Investigation on Sex Hormone-Disruption Effects of Two Novel Brominated Flame Retardants (DBDPE and BTBPE) in Male Zebrafish (Danio rerio) and Two Human Cell Lines (H295R and MVLN)

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Abstract: Decabromodiphenyl ethane (DBDPE) and 1,2-bis(2,4,6-tribromo-phenoxy) ethane (BTBPE) are novel brominated flame retardants (NBFRs) and have been detected in variety of environment and biota. Although sex endocrine-disrupting potential has been suggested in experimental studies, their adverse effects on sex steroid hormones and underlying mechanisms are largely unclear. The purpose of the present study is to investigate the sex hormone-disrupting effects of two NBFRs using in vivo and in vitro models together. For this, male zebrafish (Danio rerio) along with human adrenocortical carcinoma (H295R) and breast carcinoma (MVLN) cell lines were employed. In male zebrafish, 14-day exposure to DBDPE significantly increased 17β-estradiol (E2) concentrations. Disruption of sex hormone regulation was also suggested after exposure to BTBPE, i.e., the increasing trend of E2 levels, E2/11-ketotestosterone (11-KT) ratio, and estrogen receptor-alpha (erα) and erβ gene expression levels. In H295R cells, an E2/T ratio showed an increasing trend by DBDPE exposure, but transcriptions of major genes in steroidogenesis pathway were not affected. Taken together, our observation implies that two NBFRs could cause the sex hormone disruption potential in male zebrafish and H295R cells but probably not through alteration of steroidogenesis pathway.

Keywords: novel brominated flame retardants; DBDPE; BTBPE; sex hormones; endocrine disruption; zebrafish; H295R cell line; MVLN cell line

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. Hundreds of compounds have been used for this purpose, and among them, 75 brominated flame retardants [1] and polybrominated diphenyl ethers (PBDEs) have been used in great quantity in various commercial products. Under the Stockholm Convention, the United Nations Environmental Programme (UNEP) has listed penta-, octa-, and deca-BDE as persistent organic pollutants (POPs) because of their persistence, bioaccumulation, and adverse health effects [2,3], including endocrine-disruption potentials [4–7]. After this international recognition, alternative flame retardants including novel brominated flame retardants (NBFRs), e.g., decabromodiphenyl ethane (DBDPE) and 1,2-bis(2,4,6 tribromophenoxy) ethane (BTBPE), have been increasingly used worldwide [8].

DBDPE, structurally similar to deca-BDE, is one of the novel brominated flame retardants replacing deca-BDE that has been listed by the Stockholm Convention [1,9–12]. In several Asian countries like China and Japan, the use amount of deca-BDE has been decreasing and the use of DBDPE as a substitute has been on the rise [1,13,14]. BTBPE, which has been also suggested by the Stockholm Convention as an alternative chemical, is intended to replace octa-BDE [10]. It is approximated that about 16,710 tons of BTBPE were
in production and usage in 2001, while more recent information is not readily available [15]. Increasing use of DBDPE and BTBPE as flame retardants has resulted in their frequent and widespread occurrences in various environments [16]. DBDPE and BTBPE have been detected in Chinese wastewater at levels up to 22.3 ng/L and 2.2 ng/L, respectively [17]. The concentrations of DBDPE and BTBPE in surface sediments in China have been observed at up to 20.6 ng/g dw and 0.32 ng/g dw, respectively [18]. Furthermore, studies reported that DBDPE and BTBPE had degradation and bioaccumulation characteristics similar to those of PBDEs [19–23]. DBDPE has been suggested as persistent in the environment, e.g., no degradation for 112 days in a sunlit condition [24]. BTBPE was reported for its depuration half-life of 54.1 days in juvenile rainbow trout [25]. Considering their increasing use, widespread environmental occurrences, and potential bioaccumulation potentials, studies that look at toxicological consequences of these chemicals are necessary [3].

Considering the physicochemical similarities between PBDEs and these NBFRs, sex hormone-disruption potentials could be suspected for these two NBFRs. Most of the toxicological studies that have been conducted for DBDPE and BTBPE have been focused on the neurotoxic or thyroid hormone disrupting potential, but information on the sex hormone disrupting effects of these two NBFRs is very restricted [26,27]. In previous studies, DBDPE and BTBPE exposure in fish led to observations that were suggestive of their sex hormones-disruption potentials, e.g., up-regulation of vtg gene or increased vitellogenin (vtg), which were similar to the observations on PBDEs [28–30]. Since sex hormones play crucial roles in gonad gametogenesis and the reproductive function of animals [4,31], understanding the sex hormone-disruption potentials of emerging chemicals is warranted.

The objective of this study is to enhance understanding of the sex hormone-disruption effects and the related mechanism of two NBFRs, DBDPE and BTBPE. For this purpose, zebrafish (Danio rerio) was used. Zebrafish have been frequently used as one of the experimental models for screening endocrine disrupting chemicals because of cost-effectiveness, rapid life stage, and similarity in endocrine system with other vertebrates [32]. In addition, two human tissue cell lines, i.e., H295R (human adrenocortical carcinoma) and MVLN (human breast carcinoma) cells, were chosen to evaluate the effects on sex hormones synthesis and the related mechanisms. H295R cell line and MVLN cell line have been used to observe a potential alteration in the steroidogenesis pathway [33] and binding affinity to estrogen receptor (ER) [34–37], respectively.

2. Materials and Methods

2.1. Chemicals

DBDPE (CAS No. 84852-53-9) was purchased from Tokyo Chemical Industry (Japan, purity: >96%) and BTBPE (CAS No. 37853-59-1) from Toronto Research Chemicals (Canada, purity >98%). For both in vivo and in vitro exposures, dimethyl sulfoxide (DMSO) was used as a solvent. The physicochemical properties of DBDPE and BTBPE were described in Table S1 of Supplementary Materials.

2.2. Zebrafish Culture and Exposure

Male zebrafish were chosen to focus on the estrogenic effect of the study chemicals. 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. The 14-day fish assay was carried out according to Organization for European Economic Cooperation (OECD) Guideline No. 204 (fish prolonged toxicity test: 14-day exposure) with minor modifications [38,39]. Sixteen male zebrafish (four male fish per 3 L beaker with four replicates) were assigned to each treatment. Exposure concentrations included 0, 0.031, 0.31, and 3.1 µM for DBDPE and 0, 0.015, 0.15, and 1.5 µM for BTBPE. Based on the preliminary range-finding test, the exposure concentrations were determined. Treatments were prepared with 0.1% (v/v) DMSO as a solvent. During the exposure, the exposure media (>80%) was renewed every day and
the freshly hatched Artemia nauplii were fed twice a day. Water condition of the new and old media, i.e., temperature, dissolved oxygen, pH, and conductivity, was checked every day. After the 14-day exposure, fish were pooled for analysis of gene transcription and sex hormone levels. The liver was collected, and plasma sample was separated from blood supernatant after centrifugation (8000 rpm for 10 min at 4 °C). All collected samples were stored at −80 °C until genes and sex hormones measurement.

2.3. H295R Cell Culture and Exposure

H295R cells were used to measure concentrations of 17β-estradiol (E2) and testosterone (T) and transcriptions of several key genes related to steroidogenesis [33]. H295R cells were maintained at 37 °C with 5% CO2 condition in Dulbecco’s modified Eagle’s medium/Ham’s F-12 nutrient mixture (Sigma-Aldrich Korea, Seoul, Korea), supplemented with 2.5% Nu-Serum (BD Biosciences, Franklin Lakes, NJ, USA) and 1.2 g/L Na2CO3 (Sigma-Aldrich), and 1% ITS + Premix (BD Biosciences). For 24 h before the exposure, H295R cells were seeded at 3.0 × 105 cell/mL density and maintained in 24-well plates. The cells were exposed to DBDPE (0, 0.003, 0.01, and 0.03 µM), BTBPE (0, 0.1, 0.3, and 1 µM), and Forskolin (10 µM) as a positive control, in triplicate (n = 3) for 48 h. Exposure doses were chosen at non-cytotoxic levels (>80% cell viability), based on WST-1 cell proliferation assay (Figure S1). For sex hormone and gene transcription measurement, relevant media and the harvested cells were collected and stored at −80 °C until the analysis.

2.4. MVLN Cell Culture and Exposure

The cells were cultured in a hormone-free DMEM/F12 nutrient mixture supplemented with 1 mg/L insulin (Sigma-Aldrich), 1 mM sodium pyruvate, and 10% fetal bovine serum (Gibco®, Grand Island, NY, USA). The cells were maintained at 37 °C with a 5% CO2 atmosphere. The medium was changed to a charcoal-stripped medium at 24 h before cell plating. MVLN cells were seeded at 3.0 × 105 cell/mL density in 96-well plates for 24 h. Test doses were chosen at non-cytotoxic levels, i.e., >80% cell viability based on the WST-1 cell proliferation assay (Figure S1). The cell was exposed to DBDPE (0, 0.0003, 0.001, 0.003, 0.01, and 0.03 µM) or BTBPE (0, 0.01, 0.03, 0.1, 0.3, and 1 µM) for 72 h. After the exposure, luciferase activity was analyzed using SteadyGlo®-Luciferase Assay System (Promega Corp., Madison, WI, USA). The maximum response of E2 was set to 100%, and the relative light unit (RLU) for each well was converted to maximum induction of luciferase activity (% E2 max Luc).

2.5. Sex Hormone Extraction and Measurement

For sample extraction, 5 µL of zebrafish plasma or 500 µL of H295R cell medium were added in a vial with 400 µL of ultrapure water each. Plasma and the medium samples were extracted with 2 mL and 2.5 mL of diethyl ether twice at 2100 rpm for 10 min, respectively. After evaporating diethyl ether, the dried sample containing the plasma or the medium was diluted in 120 µL and 300 µL of buffer, respectively [40]. The sex hormone was measured using commercial kits using enzyme-linked immunosorbent assay (ELISA) (Cayman Chemical, Ann Arbor, MI, USA; E2 for both samples, T for H295R cell, and 11-ketotestosterone (11-KT) for zebrafish plasma).

2.6. Measurement of Gene Expression

In male zebrafish, the gene expression levels related to vitellogenesis (vitellogenin [vtg], estrogen receptor-alpha [era], and beta [erβ]) were analyzed in the liver. In H295R cells, the gene expression levels related to the steroidogenesis pathway (STAR, 3βHSD, CYP17, and CYP19) were measured. The primer sequences are listed in Table S2. As the housekeeping gene, 60S ribosomal protein L8 [RPL8] and beta-actin [β-ACTIN] were chosen for zebrafish and H295R cells, respectively. The primer sequences of the target genes used in the study were determined based on previous studies [36,39]. To isolate total
RNA for zebrafish liver and H295R cells, the collected samples were extracted using the RNeasy mini kit (Qiagen, Hilden, Germany). After measurement of RNA quantification and qualification of the sample by a microplate reader (Gen5 2.05, BioTek, Winooski, VT, USA), the complementary DNAs were synthesized using an iScriptTM cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). The 20 µL of solution mixed with 18 µL of pre-mix (10 µL of SYBR green, 1.8 µL of forward and reverse primer, and 4.4 µL of nano-pure water) and 2 µL of the cDNA sample was prepared for quantitative real-time PCR (qRT-PCR). The relative transcription level of the target gene was calculated as threshold cycle (Ct) value following the $2^{-\Delta\Delta Ct}$ method [41].

2.7. Statistical Analysis

To test the group difference between the control and the exposure, IBM SPSS 23.0 for Windows (SPSS Inc., Chicago, IL, USA) was used. One-way analysis of variance (ANOVA) with Dunnett’s t-test was used after confirming data normality and homogeneity of variances using Shapiro—Wilk’s test and Levene’s test. Linear regression was performed for trend analysis. If necessary, log-transformation was employed on the data. $p < 0.05$ was considered statistically significant. All data were shown as mean ± standard deviation (SD).

3. Results

3.1. In Vivo Assay with Male Zebrafish

Alteration of Sex Hormones and Gene Expressions Related to Vitellogenesis

The concentration of plasma E2 was significantly increased in male zebrafish exposed to 0.31 and 3.1 µM of DBDPE (Figure 1A), but there were no statistically significant changes on 11-KT concentration and E2/11-KT ratio (Figure 1B,C). The concentration of E2 and E2/11-KT ratio following exposure to BTBPE was shown as an increasing trend, whereas no significant change of 11-KT concentration was observed (Figure 1). For BTBPE exposure, increasing trends of era and erβ gene transcriptions were observed without any significant change of vtg gene transcription level (Figure 2). Following exposure to DBDPE, levels of vtg, era, and erβ gene transcriptions showed no significant difference from those measured in the solvent control; however, the relative expression pattern was similar to that of 11-KT (Figures 1 and 2).

![Figure 1. Cont.](image-url)
Figure 1. Effects on sex hormones of (A) 17β-estradiol (E2), (B) 11-ketotestosterone (11-KT), and (C) E2/11-KT ratio in male zebrafish exposed to DBDPE and BTBPE. The results are shown as mean ± SD of quadruplicates (n = 4). Asterisk indicates a significant difference relative to the solvent control (0.1% DMSO, p < 0.05). The value of p for trend was determined based on the linear regression analysis. If p for trend was < 0.05, a linear trend is indicated with a red dotted line.

Figure 2. Cont.
Figure 2. Transcriptional changes of (A) vtg, (B) erα, and (C) erβ genes in male zebrafish liver exposed to DBDPE and BTBPE. The results are shown as mean ± SD of quadruplicates (n = 4). Asterisk indicates a significant difference in treatment groups relative to the solvent control (0.1% DMSO, p < 0.05). The value of p for trend was determined based on the linear regression analysis. If p for trend was <0.05, a linear trend is indicated with a red dotted line.

3.2. In Vitro Assay with H295R and MVLN Cell Lines

3.2.1. Sex Hormone and the Related Gene in H295R Cell Line

In H295R cells exposed to DBDPE, the E2/T ratio showed an increasing trend (Figure 4C), whereas most of the genes related to steroidogenesis were not changed (Figure 4). Following exposure to BTBPE, a significant up-regulation of CYP19 gene at 0.3 μM and the increasing trend of 3βHSD gene was observed (Figure 4B,D).

Figure 3. Cont.
Figure 3. Effects on sex hormones of (A) 17β-estradiol (E2), (B) testosterone (T), and (C) E2/T ratio in H295R cells exposed to DBDPE and BTBPE. The results are shown as mean ± SD of triplicates (n = 3). Asterisk indicates a significant difference relative to the solvent control (0.1% DMSO, p < 0.05). The value of p for trend was determined based on the linear regression analysis. If p for trend was <0.05, a linear trend is indicated with a red dotted line.

Figure 4. Cont.
3.2.2. ER-Binding Affinity in MVLN Cell Line

In MVLN cell line assay, binding affinity to ER was observed at below 0%, which indicates that DBDPE and BTBPE do not have direct ER agonistic effects (Figure S2).

4. Discussion

To understand the effects of two NBFRs on sex hormones, male zebrafish for in vivo and H295R and MVLN cells for in vitro were employed in this study. Both in vivo and in vitro results suggest a line of evidence supporting sex endocrine-disruption by DBDPE and BTBPE. Results of male zebrafish and H295R cells showed that exposures to two NBFRs increased E2 levels partly through the transcriptional changes of steroidogenic genes. These observations are in similar agreement with some PBDEs such as deca-BDE and octa-BDE reported elsewhere [5–7].

Sex hormones play critical roles in sexual development, such as gonad gametogenesis and reproductive systems [42]. In the present observation, in male zebrafish, DBDPE and BTBPE exposure resulted in enhanced E2 levels and E2/11-KT ratios. The observations from H295R cells also showed a similar trend despite the fact that statistical significance was not evident. These results indicate that levels of sex hormone exposed to DBDPE and BTBPE might be mediated by mechanisms other than the alteration of the steroidogenesis pathway, e.g., metabolic activation from other systems [43] or hepatic metabolism and excretion [44]. Similarly, a previous study showed significant alteration of sex hormones after exposure to DE-71 in zebrafish [45], but another study reported no effects in H295R cell after DE-71 exposure [46].

In H295R cells, key genes that are translated to the enzymes related to sex hormone synthesis (STAR, 3βHSD, CYP17, and CYP19) were analyzed to explain the weak E2 increase (Figure 4). The STAR gene regulates the transfer of cholesterol into the inner mitochondrial membrane [46]. 3βHSD and CYP17 genes are translated to enzymes for steroidogenesis and androgen synthesis. The CYP19 gene, which is translated to aromatase, is responsible for a conversion of T to E2. In our study, transcription levels of all target genes in H295R cells after exposure to DBDPE did not change despite the increasing trend in the E2/T ratio. In a previous study, a few out of 19 different PBDEs showed the alteration in aromatase activity in H295R cells [47]. Following the exposure to BTBPE, up-regulations of 3βHSD and CYP19 genes were observed, although levels of E2 were only slightly increased. These results were in correspondence with a previous paper that reported no significant change in sex hormone after the exposure to octa-BDE mixture despite an increase in aromatase activity [46]. However, our result of transcriptional levels in H925R cells are not comparable to the upregulation of E2 levels in male zebrafish, but the reasons for this discrepancy are not readily available.

Figure 4. Transcriptional changes of (A) STAR, (B) 3βHSD, (C) CYP17, and (D) CYP19 genes in H295R cells exposed to DBDPE and BTBPE. The results are shown as mean ± SD of triplicates (n = 3). Asterisk indicates a significant difference in transcriptional level of treatment groups relative to the solvent control (0.1% DMSO, p < 0.05). The value of p for trend was determined based on the linear regression analysis. If p for trend was < 0.05, a linear trend is indicated with a red dotted line.
In MVLN cells exposed to DBDPE and BTBPE, ER-binding affinity was not observed while significant increases of \( er\alpha \) and \( er\beta \) gene expressions were observed in zebrafish liver. Our results on the MVLN cell line assay demonstrated that DBDPE and BTBPE may not bind directly with the ER. Likewise, a previous study reported that PBDE congeners such as BDE-209 did not alter E2-induced ER in MVLN cells [48].

Regardless of the increase in E2 levels or alteration of \( er\alpha \) and \( er\beta \) gene expression, transcription of \( vtg \) gene after exposure to DBDPE and BTBPE was not altered in the fish. Vitellogenin has been a well-known indicator of endocrine disruption in male fish [49,50]. It is developed in the liver of female fish by E2 signal and is an essential protein for oocyte maturation and reproduction in female fish. Our observations were not comparable with some previous reports, which showed altered \( vtg \) gene regulation or VTG levels after exposure to DBDPE or BTBPE in certain fish species [28–30]. Nevertheless, several possible reasons could explain the present observations. One possible reason may be that the levels of E2 were not sufficiently high to induce vitellogenesis, which is in agreement with previous studies [51,52]. Secondly, hormone metabolic breakdown or clearance can be considered [43]. In a previous report, E2 metabolites and conjugates e.g., estradiol-sulfate or glucuronide in liver and plasma, may affect binding affinity of E2 with ER in rainbow trout and Siberian sturgeon [53]. Lastly, effects of 11-KT on ovarian development and \( vtg \) are also possible. Several works showed that androgens could alter the levels of \( vtg \) gene, VTG synthesis, or binding to ERs in both in vitro and in vivo fish studies [54–56].

Our observations imply that DBDPE and BTBPE may possess weak sex hormone-disruption potency in male zebrafish and H295R cells, while steroidogenic gene alterations and sex hormone receptor activation could not explain the observation. This study has several limitations. First, only the genes related to the steroidogenic pathway in H295R cells and the vitellogenesis in male zebrafish were evaluated, and therefore, different modes of action that may cause sex hormone disruption, such as altered hepatic metabolisms [44], could not be tested. Secondly, our study employed only male zebrafish, thus the potential effects on female zebrafish remain to be investigated in the future. Lastly, exposure levels of the present study are higher than the levels of environmental occurrences. Therefore, studies of the consequences of long-term exposure at environmentally relevant concentration are warranted to understand the reproduction process and its detailed mechanisms in the aquatic ecosystem.

5. Conclusions

The present observations suggest that both DBDPE and BTBPE possess weak estrogenic potentials. A significant, increasing trend of E2 levels and changes in the related gene expression in male zebrafish and H295R cells may support that two NBFRs have sex hormone disrupting effects which are probably not mediated by steroidogenic alterations. Given the crucial role of sex hormones on sexual development and reproduction and increasing use of these NBFRs, studies to evaluate the consequence of endocrine disruption are needed.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/app11093837/s1, Table S1: Physicochemical properties of DBDPE and BTBPE; Table S2: Information of primer sequence of zebrafish and H295R cell lines used in the study; Figure S1: Preliminary range finding of DBDPE and BTBPE. Cell viability of (A) H295R and (B) MVLN cell lines were measured by WST-1 assay; Figure S2: Binding affinity of DBDPE and BTBPE with ER in MVLN cell luciferase assay.

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