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Toxicological responses following short-term exposure to Dechlorane Plus in zebrafish (*Danio rerio*)

Dechlorane Plus의 단기노출에 따른 제브라피쉬의 독성반응

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Abstract

Toxicological responses following short-term exposure to Dechlorane Plus in zebrafish (*Danio rerio*)

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Dechlorane Plus (DP) is a widely used chlorinated flame retardant, and has been detected in various environmental matrices and biota including humans. Although DP is globally distributed in high frequency, there is limited information on its toxicity. In order to identify toxicity of DP in vertebrate, zebrafish were employed to evaluate possible toxicological responses including oxidative stress and endocrine disruption. DP was dissolved in corn oil, and delivered to adult male zebrafish via oral gavage feeding with doses

of 0, 0.3, 1, and 3 μg/g zebrafish wet weight. Fish were fed with DP twice (days 0 and 2). On day 6, blood, liver, testis, and brain were collected and were evaluated for oxidative stress and endocrine disruption. Following DP exposure, catalase activity in liver showed a significant dose-dependent increase, while superoxide dismutase activity was not affected. In addition, plasma thyroxine (T4) concentrations increased following DP exposure. Transcriptions of *corticotropin releasing hormone* (*crh*) and *thyroid stimulating hormone β* (*tshβ*) gene in brain increased in a dose-dependent manner, explaining the changes of plasma T4 level. Transcriptional responses of sex hormone related genes in brain including aromatase gene (*cyp19b*) and estrogen receptor genes also showed sex hormone disruption potentials of DP in fish. Effects of water-borne DP exposure on thyroid hormone and development of early life stage of zebrafish were also evaluated. In contrast to results of adult fish, DP did not show any toxic effect on embryos/larvae. Our observations suggest that DP may have thyroid and sex endocrine disrupting potential by alternating hormonal regulatory systems in fish.

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Keywords: Dechlorane Plus; flame retardant; endocrine disruption; oxidative stress; *Danio rerio*

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Contents

List of Figures

List of Tables

Abbreviations

CAT: catalase

crh: corticotropin releasing hormone

cyp: cytochrome P450

dio: deiodinase

DP: Dechlorane Plus

er: estrogen receptor

fsh: follicle stimulating hormone

gnrh: gonadotropin-releasing hormone

hpf: hour post-fertilization

lh: luteinizing hormone

SOD: superoxide dismutase

T3: triiodothyronine

T4: thyroxine

tsh: thyroid stimulating hormone

tg: thyroglobulin

tr: thyroid hormone receptor

trh: thyrotropin-releasing hormone

tshr: thyroid stimulating hormone receptor

qRT-PCR: quantitative real-time polymerase chain reaction

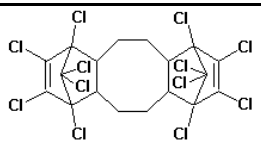
1. Introduction

Dechlorane plus (DP) is a highly chlorinated flame retardant which has been produced since 1960s. DP has been widely used in electrical devices and furniture (Sverko et al., 2011) as a replacement of Dechlorane. Dechlorane is an earlier type of chlorinated flame retardant, which had been banned in 1970s due to its toxicity to organisms (Kaiser, 1978; Qiu and Hites, 2007; Wang et al., 2010). Recently, flame retardants have been actively studied in terms of their environmental issues and health problems. In spite of its high amount of production (Betts et al., 2006), DP had received little attention in environmental field until its first detection in environmental matrices in the Great Lakes region, USA, where a DP manufacturing plant was located (Hoh et al., 2006). Since then, several studies have reported widespread contamination of DP in surface water, sediment, soil, and ambient air worldwide. DP was detected in surface water up to 2.40 ng/L (Qi et al., 2010), and occurred in marine and lake sediment (Fang et al., 2014; Sverko et al., 2009) and soil (Syed et al., 2013; Wang et al., 2010) up to 13.4 μg/g dry weight near DP manufacturing plant (Wang et al., 2010). Also, DP is persistent in ambient air, distributed in the entire world; it exists not only in urban air in Korea (Baek et al., 2013) or China (Ren et al., 2008), where DP is widely used or produced, but also in remote air such as Arctic and Antarctic air (Möller et al., 2010).

DP consists of two isomers, i.e., *syn*- and *anti*-form, and is expected to have

relatively high $\log K_{ow}$ (Table 1). This characteristic explains its bioaccumulative property (estimated log bioaccumulation factor: 2.4-4.5; Feo et al. (2012). Aquatic biota are contaminated with DP at concentrations ranging from pg/g to ng/g lipid weight (lw) in various regions (Feo et al., 2012) including Korea (Kang et al., 2010). Especially, fish caught near the DP manufacturing plant showed much higher levels of DP up to 1110 ng/g wet weight (ww) (Wang et al., 2013). Considering low levels of DP in water, food web transfer could be an important exposure route of DP for fish, while reports indicating otherwise are available (Peng et al., 2014; Tomy et al., 2007; Wu et al., 2010). DP has also been detected in human in high levels and frequencies. In general population of Canada, the median serum concentration of DP was reported at 2.37 ng/g lw (He et al., 2013), which is comparable to the reported levels of polybrominated diphenyl ether (PBDE) in general population of USA (Frederiksen et al., 2009). The levels are greater among the workers in DP-related workplace such as DP manufacturing plants (Zhang et al., 2013) and e-waste recycling plants (Yang et al., 2013; Zheng et al., 2010). Among the workers of DP manufacturing plant, the median DP level in blood was 857 ng/g lw with the maximum level of 2958 ng/g lw (Zhang et al., 2013).

Table 1. Physico-chemical properties of DP

Compound	CAS No.	IUPAC name	Structure	$logK_{ow}$ [a)]	Water solubility [b)]	Molecular weight
Dechlorane Plus (DP)	13560-89-9	1,2,3,4,7,8,9,10,13,13,14,14-dodecachloro-1,4,4a,5,6,6a,7,10,10a,11,12,12a-dodecahydro-1,4,7,10-dimethanodibenzo[a,e]cyclooctene		11.3	0.044 - 249 μg/L	653.7

a) Zhou et al. (2011)
b) Xian et al. (2011)

In spite of global distribution of DP in the environment and human, only limited information is available about its toxicity. Oxidative stress has been observed in various species (Li et al., 2013b; Liang et al., 2014; Wu et al., 2012; Zhang et al., 2014), which may lead to oxidative DNA damage (Wu et al., 2012). Only general toxicological responses such as disruption in metabolism and signal transduction (Wu et al., 2012), and cell proliferation and apoptosis effects (Liang et al., 2014) have been identified as toxicity of DP. This lack of knowledge is partly due to the fact that until now most studies employing vertebrate have focused on liver as a target organ of DP. However, liver may not be the best choice for toxicological responses such as endocrine disruption. So far, endocrine disrupting potentials of various flame retardants were suggested (Kim et al., 2014; Liu et al., 2012), but there is no toxicological information regarding endocrine disrupting potential of DP. Endocrine disrupting potentials of DP have been suggested in human epidemiological studies. Association between thyroid hormone level and DP concentration in sera was observed in a cross-sectional study (Ben et al., 2014). However no experimental study is available to date on endocrine disruption of DP.

Very few experimental studies employing fish have been reported for DP, because of its very low water solubility. To date, only one study reported toxicity of DP in fish (Liang et al., 2014). In this study, DP was delivered to juvenile Chinese sturgeon (*Acipenser sinensis*) through intraperitoneal injection.

In the present study, endocrine disruption effects of DP were evaluated using zebrafish (*Danio rerio*). For delivery of DP, non-invasive oral gavage feeding method (Collymore et al., 2013) was applied. Alterations in transcription of the genes associated with regulation of thyroid and sex steroid hormone systems were measured. In addition, antioxidant enzyme activity in liver, and hormonal alteration in blood were observed (Fig. 1). In addition, zebrafish embryos/larvae test was conducted to verify observed thyroid disrupting effects in adult fish. The results of this study will help understand the endocrine disruption of DP among ecosystem.

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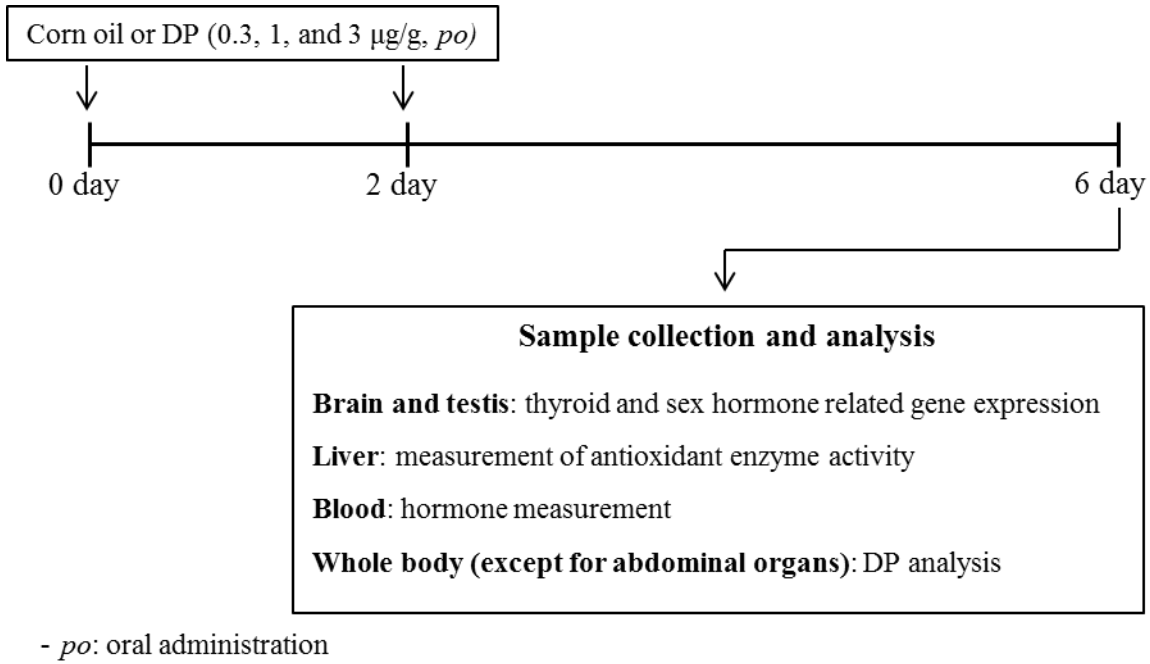


Figure 1. Overall schematic of the adult fish exposure.

2. Materials and Methods

2.1 Chemicals

DP-25 (a commercial mixture of DP) was purchased from Jiangsu Anpon Electrochemical Co., Ltd (Jiangsu Province, China). Corn oil was purchased from Sigma–Aldrich (St. Louis, MO, USA), and acetone was purchased from Merck (Darmstadt, Germany). In chemical analysis, target compound were *anti*- and *syn*-DP (Wellington Laboratories, Guelph, ON, USA). Internal standards (^{13}C-*syn*-DP and ^{13}C-*anti*-DP) were obtained from Cambridge Isotope Laboratories (Cambridge, MA, USA). All solvents used in chemical anaylsis (dichloromethane, hexane, and methanol) were of ultra residue-analysis grade (J.T. Baker, Phillipsburg, NJ, USA).

2.2 Chemical analysis

Over 5 g of sample was used for each analysis by pooling several individual fish samples into on sample. Each pooled sample was homogenized and spiked with ^{13}C-*syn*-DP and ^{13}C-*anti*-DP and extracted in a Soxhlet apparatus with 200 mL of 25% dichloromethane in hexane for 16 h. The extracts were cleaned by passing through a multi-layer silica gel column with 150 mL of 15% DCM in hexane, and the eluants were dissolved in methanol prior to analysis by liquid chromatography–tandem mass spectrometry (LC-MS/MS). Water samples were spiked with internal standards (^{13}C-*syn*-DP and ^{13}C-*anti*-DP)

and shaken in an orbital shaker at 250 rpm for 30 min, twice. The samples were extracted in methanol and analyzed by LC-MS/MS.

DP analysis was conducted using an Agilent 1290 Infinity HPLC system (Agilent Technology, Waldbronn, Germany) liquid chromatograph and Micromass Quattro tandem mass spectrometer (MS/MS) in the negative ionization in atmospheric pressure chemical ionization mode with multiple reaction monitoring (MRM; ABSciex Qtrap 4500; AB Sciex, Toronto, ON, Canada). Chromatographic separation was achieved with a 4.6 × 100 mm Agilent Eclipse C18 (particle size: 3.5 μm) with Guard column. The mobile phase comprised 15% H_2O in methanol (A solution) and 100% of methanol (B solution). For 6 min, A solution flowed with the rate of 400 μg/min, then B solution flowed with the same rate for 4.1 min. For the final 9.9 min, flow rate was changed to 500 μg/min. A volume of 5 μl of each sample was injected. Standard solutions for calibration curve were prepared with 1, 5, 10, 50, 100, 500, 1000, and 2000 ng *syn*-DP or *anti*-DP/ml methanol. The linearity of each standard curve was confirmed ($R^2 > 0.99$ for both *syn*-DP and *anti*-DP). Limit of quantification was 0.5 and 0.1 ng/ml for *syn*-DP and *anti*-DP, respectively. Average recovery ranged 87.9-101.0 % for ^{13}C-*syn*-/*anti*-DP in fish and water samples. MRM transitions and collision energy employed in the analysis are shown in Table 2.

Table 2. LC-MS/MS parameters for analysis of *syn*-/*anti*-DP and ^{13}C-*syn*-/*anti*-DP

Parameters	*syn*-DP	*anti*-DP	^{13}C-*syn*-DP	^{13}C-*anti*-DP
1st MRM	632.7→560.8	632.7→560.8	642.6→570.8	642.6→570.8
2nd MRM	634.7→562.6	634.7→562.6	642.6→568.7	642.6→568.7
Declustering potential (V)	-20	-55	-5	-5
Entrance potential (V)	-10	-10	-10	-10
Collision energy (V)	-18	-22	-18	-20
Collision cell exit potential (V)	-9	-15	-23	-25

2.3 Zebrafish culture and exposure

Wild-type male zebrafish (~ 6 months old) were obtained from a local supplier (Gangnam Aquaria, Suwon, Korea). Prior to exposure, fish were acclimated using dechlorinated tap water for 11 days. During acclimation period, fish were fed with newly hatched *Artemia* nauplii twice a day and maintained at 26℃ and under 15:9 h light:dark photoperiod. After acclimation, zebrafish were exposed to DP-25 by oral gavage twice at day 0 and day 2, following previously published method (Collymore et al., 2013) with minor modifications. Briefly, zebrafish were anesthetized with ice-cold water. Zebrafish were fed with 1.5 μl of corn oil (0, 0.02, 0.07 or 0.20 μg/μl of DP-25) per 100 mg of zebrafish using a catheter (Micro-Renathane *Braintree Scientific* O.D.X.012 I.D., cat# MRE-025) attached to 27-gauge needle (Hamilton, RN NDL (27/2”/3)S, cat# 7762-01). These gavaging doses correspond to 0, 0.3, 1, or 3 μg/g of zebrafish ww. After dosing, fish of each group were placed in 10 L of dechlorinated tap water. During exposure period, fish were fed with *Artemia* nauplii twice a day and maintained at 26℃ and under 15:9 h light:dark photoperiod. Water renewal and water quality parameters (DO, pH, temperature, and conductivity) check were conducted every other day. After 6 days of the first dosing (at day 6), fish were anesthetized with ice-cold water. Liver, brain, and testis were dissected out from fish, and blood was also collected for evaluation of toxicological responses. Whole body (except for abdominal organs) was collected to

measure body residue of DP in fish. These samples were stored at -80℃ until used in analyses.

Embryos/larvae test was conducted to verify observed response in the adult experiment. For this experiment, eggs spawned by zebrafish raised in the laboratory were used. The 20 embryos within 4 h post-fertilization (hpf) were placed into 50 L of each test beakers. Test solutions contained acetone as a solvent and DP-25 with different concentrations solutions (control, acetone control (0.4% v/v), DP-25 0.4, 0.8, or 1.6 mg/L), each of which consisted of 6 replicates. Test solutions were prepared in the conditioned water containing sodium bicarbonate (75 mg/L), calcium sulfate (15 mg/L), and sea salt (8.4 mg/L) in deionized water (Nusslein-Volhard and Dahm, 2002). The treatment solution was renewed, and water quality parameters (DO, pH, temperature, and conductivity) were recorded every other day. During exposure, time to hatch was recorded, and at 144 hpf, malformation rate (yolk sac edema, bent spine, and tail malformation) and survival rate were checked. Finally, larvae were collected to analyze gene transcription.

2.4 Gene transcription analyses

Gene transcription levels in the tissue samples or larvae samples were analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). For one larvae sample, 14 larvae were pooled. Total RNA in the samples was extracted using Maxwell® 16 LEV simplyRNA Purification Kit (Promega,

Madison, WI, USA) according to manufacturer's instruction. After quality and quantity of RNA were checked with an EPOCH microplate spectrophotometer (BioTek, Winooski, VT, USA), cDNA was synthesized from total RNA by iScript cDNA Synthesis Kit (BIORAD, Hercules, CA, USA). For qRT-PCR, the 20 μl of PCR reaction mixture contained 2 μl of cDNA template (75 ng/μl), 2 μl of each PCR primer (10 μM), 10 μl of LightCycler-DNA Master SYBR green Ⅰ mix (Roche Diagnostics Ltd, Lewes, UK), and 4 μl of nuclease-free water. LightCycler 480 (Roche Applied Science Indianapolis, IN, USA) was used for this reaction and detection of fluorescence. The thermal cycle profile consisted of pre-incubation at 95℃ for 10 min, followed by 45 cycles of amplification at 95℃ for 10 s, 55℃ for 20s, and 72℃ for 20s. Used primers are listed in table 3. These primers were validated by checking efficiency and melting curve. For quantification of gene transcription, the threshold cycle (*Ct*) was determined by Roche LightCycler 480 software version 1.5. By *ΔΔCt* method (Livak and Schmittgen, 2001), each target gene was normalized to the housekeeping gene (18S rRNA).

Table 3. Primer information for qRT-PCR and LA-QPCR

target	accession number	primer sequences (5' - 3')	
		forward	reverse
qRT-PCR			
reference gene			
18S rRNA	FJ915075	acgcgagatggagcaataac	cctcgttgatgggaaacagt
sex steroid hormone related genes			
cytochrome P450 17 (cyp17)	NM_212806	ggactccagtgttggtgaataca	gggttcttccattccttctcatcat
cytochrome P450 19b (cyp19b)	AF183908	gtcgttacttccagccattcg	gcaatgtgcttcccaacaca
cytochrome P450 11a (cyp11a)	NM_152983	ggcagagcaccgcaaaa	ccatcgtccagggatcttattg
estrogen receptor α (erα)	NM_152959	cagactgcgcaagtgttatgaag	cgccctccgcgatctt
estrogen receptor 2β (er2β)	NM_174862	ttcacccctgacctcaagct	tccatgatgccttcaacacaa
androgen receptor (ar)	NM_001083123	tctgggttggaggtcctacaa	ggtctggagcgaagtacagcat
gonadotropin-releasing hormone 2 (gnrh2)	AY657018	ctgagaccgcagggaagaaa	tcacgaatgagggcatcca
gonadotropin-releasing hormone 3 (gnrh3)	NM_182887	ttgccagcactggtcatacg	tccatttcaccaacgcttctt
follicle stimulating hormone β (fshβ)	NM_205624	gctgtcgactcaccaacatctc	gtgacgcagctcccacatt
luteinizing hormone β (lhβ)	NM_205622	ggctgctcagagcttggttt	tccaccgataccgtctcattta
thyroid hormone related genes			
corticotropin releasing hormone (crh)	NM_001007379	ttcgggaagtaaccacaagc	ctgcactctattcgccttcc
thyroid stimulating hormone β (tshβ)	AY135147	gcagatcctcacttcactacc	gcacaggtttggagcatctca
deiodinase 1 (dio1)	BC076008	gttcaaacagcttgtcaaggact	agcaagcctctcctccaagtt
deiodinase 2 (dio2)	NM_212789	ttctccttgcctcctcagtg	agccacctccgaacatcttt

Table 3. Continued

target	accession number	primer sequences (5' - 3')	
		forward	reverse
thyroid hormone receptor α (trα)	NM_131396	caatgtaccatttcgcgttg	gctcctgctctgtgttttcc
thyroid hormone receptor β (trβ)	NM_131340	tgggagatgatacgggttgt	ataggtgccgatccaatgtc
thyroid stimulating hormone receptor (tshr)	NM_001145763	gctccttgatgtgtccgaat	cgggcagtcaggttacaaat
thyroglobulin (tg)	XM_001335283	ccagccgaaaggatagagttg	atgctgccgtggaatagga

2.5 Measurements of activity of antioxidant enzymes

For the measurement of activities of superoxide dismutase (SOD) and catalase (CAT) in liver, protein was extracted in liver samples as soon as they were collected. The concentration of the extracted protein was measured by BCA assay (Smith et al., 1985). Activity of SOD was determined by indirectly measuring inhibition of cytochrome-C reduction (McCord and Fridovich, 1969), while activity of CAT was determined by the decrease in absorbance at 240 nm following H_2O_2 consumption (Aebi, 1974). For statistical analysis, measured activities of SOD and CAT were normalized by protein contents.

2.6 Hormone measurement

Plasma was immediately separated from the collected blood samples through centrifugation at 5000g for 7 min. For one replicate, 3-4 separated plasma samples were pooled. Thyroxine (T4) level in plasma was measured using commercially available enzyme-linked immunosorbent assay kit (Cat No. E0452Ge), following the manufacture's instruction.

2.7 Statistical analysis

All statistical analyses were performed using SPSS 18.0 K for Windows® (SPSS, Chicago, IL, USA). Prior to comparisons among exposure groups, normality and homogeneity of each variance were tested by the Shapiro–Wilk test and Levene's test, respectively. In case of normal distribution, group

comparison was conducted by one-way analysis of variance followed by Dunnett's test. Otherwise, Kruskal-Wallis combined with Dunn's test was used. For comparisons between control and acetone control group, *t*-test was used. For trend analyses of measurements, Spearman's rank correlation was performed. In the all statistical analysis, p values less than 0.05 was considered to be statistically significant.

3. Results

3.1 Concentration of DP in exposed fish and exposure media

Body residue of DP in adult fish after 6 days exposure and concentrations of DP in exposure media of embryos/larvae test were measured. The results are represented in Table 4. For simplicity, nominal concentrations were used for the data presentation.

Table 4. Nominal and measured concentrations of DP in adult fish and exposure media

	Nominal concentration	Measured concentration[a)]		
	DP-25	*syn*-DP	*anti*-DP	total DP
adult fish (ng/g ww) on day 6	0	0.44±0.06	4.53±0.06	4.97±0.10
	300	5.63±0.50	24.4±0.45	30.0±0.69
	1000	8.74±0.59	35.7±1.28	44.4±0.80
	3000	100±4.25	320±19.4	420±23.0
exposure media (μg/L) at 0 hr	control	n.d.	n.d.	n.d.
	acetone control	n.d.	n.d.	n.d.
	400	33.3	107	140
	800	58.9	189	248
	1600	66.0	201	267
exposure media (μg/L) at 48 hr	control	n.d.	n.d.	n.d.
	acetone control	n.d.	n.d.	n.d.
	400	7.13	21.6	28.8
	800	16.5	54.1	70.5
	1600	16.8	54.9	71.7

n.d.: Below limit of detection (0.5 ng/ml for *syn*-DP, 0.1 ng/ml for *anti*-DP)

a) Data represent mean ± standard deviation (SD) of three replicates or mean of two replicates for adult fish or exposure media, respectively.

3.2 Hepatic antioxidant enzyme activity

In order to identify effects of DP exposure on antioxidant activity, CAT and SOD activity in liver were measured. CAT activity significantly increased following DP exposure (Fig. 2A). On the contrary, there was no effect on SDO activity (Fig. 2B).

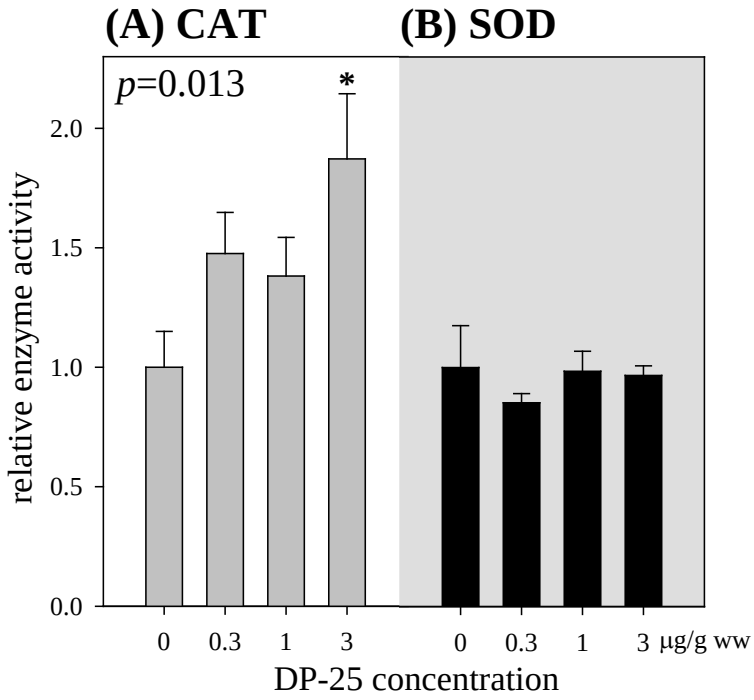


Figure 2. Effects on activity of (A) CAT and (B) SOD in zebrafish liver. The results represent mean ± standard error (SE) of 6 replicates (CAT) or 5 replicates (SOD). Asterisk indicates significant difference from corn oil control. The *p* value with statistical significance based on Spearman's rank correlation was represented.

3.3 Thyroid hormone disruption

Plasma T4 level showed monotonously increasing trend, but there was no statistical significance (Fig. 3). In brain, genes related to thyroid hormone regulation were evaluated (Fig. 4). Among them, transcription of *crh* and *tshβ* were up-regulated (Fig. 4A, B) in dose-dependent manners. Other thyroid hormone-related genes were not altered in brain (Fig. 4C-F).

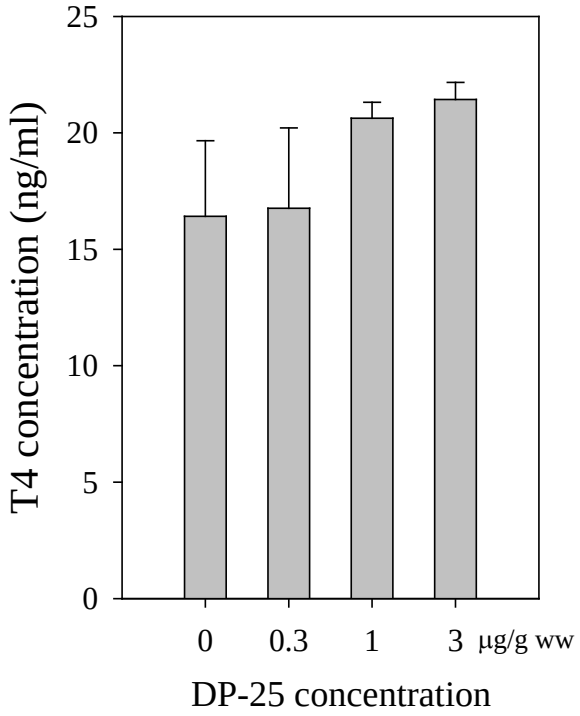


Figure 3. Effects on T4 levels in zebrafish plasma. The results represent mean ± SE of 3 replicates. For one replicate, plasma samples of 3-4 zebrafish were pooled. There was no significant difference or trend among dose groups.

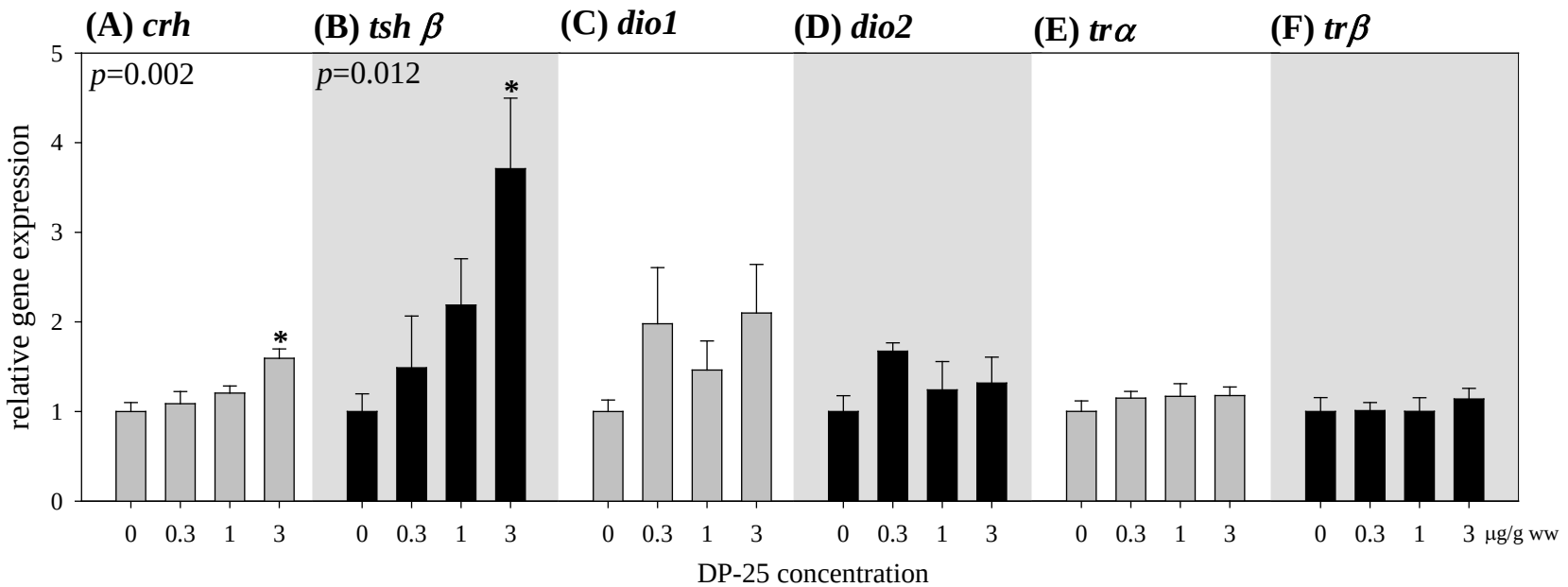
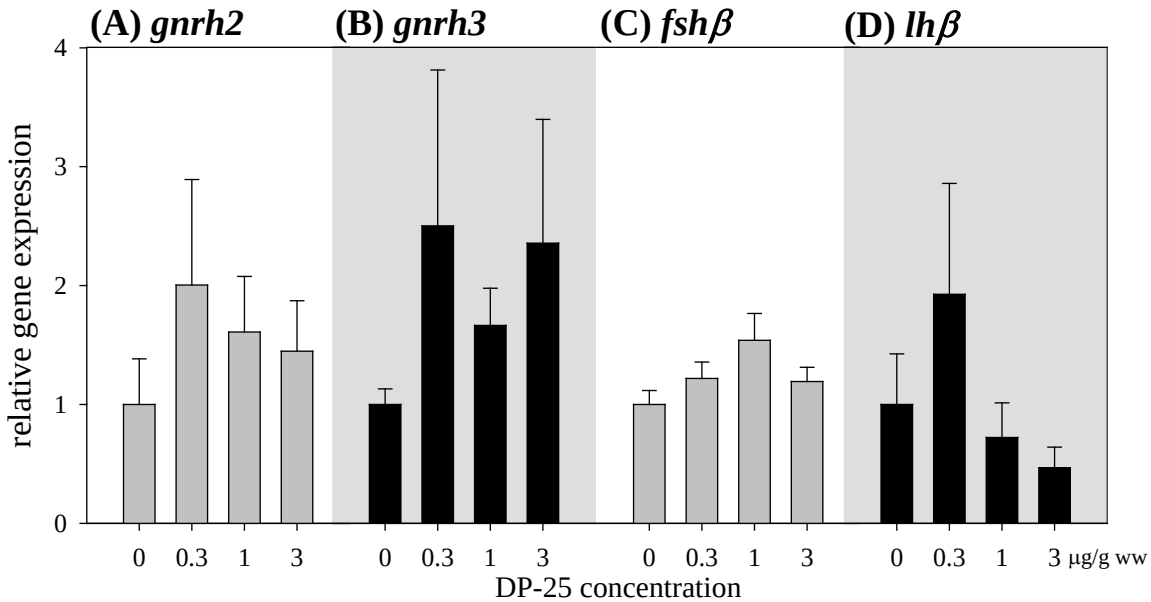


Figure 4. Effects on gene transcription of (A) *crh*, (B) *tshβ*, (C) *dio1*, (D) *dio2*, (E) *trσ*, and (F) *trβ* in zebrafish brain. The results represent mean ± SE of replicates. Asterisk indicates significant difference from corn oil control. The *p* values with statistical significance based on Spearman's rank correlation were represented.

3.4 Sex hormone disruption

In brain, transcription of *erσ* significantly increased in a lower DP exposure group but not in higher exposure group (Fig. 5F). Increasing trends were observed for *erβ* and *ar* in brain (p=0.106 and p=0.0123, respectively; Fig. 5G, H). In addition, *cyp19b* was significantly up-regulated (Fig. 5E), while other genes related to sex hormone regulation were not affected (Fig. 5). Besides, estrogen and androgen receptors genes and steroidogenesis genes were evaluated in testis, but there was no significant alteration in these genes (Fig. 6).


(A) gnrh2
(B) gnrh3
(C) fshβ
(D) lhβ
relative gene expression
0
1
2
3
4
0 0.3 1 3 0 0.3 1 3 0 0.3 1 3 0 0.3 1 3 µg/g ww
DP-25 concentration

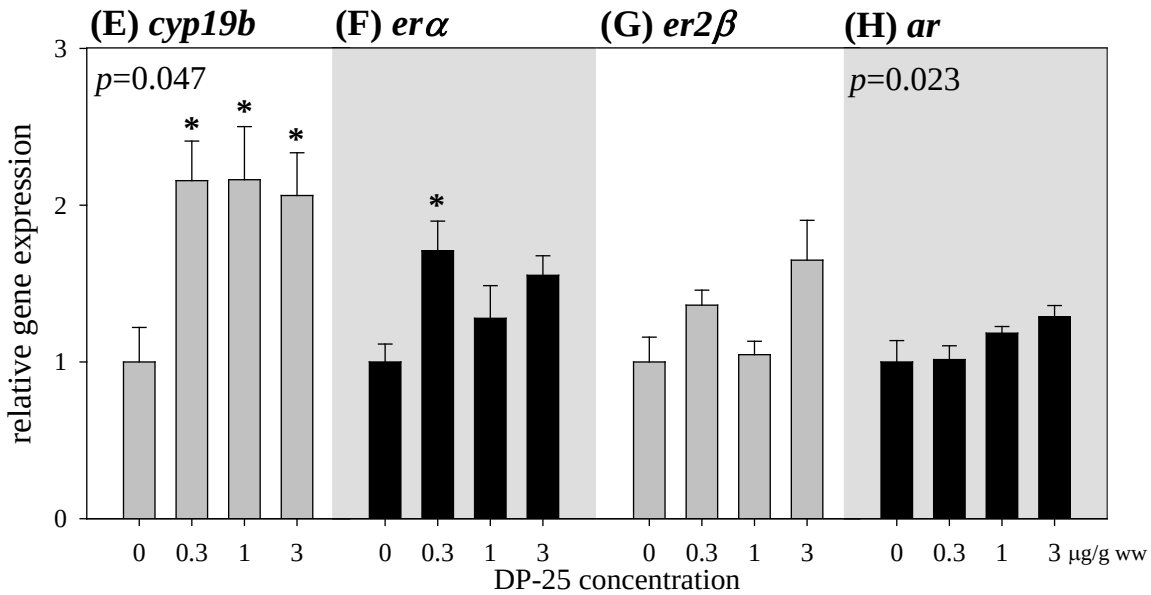


Figure 5. Effects on gene transcription of (A) *gnrh2*, (B) *gnrh3*, (C) *fshβ*, (D) *lhβ*, (E) *cyp19b*, (F) *erσ*, (G) *er2β*, and (H) *ar* in zebrafish brain. The results represent mean ± SE of 5 replicates. Asterisk indicates significant difference from corn oil control. The *p* values with statistical significance based on Spearman's rank correlation were represented.

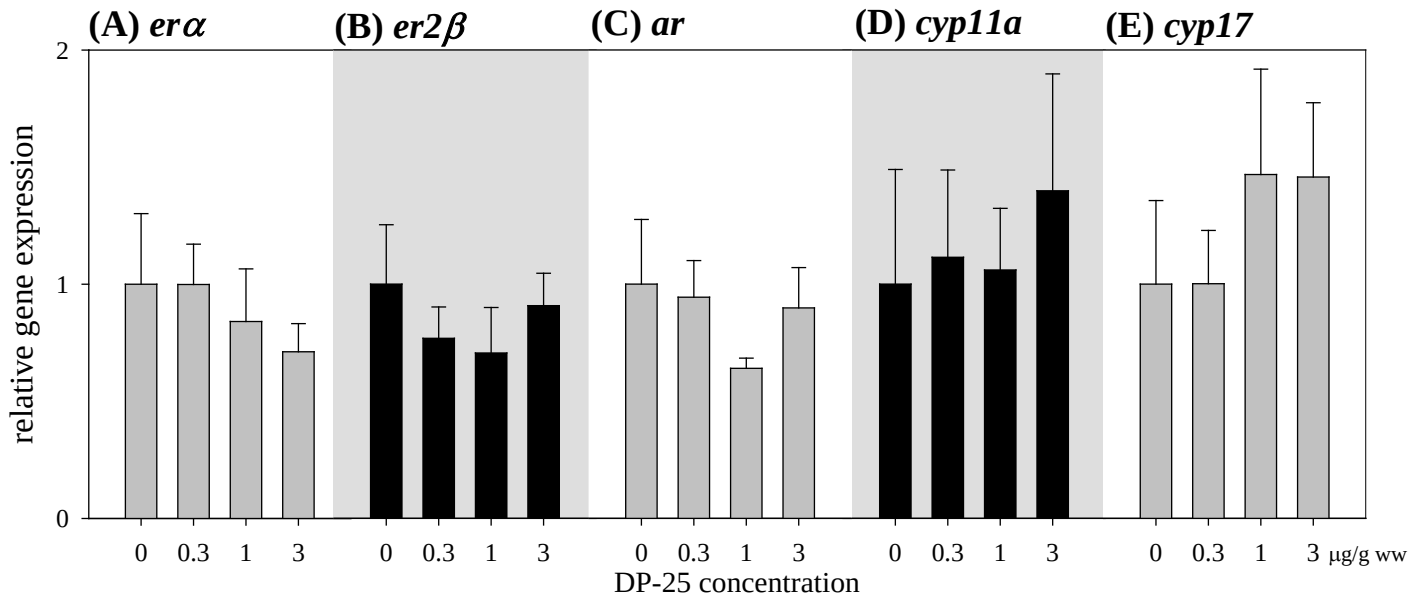


Figure 6. Effects on gene transcription of (A) *erσ*, (B) *er2β*, (C) *ar*, (D) *cyp11a*, and (E) *cyp17* in zebrafish testis. The results represent mean ± SE. Due to experimental mistakes, different number of replicates (3-5) by exposure groups were used. There was no significant difference or trend among dose groups.

3.5 Embryos/larvae toxicity

To confirm whether thyroid disruption observed in adult fish occurs in developmental stage, embryos/larvae test was conducted. Since DP is rarely dissolved in water, 0.4 % (v/v) acetone was used as solvent. There was no significant difference in observed end point between control and acetone control except for time to hatch. In acetone control, time to hatch was significantly delayed compared with control group. Although applied acetone concentration was effective on hatching, among DP-treated groups, there was no significant effect on hatching, survival, and malformation compared with acetone control (Table 5). Also, no significant effect on transcriptional change regarding thyroid hormone was observed (Table 6).

Table 5. Effect of DP-25 on hatching, survival, and malformation in embryos/larvae

Concentration (mg/L)	Hatchability (%)	Time to hatch (hpf)	Larval survival (%)	Malformation rate (%)
Acetone control	92.50±5.24	42.54±3.96	97.32±7.63	2.84±3.13
0.4	95.83±4.92	44.31±3.41	95.65±3.82	4.75±5.76
0.8	93.33±6.06	49.22±8.97	96.33±4.53	10.2±9.01
1.6	88.33±8.16	45.07±4.83	96.43±4.22	5.86±3.55

The results represent mean ± SD of 6 replicates. There was no significant difference or trend among groups for all the observed end points.
a) To dissolve DP up to concentration of 1.6 mg/L, 0.4% (v/v) acetone was used. No significant difference except for time to hatch was observed between control and acetone control groups.

Table 6. Relative expression of thyroid hormone related genes in embryos/larvae following 6 days of DP-25 exposure

Concentration (mg/L)	***crh***	***tshβ***	***tshr***	***tg***
Acetone control	1.00±0.06	1.00±0.08	1.00±0.06	1.00±0.03
0.4	0.88±0.01	0.93±0.03	0.85±0.06	0.86±0.09
0.8	1.13±0.12	0.93±0.13	0.86±0.09	0.74±0.05
1.6	1.08±0.04	1.02±0.11	0.80±0.02	0.92±0.06

The results represent mean ± SE of 4 replicates. There was no significant difference or trend among groups for all the evaluated genes.

4. Discussion

The results of this study suggest that DP have thyroid and sex steroid hormone disrupting potential in zebrafish. Previously, several studies investigated toxicity of DP in molecular levels using vertebrates models including rodents (Li et al., 2013a; Wu et al., 2012), birds (Crump et al., 2011; Li et al., 2013b), and fish (Liang et al., 2014). However the organs employed in these studies were inappropriate for testing endocrine disruption. This study, for the first time, reports molecular responses in brain and gonad which play crucial roles in endocrine feedback loops following DP exposure. The responses observed in this study warrants further studies on endocrine disrupting mechanism of DP.

Alterations in genes associated with thyroid hormone regulation and plasma T4 level in adult zebrafish imply thyroid hormone disrupting potential of DP. Thyroid hormone is an important factor for development and maintenance of physiological functions. Synthesis and secretion of thyroid hormone are controlled by hypothalamic-pituitary-thyroid gland axis. Thyroid hormone is synthesized and secreted in thyroid gland, and this process is stimulated by TSH released from pituitary gland. In mammals, secretion of TSH to blood is regulated by thyrotropin-releasing hormone (TRH) synthesized in hypothalamus. However, CRH rather than TRH is believed to be more potent stimulator of TSH secretion in fish (De Groef et al., 2006). In this study, T4

level in plasma showed an insignificant but increasing trend according to DP exposure (Fig. 3). The elevated transcriptions of *crh* and *tsh β* (Fig. 4A, B) partly explain increased plasma T4 level. These observations are in line with a human observation (Ben et al., 2014). Among mothers who had lived near an e-waste recycling plant over 20 years, significant positive association between DP concentration and total triiodothyronine (T3) level was observed (Ben et al., 2014). In contrast to *crh* and *tsh β*, DP-25 did not significantly alter transcription of deiodinase and thyroid hormone receptor genes in brain (Fig. 4C-F), suggesting alteration in *crh* and *tsh β* are independent of deiodinase or thyroid hormone receptor. Taken together, it is suggested that DP may have thyroid hormone disrupting effects, and mechanism underlying thyroid hormone disruption of DP warrants further studies.

Elevations in transcription levels of estrogen receptors and aromatase in male zebrafish brain after DP exposure imply possibility of sex hormone disruption of DP. Fish have two aromatase genes, which have distinct function according to their expressed organs: *cyp19a* (aromatase A) activated in gonad and *cyp19b* (aromatase B) activated in brain (Le Page et al., 2010). In brain, CYP19B activity peaks at adult stage and plays roles in brain growth and reversible brain sexualization in the entire life. Expression of *cyp19b* is strongly up-regulated by estradiol and several testosterones via activation of estrogen receptors (Le Page et al., 2010). Therefore, clear induction of *cyp19b* in brain (Fig. 5E) implies influence of certain estrogenic signal to brain, and

this is supported by elevated estrogen receptor expressions (Fig. 5F, G). These observations may result from elevated estrogen level following DP exposure. There is also possibility that up-regulation of *cyp19b* in brain was a direct effect of DP, considering blood-brain barrier permeability of DP (Zhang et al., 2011; Zheng et al., 2014). In this study, we could not measure sex hormone levels, owing to limited amount of blood sample. Hormonal alteration data would help understand the mechanism of transcriptional alteration of sex hormone related genes in brain. In contrast to aromatase and estrogen receptors genes, concentration of DP could not alter other sex hormone related genes evaluated in brain and testis were not influenced (Fig. 5A-D and Fig. 6), showing that elevated transcriptions of aromatase and estrogen receptors could not affect hormone regulation genes in hypothalamic–pituitary–gonadal axis.

Measured concentration of total DP in adult zebrafish exposed to DP-25 showed clear increasing trend according to nominal concentration of DP-25 (table 4), and this level of body burden is comparable to DP levels in fish in hot spot area. Near the DP manufacturing plant in China, total DP level in fish ranged 56.8 – 1110 ng/g ww (Wang et al., 2013). Also, among fish species caught near the e-waste recycling plants in China, body residue of total DP reached up to 1708 ng/g lw, which corresponds to approximately 50 ng/g ww considering lipid contents of the fish (Wu et al., 2010). Compared to DP levels in the fish in these hot spot areas, body burden of DP in zebrafish (table 4) can

be regarded as an environmentally relevant level. Therefore, endocrine disruption in molecular level observed in adult zebrafish in this study implies that aquatic biota in highly DP-contaminated sites may have risk of endocrine disruption.

We exposed zebrafish embryos to DP-25 to assess effect of DP on development and thyroid of zebrafish embryo/larvae. Time to hatch was significantly delayed in acetone control group (Table 5). Nevertheless, 0.4 % (v/v) acetone was used to increase DP concentration to increase solubility of DP. However, there was not significant adverse effect on survival and developmental indices in exposed DP levels (Table 5). Moreover, in contrast to the results of adult fish, *crh* and *tsh β* was not altered (Table 6). Transcription of *tshr* and *tg*, which, respectively, code receptor of TSH and thyroglobulin a protein precursor of thyroid hormone in thyroid gland were not affected (Table 6). Generally, rapidly developing phases are considered to be more sensitive to toxicants than adult phases. Therefore, the different responses between adult fish and larvae appear to have resulted from differences in exposure routes and/or amount of DP. Measured concentrations of DP in exposure media were much lower than their nominal concentrations, which supports that different exposure amount could result in different responses between the two experiments.

Dose-dependent increase in hepatic CAT activity observed in the present study (Fig. 2A) is in line with the previously reported researches (Li et al.,

2013b; Wu et al., 2012; Zhang et al., 2014), which reported induction of antioxidant enzyme activity following DP exposure. Previous studies reported other apparent evidences of induction of oxidative damage by DP such as formation of malondialdehyde or 8-hydroxy-2'-deoxyguanosine (Wu et al., 2012; Zhang et al., 2014). In addition, DP induced proteomic responses related to oxidative damage (Liang et al., 2014). Although SOD activity in liver was not affected by DP exposure (Fig. 2B), bearing in mind previous results, change in hepatic CAT activity can be interpreted as a line of evidence for induction of oxidative damage by DP.

To our knowledge, this is the first study to expose zebrafish to environmental toxicants via oral gavage feeding. Mortality rate after the exposure of adult fish to DP-25 was 3.6%. Since all deaths were observed within 24 h post dosing, observed lethal effect was considered to be resulted from dosing stress. Dosing success rate in this study was relatively higher (91.7%) than that of previous study (82%) for male zebrafish (Collymore et al., 2013). Other methods currently used to deliver insoluble chemicals to fish have inaccuracy in the administered dose (Collymore et al., 2013) or have an invasive nature. Our experience suggests that oral gavaging can be an alternative useful tool for aquatic toxicology studies on persistent insoluble contaminants like DP.

In summary, the results of this study suggest possibility of endocrine disruption of DP on thyroid and sex hormones, including oxidative damage, in

fish with environmentally relevant body burden of DP. Considering its wide-spread contamination in the environment, endocrine disrupting potential of DP is of concern to human and aquatic organisms. Further studies are warranted to understand the mechanisms of the endocrine disruption of this emerging chemical.

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국문초록

Dechlorane Plus의 단기노출에 따른 제브라피쉬의 독성반응

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Dechlorane Plus(DP)는 최근 널리 사용되고 있는 염화 난연제로 인체를 포함한 여러 환경 시료에서 높은 빈도로 검출되고 있다. 전세계적인 검출에도 불구하고 DP에 대한 독성학적인 정보는 부족한 실정이다. 본 연구에서는 DP의 독성을 규명하기 위하여 제브라피쉬를 이용하여 산화손상과 내분비계 교란을 비롯한 가능성있는 독성 영향을 평가하였다. 우선 수컷 성어 제브라피쉬에 corn oil에 녹인 DP를 경구투여하여 노출하였다. 0일과 2일에 각각 한 번씩, 총 두 번에 걸쳐 0, 0.3, 1, 3 μg/g zebrafish wet weight의 용량으로 경구투여하였다. 첫 번째 노출일로부터 6일 후에 제브라피쉬에서 혈액, 간, 정소, 뇌 시료를 수집하여 독성평가에 사용하였다. 산화손상을 확인하기 위한 지표 중에서 간에서의 catalase 활성이 DP 노출에 따라 용량의존적으로 유의하게 증가한 반면 superoxide dismutase의 활성에는 영향이 없었다.

또한, 혈장 중 갑상선 호르몬인 thyroxine(T4)의 농도가 DP노출에 따라 증가하는 양상을 나타냈다. 이러한 T4 농도의 변화는 뇌에서 증가한 *corticotropin releasing hormone*과 *thyroid stimulating hormone β* 유전자의 발현으로 일부 설명된다. 또한 aromatase와 에스트로젠 수용체를 포함한 뇌에서의 성호르몬 관련 유전자들의 발현 변화는 성호르몬 시스템에도 DP가 영향을 줄 수 있음을 보여주었다. 제브라피쉬의 초기 성장 단계에서 DP의 갑상선 및 발달 영향을 평가하기 위하여 제브라피쉬의 수정란과 치어에 DP를 노출시켰지만 성어에서의 결과와 다르게 유의한 영향이 나타나지 않았다. 본 연구의 결과는 DP가 간에서 산화손상을 일으킬 수 있을 뿐 아니라 갑상선 및 성호르몬 교란을 일으킬 수 있음을 제시하다.

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주요어: Dechlorane Plus; 난연제; 내분비계; 산화손상; 제브라피쉬

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