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Effects of several low molecular weight phthalates and their metabolites on sex hormone system in male zebrafish *(Danio rerio)* and H295R cell line

제프라피쉬 *(Danio rerio)*와 세포주 H295R을 이용한
일부 저분자량 프탈레이트와 그 대사체의 성호르몬 교란
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ABSTRACT

Effects of several low molecular weight phthalates and their metabolites on sex hormone system in male zebrafish (*Danio rerio*) and H295R cell line

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Phthalates are commonly used to increase flexibility of plastics, and are widely used in PVC, commercial products, wrapping food, cosmetics, medical devices and personal care products. Some phthalates with low molecular weight such as diethyl phthalate (DEP), benzyl butyl phthalate (BBzP), or diisobutyl phthalate (DiBP) are suspected as an endocrine disruptor. It is generally known that they possess higher toxicity than other long chain phthalates and can be quickly metabolized in organism. However, their adverse effects on sex steroid production and underlying mechanism are not fully understood.

The aim of this study is to investigate the effects of three low molecular weight phthalates (LMWPs), DEP (0, 0.08, 0.4, 2, or 10 mg/L), BBzP (0, 0.02, 0.1, 0.5, or 2.5 mg/L), and DiBP (0, 0.0008, 0.004, 0.02, or 0.1 mg/L), on sex steroid hormone system in male zebrafish. In addition, these three phthalates as well as their major metabolites, monoethyl phthalate (MEP), monobenzyl phthalate (MBzP), or

monoisobutyl phthalate (MiBP), were individually exposed to a human adrenocortical carcinoma (H295R) cell to understand their effects on sex steroidogenesis. In both zebrafish and H295R cells, changes in sex steroid hormone and gene transcriptions involved in steroidogenic pathway were evaluated.

For male zebrafish, 14 days exposure to DEP, BBzP, or DiBP significantly decreased testosterone (T) concentrations at higher exposure concentrations of each compound. For DEP, 17β-estradiol (E2) concentrations were also significantly decreased at 10 mg/L. In addition, transcriptions of the genes involved in steroidogenesis pathway were also affected. DEP or DiBP exposure led to significant down-regulation of *star* gene and similar decreasing trend was also found in male zebrafish exposed to BBzP. All test compounds significantly up-regulated *cyp19a* gene transcription in a concentration dependent manner.

In H295R cell bioassay, five test compounds, i.e. DEP, BBzP, DiBP, MEP, or MBzP, decreased T concentrations, except MiBP which showed opposite direction of change. E2 levels were slightly decreased by MEP, and increased by BBzP, or MBzP in H295R cells. Changes in gene transcription level were in good agreement with the zebrafish exposure data where significant down-regulation of *StAR* gene and up-regulation of *CYP19A* gene were observed. On the other hand, DEP and MEP exposure significantly down-regulated the transcriptions of *3βHSD2* gene respectively in H295R cells unlike in male zebrafish.

The results of our present study show that some LMWPs could affect sex hormone system in both zebrafish and cell lines by alteration of steroidogenesis pathway.

Keywords: low molecular weight phthalates (LMWPs); endocrine disruption; steroidogenesis; H295R cell line

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1. Introduction

Phthalates are group of synthetic compounds which are key additives used for softening PVC, commercial products, and personal care products (Barse et al., 2007; Okita, 1992; Wan et al., 2013). Phthalates can be divided into two groups; "low molecular weight phthalates (LMWPs)" which contain carbon chain less than 6, and "high molecular weight phthalates (HMWPs)" which have carbon side chains more than 6 (Bradlee and Thomas, 2003). Especially LMWPs including diethyl phthalate (DEP), benzyl butyl phthalate (BBzP), and diisobutyl phthalate (DiBP) (Table 1), are widely used in toys, childcare products, personal-care products, some cosmetics, fragrances, lacquers, and varnishes (Jurewicz et al., 2013). Koniecki et al. (2011) detected DEP, di-n-butyl phthalate (DnBP), DiBP, and dimethyl phthalate (DMP) in cosmetics and personal care products. Especially, DEP was detected 103 products out of 252. Also, Koo and Lee (2004) detected DEP, BBzP, and dibutyl phthalate (DBP) in cosmetics, particularly DEP was frequently detected as high concentration in perfume.

DEP, BBzP, and DiBP can migrate into the environment through various pathways (Qu et al., 2015). Table 2 summarized the occurrence of DEP, BBzP, and DiBP in environments. In Guangzhou river, DiBP was detected up to 2,860 ng/L (Zeng et al., 2009). Also, DEP, BBzP were detected in France river water with very high concentration. DEP and BBzP were detected up to 1,870 ng/L and 12,100 ng/L respectively (NORMAN, 2011 and 2012).

After LMWPs enter into body, they can be easily metabolized. DEP, BBzP, and DiBP are hydrolyzed and transformed into monoester form of monoethyl phthalate (MEP; metabolism ratio; 73%), monobenzyl phthalate (MBzP; 69%), and monoisobutyl phthalate (MiBP; 73%), respectively (Table 1) (Koch and Calafat, 2009). Therefore, MEP, MBzP, and MiBP have been frequently detected in human

urine and serum samples (Table 3). Also, MEP, a major metabolite of DEP, was detected 100% of breastmilk samples in Korean women (Kim et al., 2015).

A few epidemiological study suggested that there are associations between urinary phthalate and potential effects of phthalate exposure on the male reproductive system. In previous study, urinary phthalate metabolites showed negative association with anogenital index (AGI), semen quality, and reproductive hormones (Kay et al., 2014; Latini et al., 2006; Lottrup et al., 2006). Especially MBzP is associated with decreasing of semen quality (Pan et al., 2006). Also, urinary MEP concentration is associated with DNA damage rate, and decreasing of motility (Duty et al., 2003; Hauser et al., 2007; Jonsson et al., 2005). Although the adverse effects of LMWPs metabolites have been conducted in epidemiological studies, available information on mechanisms of them are very limited. Moreover, despite high metabolism rate of LMWPs, toxic effects of phthalate metabolites have been rarely investigated.

Nevertheless the regulation of LMWPs has started recently. Among LMWPs, BBzP and DiBP were classified as substances of very high concerns (SVHC) in REACH. European authorities classified them in Category 1B, substances considered as toxic to reproduction and the use of these phthalates is prohibited in toys and child care products (Ventrice et al., 2013). In Europe and Korea, among three LMWPs only BBzP is banned from childcare products, toy, and PVC packaging (KFDA, 2009; Lee et al., 2014).

And also LMWPs known to be more toxic than HMWPs (Bradlee and Thomas, 2003). Anti-androgenicity of LMWPs among *in vitro* and *in vivo* study was reported (Hannas et al., 2011; Hotchkiss et al., 2004; Howdeshell et al., 2008; Mankidy et al., 2013; Jobling and Sumpter, 1995). However, the endocrine disrupting effects of LMWPs using aquatic organisms are not studied well. Only a few acute studies were conducted about phthalates (Staples et al., 1997; Adams et

al., 1995). And among LMWPs only BBzP were observed the estrogenic activity using transgenic medaka (Chen et al., 2014). Therefore our understandings on toxicological effects of LMWPs and their underlying mechanisms are very limited.

The aim of this study is to determine the effects of LMWPs and their metabolites on sex steroid hormone system of male zebrafish. In addition, *in vitro* study conducted to clarify the toxicological effects of metabolites in steroidogenic pathway and to investigate mechanisms of LMWPs. The results of this study will help to understand the sex hormone disrupting potentials and underlying mechanisms of LMWPs.

Table 1. Physicochemical properties of the studied three LMWPs and their metabolites

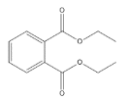
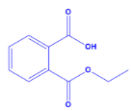
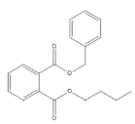
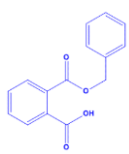
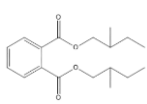
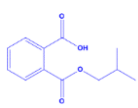
Compounds	CAS number	Structure	Usage	logKow	Water solubility (g/L)	Molecular weight (g/mol)
Diethyl phthalate (DEP)	84-66-2		cosmetics, fragrances	2.42	1.08	222.24
Monoethyl phthalate (MEP)	2306-33-4		metabolite of DEP	1.86	1.21	194.18
Benzylbutyl phthalate (BBzP)	85-68-7		PVC, floor tiles	4.7	3.8×10^{-3}	312.39
Monobenzyl phthalate (MBzP)	2528-16-7		metabolite of BBzP	3.07	0.052	256.3
Diisobutyl phthalate (DiBP)	84-69-5		cosmetics, nail polish	4.11	2.0×10^{-5}	278.35
Monoisobutyl phthalate (MiBP)	30833-53-5		metabolite of DiBP	2.77	0.98	222.24

Table 2. Environmental concentrations of DEP, BBzP, and DiBP

Compounds	Matrix	Country	Location	No. of sampling site	Sampling date	Detection frequency (%)	Median (ng/L)	Lowest level (ng/L)	Maximum level (ng/L)	Reference
DEP	Surface water	China	Songhua river	10	Oct., Dec., 2011	100	2.35	1.33	6.67	Gao et al. (2014)
		China	Guangzhou river	15	May, 2005	100	31	15	320	Zeng et al. (2008)
BBzP		China	Songhua river	10	Oct., Dec., 2011	18	2.49	n.d.	4.39	Guo et al. (2013)
DiBP		China	-	13	Aug., 2009	-	37	8.3	200	Hu et al. (2013)
		China	Guangzhou river	15	Apr., 2006	100	495	281	1750	Zeng et al. (2009)
				15	Aug., 2006	100	1280	884	2860	
				15	Dec., 2006	100	321	161	971	

Table 2. (Continued)

Compounds	Matrix	Country	Location	No. of sampling site	Sampling date	Detection frequency (%)	Median (ng/g)	Lowest level (ng/g)	Maximum level (ng/g)	Reference
DEP	Sediment	China	Songhua river	6	Oct., Dec., 2011	100	31.51[a]	26.7	38.24	Gao et al. (2014)
		China	Qiantang river	23	Apr., 2011	95.65	7	n.d.	218	Sun et al. (2013)
BBzP		China	Songhua river	6	Oct., Dec., 2011	33	62.36[a]	n.d.	96.32	Guo et al. (2013)
		China	Guangzhou river	15	May, 2005	73	34	n.d.	280	Zeng et al. (2008)
DiBP		China	Zhujiang river	11	-	100	2.29	0.561	12.4	Liu et al. (2014)
		China	Dongjiang river	21	-	100	0.811	0.108	5.28	
		China	Qiantang river	23	-	100	0.118	0.019	0.769	Sun et al. (2013)

n.d.: not detected; - : not provided; [a]Mean

Table 3. Biomonitoring studies about MEP, MBzP, and MiBP reported worldwide

Compounds	Country	Age (year)	Sampling size	Sampling date	Detection frequency (%)	Median (μg/L)	Lowest level (μg/L)	95th percentile (μg/L)	Maximum level (μg/L)	Reference
MEP	Korea	19-27	40 (male)	Jul. 2011	100	9.9	-	53.3	-	Lee et al. (2013)
			40 (female)		100	22.4	-	227	-	
	Norway	8.8-12.5	623	2001-2004	100	56.7	8.5	360.2	6006	Bertelsen et al. (2013)
	Germany	6.8±0.6	104	2007-2009	100	33.6	2.7	391	1787	Kasper-Sonnenberg et al. (2012)
		39.2±4.6	103		100	53.8	5.1	265	756	
	USA	18-35	300	1998-2006	100	143.5	-	335.2*	-	Whyatt et al. (2014)
MBzP	Norway	8.8-12.5	623	2001-2004	100	29.3	2.1	128.7	6710	Bertelsen et al. (2013)
	Mexico	813	49 (male)	2010	98	3.4	-	46.8	32.5	Lewis et al. (2013)
			50 (female)		100	2.9	-	29.6	48.4	
MiBP	Korea	19-27	40 (male)	Jul. 2011	-	8.1	-	22.2	-	Lee et al. (2013)
			40 (female)		-	9.6	-	22.4	-	

Table 3. (Continued)

MiBP	Norway	8.8-12.5	623	2001-2004	100	49.2	3.1	231	1480	Bertelsen et al. (2013)
	Germany	6.8±0.6	104	2007-2009	100	68.7	1.8	340	1284	Kasper-Sonnenberg et al. (2012)
		14-60	23 (male)	Apr.-Oct. 2005	100	47.3	23.1	107.2	119.7	Fromme et al. (2007)
		14-60	27(female)	Apr.-Oct. 2005	100	36.1	15.7	109.1	163.8	
	Mexico	813	49 (male)	2010	96	2.1	-	7.3	39.4	Lewis et al. (2013)
			50 (female)		98	2.2	-	12.1	33.9	
	USA	24 ± 6.2	382	1998-2002	97.4	6.2	LOD	12*	131	Wolff et al. (2008)

* 75th percentile; -: not provided; LOD<0.26

2. Materials and Methods

2.1. Chemicals

DEP (CAS No. 84-66-2) purchased from Sigma-Aldrich (St. Louis, MO, USA). MEP (CAS No. 2306-33-4), BBzP (CAS No. 85-68-7), MBzP (CAS No. 2528-16-7), DiBP (CAS No. 84-69-5), and MiBP (CAS No. 30833-53-5) purchased from AccuStandard (New Haven, CT, USA). Dimethyl sulfoxide (DMSO) was used as solvent in both *in vitro* and *in vivo* studies. The final concentration of solvent in the exposure media was 0.005% (v/v) for zebrafish exposure, and 0.1% (v/v) for H295R cell assays, respectively.

2.2. Zebrafish culture and exposure

Wild type adult male zebrafish (~4 months old) were obtained from commercial vendor (Gangnam aquaria, Suwon, Korea). Before exposure, fish were acclimated in dechlorinated tap water for 7 days.

For DEP or BBzP exposure, 16 male zebrafish were divided into four replicates with four fish per replicate. Each replicate was kept in a 2 L beaker. For DiBP exposure, 9 male zebrafish were divided into three replicates with three fish per replicate. A various concentrations of exposure media (0.08, 0.4, 2, or 10 mg/L DEP; 0.02, 0.1, 0.5, or 2.5 mg/L BBzP; 0.0008, 0.004, 0.02, or 0.1 mg/L of DiBP) were prepared with dechlorinated water. The exposure concentration was determined based on the preliminary range finding test. Followed by range finding test, the maximum concentration which did not showed mortality was chosen as highest concentration. The exposure media were renewed every other day. The experiments were carried out for 14 days. This period was recommended by OECD guidelines for the testing of chemicals test No. 204: fish prolonged toxicity test: 14 day study (OECD, 1984). Fish were fed with freshly hatched *Artemia nauplii* twice

a day and fish are maintained at 26±1℃ and under 14:10 h light:dark photoperiod. Water quality parameters including dissolved oxygen, pH, conductivity, and temperature are monitored routinely.

After 14 days of exposure, liver and testis were dissected out from each fish. Blood samples were collected using a glass capillary tube from caudal vein and samples from three fish were pooled for each replicate. Plasma was collected after blood centrifugation (8,000 rpm for 10 min at 4℃) and then the supernatant was collected and stored at -80℃ until sex steroid hormone analysis. No mortality was observed during the fish exposure.

2.3. H295R cell culture and exposure

H295R cells were cultured in a mixture of Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mixture (DMEM/F12) (Sigma D-2906; Sigma-Aldrich) supplemented 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). Medium was renewed every other day, and cells were subcultured when cell confluence was over 80%. Cells were cultured at 37 ℃ in a 5 % CO_2 atmosphere (Hilscherova et al., 2004). Prior to the chemical exposure, cytotoxicity tests conducted with WST-1 cell proliferation reagents (Roche Applied Science). The exposure concentrations were determined on the basis of 80% cell survival compared to solvent control. For the cell bioassay, H295R cells were seeded with a density of 3.0 x 10^5 cells/mL per each well and incubated in 24-well plates for 24 hours, and the cells were dosed with various LMWPs (0.004, 0.04, or 0.4 M DEP, MEP; 0.001, 0.01, or 0.1 M BBzP, BBzP; 0.0001, 0.001, or 0.01 M DiBP, MiBP). Each treatment included three replicates and exposure duration was 48 hours. Media were collected in each treatment to measure sex steroid hormone. Cells are homogenized and collected for measuring gene transcriptions.

2.4. Measurement of sex hormones and gene transcriptions

In both fish plasma and H295R medium, sex steroid hormones were measured by enzyme-linked immunosorbent assay (ELISA) with test kits (Cayman chemical; 17β-estradiol [Cat No. 582251], and testosterone [Cat No. 582701]). Before measuring, sex hormone was extracted from H295R media and fish plasma, respectively. 4 μl of plasma sample and 500 μl of H295R media with 400 μl UltraPure water was extracted with 2 ml diethyl ether twice at 2000 g for 10 min. After the diethyl ether was evaporated, the residues were dissolved in 120 μl (zebrafish), 300 μl (H295R cell) of EIA buffer for ELISA assay (Ji et al., 2010).

H295R cells and fish organ samples are collected and homogenized. Total RNA was extracted using RNeasy mini kit (Qiagen). RNA quality and concentration are determined by a use of a Gen5 2.05 (BioTek, Winooski, VT, USA). Isolated RNA was diluted to 100 ng/μL, and iScriptTM cDNA synthesis kit (BioRad, Hercules, CA, USA) was used to synthesize complementary DNA following the manufacture's protocol. Quantitative real-time PCR (qRT-PCR) was performed with the 20 μL of qRT-PCR reaction mix consisting of 10 μL of LightCycler-DNA Master SYBR Green I mix (Roche Diagnostics Ltd, Lewes, UK), 4.4 μL of nuclease free water, and 1.8μL of forward, reverse primer (10 pmol). After seeding 18 μL of pre-mix, 2 μl cDNA templates are added into each well and Light Cycler 480 (Roche Applied Science, Indianapolis, IN, USA) was used to perform qRT-PCR. The relative transcription level of target gene was calculated with the threshold cycle (Ct) value according to the 2ΔΔCt method (Livak and Schmittgen, 2001). Only *StAR*, *3βHSD2*, and *CYP19A* which showed significant change in male zebrafish study were observed in H295R cell assay. As a housekeeping gene, *18s rrna* and *β-actin* were used for zebrafish and H295R cell, respectively. Primer sequences for selected genes and a housekeeping genes are listed in Table S1.

2.5. Chemical analysis

The analyses for measuring LMWPs and their metabolites in zebrafish exposure media were performed on a high performance liquid chromatography (Nanospace SI-2, Shiseido) with triple quadrupole tandem mass spectrometry (LC/MS/MS; API 4000, Applied Biosystems) as detailed in Kho et al. (2008). Before measurement, 100 μl of water sample were added and voltexed during 10 seconds. On-line clean-up and separation of phthalate metabolites was accomplished using the switching-column technique with a pretreatment column (Shiseido MF C8, 50_4.6 mm, 5 mm), trap column (Imtackt Cadenza CD-C18, 30_2.0 mm, 5 mm) and analytical column (Imtackt Cadenza CD C18, 75_2.0 mm, 3 mm). 0.1 % acetic acid in water (A) and 0.1 % acetic acid in ACN (B) were used as mobile phase. The quantification of LMWPs and their metabolites were performed using electrospray ionization (ESI) positive ion mode. LOQ was estimated to be 0.376 μg/L for DEP, 0.037 μg/L for MEP, 0.079 μg/L for BBzP, 0.072 μg/L for MBzP, 0.184 μg/L for DiBP, and 0.177 μg/L for MiBP

2.6. Statistical analysis

Normality of data and homogeneity of variances were analyzed by the Shapiro-Wilk's test and Levene's test, respectively. When necessary, log-transformation was conducted. For group comparison, data are analyzed by one way analysis of variance (ANOVA) followed by a Dunnett's t-test. *P*-values less than 0.05 were considered significant. SPSS 20.0 for Windows (SPSS Inc., Chicago, IL, USA) was used. Trend analysis was applied based on the linear regression.

Table 4. Primer sequences used for the quantitative RT-PCR analysis in this study

Assay	Gene name		Primer sequence (5'-3')
In vivo D. rerio	*18s rrna*	forward	catggccgttcttagttggt
		reverse	cggacatctaagggcatcac
	star[a]	forward	ggtctgaggaagaatgcaatgat
		reverse	ccaggtccggagagcttgt
	cyp11a	forward	ggcagagcaccgcaaaa
		reverse	ccatcgtccagggatcttatt
	3β hsd	forward	aggcacgcaggagcacatct
		reverse	ccaatcgtctttcagctggtaa
	17β hsd	forward	tgcatctcgcatcaaatcca
		reverse	gtccaagttccgcatagtagca
	cyp17	forward	ggactccagtgttggtgaataca
		reverse	gggttcttccattcctcctca
	cyp19a	forward	tctgcttcagaagattcataaatacttt
		reverse	cctgcaactcctgagcatctc
	vtg	forward	aagacccctgtcgttccaatc
		reverse	aaactcgtactgcagggatcc
***In vitro* H295R cell**	*β-actin*	forward	ggacttcgagcaagagatgg
		reverse	agcactgtgttggcgtacag
	StAR	forward	aagagggctggaagaacgag
		reverse	tctccttgacattggggttc
	3βHSD2	forward	cgggcccaactcctacaag
		reverse	tttagaggctcttcttcg
	CYP19A[b]	forward	aggtgctattggtcatctgctc
		reverse	tggtggaatcgggtctttatgg

[a] Jo et al. (2014);[b] Hilscherova et al. (2004); other primer sequences were designed using Primer 3 online software ver.4.0.0 (http://primer3.ut.ee/).

3. Results

3.1 Zebrafish 14 days exposure test

3.1.1 Effects of three LMWPs on plasma sex hormones

The effects of DEP, BBzP, and DiBP on 17β-estradiol (E2), testosterone (T), and E2/T ratio in male zebrafish were shown in Fig. 1. All three test compounds significantly decreased the concentration of T (Fig. 1B). Following exposure to DEP, not only T but also E2 levels were significantly decreased at 10 mg/L DEP. For BBzP exposure, T levels decreased at 0.1, 0.5 and 2.5 mg/L BBzP while E2 levels were not altered. However, statistically negative trend was shown in E2 concentrations. Therefore E2/T ratio was significantly increased by BBzP (Fig. 1C). After exposure to DiBP, only T concentrations decreased at 0.02 and 0.1 mg/L but no significant changes were found in E2 concentrations.

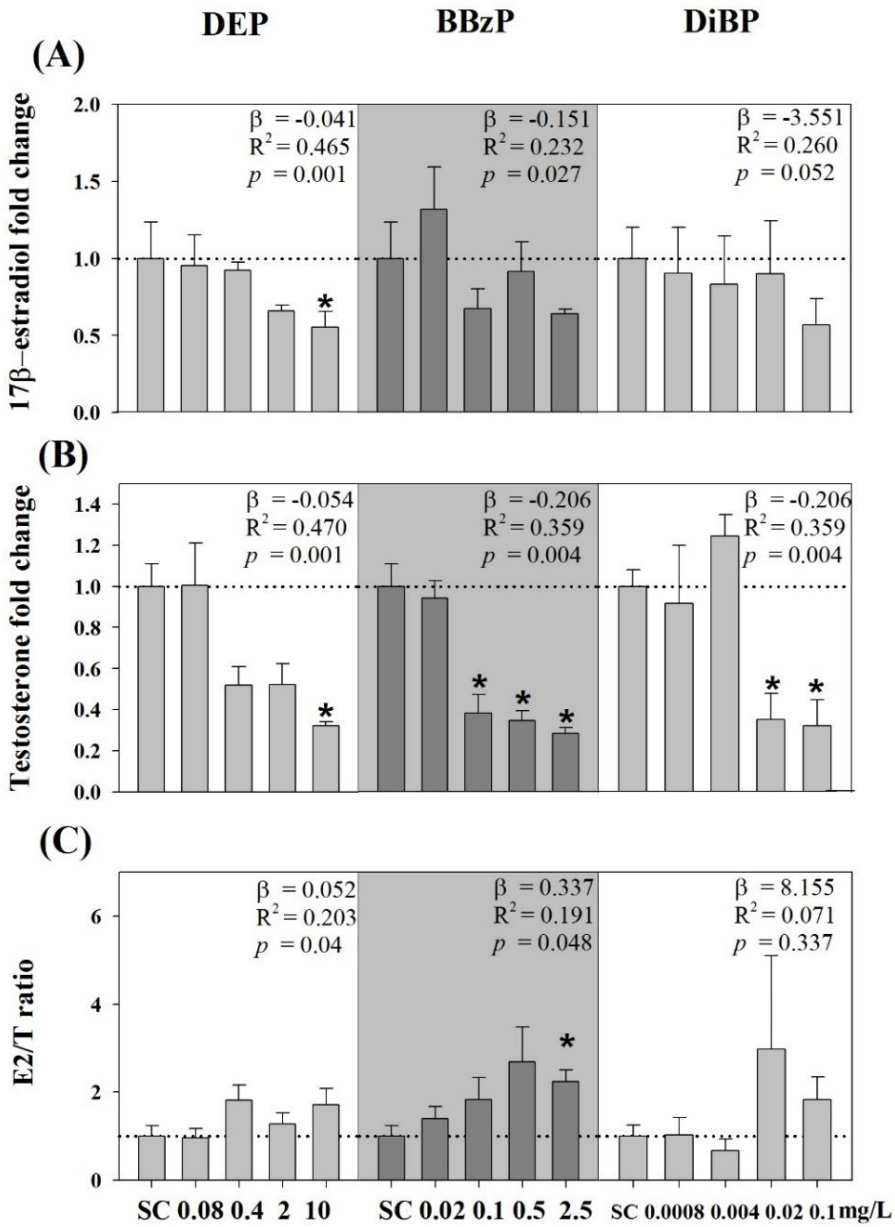


Figure 1. Effects on plasma (A) 17β-estradiol (E2) concentration, (B) testosterone (T) concentration, and (C) E2/T ratio in male zebrafish after exposure to DEP (0.08–10 mg/L), BBzP (0.02-2.5 mg/L), and DiBP (0.008–0.1 mg/L) for 14 days. The results are shown as mean ± SD of three or four replicates. The * indicates significant difference from solvent control (SC, treated with 0.005% DMSO, $p<0.05$).

3.1.2 Effects of three LMWPs on gene transcriptions

Following exposure to DEP, BBzP, or DiBP, transcriptions of several genes involved in steroidogenesis were significantly changed in male zebrafish gonad (Fig. 2). The transcription of steroidogenic acute regulatory protein (*star*) gene was significantly down-regulated after exposure to 10 mg/L DEP and 0.1 mg/L DiBP (Fig. 2A). Similarly, down-regulation trend of *star* gene transcription by BBzP was observed in male gonad although not statistically significant.

Also negative trends in transcriptional level of 3β-hydroxysteroid dehydrogenase gene (*3β hsd*) observed by all three LMWPs exposure (Fig. 2C). Gene transcription of 17β-hydroxysteroid dehydrogenase (1*7β hsd;* Fig. 2D) was only influenced by 0.1 mg/L of DiBP. Any significant change was found in transcriptions of cholesterol side-chain cleavage enzyme gene (*cyp11a;* Fig. 2B) and steroid 17-alpha-monooxygenase gene (*cyp17;* Fig. 2E). However, negative trend were shown in *cyp11a* gene transcriptions by DEP exposure, and *cyp17* gene transcriptions by DiBP exposure. Also, as shown in Fig. 2F, transcription of *cyp19a* gene encoding aromatase protein was significantly up-regulated after exposure to DEP, BBzP, and DiBP. In liver, gene transcription of *vtg* was down-regulated after exposure to 10 mg/L of DEP but no significant change was observed by BBzP or DiBP exposure (Fig. 3).

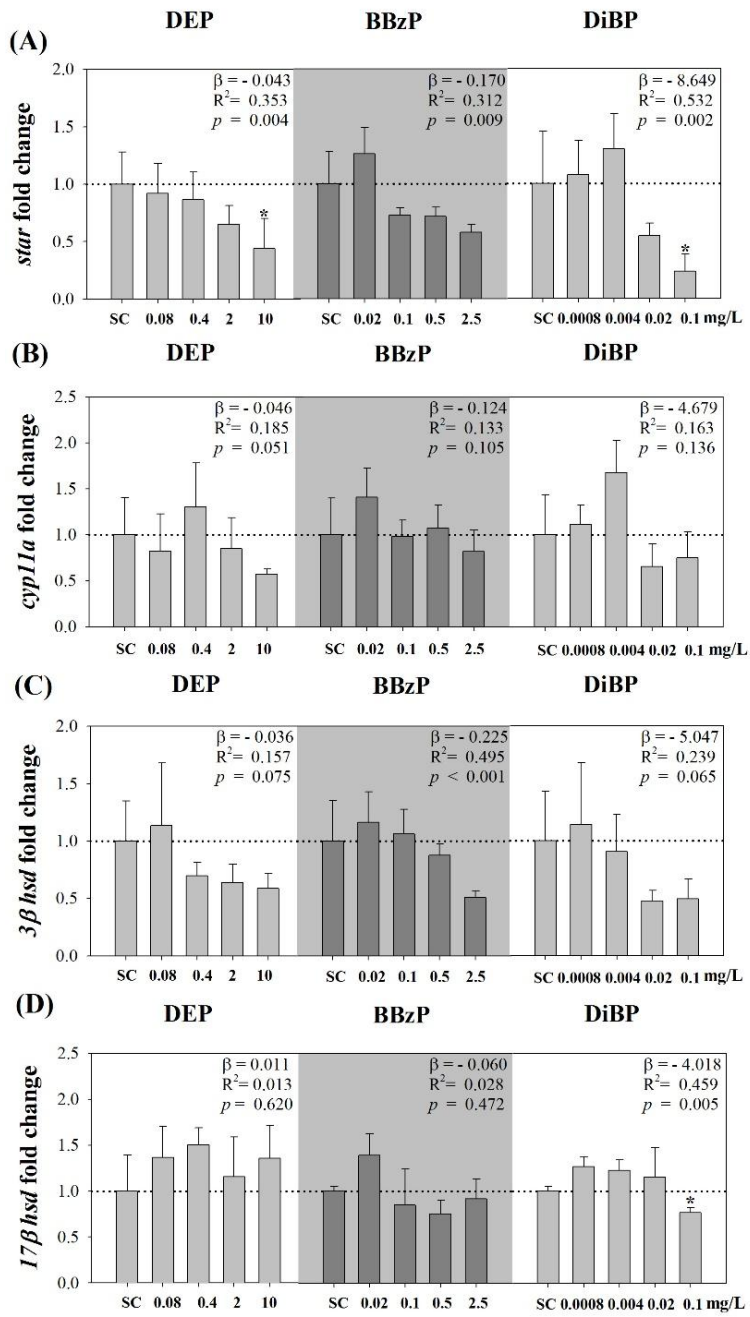


Figure 2. Effects on (A) *star*, (B) *cyp11a*, (C) *3β hsd*, (D) *17β hsd*, (E) *cyp17*, (F) *cyp19a* gene transcription in male zebrafish gonad exposed to DEP (0.08-10 mg/L), BBzP (0.02-2.5 mg/L), DiBP (0.0008-0.1 mg/L) for 14 days. The results are shown as mean ± SD of three or four replicates. * indicates significant difference from solvent control (SC, treated with 0.005% DMSO).

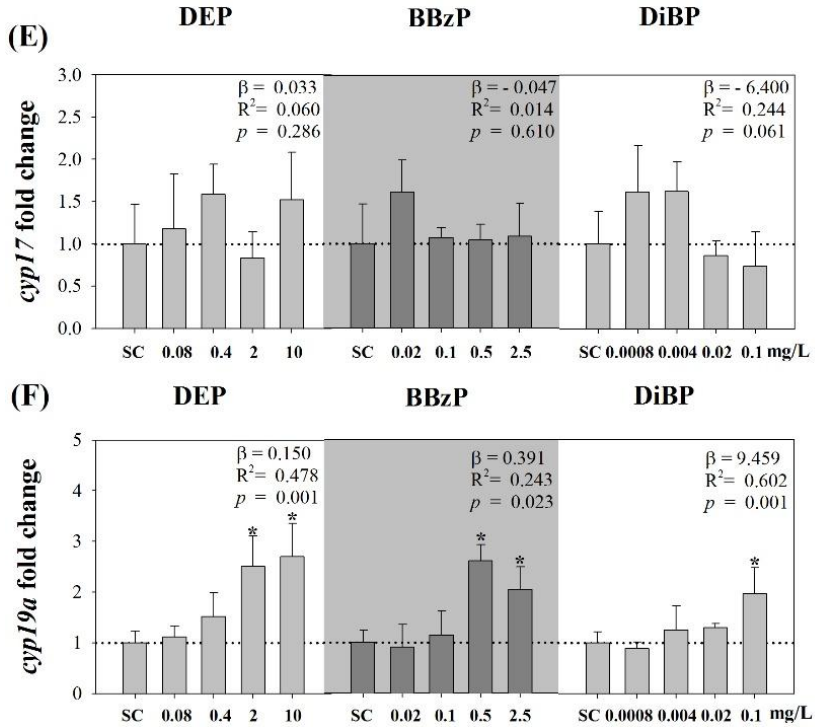


Figure 2. (Continued).

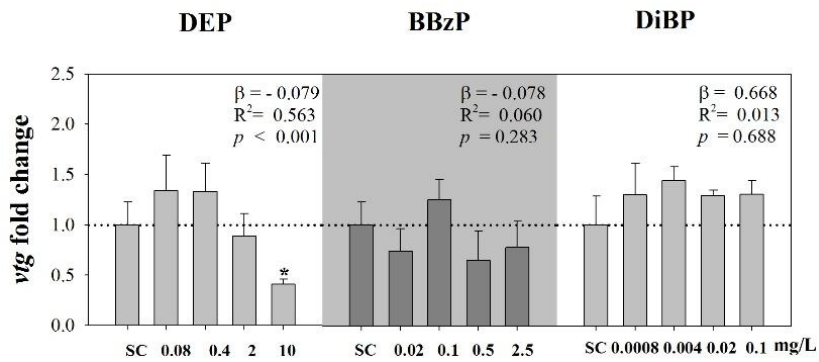


Figure 3. Effects on *vtg* gene transcription in male zebrafish liver exposed to DEP (0.08-10 mg/L), BBzP (0.02-2.5 mg/L), DiBP (0.0008-0.1 mg/L) for 14 days. The results are shown as mean ± SD of three or four replicates. * indicates significant difference from solvent control (SC, treated with 0.005% DMSO).

3.2 H295R cell line assays

3.2.1 Effects of three LMWPs and their metabolites on sex hormones

In H295R cells, the alterations of E2 and T production were observed as well as E2 to T ratio (E2/T) when exposed to three LMWPs and metabolites i.e. DEP, BBzP, DiBP, MEP, MBzP, and MiBP (Fig. 4). All test compounds concentration-dependently decreased T levels except MiBP (Fig. 4B). Following exposure to DEP, T concentration was significantly decreased from 0.004 to 0.4 M (Fig. 4B) whereas no significant alteration was found in E2 levels (Fig. 4A), which resulted in the significant increase of E2/T ratio (Fig. 4C). MEP, a metabolite of DEP, led to significant decrease in T at 0.04 and 0.4 M. Combined with slight decrease in E2 concentrations, E2/T ratio was significantly increased at 0.4 M MEP. Similarly, other test compounds such as BBzP, MBzP, and DiBP, showed notable decrease in T (Fig. 4B) and increasing of E2/T ratio (Fig. 4C) in H295R cells with an exception of MiBP. Following exposure to MiBP, T level was significantly increased at 0.001 and 0.01 MiBP (Fig. 4B) so that different E2/T ratio pattern was found from other compounds (Fig. 4C). In general, concentrations of E2 were influenced only at the highest exposure concentration, or little influenced (Fig. 4A).

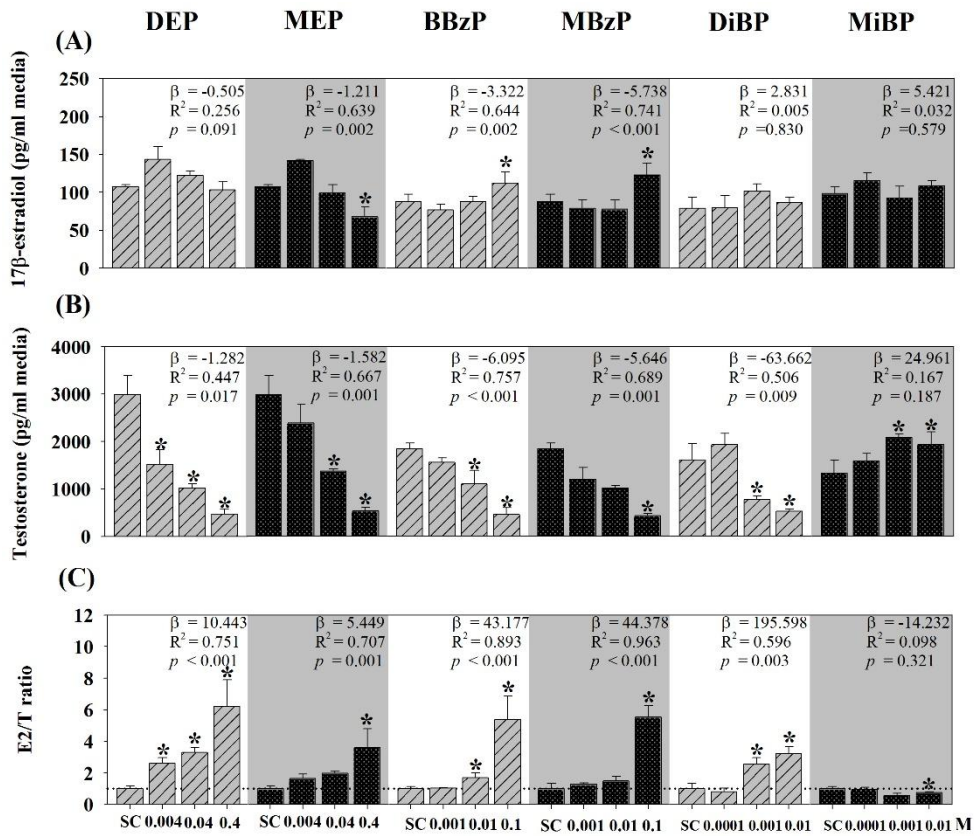


Figure 4. Effects on steroid hormone (A) 17β-estradiol (E2) concentration, (B) testosterone (T) concentration, and (C) E2/T ratio in H295R cell line after exposure to DEP, MEP (0.004-0.4 M), BBzP, MBzP (0.001-0.1 M), DiBP, MiBP (0.0001-0.01 M) for 48 h. The results are shown as mean ± SD of three replicates. * indicates significant difference from solvent control (SC, treated with 0.1% DMSO, $p<0.05$).

3.2.2 Effects of three LMWPs and their metabolites on gene transcriptions

Significant changes in transcription level of several genes were observed in H295R cells exposed to six compounds, respectively (Fig. 5). *StAR* gene was significantly down-regulated by from 0.004 to 0.4 M DEP or MEP, or 0.1 M of BBzP or MBzP exposure (Fig. 5A). Gene transcription of *StAR* was also shown negative trend in DiBP, but not statistically significant in MiBP. Also, DEP or MEP exposure significantly down-regulated *3βHSD2* gene transcription (Fig. 5B). *CYP19A* gene transcriptions were significantly up-regulated in all test LMWPs and their metabolites except MiBP (Fig. 5C). Unlike other, 0.001, 0.01, or 0.1 M of MiBP significantly down-regulated *CYP19A* gene transcription.

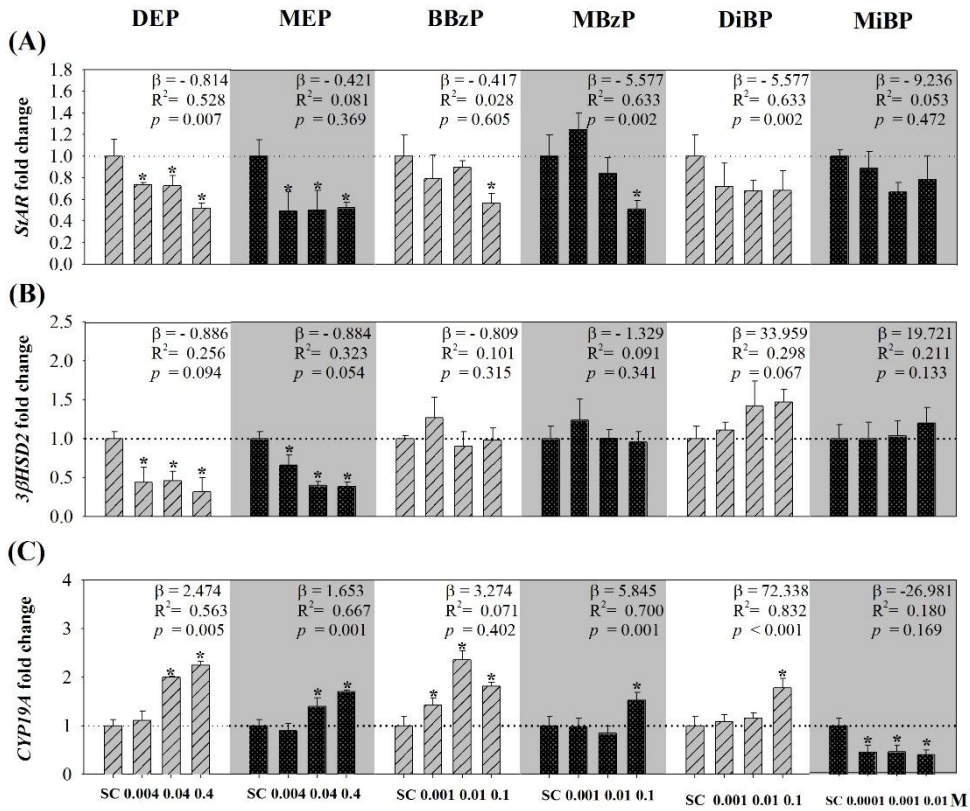


Figure 5. Effects on (A) *StAR*, (B) *3βHSD2*, (C) *CYP19A* gene transcription in H295R cell line after exposure to DEP, MEP (0.004-0.4 M), BBzP, MBzP (0.001-0.1 M), DiBP, MiBP (0.0001-0.01 M) for 48 hours. The results are shown as mean ± SD of three replicates. * indicates significant difference from solvent control (SC, treated with 0.1% DMSO, $p<0.05$).

3.3 Chemical analysis

The measurement of zebrafish exposure media, DEP, BBzP were only detected 0 hr. However, in DiBP exposure media, DiBP detected both 0 and 48 hr. Metabolites were detected 2, 10 mg/L of DEP, 0.5 mg/L of BBzP exposure media (Table 5).

Table 5. Measured concentrations of low molecular weight phthalates and their metabolites for given nominal concentrations in test waters before and after the exposure with zebrafish (*Danio rerio*)

Exposure chemical	Nominal concentration (mg/L)	Exposure time (h)	Measured concentration (parents; mg/L)	Measured concentration (metabolites; mg/L)
DEP	0	0	ND	ND
		48	ND	ND
	0.08	0	0.070±0.027	ND
		48	ND	ND
	0.4	0	0.338±0.071	ND
		48	ND	ND
	2	0	1.327±0.248	0.014±0.005
		48	ND	ND
	10	0	6.253±1.330	0.417±0.316
		48	ND	ND
BBzP	0	0	ND	ND
		48	ND	ND
	0.02	0	0.010±0.029	ND
		48	ND	ND
	0.1	0	0.066±0.014	ND
		48	ND	ND
	0.5	0	0.439±0.072	0.001±0.003
		48	ND	ND
	2.5	0	1.673±0.221	ND
		48	ND	ND
DiBP	0	0	0.018±0.011	ND
		48	0.012±0.008	ND
	0.0008	0	0.016±0.009	ND
		48	0.008±0.004	ND
	0.004	0	0.023±0.014	ND
		48	0.016±0.005	ND
	0.02	0	0.049±0.020	ND
		48	0.009±0.005	ND
	0.1	0	0.197±0.128	ND
		48	0.024±0.017	ND

ND*: not detected; DiBP, MiBP; μg/L); The results are shown as mean ± SD of three replicates.

4. Discussion

The results of our present study demonstrate that three LMWPs and their metabolites altered the levels of plasma sex hormones and gene transcriptions involved in steroidogenesis in male zebrafish and H295R cells, respectively. The synthesis of T exposed to all three LMWPs significantly decreased in a dose dependent manner (Fig. 1B). A few studies were reported that LMWPs affects T concentration. In Kumar et al. (2015) suggested that DEP inhibited the T induced androgen receptor transactivation in Chinese hamster ovary (CHO) cell. Also serum T level is significantly decreased in DEP treated rat. And DiBP, BBzP exposure can increase fetal mortality and reduce fetal T level (Howdeshell et al., 2008). Other studies about the endocrine disrupting effects of LMWPs were conducted using rat model (Borch et al., 2006; Hannas et al., 2012; Hannas et al., 2011).

Following exposure to DEP, also E2 level was significantly decreased at 10 mg/L DEP. Down-regulation of *vtg* gene transcription was also observed in liver following 10 mg/L of DEP exposure. *vtg* production is caused by E2 level (Sumpter and Jobling. 1995). Therefore, in our study, down-regulation of *vtg* gene transcription caused by decreasing of E2 level. Alteration of E2 levels and gene transcriptions of *vtg* can affect reproductive disruption (Levi et al., 2009). Following exposure by mixture of contaminants including DEP to mice, they failed to pregnancy and delivery (Hayashi et al., 2010). Also in Swiss CD-1 mice study, delayed sexual maturation, sperm abnormality, or decreasing number of pups were observed (Fujii et al., 2005).

Alteration of E2 and T level, increasing of E2/T ratio was occurred by BBzP exposure. This result implies that estrogenic activity of BBzP in male fish. Estrogenicity of LMWPs is also reported (Table 5). Harris et al. (1997) conducted recombined yeast screening assay and revealed that among various phthalates,

BBzP and DBP are estrogenic compounds.

Also, in DiBP exposure, T level was significantly decreased in *in vivo* test. Borch et al. (2006) revealed that testosterone level, anogenital distance were decreased when rat exposed to DiBP. These results are coincided with our results.

According to change of hormone levels in zebrafish, major steroidogenic gene transcriptions alteration such as *star, 3β hsd, 17β hsd,* and *cyp19a* was observed. *Star* gene is involved in the first part of steroidogenic pathway which is responsible for the transport of cholesterol into the mitochondrial membrane (Clewell et al., 2010). Previous study reported that down-regulation of *star* gene transcription could affect cholesterol uptake in cells (Rone et al., 2009). Therefore, in our study, down-regulation of *star* gene transcriptions indicated that three LMWPs can disrupt uptake of cholesterol in cells. Also previous study suggested that some phthalates can cause down-regulation of *star* gene in male Sprague Dawley (Hannas et al., 2012). *3β hsd* is a steroid-metabolizing enzyme that is essential for mineralocorticoids and glucocorticoids (Feltus et al., 2002). The decreasing trend of *3β hsd* gene transcription may be followed by down-regulation of *star* gene transcription. It suggests the possibility that LMWPs can affect cortisol production (Liu et al., 2012). *Cyp19a*, which is involved in conversion of T to E2, up-regulated by DEP, BBzP, DiBP exposure. According to gene transcriptions, decreasing of E2 and T level can be explained by down-regulation of *star* gene transcription. Also T levels are showed good agreement with up-regulation of *cyp19a*.

To notice the toxicological effect of metabolites, H295R cell assays were conducted using three LMWPs and their metabolites. LMWPs and their metabolites can alter sex hormones in H295R cell assay. Also, similar trends were observed between male zebrafish exposure test and H295R assays.

In DEP exposure, E2 level was not changed, while T level was statistically significant decreased in all exposure groups. Accordingly, E2/T ratio is

significantly increased. On the other hand, E2 and T level was decreased by exposure of MEP. Therefore, increasing pattern of E2/T ratio was also shown. Especially MEP showed significant decrease of E2 in H295R cell assay, therefore MEP can alter E2 level when fish is exposed to DEP. MEP were detected in 2, 10 mg/L of DEP exposure media in zebrafish test (Table 5). Therefore in our zebrafish study, fish were affected by both DEP and MEP when they exposed to DEP. In BBzP and MBzP exposure, both parent compound and metabolites were showed similar patterns. E2 level was slightly increased at the highest concentration (0.1 M). However, T levels decreased as dose dependent manner which is similar pattern those of zebrafish. In Mankidy et al. (2013), E2 level in H295R exposed by DEP was increased in 10 mg/L (45 μM). While T level was decreased in 0.1, 1, 10 mg/L (0.45, 4.5, 45 μM). Also neither E2 nor T levels were not changed by BBzP. This is because the concentration of exposure media is lower than our study. Therefore high concentration of DEP and BBzP exposure can alter sex hormone change in H295R cells. E2 levels are changed significantly in both DiBP and MiBP. However, T level was shown different pattern. T level decreased in DiBP 0.001, 0.01 M. Following MiBP exposure, T level was increased 0.01, 0.1 M. As a result, E2/T ratio showed different patterns between DiBP and MiBP. Metabolism of DiBP is quite different from other LMWPs which is used in our study. DEP and BBzP metabolized through hydrolysis and produce only hydrolytic monoester i.e. MEP, MBzP. However, DiBP generate several metabolites after hydrolysis and oxidation (Koch and Calafat, 2009). Therefore, DiBP is likely to have a specific mechanism differently from that of other LMWPs and their metabolites. OH-MiBP are formed after the oxidation of DiBP (Koch and Calafat, 2009). In our study, small amount of MiBP were detected in DiBP exposure media (Table 5). It is quite different result which are not detected in DEP, BBzP exposure media. Also there are some limitation that we did not measure OH-MiBP which is the secondary

metabolites of DiBP. However in H295R study, the endocrine effects of MiBP were confirmed, therefore, further studies about the metabolites of DiBP will be needed.

The change of sex hormone in H295R cell can be explained by alteration of steroidogenic gene transcription. Up-regulation of *CYP19A* by DEP and MEP can explain decreases of T, however, due to down-regulation of *StAR* gene transcription, E2 probably could not increased. Down-regulation of *3βHSD2* gene transcription was also followed by down regulation of *StAR* gene transcription. In BBzP and MBzP, *CYP19A* gene lead to increasing E2 and decreasing T. Meanwhile DiBP showed similar hormonal change pattern with DEP. However gene transcription changes in DiBP are not sensitive as for DEP. Because reduction of hormonal change in DEP exposure is much bigger than DiBP. The transcriptional levels of *CYP19A* were down-regulated when only exposed to MiBP which is closely related to increased T levels. However, several factors can affect the altering of sex hormones further studies about mechanisms of LMWPs and their metabolites are needed.

The results of our study indicate that LMWPs and their metabolites are likely to disrupt sex hormone system in both male zebrafish and H295R cell lines. Thus, the changes in gene transcriptions involved in steroidogenesis were also observed. This result is similar as previous phthalates studies. Previous studies of LMWPs on endocrine system were organized in Table 5. Previous studies of LMWPs were conducted by using rat, and observed T level and related gene transcriptions (Borch et al., 2006; Hannas et al., 2012). Decreasing of T level caused by DiBP in wister rat (Borch et al., 2006). Also, Hannas et al. (2011) suggested exposed to DiBP lead to decreasing of *star, cyp11a* gene transcriptions which is take part in steroidogenesis. These results are similar with our study.

On the other hand, several studies about endocrine disrupting effects of HMWPs in aquatic organism were reported. The most well known phthalate, bis (2-

ethylhexyl) phthalate (DEHP) and it's metabolites, mono(2-ethylhexyl) phthalate (MEHP) were reported that these chemicals can affect male reproduction system in aquatic organisms. When DEHP and MEHP exposed marine medaka, the number of egg production, fertilization success was decreased (Ye et al., 2014). Especially Hannon et al. (2015) showed that MEHP is more potent endocrine disrupting chemical than DEHP by decreasing T level. Also, Li et al. (2015) and Saillenfait et al. (2013) revealed that diisononyl phthalate (DiNP) and diisooctyl phthalate (DiOP) reduced T levels and affected steroidogenesis gene transcriptions in rat. Although people can exposed to LMWP persistently, and concerned the potential toxicity of LMWPs, the endocrine disrupting effects of LMWPs are less studied. However, in our study revealed that LMWPs and their metabolites can reduce T levels and related gene transcriptions can altered, like HMWPs.

In summary, our study suggest that the LMWPs and their metabolites have the potential to disrupt endocrine system through steroidogenesis pathway. Based on these result, further study would be needed to elucidate the mechanism more clearly.

Table 6. Previous reports on endocrine disruption and related mechanism of the studied LMWPs

Chemicals	Cells/organisms	Results	Reference
DEP	H295R cell	T concentration ↓	This study
	Sertoli cell	Androgen receptor activity ↓	Kumar et al. (2015)
	H295R cell	E2 concentration ↑ T concentration ↓	Mankidy et al. (2013)
	Zebrafish (male)	T concentration ↓ E2 concentration ↓	This study
	Common Carp (male)	VTG ↑	Barse et al. (2007)
MEP	H295R cell	T concentration ↓ E2 concentration ↓	This study
BBzP	H295R cell	T concentration ↓ E2 concentration ↑	This study
	Yeast	Estrogenic activity ↑	Harris et al. (1997)
	MCF-7 cell	Estrogenic activity ↑	Jobling et al. (1995)
	MVLN cell	ER transactivation ↑	Mankidy et al. (2013)
	Zebrafish (male)	T concentration ↓	This study
	Transgenic medaka	Estrogenic activity ↑	Chen et al. (2014)
	Sprague-Dawley rat (female)	Fetal T concentration ↓	Howdeshell et al. (2008)
	Fathead minnow	Number of spawning ↓	Harries et al. (2000)

Table 6. (Continued)

	Sprague-Dawley rat (male)	*Insl3* ↓ T concentration ↓	Wilson et al. (2004)
	Albino rat	Sperm count ↓ T concentration ↓	Ahmad et al. (2014)
	Sprague-Dawley rat (male)	T concentration ↓ AGD ↓	Hotchkiss et al. (2004)
	Wister rat (female)	LH secretion ↓	Kawaguchi et al. (2002)
	Sprague-Dawley rat	T concentration ↓ AGD ↓	Nagao et al. (2000)
MBzP	H295R cell	T concentration ↓ E2 concentration ↑	This study
DiBP	H295R cell	T concentration ↓	This study
	Yeast	Estrogenic activity ↑	Harris et al. (1997)
	Zebrafish (male)	T concentration ↓	This study
	Sprague-Dawley rat	T concentration ↓	Hannas et al. (2012)
	Sprague-Dawley rat, Wister rat	T concentration ↓ *star* ↓ *cyp11a* ↓	Hannas et al. (2011)
	Wister rat	Testicular weight ↓ AGD ↓	Borch et al. (2006)
MiBP	H295R cell	T concentration ↑	This study

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국문초록

제프라피쉬 *(Danio rerio)*와 세포주 H295R을 이용한 일부 저분자량 프탈레이트와 그 대사체의 성호르몬 교란 영향과 기전

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프탈레이트는 플라스틱을 유연하게 만드는 물질로 PVC, 포장재, 화장품, 의료기기, 개인생활용품에 널리 사용되는 물질이다. 다양한 프탈레이트 물질 중에서도 일반적으로 저분자량 프탈레이트인 DEP, BBzP, DiBP는 고분자량 프탈레이트보다 더 독성이 강하다고 알려져 있다. 그러나 이러한 저분자량 프탈레이트의 잠재적인 독성연구, 특히 성호르몬 생합성에 관한 연구는 거의 진행되지 않았다. 또한 저분자량 프탈레이트는 체내에 유입되면 빠르게 대사된다. 이러한 빠른 대사율에도 불구하고 프탈레이트 대사체의 독성연구 또한 많이 진행되지 않았다.

따라서 본 연구는 일부 저분자량 프탈레이트와 그 대사체가

물고기의 성호르몬의 변화에 미치는 영향과 성호르몬 합성 기전에 미치는 영향을 파악하기 위한 목적으로 수행되었다. 본 연구에서는 수컷 성어 제브라피쉬를 저분자량 프탈레이트인 DEP, BBzP, DiBP에 각각 14일 동안 노출시킨 후 성호르몬의 변화와 성호르몬 생합성 기전에 영향을 미치는 영향을 분자적 수준에서의 변화로 관찰하였다. 또한 저분자량 프탈레이트의 구체적인 기전을 파악하기 위해 H295R 세포에서 성호르몬의 변화와 성호르몬 합성에 미치는 유전자의 변화를 관찰하였다.

DEP를 노출시킨 수컷 제브라피쉬에서는 17β-에스트라디올과 테스토스테론이 감소하였고 BBzP와 DiBP를 노출시킨 수컷 제브라피쉬에서는 테스토스테론이 감소하였다. 또한 저분자량 프탈레이트에 의해 성호르몬 생합성에 관여하는 유전자 전사수준도 변화되었다. 성 호르몬 생합성의 첫 단계 중 콜레스테롤 동원 관여하는 *star* 유전자가 DEP, BBzP 노출군에서 감소하였다. *3β hsd* 는 유의하지는 않았지만 감소하는 패턴을 보였고 테스토스테론을 17β-에스트라디올로 변환하는 *cyp19a*는 증가하였다.

H295R 세포에서는 MiBP를 제외한 모든 프탈레이트와 그 대사체에서 테스토스테론이 감소하였다. 한편 17β-에스트라디올은 MEP에서 감소, BBzP, MBzP노출에서 증가하였다. 이에 따라 MiBP를 제외한 프텔레이트와 그 대사체에서 E2/T 비율이 증가하였다. 유전자 전사 수준에서는 *StAR, 3βHSD*가 감소하고 *CYP19A*가 증가하였다. 이는 수컷 제브라피쉬의 결과와 동일하다.

본 연구를 통해 저분자량 프탈레이트 DEP, BBzP, DiBP와 그 대사체가 생체 내 성 호르몬의 불균형과 성호르몬 생합성에 관여하는

유전자 전사 수준 변화를 초래한다는 것이 확인되었다.

주요어: 저분자량 프탈레이트; 내분비계 교란; 성호르몬 생합성; H295R 세포주

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