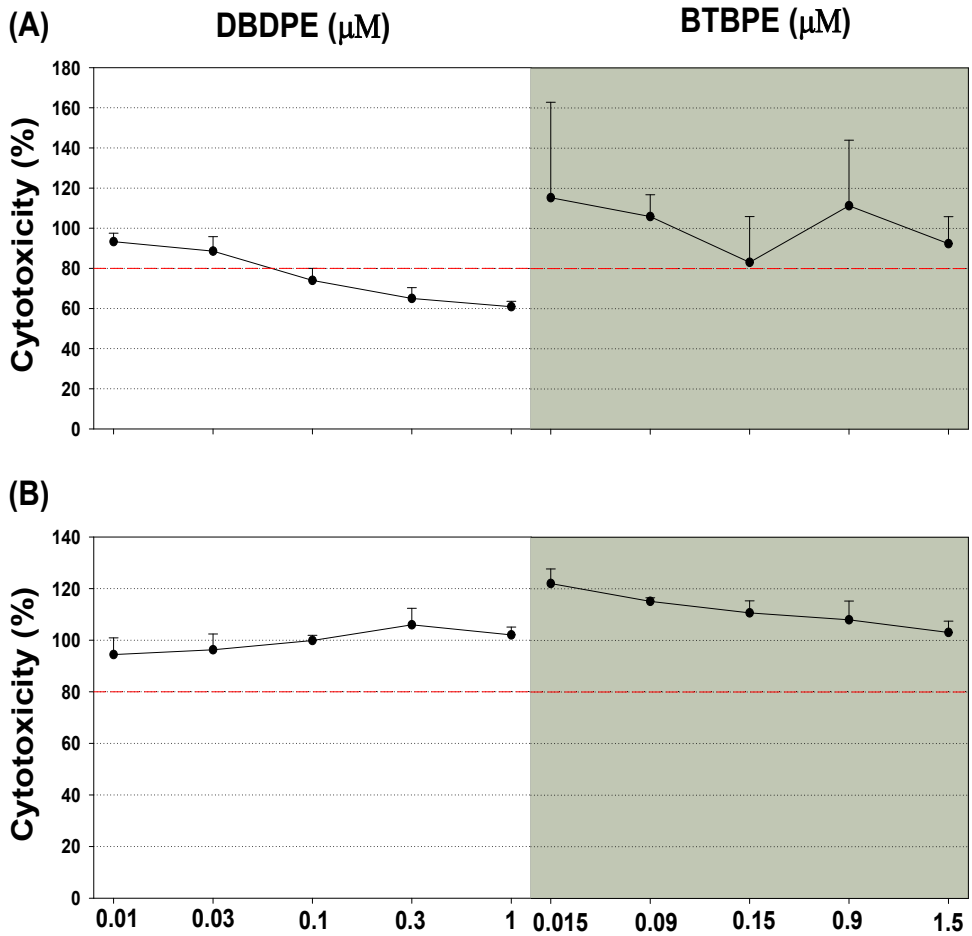


Figure 3. Preliminary range finding test of two NBFRs. (A) H295R and (B) MVLN cytotoxicity were measured following exposure to DBDPE (0.01-1 μM) and BTBPE (0.015-1.5 μM) using WST-1 assay. The cell cytotoxicity (%) was normalized to that of solvent control (0.1%, DMSO). The results are shown as mean ± SD of three replicates (n=3).

3.2.2. Effects on sex hormone and transcriptional changes in H295R cell

For exposure to DBDPE in H295R cells, concentrations of E2/T ratio showed an increase in trend due to less alteration of T levels (Figure 4C). Following the measured steroidogenesis genes, exposure to BTBPE showed a significant up-regulation of *cyp19* gene and increasing trends in *3βhsd* genes (Figure 5B and D). The transcription level in *cyp19* significantly increased at 0.3 μM (Figure 5D). For DBDPE exposure, all gene expression levels were not altered (Figure 5).

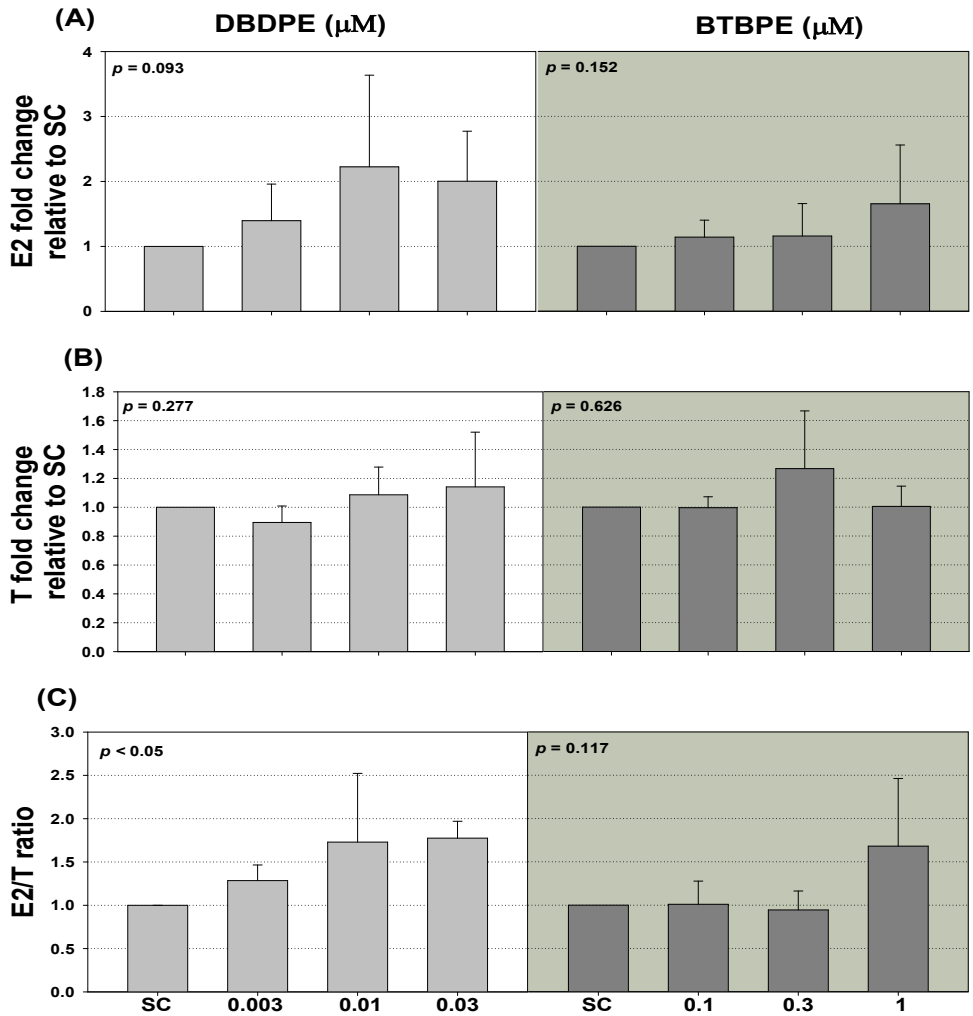


Figure 4. Effects on sex hormone (A) 17β-estradiol (E2) concentration, (B) testosterone (T) concentration, and (C) E2/T ratio in H295R cell line after exposure to DBDPE (0.003-0.03 μM) and BTBPE (0.1-1 μM) for 48h. The results are shown as mean ± SD of three replicates (n=3). Asterisk (*) presents significant difference from solvent control (SC, treated with 0.1% DMSO, $p<0.05$). The p-value for trend were observed based on linear regression analysis.

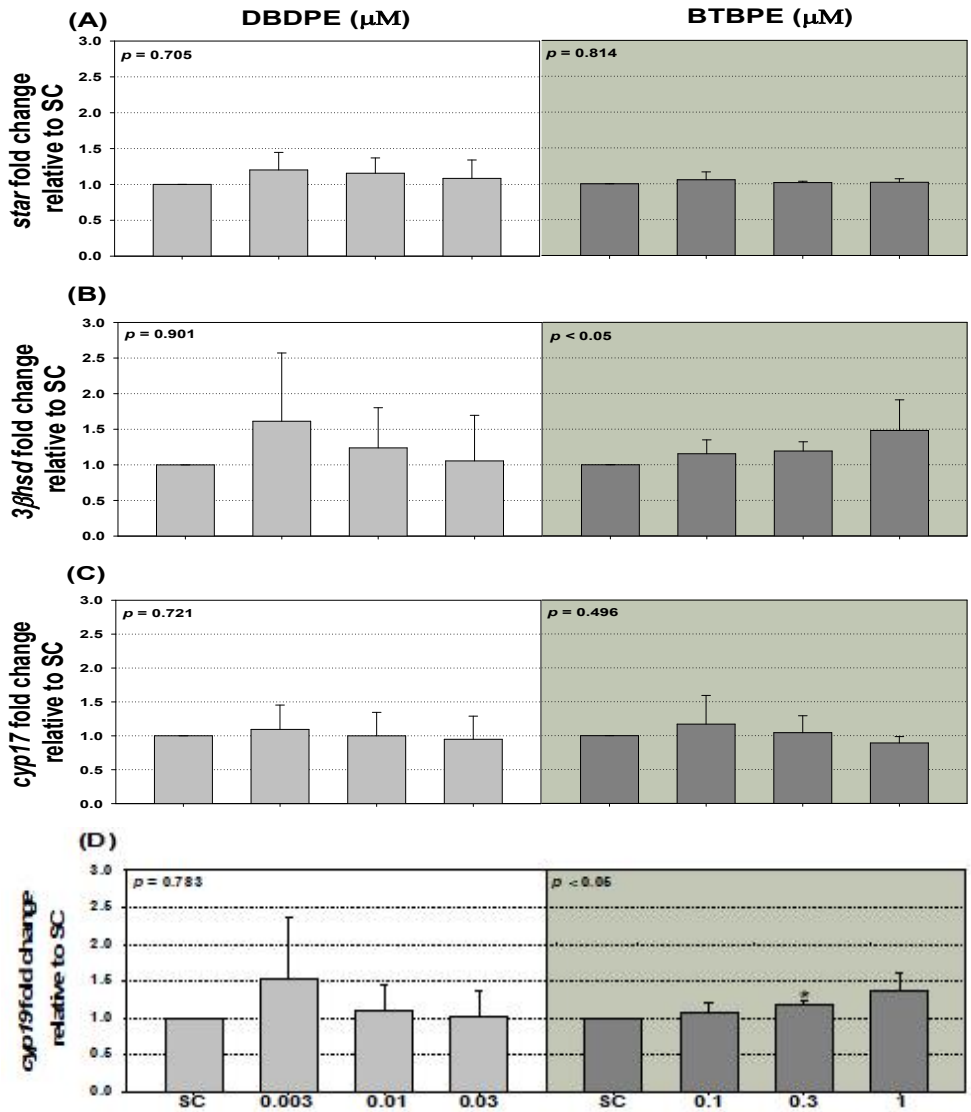


Figure 5. Effects on (A) *star*, (B) *3βhsd*, (C) *cyp17*, (D) *cyp19* gene expression in H295R cell line after exposure to DBDPE (0.003-0.03 μM) and BTBPE (0.1-1 μM) for 48h. The results are shown as mean ± SD of three replicates (n=3). Asterisk (*) presents significant difference from solvent control (SC, treated with 0.1% DMSO, $p<0.05$). The p-value for trend were observed based on linear regression analysis.

3.2.3. MVLN luciferase assay

Binding affinity to ER was measured at below 0 %, indicating that DBDPE and BTBPE did not affect ER agonistic effects in MVLN cell lines (Figure 6).

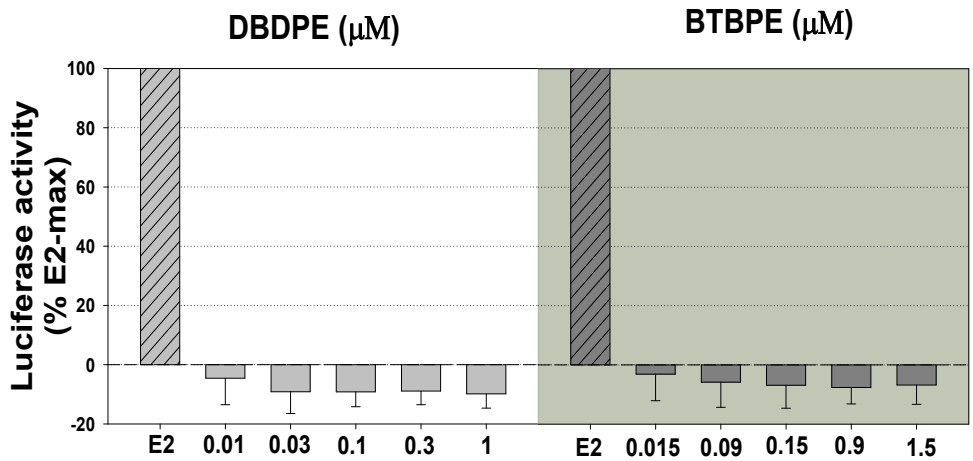


Figure 6. Effects on DBDPE and BTBPE in the MVLN cell luciferase assay. The results are shown as mean ± SD of three replicates (n=3). The E2 concentration for the calculation uses 3.7×10^{8} μM, which shows maximum luciferase value.

4. Discussion

In the present study, a significant up-regulation of E2 levels following exposure to DBDPE and an increasing trend of E2 levels following exposure to BTBPE in male zebrafish indicates that two NBFRs have potential sex hormone disrupting effects (Figure 1). Alteration in some gene transcriptions of male zebrafish and H295R cell lines also confirmed endocrine disrupting potentials (Figures 2 and 5). These results suggest that both DBDPE and BTBPE possess sex hormone disrupting potentials similar to PBDEs such as deca-BDE and octa-BDE (Kim et al., 2009; Chen et al., 2006; Johnson., 2010).

4.1. Sex hormones disrupting effects of two NBFRs

In male zebrafish 14 days exposure, DBDPE showed a significant increase of E2 concentrations, and BTBPE showed an increasing trend of E2 and E2/11-KT ratio levels (Figure 1). Based on E2/11-KT ratio, exposure to DBDPE and BTBPE resulted in estrogenicity in male zebrafish. This observation supports estrogenicity of both flame retardants similar to PBDEs (Kim et al., 2009; Chen et al., 2006; Johnson., 2010). Observations from the H295R cell assays also show similar trends that were observed in male zebrafish following exposure to DBDPE and BTBPE, while statistical significance was not observed. In the previous study, exposure to DE-71 in zebrafish altered sex hormones, but no effects in H295R cell lines (Yu et al., 2014; Song et al., 2008), suggesting that sex

hormone regulation by DBDPE and BTBPE exposure may be initiated by mechanisms other than steroidogenesis, e.g., metabolic activation from other systems (Zacharewski et al., 1998).

4.2. Transcriptional changes related to vitellogenesis of two NBFRs in male zebrafish

Vitellogenin is a crucial protein for oocyte maturation and reproduction of female fish, which is produced in liver of female fish following the estrogen production and binding with estrogen receptor. Therefore, vitellogenin has been recognized as a known indicator of sex hormone disruption in male fish (Leaños-Castañeda et al., 2007; Liu et al., 2009). However, DBDPE and BTBPE did not induce *vtg* gene transcription in spite of higher E2 levels and upregulation of *era* and *erβ* gene levels. Our observation is not comparable to several previous reports that exposure to DBDPE or BTBPE in some fish species altered *vtg* gene transcription or vitellogenin levels (Nakari et al., 2009; Giraudo et al., 2017; de Jourdan et al., 2011). Nevertheless, One possible reason for this observation may be that E2 levels in this study is not enough to induce vitellogenin, which is corresponded to the previous studies (Spanò et al., 2004; Sanderson et al., 2001). Another possible explanation may be found from hormone breakdown or clearance (La Merrill et al., 2019). Metabolites and conjugated form of estradiol, e.g., estradiol-sulfate or glucuronide, present in plasma may not bind with estrogen receptor in rainbow trout and siberian sturgeon (Latonnelle et al., 2002).

4.3. Transcriptional changes in steroidogenesis genes of two NBFRs in H295R cell line

To understand the change of sex hormone concentration, several major gene transcriptions related to sex hormone synthesis (*star, 3β hsd, cyp17, cyp19*) were also quantified in H295R cell line. According to the previous review (Payne et al., 2004), change in *star* gene transcription may affect transfer of cholesterol into inner mitochondrial membrane, which is the beginning of steroidogenesis. Transcriptional alteration of *3βhsd* or *cyp17* genes that encode steroid metabolizing enzymes contributes to the conversion to androstenedione. Transcription of *cyp19* is essential for conversion of T to E2. For DBDPE exposure in H295R cells, no genes related to steroidogenesis were observed in spite of an increasing trend in E2/T ratio levels. The previous study did not observe the change of *cyp19* level exposed to some PBDEs in H295R cell lines (Cantón et al., 2005). For BTBPE exposure in H295R cells, while slightly increasing patterns in hormone levels were shown, a positive trend of *3βhsd* and a significant up-regulation of *cyp19* gene were observed. A previous study demonstrated that the exposure to octa-BDE mixture caused the enhanced aromatase activity, which increased female sex hormones (Song et al., 2008). However, DBDPE and BTBPE exposure did not cause regulatory changes in steroidogenesis related genes, which cannot explain the phenomenon of the increase in E2 level in male zebrafish. In a previous study, Zhang (2011) observed that inhibition of E2 metabolism, i.e., influencing activities of E2 sulfotransferase and E2-glucuronidase, was the primary mechanism causing increased E2 levels in the cell medium rather than aromatase

(CYP19) activity. Therefore, further studies are warranted to understand possible molecular mechanisms that cause high E2 levels.

4.4. Estrogen receptor affinity in MVLN cell line

In MVLN cell assays, estrogenic responses were not observed, following exposure to DBDPE and BTBPE, while *er*s genes were upregulated in male zebrafish. A previous study showed that PBDE congeners including BDE-209 did not induce E2-induced estrogen receptor (ER) response in MVLN cell bioassay (Villeneuve et al., 2009). However, since ERs are present in many organs such as liver, testis, etc, the present observation may not be the case in other types of cells and in *in vivo* assays (Folmar et al., 2002; Goodin et al., 2002).

5. Conclusion

DBDPE and BTBPE showed similar disrupting effects on sex hormone of male zebrafish and H295R cell. This observation supports that NBFRs have potential endocrine disruption toxicity similar to PBDEs. However, since the mechanisms of steroidogenesis and vitellogenin synthesis are unclear, studies on adverse endocrine responses and associated mechanisms following long term exposure are warranted.

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7. Supplementary Information

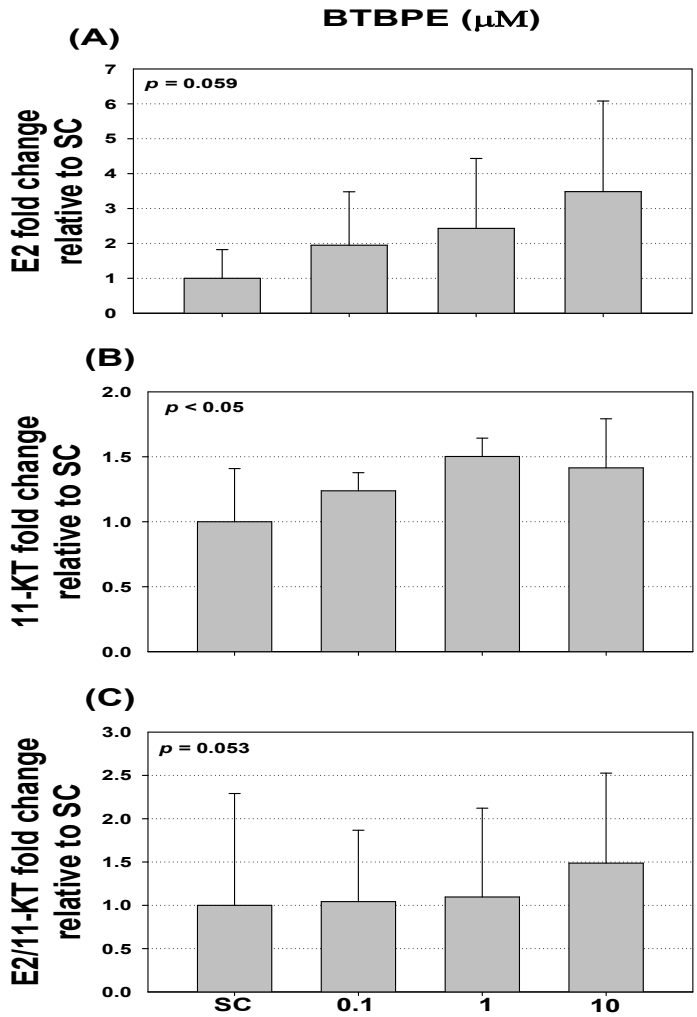

Figure S1. Effects on plasma sex hormone (A) 17β-estradiol (E2) concentration, (B) 11-ketotestosterone (11-KT) concentration, and (C) E2/11-KT ratio in male zebrafish after exposure to BTBPE (0.1-10 μM) for 14d on repeated experiment. The results are shown as mean ± SD of four replicates (n=4). The p-value for trend was observed based on linear regression analysis.

국문 초록

수컷 제브라피쉬(*Danio rerio*)와 H295R, MVLN 세포주를 이용한 신종 브롬계난연제(NBFRs)의 성 호르몬 교란 영향 연구

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Decabromo diphenyl ethane (DBDPE)과 1,2-bis(2,4,6 tribromo-phenoxy) ethane (BTBPE)는 스톡홀름 협약에서 제안한 신종 브롬계난연제(Novel brominated flame retardants, NBFRs) 중 하나이며 환경과 생물체까지 다양하게 발견되었다. DBDPE와 BTBPE에 대한 성 호르몬 교란성 연구는 몇몇 보고 되었지만, 대부분 *vtg* 유전자와 난황전구체(vitellogenin) 중심의 연구이므로 성호르몬 및 관련 기전연구는 제한적이다.

본 연구에서는 수컷 제브라피쉬와 두 세포주는 이용하여 DBDPE와 BTBPE의 성호르몬 교란성과 관련 메커니즘을 확인하였다. 수컷 제브라피쉬를 이용하여 14일 동안 노출 후 성호르몬 변화(E2, 11-KT)와 난황전구체 생성 기전에 미치는 유전자의 변화를 관찰하였다. 또한 신종 브롬계난연제의 구체적인 기전을 파악하기 위하여 H295R 세포에서의 성호르몬 변화(E2, T) 및 성호르몬 합성에 영향을 주는 유전자의 변화를 확

인하였다. MVLN 세포에서는 에스트로젠 수용체와의 결합 친화력을 형광발현도를 통해 확인하였다.

수컷 제브라피쉬 14일 노출에선, DBDPE의 경우 E2 농도가 통계적으로 유의하게 증가하였고, BTBPE의 경우 E2 농도 및 E2/11-KT 비율 수준이 증가하는 경향성을 보였다. 또한 BTBPE의 경우 간에서 난황전구체 생성을 위해 E2와 결합하는 *era* 와 *erβ* 유전자 모두 증가하는 경향성을 보인 반면 *vtg* 유전자는 아무런 변화가 없었고, DBDPE는 해당 유전자들에서 아무런 변화도 보이지 않았다. H295R 세포에서의 노출의 경우 몇몇 결과만 통계적으로 유의하지만, 전반적으로 DBDPE와 BTBPE 모두 수컷 제브라피쉬 결과와 비슷한 경향성이 관찰되었다. MVLN 세포의 경우 DBDPE와 BTBPE 모두 에스트로젠 수용체와의 친화도가 보이지 않았다.

본 연구를 통하여 신종 브롬계 난연제 DBDPE와 BTBPE의 수컷 성어 및 세포 수준에서의 잠재적 성호르몬 교란성을 확인하였다. 또한 본 연구는 이러한 물질들로 인한 호르몬 영향이 성호르몬 생합성 경로가 아닌 다른 메커니즘의 가능성을 시사한다. 성호르몬 조절은 사람과 동물 모두 생식기 발달과 번식활동 등에 있어 중요한 역할을 하기 때문에 번식 과정 및 좀 더 구체적인 기전을 확인하기 위해 추후 만성 연구가 필요하다.

주요어: 신종 브롬계난연제, DBDPE, BTBPE, H295R 세포주, MVLN 세포주, 제브라피쉬, 성호르몬, 내분비계교란

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