보건학석사 학위논문

Effect of *p,p'*-DDE on thyroid hormone, neurodevelopment, and kidney in embryo-larval and adult zebrafish (*Danio rerio*)

제브라피시 배아/자어와 성어에서

p,p'-DDE의 갑상선호르몬 교란과 신경 및 신장 영향 연구

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Abstract

Effect of *p,p'*-DDE on thyroid hormone, neurodevelopment, and kidney in embryo-larval and adult zebrafish (*Danio rerio*)

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Exposure to *p,p'*-DDE (*1-dichloro-2,2-bis (p-chlorophenyl) ethylene*), has been reported to be associated with thyroid hormone disruption or kidney dysfunction. However, such outcomes have mostly been studies in separation, and have rarely been investigated for fish especially at early life stage.

In this study, I evaluated thyroid hormone disrupting effects of *p,p'*-DDE, and screened for potential for its neurological and renal effects using embryo/larval and adult zebrafish. For each outcome, regulatory changes of key genes were chosen and quantified following the exposure.

In the larvae, thyroid hormone production and synthesis related genes such as *crh*, *tshβ*, *nkx2.1* and *tg* showed up-regulations after the exposure. Thyroid hormone transformation related genes such as *dio1* and *dio2* were also significantly up-regulated. Moreover, *mpb*, *gfap*, and *syn2a* genes, responsible for myelination and synapse formation, were significantly down-regulated in all exposure groups ($p< 0.05$). The *c-fos* gene, neuronal activation marker, was up-regulated in all exposure groups. Among the genes related to renal function, *podocin* gene was markedly down-regulation, but *wt1a* gene was significantly increased at 1uM ($p< 0.05$).

In adult fish, *crh, tshr, nis, tg, dio1*, and *dio2* genes tended to up-regulate, and T3 hormones also significantly increased ($p< 0.05$). Changes of the genes and hormones are likely to stimulate thyroid synthesis following *p,p'*-DDE exposure. In the brain, the expression of *gfap*, *mbp*, and *c-fos* genes showed increased pattern. In the kidney, *kim-1* gene was up-regulated at 0.1 μM *p,p'*-DDE ($p< 0.05$).

The present observation indicates that *p,p'*-DDE exposure on embryo/larval and adult zebrafish can disrupt thyroid, neurological and renal system. Further investigation on mechanistic link on the neurological and renal effects in association with thyroid hormone disruption is warranted.

Keywords: *p,p'*-DDE, zebrafish, thyroid hormone, endocrine disruption, kidney, neurological effects, screening

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1. Introduction

p,p'-DDE (2,2-Bis(4-chlorophenyl)-1,1-dichloroethylene) is a major breakdown product of DDT (dichloro-diphenyl-trichloroethane). DDT has been banned for use since 1970's due to its toxicity and extremely long persistence. The only exception is for specified purpose including control of mosquito-borne disease (US ATSDR, 2002). Physicochemical properties of DDT and *p,p'*-DDE are summarized in Table 1. Because of persistent nature of DDT and DDE, these chemicals have been ubiquitously found in aquatic systems (Nash et al., 2008; Saadati et al., 2012; Torres et al., 2015; Carrizo et al., 2017). In Asia, *p,p'*-DDE is frequently detected in natural inland waters, at concentrations up to 203 μg/L (Samoh and Ibrahim, 2008). In human, levels of 16.12 and 7.1 ng/g lipid were reported for adult male and female, respectively. Furthermore, in babies, *p,p'*-DDE concentration in serum is up to 1377 ng/g lipid (Guo et al., 2014; Verner et al., 2018). The estimated half-life of *p,p'*-DDE is about 8.6 years in plasma (Wolff et al., 2000).

Several studies have also documented that *p,p'*-DDE is suspect to be a thyroid disrupting chemical (O'Connor et al., 1999; Mortensen et al., 2006; Kim et al., 2015). For instance, *p,p'*-DDE might lower circulating T4, by decreasing thyroid hormone transporters and elevating hepatic enzyme activities (Liu et al., 2011). Additionally, exposure to *p,p'*-DDE could result in tissue-specific expression of thyroid hormone receptors in amphibians (Arukwe and Jenssen, 2005).

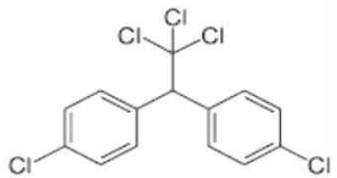
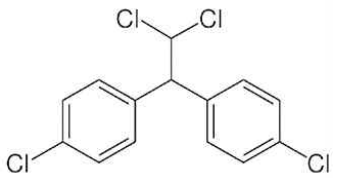
In vertebrates, THs play a key role in the endocrine system, regulating early development (Cheng et al., 2017). Changes in TH balance may affect diverse target organs. THs are especially critical for neuro-development, and therefore the neurogenesis, neuronal migration, myelination and synaptogenesis are most susceptible to thyroid disrupting chemicals (Juan Bernal, 2015). In the epidemiological studies, *p,p'*-DDE exposure at early stages can cause neurological effects such as hyperactivity or lack of concentration (Sagiv et al., 2014; Rosenquist et al., 2017).

Although limited, there are experimental evidence for neurological effects of *p,p'*-DDE. Both *in vitro* and in vivo evidences are available for neurological changes induced by *p,p'*-DDE treatment (Tiedeken and Ramsdell, 2010; Poulsen et al., 2012; Wang et al., 2014). Although the abnormal effects on nervous system were reported to human and animals, studies on neurological effects on *p,p'*-DDE exposure in the transcriptional level are still under investigation.

Several research recently suggest a link between thyroid hormone changes and impaired kidney function (Zhang et al., 2018). THs affect renal development, glomerular filtration rate and sodium and water homeostasis (Iglesias et al., 2017). Kidney plays a certain role to metabolize THs by conversion of thyroxine (T4) to triiodothyronine (T3) as well (Prajapati et al., 2013). Epidemiological studies have also shown an association with impaired kidney function via chemical exposures e.g. OCPs, OPEs and PFASs (Siddharth et al., 2012; Stanifer et al., 2018; Kang et al., 2019). *p,p'*-DDE also have been reported to be associated with lowered estimated glomerular filtration rate (eGFR) (Siddharth et al., 2012; Ghosh et al., 2017; Jayasinghe et al., 2018). However, there remains knowledge gap on mechanism of kidney toxicity following exposure to thyroid toxicant.

The aim of this study is to compare the thyroid hormone disrupting effects, neurological and renal effects of *p,p'*-DDE exposure in zebrafish embryo-larvae and adult. The result of this study will identify thyroid hormone disrupting effects, neurological and renal effects following *p,p'*-DDE exposure in fish at early and adult life stages.

Table 1. Physicochemical properties of DDT and *p,ps*-DDE

Compounds	CAS #	Structure	Use	LogKow	Water solubility (μ g/L)	Molecular weight (g/mol)	Vapor pressor (Torr)
DDT	50-29-3		Insecticide & disease vector	6.91	5.5	354.49	0.0001
p,p'-DDE	72-55-9		Insecticide	6.51	40	318.018	0.0045

2. Materials and Methods

2.1. Chemicals

p,p'-DDE (CAS No. 72-55-9, purity, 99 %) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) (CAS No. 67-68-5, purity, 99.8 %) was purchased from Junsei Chemical Co. Ltd. and used as solvent in all exposure media. The concentration of the solvent in the media was set at 0.01% (v/v) for adult test, 0.1% (v/v) for embryo-larvae test.

2.2. Maintenance of fish and experimental design

2.2.1. Fish maintenance

For embryo/larvae fish test, adult zebrafish were used, which were cultured in-house at Environmental Toxicology Laboratory, Seoul National University (Seoul, Korea). Fertilized eggs were collected by mating sexually mature adult fish. Wild type adult zebrafish (*Danio rerio*) and embryos were maintained at a temperature-controlled room (25±1℃) under a 14 h light/10 h dark cycle. For adult fish test, adult male zebrafish (*Danio rerio*) were purchased from a commercial vendor (Green Fish, Seoul, Korea). The adult fish were about six months old and acclimated in the laboratory at least for seven days before the experiment.

Before the animal experiments, the experiments were approved and performed according to the recommendations in the Institutional Animal Care and Use Committee by Seoul National University (SNU-190114-5).

2.2.2. Embryo-larvae fish test

Within two hour-post-fertilization (hpf), eggs were randomly pooled into the glass 50 mL beakers with 20 eggs per replicate, and five replicates were used for each group. The final concentrations, i.e., 0.01, 0.03, and 0.1 μM *p,p'*-DDE with (0.1 % v/v) DMSO, were determined based on the preliminary range finding tests. The exposure duration was set until six dpf.

Embryo and larval survival, hatchability, malformation rate and time to hatch were observed every day. Embryo survival was the percentage of surviving embryos among total fertilized eggs, and larval survival was that of surviving larvae among the hatched. Malformation rate was the percentage of malformed individuals, including dead fish. Hatchability represents the percentage of hatchling among the live embryos.

For investigation of neurological effects, within 2 hpf, eggs were randomly pooled into the glass 50 mL beakers with 25 eggs per replicate and five replicates were used for each group. The test concentration, i.e., 0, 0.1, 0.3, 1, and 3 μM *p,p'*-DDE with (0.1 % v/v) DMSO, were determined. Duration for exposure was set at 120 hpf.

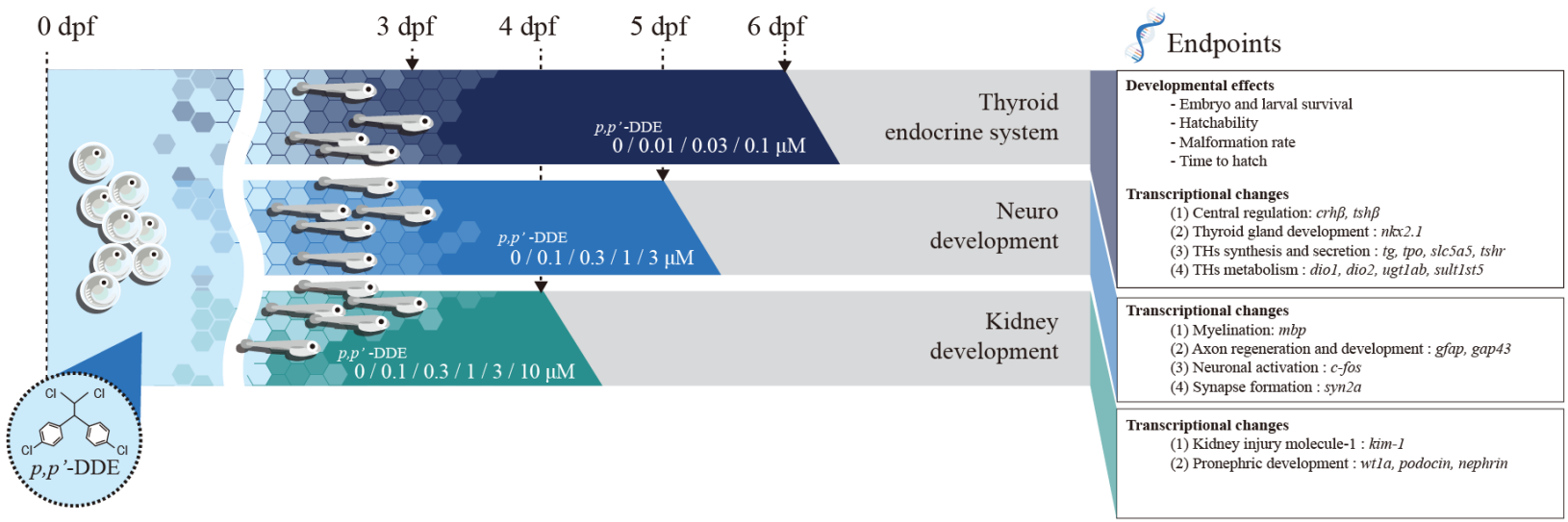
For investigation of renal effects, within 2 hpf, eggs were randomly pooled into the glass 50 mL beakers with 25 eggs per replicate and five replicates were used for each group. The test concentration, i.e., 0, 0.03, 0.1, 0.3, 1, 3, and 10 μM *p,p'*-DDE with (0.1 % v/v) DMSO, were determined. The exposure duration was set at 96 hpf.

On day 4-6 after fertilization, 15-25 larvae were randomly collected, anesthetized in ice-cold water, and the whole body samples were immediately stored at -80°C until further analysis. Throughout the test duration, exposure solution was

renewed daily. Water quality parameters such as conductivity, temperature, pH and dissolved oxygen, were measured regularly after renewal of exposure media. Embryo-larvae test designs are illustrated in Figure 1.

Figure 1. Summary of embryo-larvae test design

*dpf: day-post-fertilization



2.2.3. Adult fish test

In this study, six male zebrafish were kept in 5L exposure solution (0, 0.03, 0.1, 0.3 or 1 μM *p,p'*-DDE) for 21 days. After 21 days exposure, 5 fish from each beaker were pooled as a replicate for further analysis. Each group has four replicates (n=4). The exposure solution was renewed every day. Fish were fed with *Artemia* twice daily. Conductivity, pH, temperature, and dissolved oxygen were recorded three times a week. After the 21-day exposure, the fish were sacrificed, and the blood samples were collected from the caudal vein using heparinized capillary tubes. To obtain enough volume for hormone measurement, the blood samples from five fish were pooled, and the plasma was separated by centrifugation (7000 xg for 10 min at 4℃). For investigation of thyroid hormone regulation, neurological effects and renal effects in the transcriptional level, brain, thyroid, liver and kidney tissues were collected from three male zebrafish from each replicate and pooled for measurement. The plasma and tissue samples were kept at -80℃ until hormone and gene transcription analysis.

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

Several genes related to thyroid endocrine system, neuro/renal development and neuro/renal toxic markers were selected and evaluated in the whole larval homogenate and tissue homogenate. The samples were grinded, and the mRNA was extracted using RNeasy mini kit (Qiagen) following the manufacturer's instruction. Quality and quantity of RNA were checked with an Epoch Take 3 microplate spectrophotometer (BioTek, Bad Friedrichshall, Germany). Complementary DNAs (cDNAs) was synthesized from total RNA from the total RNA (100 ng/μL) using iScript cDNA Synthesis Kit (BIORAD, Hercules, CA, USA). Quantitative real-time PCR (RT-PCR) was performed with Light Cycler-DNA Master SYBR Green I mix (Roche Diagnostics Ltd, Lewes, UK) using Light Cycler 480 (Roche Applied Science, Indianapolis, IN, USA). The protocol of thermal cycle is as follows; pre-incubation at 95° C for 10 min, followed by 40 cycles of amplification at 95° C for 10 s, 55° C for 20 s, and 72° C for 20 s. Primer sequences are listed in Table 2. For quantification of PCR results, the threshold cycle (Ct) was determined for each reaction. Ct value for each target gene was normalized to the housekeeping genes; *18s rRNA, rpl8* and *β-actin* using the ΔΔCt method (Livak and Schmittgen, 2001).

Table 2. Primer sequence information

Category	Gene	Primer Sequence (5'-3')	Accession No.	Function
Reference	*18s*	F: acgcgagatggagcaataac	FJ915075	Housekeeping
	rRNA	R: cctcgttgatgggaaacagt		genes
	rpl8	F: tgttggtgttgttgctggt	-	
		R: ggatgctcaatgcagggttcat		
	β-actin	F: tgctgttttcccctccattg	NM_131031	
		R: tcccatgccaaccatcact		
Thyroid	*crhβ*	F: ttcgggaagtaaccacaagc	NM_001007379.1	Central regulation
hormone	(larvae)	R: ctgcactctattcgccttcc		of thyroid hormone
regulation	*tshβ*	F: gcagatcctcacttcacctacc	-	
	(larvae)	R: gcacaggtttggagcatctca		
	trh	F: gctctctccgtcggtctgtt	NM_001012365	
	(adult)	R: gcgagatccgtgctgatga		
	nkx2.1	F: aggacggtaaaccgtgtcag	BC162296.1	Thyroid gland
		R: caccatgctgctcgtgtact		growth
	tshr	F: gcgccaaccctttctgtat	EF487539.1	Thyroid stimulating
	(larvae)	R: ctcgtttgctcctgtttgct		hormone receptor
	tshr	F: gcgagaagggagaggaggtt	NM_001145763	
	(adult)	R: tcctcgcaagggttgaactc		
	tg	F: ccagccgaaaggatagagttg	XM 001335283	Thyroid hormone
	(larvae)	R: atgctgccgtggaatagga		synthesis
	tg	F: gtctcttgagtgttcgaatgacaag	XM_689200	
	(adult)	R: aaaggcgggccattaagg		
	tpo	F: gttcggtctgccaggacact	EU267076	
	(adult)	R: tccaagcgcttcagcagagt		
	nis	F: aatcaagccacaggcctgaa	NM_001089391	
	(adult)	R: aatgtgcagatgagcccagtt		
	dio1	F: gttcaaacagcttgtcaaggact	BC076008	Thyroid hormone
	(larvae)	R: agcaagcctctcctccaagtt		transformation
	dio1	F: aacttggaggagaggcttgct	BC076008	
	(adult)	R: agggcatggagggtcttctt		
	dio2	F: ttctccttgcctcctcagtg	NM_212789.4	
	(larvae)	R: agccacctccgaacatcttt		
	dio2	F: cgcgaaatgggcttgct	NM_212789	

	(adult)	R: ccaggcaaaatctgcaaagtta		
	mct8 (larvae)	F: tgatgtccatgatgattcc R: ccataatcgtgatgaaacag	NM_001258230	Thyroid hormone transport
	ttr	F: gcacaacttgatcacggagc R: tgtggtgtacgagaaagggc	NM_001005598.2	
	ugt1ab (larvae)	F: ccaccaagtctttccgtgtt R: gcagtccttcacaggctttc	NM_213422	Thyroid hormone elimination
	ugt1ab (adult)	F: gccagctttgatgaacttgcc R: aactcctccagttccttggtt	NM_213422.2	
	sult1st5 (larvae)	F: gtgcgcatgccgtttttaga R: cgggccacatatataaccttgc	NM_001199903	
	sult1st5 (adult)	F: cccatccaacttttgcctcg R: ggatccccataccaattgtcct	NM_001199903.1	
Neuro-development	*mbp*	F: cagcaggttcttcggaggag R: acgaggagaggacacaaagc	XM_001340280.4	Myelination
	gap43	F: aaatagacaaaccagacgctgc R: cgaacataaagcaggctgtcg	NM_131341.1	Axon extension
	gfap	F: ggatgcagccaatcgtaat R: ttccaggtcacaggtcag	NM_131373	Axon regeneration
	c-fos	F: tgcagcacggcttcaccgag R: cgggcatgaagagatcgccgt	NM_205569.1	Neuronal activation
	syn2a	F: gttctgatccggcaacatgc R: cagacatgcaaatgcccagg	NM_001002597.2	Synapse formation
Kidney injury and development markers	*kim-1* (adult)	F: ttgtgtcagactccaccacac R: ccgcagtctgaggaaaagtgc	XM_003200873.5	Kidney injury molecule
	kim-1 (larvae)	F: atgctgggatgtacgtctgc R: tgcggacatcatcttgtttttcc	XM_009295516.3	
	podocin (adult)	F: agatataagaccactgcagaaaacc R: ctcttttgcagaaggcgatgg	XM_009296250.3	Kidney development
	podocin (larvae)	F: aggaccgaaacagaaacatctc R: aggtccctcagtctccaataa	-	
	wt1a	F: cagcaagccaacctccac R: gcaaccgtgccgtaacct	NM_131046.2	
	nephrin	F: tccacttattatgagggaagagca R: cctaaacctgacctgagcaga	XM_017351014.2	

2.4 Measurement of thyroid hormone

For thyroid hormone analysis in adult fish, commercial kit based on the enzyme-linked immunosorbent assay method (Cloud-Clone Cor.: Triiodothyronine (T3) [Cat No. CEA453Ge]) was used. Protocol provided by the manufacturer was followed. The limit of detection for T3 was 51.7 pg/mL. Intra-assay and inter-assay variations were below 10% and 12%, respectively.

2.5. Statistical analysis

Statistical analysis was performed using SPSS 23.0 K for Windows (SPSS, Chicago, IL, USA). Data homogeneity of variances was analyzed by the Levene's test. Data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's t-test post hoc test. Spearman's correlation was applied for trend analysis. The data presented in this study were expressed as mean ± standard deviation. The criterion of $p < 0.05$ was used for statistical significance.

3. Results

3.1. Transcriptional and morphological changes in zebrafish embryo/larvae

3.1.1. Thyroid endocrine system

No significant effects were observed on embryo/larvae survival, hatchability and malformation rate following exposure *p,p'*-DDE (0.01, 0.03, 0.1 and 0.3 μM) at 6 dpf (Table 3). The exposure to *p,p'*-DDE influence the expressions of corticotrophin-releasing hormone (*crh*), thyroid-stimulating hormone beta (*tshβ*), thyroglobulin (*tg*), NK2 homeobox 1 (*nkx2.1*), deiodinase1 (*dio1*) and, deiodinase (*dio2*) were up-regulated at 1 μM (>2 fold change) in zebrafish larvae. Thyroid-stimulating hormone receptor (*tshr*) gene was down-regulated at 1 μM (<0.6 fold change) but statistical significance was not observed (Fig. 2).

Table 3. Developmental index of zebrafish larvae after exposure to *p,p'*-DDE (0, 0.01, 0.03, 0.1 and 0.3 μM) at 6 dpf.

***p,p'*-DDE**	**Control**	**Solvent control (DMSO 0.1% v/v)**	**0.01**	**0.03**	**0.1**	**0.3**
Embryo survival (%)	95.2±4.2	95.7±3.5	98.0±2.4	99.0±1.3	94.5±6.6	97.8±2.6
Hatchability (%)	98.9±1.4	100.0±0.0	99.5±1.1	98.0±3.3	99.5±1.1	100.0±0.0
Larval survival (%)	1.2±2.5	1.2±2.5	2.1±4.7	1.0±1.4	0±0.0	5.0±5.6
Malformation rate (%)	100.0±0.0	100.0±0.0	99.4±1.1	99.4±1.1	98.9±1.5	56.0±13.2

The values represents mean ± standard deviation of two independent experiments (n=2).

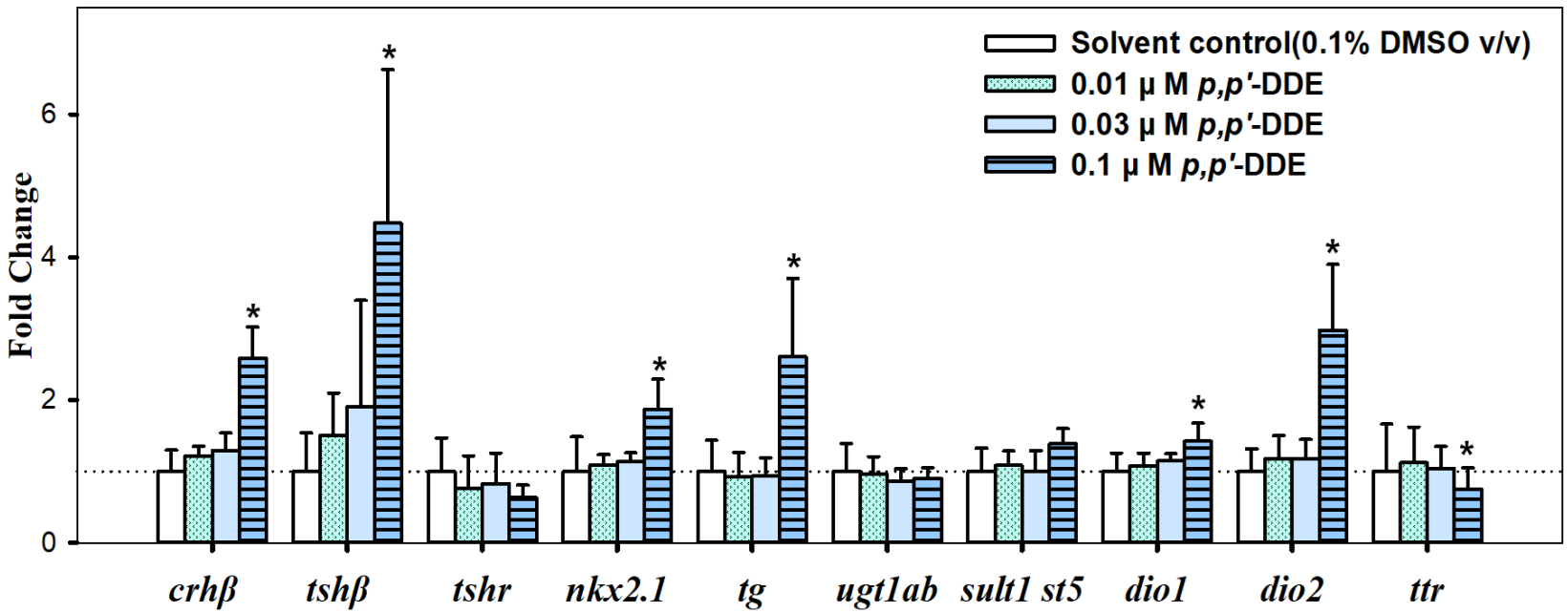


Figure 2. Transcriptional changes of thyroid endocrine system regulated genes in zebrafish embryo/larvae after exposure to 0, 0.01, 0.03, 0.1 μM *p,p'*-DDE for 6 dpf. The error bar represents the standard deviation of two independent experiments (n=2). Asterisks ($p^* < 0.05$) indicate significant difference compared to solvent control (SC, DMSO 0.1 %).

3.1.2. Neurological effects

The gene transcription levels for myelin basic protein (*mbp*), growth associated protein 43 (*gap43*), glial fibrillary basic protein (*gfap*), v-fos FBJ murine osteosarcoma viral oncogene homolog Ab (*fosab* or *c-fos*) and synapsin2 (*syn2a*) genes were analyzed. *gap43, gfap* and *syn2a* gene were significantly down-regulated in *p,p'*-DDE treatment groups and *c-fos* was up-regulated in dose-dependent manner (Fig. 3).

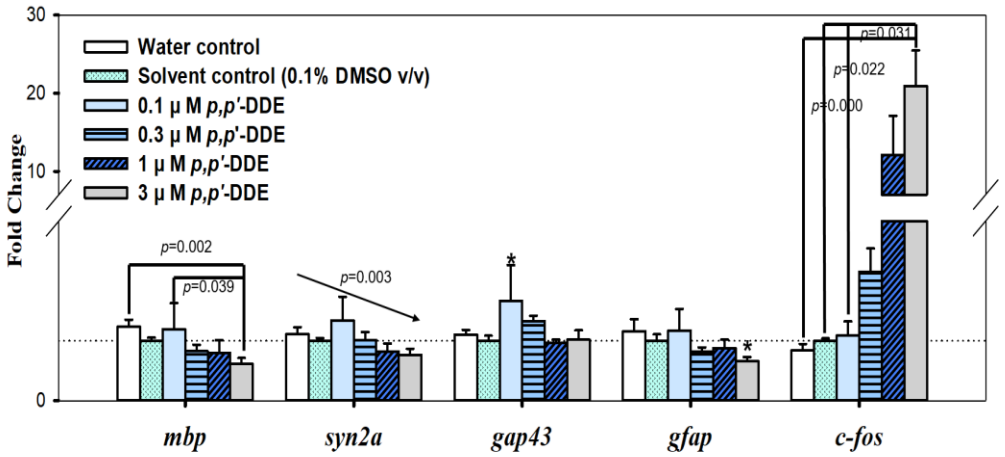


Figure 3. Neurological effects related genes in zebrafish embryo/larvae after exposure to 0-3 μM *p,p'*-DDE for 5 dpf. The error bar represents the standard deviation. Asterisks (p^* <0.05) indicate significant difference compared to solvent control (SC, DMSO 0.1 %).

3.1.3. Renal effects

The gene transcription levels for kidney injury molecule-1 (*kim-1*), *nephrin*, wilms' tumor suppressor (*wt1a*) and *podocin* genes were analyzed. *podocin* gene was attenuated in DDE treatment groups but *wt1a* was induced in 1 μM *p,p'*-DDE (Fig. 4).

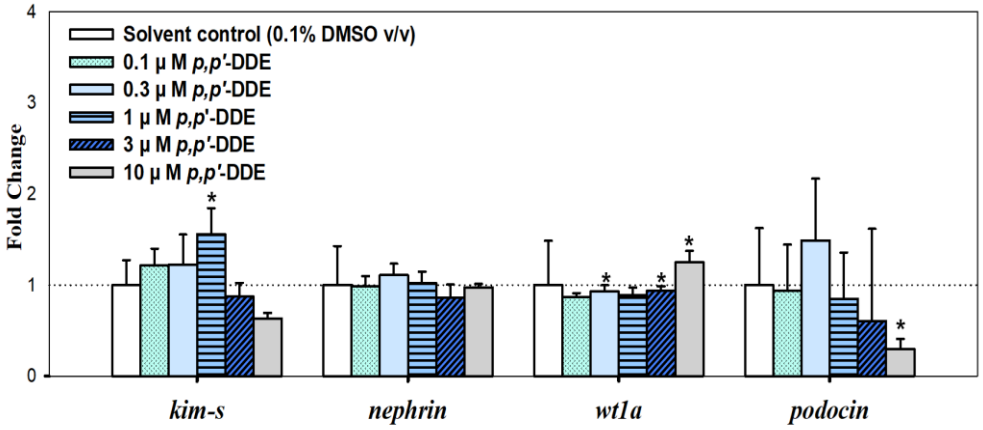


Figure 4. Renal effects related genes in zebrafish embryo/larvae after exposure to 0-10 μM p,p'-DDE for 4 dpf. The error bar represents the standard deviation. Asterisks (p* <0.05) indicate significant difference compared to solvent control (SC, DMSO 0.1 %).

3.2. Transcriptional and hormone changes in adult zebrafish

3.2.1. Transcriptional changes on thyroid endocrine system

In the brain tissue, the gene transcription levels for *crhβ, trh,* and *mct8* genes were analyzed. *crhβ* genes were up-regulated at 0.3 and 1 μM *p,p'*-DDE and *trh* gene was down-regulated but there was no statistical significance (Fig. 5A).

In the thyroid tissue, the gene transcription levels for *tshr, tg, nis, tpo* and *dio1* genes were analyzed. All genes showed up-regulation in the *p,p'*-DDE exposure group but not statistically significant (Fig. 5B).

In the liver tissue, the gene transcription levels for *sult1 st5, ugt1ab* and *ttr* genes were analyzed. *ugt1ab* gene showed up-regulation in the *p,p'*-DDE exposure group but there was no statistical significance. *ttr* genes indicated down-regulation in the *p,p'*-DDE exposure group (Fig. 5C).

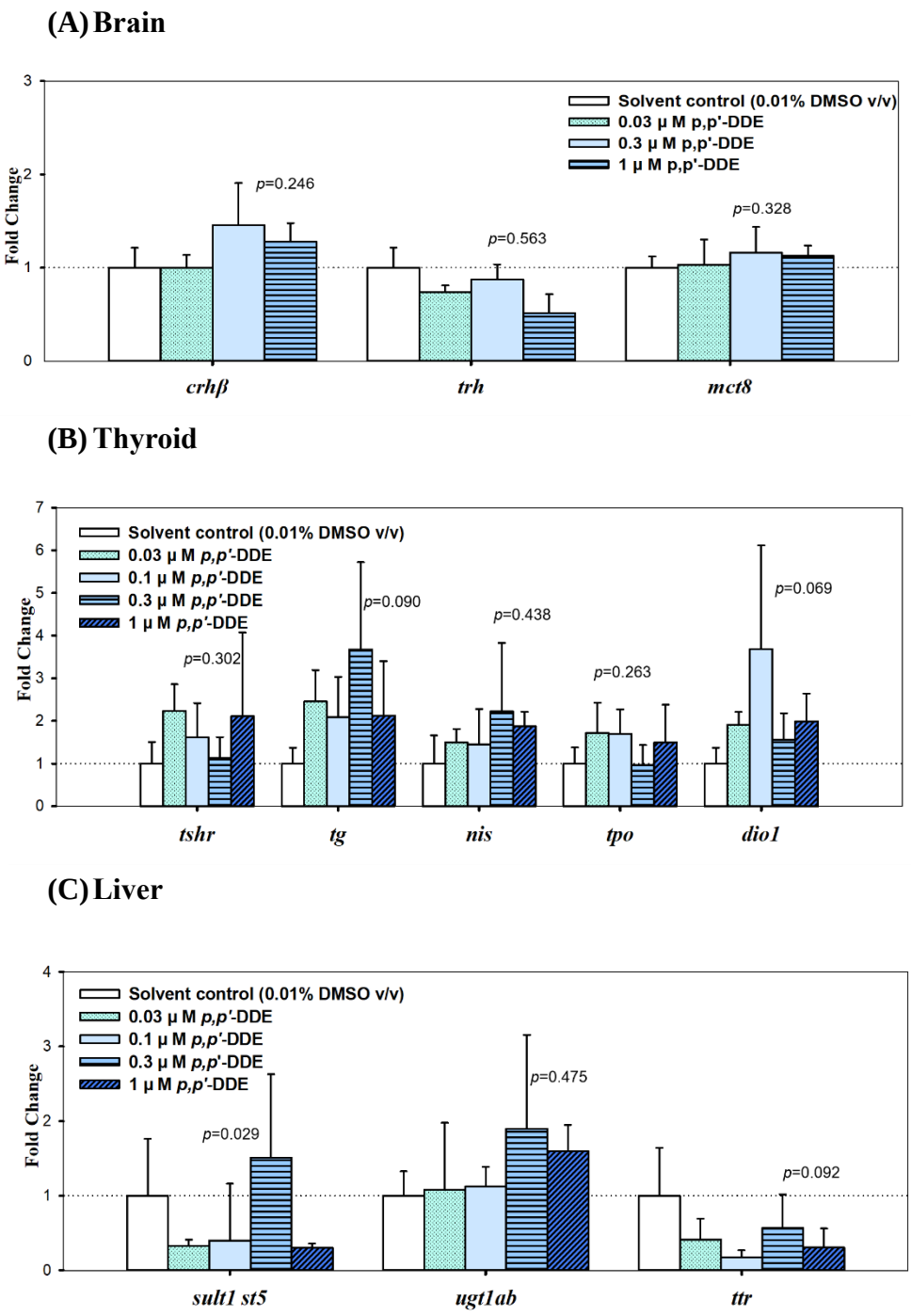


Figure 5. Transcriptional changes of thyroid endocrine system regulated genes in (A) brain, (B) thyroid, and (C) liver of zebrafish embryo/larvae after exposure to 0, 0.03, 0.1, 0.3, and 1 μ M *p,p'*-DDE for 21 days.

3.2.2. Thyroid hormone changes

Exposure to *p,p'*-DDE until 21 days significantly increased total T3 levels at 0.3 μM in adult male zebrafish (Fig. 6).

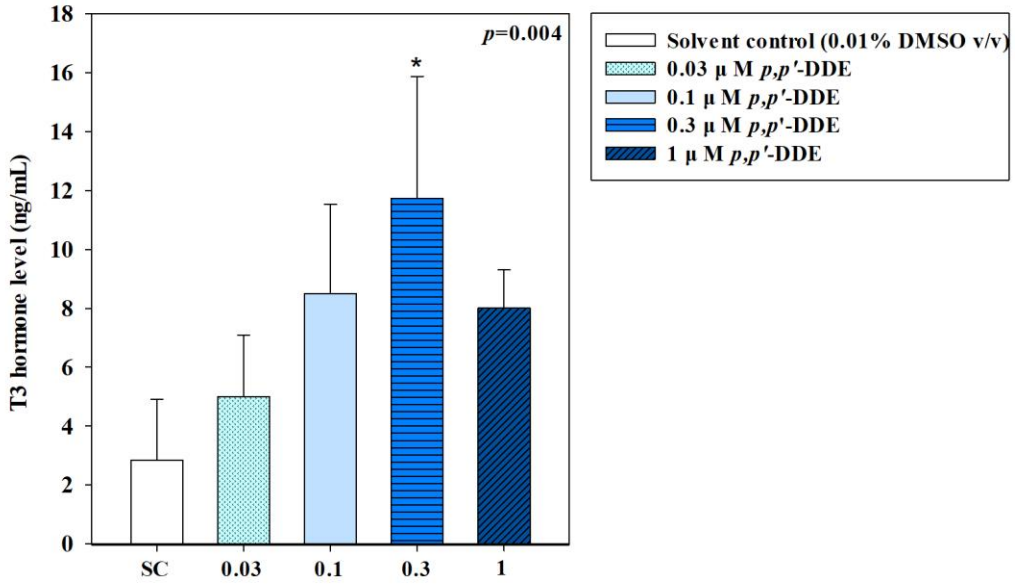


Figure 6. T3 levels in male adult zebrafish after exposure to 0, 0.03, 0.1, 0.3, and 1 μM *p,p'*-DDE for 21 days. The error bar represents the standard deviation of four replicates (n=4). Asterisks (p* <0.05) indicate significant difference compared to solvent control (SC, DMSO 0.01 %).

3.2.3. Neurological effects

In the brain tissue, the gene transcription levels for *mbp*, *gap43*, *gfap*, *c-fos* and *syn2a* genes were analyzed. *mbp*, *gap43, gfap* and *c-fos* gene showed up-regulation in *p,p'*-DDE treatment groups and only *gfap* gene was up-regulated at 0.1 μM *p,p'*-DDE (Fig. 7).

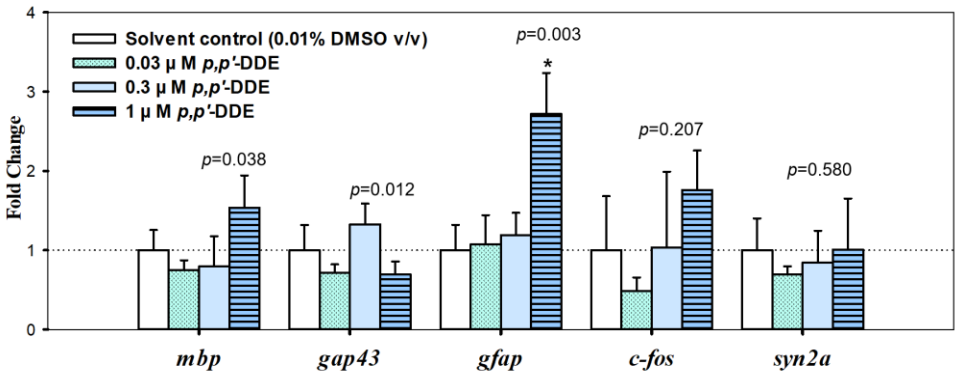


Figure 7. Neurological effects related genes in adult zebrafish after exposure to 0, 0.03, 0.1, 0.3, and 1 μM *p,p'*-DDE for 21 days. The error bar represents the standard deviation of four replicates (n=2-4). Asterisks ($p^* < 0.05$) indicate significant difference compared to solvent control (SC, DMSO 0.01 %).

3.2.4. Renal effects

In the kidney tissue, the gene transcription levels for *kim-1*, *nephrin* and *podocin* genes were analyzed. *kim-1* and *podocin* genes were significantly up-regulated in *p,p'*-DDE treatment groups. In addition, *nephrin* gene was up-regulated but there was no statistical significance (Fig. 8).

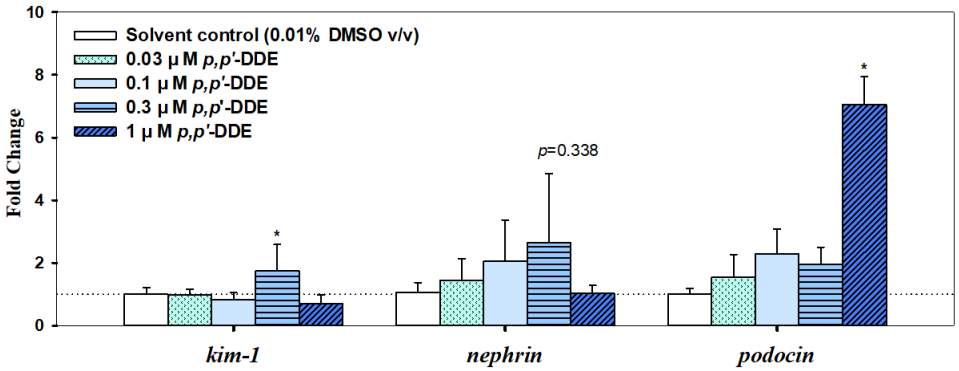


Figure 8. Renal effects related genes in adult zebrafish after exposure to 0, 0.03, 0.1, 0.3, and 1 μM *p,p'*-DDE for 21 days. The error bar represents the standard deviation of four replicates (n=3-4). Asterisks (p* <0.05) indicate significant difference compared to solvent control (SC, DMSO 0.01 %).

4. Discussion

4.1. Thyroid endocrine system

Thyroid hormone disrupting effects of *p,p'*-DDE has been reported in humans and various experimental models such as rodents, fish, amphibians. However, animals and epidemiological studies on thyroid hormone disrupting effects are inconsistent (Table S1 and S2). Therefore, we evaluated several genes regulating THs in zebrafish embryo/larvae and adults after *p,p'*-DDE exposure and analyzed T3 hormone levels in adult zebrafish.

Expression of *crhβ* and *tshβ* in embryo/larvae was significantly up-regulated after exposure to *p,p'*-DDE (Fig. 2). In adult fish, *crhβ* gene was also significantly up-regulated (Fig. 5). In vertebrates, *crhβ* gene would stimulate TSH excretion, and subsequently synthesis of thyroid hormones (De Groef et al., 2006). Additionally, expression of *tshβ* in larvae also significantly up-regulated at 1 μM. A number of studies reported decreased thyroid hormones along with up-regulation of *crhβ* and *tshβ* genes in zebrafish following exposure to thyroid disrupting chemicals (TDCs) including decabromodiphenyl ether (BDE-209), bisphenol A, DE-71 and tris (1,3-dichloro-2-propyl) phosphate (TDCPP) (Chen et al., 2012; Guo et al., 2019; Wang et al., 2013; Yu et al., 2010). Therefore, it may be due to a feedback regulation in HPT axis to increase thyroid hormones, which was compensated for the decreased thyroid hormones levels.

The *tg, tpo* and *nis* genes were significantly up-regulated in adult fish following exposure to *p,p'*-DDE, which might be secondary response to the decrease in thyroid hormone synthesis and release. Similarly, decreased whole-body T4 was

accompanied with up-regulated the expression of the *tg* and *nis* genes in adult zebrafish after exposure to fipronil (FIP) (Xu et al., 2019b). *nkx2.1* is expressed by the thyroid gland, and plays critical role in thyroid growth and differentiation in zebrafish (Rohr and Concha, 2000; Porazzi et al., 2009). In this study, *nkx2.1* was significantly up-regulated in the highest exposure group of the larvae, which indicates that they compensate for the damage to thyroid gland by exposure to *p,p'*-DDE.

The deiodinases in peripheral tissues are a major determinant of plasma T3 concentration. In this study, we analyzed two types of thyroid hormone deiodinase genes; *dio1* and *dio2*. *dio1* is a major converter of T4 to T3, which is found predominantly in liver and kidney. *dio2* maintains the level of intracellular T3 and expressed in the brain and pituitary gland in fish (Wu et al., 2019). In this study, both *dio1* and *dio2* genes were significantly up-regulated at the highest exposure concentration in larvae (Fig. 2). Additionally, adult fish showed increased *dio1* expression level (Fig. 5). Similarly, following the exposure to DEHP and MEHP, up-regulation of *dio1* and *dio2* was observed together with increased whole-body T3 contents in zebrafish (Zhai et al., 2014; Jia et al., 2016), showing that transformation of T4 into T3 was facilitated by up-regulation of *dio1* gene expression.

Organochlorine pesticides are known for competing with or binding to THs transport proteins, including TBG and TTR, changing THs levels (Freire, 2016). In this study, *ttr* gene showed down-regulation in adult fish although there was no statistical significance. Among DDT analogues, *o,p'*-DDD has been reported to alter TH levels through interrupting with THs transporters in experimental studies (Wu et al., 2019). Many studies also reported the down-regulation of *ttr* tre down-regulation oftatistical significance. hormonated the expression of the \l "bib0320"pyrene (Wu et

al., 2019). In contrast, an increased *ttr* gene expression and an elevated T4 levels following exposure to *o,p'*-DDT, DE-47, perfluoroalkyl phosphinic acid and arsenite, were reported in zebrafish (Yu et al., 2011; Liu et al., 2019; Sun et al., 2015).

In the present study, T3 levels significantly increased at 0.3 μM in adult fish (Fig. 6). The results of the transcriptional and hormone changes are similar to Zhai et al. (2014), which showed increased T3 levels and decreased T4 levels in zebrafish larvae after exposure to MEHP. Although T4 levels were not analyzed in larvae and adult, the present results demonstrated that exposure to *p,p'*-DDE might disrupt thyroid endocrine system in fish via increased T3 levels and change of genes transcription involved in the HPT axis.

4.2. Neurological effects

Certain changes of THs related genes or proteins have been associated with neurological effects. For example, *mct8* plays an important role on T3 uptake in neurons and oligodendrocytes (de Vrieze et al., 2014). Human mutations of the gene, *SLC16A2*, encoding MCT8, caused hypo-myelination disorder (Lee et al., 2017). In addition, blocking MCT8 via pharmacology and genetics could lead apoptosis in human oligodendrocytes, which damages myelination (Lee et al., 2017). *Dio2* is expressed in astrocytes throughout the brain and oligodendrocytes precursor cells, generating T4 to T3. Severe motor impairment is reported in Knockout (KO) of Dio2 adult mice, compared to minimal neurological impairment at young ages (Bárez-López et al., 2019). However, there are limited studies on comparison between thyroid system and neuronal effects. A lot of the research, behavioral changes could

be relative to the specific biomarkers involved in regenerative response, synaptic vesicle cycling and axonal function. For example, *mbp* is a marker of myelination, highly expressed in oligodendrocytes (Yoshida and Macklin, 2005). *gap43* plays a crucial role on axonal extension and regeneration, which is largely expressed in axon and presynaptic terminal (Gu et al., 2019). *gfap* is a great extent in the central nervous system (CNS), especially expressed in reactive astrocytes and ependymal cells (Singh et al., 2003). *c-fos* is a reliable biomarker of neuronal activation because *c-fos* mRNA and protein were used in a variety of species such as human, rodent and zebrafish (Adam Douglas Collier, 2017). Lastly, *syn2a* is a marker of synapse formation.

Therefore we confirmed both thyroid disrupting effects and neurological effects following *p,p'*-DDE exposure with candidate genes which are associated with neuro-development markers including *mbp*, *gap43*, *gfap*, *c-fos* and *syn2a*.

In the present study, regulatory change of the genes related to neuronal effects was not observed in adult zebrafish, except for *mbp, gfap*, and *c-fos* (Fig. 7). However, in larvae, expression levels of *syn2a*, *gfap* and *mbp* were down-regulated in *p,p'*-DDE exposure group (Fig. 3), suggesting that neurological effects of early stages are more susceptible for *p,p'*-DDE exposure than later stages. Many studies have revealed that the gene expression of *mbp*, *gfap*, and *syn2a* was down-regulated in zebrafish embryo/larvae after exposure to thyroid hormone disrupting chemicals such as BPS, triclosan (TCS), chlorpyrifos (CPF), organophosphate esters and ibuprofen (Giordani and d'Amora, 2018). Interestingly, exposure to caffeine combined with alcohol in adult zebrafish revealed that c-fos protein expression was induced and freezing duration was significantly increased compared to control, arguing that alteration of those gene expression could be related to change of behavior patterns.

4.3. Renal effects

Interaction between thyroid and kidney is known for few decades. For example, nephrotic syndrome is related to change of THs and glomerulonephritis disease might be associated to hypo-thyroidism (Iglesias et al., 2017). However, experimental evidence supporting the link between thyroid and kidney remains unclear. *p,p'*-DDE has been reported to be associated with kidney function, e.g., lowered eGFR and THs changes in epidemiological studies (Siddharth et al., 2012; Ghosh et al., 2017; Jayasinghe et al., 2018). Renal effects on *p,p'*-DDE is not well understood at the molecular levels.

In the present study, transcriptional change of *podocin* gene, which is responsible for formation of pronephros, was down-regulated in zebrafish embryo/larvae (Fig. 4). Additionally, the transcriptional level of *kim-1* gene, which is well-known for kidney injury marker, was up-regulated (Fig. 4). These results suggest that the development of pronephros were affected by exposure to *p,p'*-DDE. Similarly, zebrafish embryo/larvae exposed to adriamycin and aristolochic acid, which are anticancer medicine and herbal medicine, respectively, observed similar patterns in the transcriptional changes, e.g. down-regulation of *nephrin* and *wt1a* genes (Ding et al., 2012; Zennaro et al., 2014; Wang et al., 2016). In contrast, adult zebrafish showed different expression patterns of several genes. Expression of *podocin*, *nephrin* and *kim-1* genes were up-regulated in *p,p'*-DDE exposure groups (Fig. 8). Age-dependent differences in transcription of kidney injury makers warrant further investigations.

kim-1 is an indicator of tubular damage. It is not expressed in normal healthy condition, and it is known to be mainly expressed in proximal tubules when tubular

injury occurs. A various species, including humans, monkeys, rats, and zebrafish, shares *Kim* homologues (Yin et al., 2016). In humans, a marked increase in the expression of *KIM-1* was first reported in patients with acute tubular necrosis. High levels of *KIM-1* were found in the urine of acute kidney injury (AKI) patients (Han et al., 2002). In animals, *kim-1* was also associated with kidney injury. Tubular damage and nephritis were observed in *Kim-1* overexpressed transgenic mice (Yin et al., 2016). Zebrafish has four types of *kim* family. Among them, *kim-1* is structurally and functionally similar to mammalian *Kim-1*. The kim-1 gene is mostly expressed in the zebrafish kidney after exposure to gentamicin, which also cause reduced glomerular filtration rate (GFR) and edema (Yin et al., 2016).

The Wilms' tumor suppressor gene, i.e., *wt1*, which is a key regulator of kidney development, encodes a zinc-finger transcription factor. In developing zebrafish, two types of *wt1* genes exist, i.e., *wt1a* and *wt1b*. Inactivation of wt1a is reported to be associated with failure of glomerular differentiation and morphogenesis, leading to the pericardial and yolk sac edema (Perner et al., 2007). Knockdown of *wt1* in renal tissue culture allows inhibition of nephron formation along with increased apoptosis in the organ periphery and proliferation of surrounding cells (Davies et al., 2004). The inactivation of *Wt1* in mice also cause organ abnormalities affecting the kidneys. Moreover, in human, inactivation of *WT1* affects to pediatric kidney cancer (Call et al., 1990; Gessler et al., 1990). There are several studies on the mechanism by *wt1a*, which controls podocyte formation and has the target genes like *podocin* and *nephrin* in the zebrafish pronephros (Miceli et al., 2014).

Podocin practically functions as a filter in glomerulus with nephrin, so it is a reliable biomarker for proteinuria. In focal segmental glomerulosclerosis (FSGS)

patients, expression of *podocin* was decreased (Pereira et al., 2019). Animal models showed the same pattern. Mice lacking podocin was reported that they developed proteinuria before birth and died due to uremia after a few days (Caridi et al., 2005)

Nephrin is a podocyte-specific transmembrane protein and is important for proper blood filtration (Kotb et al., 2016). If nephrin is absent, proteins can pass through the glomerular filtration barrier. It is mainly expressed in the zebrafish pronephric glomerulus and mammalian mesonephric glomerulus as well (Fukuyo et al., 2014). In zebrafish, nephrin expressing regions emerged in 2 dpf, and the expression was preserved within the glomerulus in 3-4 dpf (Ichimura et al., 2013). Loss of nephrin by morpholino knockdown zebrafish leaded to change of podocyte morphology and nephrosis at 4 dpf. Additionally, Morpholino disruption of nephrin expression resulted in morphological changes such as curved or shorter body in zebrafish embryos (Sohn et al., 2009). In rodent model, adult mice with long term exposure (>20 weeks) nephrin knockdown occurred to mild proteinuria and podocyte apoptosis (Li et al., 2015).

In the present study, age dependent differences were observed for the regulation of the genes associated with neurological and renal effects in zebrafish. The difference might be explained by age-dependent difference in sensitivity. Several important organs are formed in early developmental period, and hence the response to the chemical exposure could be different from those observed for adults. Further studies are warranted to elucidate mechanisms underlying the different response by developmental stage.

5. Conclusion

The present observation indicates that *p,p'*-DDE exposure on zebrafish embryo/larvae and adult can disrupt thyroid, neurological and renal system. Age-dependent differences are observed for renal outcomes following the exposure to *p,p'*-DDE. Potential link among these outcomes is suggested. Further investigation on mechanistic link on the neurological and renal effects in association with thyroid hormone disruption is warranted.

Supporting information

Table S1. Epidemiological studies on thyroid hormone changes of *p,p'*-DDE

Population characteristics	Compound	Period	Sample type	Sample size	Endpoints	Results	References
Sweden (adult men)	*p,p'*-DDE	2000	Serum	1500	TSH levels	Increase	Rylander et al., 2006
Spain (male infant)	*p,p'*-DDE	2000-2002	Cord blood	220	TSH levels	Increase	Freire et al., 2011
USA (adult men)	*p,p'-DDE*	2000-2003	Serum	341	TSH levels Total T3 Total T4	Decrease Increase Increase	Meeker et al., 2007
Europe[a] (infant)	*p,p'-DDE*	2002-2006	Cord plasma	1784	TSH levels	Decrease	de Cock et al., 2017
Thailand (infant)	*p,p'-DDE*	2003-2004	Cord serum	39	TSH levels Total T4 Free T4	- Decrease -	Asawasinsopon et al., 2006
Spain (pregnant)	*p,p'-DDE*	2003-2005	Serum	157	TSH levels Total T3 Free T4	Increase - Decrease	Lopez-Espinosa et al., 2009
Korea (infant)	*p,p'-DDE*	2011	Cord serum	118	Total T3 Total T4	Decrease Decrease	Kim et al., 2015

Table S2. Experimental studies on thyroid hormone disruption of *p,p'*-DDE

Species	Stages/ Sex	Exposure route	Exposure Conc.	Exposure Duration	Endpoints	Results	References
Crl:CD IGS BR rat	63 days old/ Male	IP	0 - 300 mg/kg/day	15 days	Serum T4 level	Decrease	O'Connor et al., 1999
LE rat	63 days old/ Male	IP	0 - 300 mg/kg/day	15 days	Serum TSH level	Increase	O'Connor et al., 1999
LE rat	63 days old/ Male	IP	0 - 300 mg/kg/day	15 days	Serum T4 level	Decrease	O'Connor et al., 1999
SD rat	Male	IP	0 - 100 mg/kg	10 days	Serum total T4, free T4	Decrease	Liu et al., 2011
SD rat	Male	IP	0 - 100 mg/kg	10 days	Serum TTR	Decrease	Liu et al., 2011
SD rat	Male	IP	0 - 100 mg/kg	10 days	*Trα*, *Trβ*, *Udpgt* mRNA expression	Increase	Liu et al., 2011
European common frog	Stage 36	Water	0 - 0.01 ppm	3 days	*tshβ*(brain), *trβ*(tale) mRNA expression	Decrease	Mortensen et al., 2006
European common frog	Adult/ Male	SC	0 - 10 mg/kg	2 weeks	*trβ* mRNA expression	Decrease (liver) Increase (brain)	Wang et al., 2014
SD rat	3 weeks/ Male	IP	32 mg/kg (PCB) +20 - 100 mg/kg (DDE)	5 days	Serum TBG, TTR	Decrease	Liu et al., 2014
SD rat	3 weeks/ Male	IP	32 mg/kg (PCB) +20 - 100 mg/kg (DDE)	5 days	*D2*	Decrease	Liu et al., 2014
SD rat	3 weeks/ Male	IP	32 mg/kg (PCB) +20 - 100 mg/kg (DDE)	5 days	*UDPGT* mRNA expression	Increase	Liu et al., 2014

References

Al-Daghri, N., Abd-Alrahman, S. H., Wani, K., Panigrahy, A., McTernan, P. G., Al-Attas, O. S., & Alokail, M. S. (2019). Biomonitoring and risk assessment of organochlorine pesticides among Saudi adults. Arabian Journal of Chemistry, 12(7), 1795-1801.

Arukwe, A., & Jenssen, B. M. (2005). Differential organ expression patterns of thyroid hormone receptor isoform genes in p,p′ -DDE-treated adult male common frog, Rana temporaria. Environmental toxicology and pharmacology, 20(3), 485-492.

Asawasinsopon, R., Prapamontol, T., Prakobvitayakit, O., Vaneesorn, Y., Mangklabruks, A., & Hock, B. (2006). The association between organochlorine and thyroid hormone levels in cord serum: a study from northern Thailand. Environment international, 32(4), 554-559.

Bárez-López, S., Grijota-Martínez, C., Ausó, E., Fernández-de Frutos, M., Montero-Pedrazuela, A., & Guadaño-Ferraz, A. (2019). Adult mice lacking Mct8 and Dio2 proteins present alterations in peripheral thyroid hormone levels and severe brain and motor skill impairments. Thyroid.

Bernal, J. (2015). Thyroid hormones in brain development and function. In *Endotext [Internet]*. MDText. com, Inc..

Caridi, G., Perfumo, F., & Ghiggeri, G. M. (2005). NPHS2 (Podocin) mutations in nephrotic syndrome. Clinical spectrum and fine mechanisms. Pediatric research, 57(5 Part 2), 54R.

Carrizo, D., Sobek, A., Salvadó, J. A., & Gustafsson, O. (2017). Spatial distributions of DDTs in the water masses of the Arctic Ocean. Environmental science & technology, 51(14), 7913-7919.

de Cock, M., de Boer, M. R., Govarts, E., Iszatt, N., Palkovicova, L., Lamoree, M. H., ... & van de Bor, M. (2017). Thyroid-stimulating hormone levels in newborns and early life exposure to endocrine-disrupting chemicals: analysis of three European mother–child cohorts. Pediatric research, 82(3), 429.

de Morais Pereira, L. H., da Silva, C. A., dos Reis Monteiro, M. L. G., Araújo, L. S., Rocha, L. P., da Rocha Reis, M. B., ... & Machado, J. R. (2019). Podocin and uPAR are good biomarkers in cases of Focal and segmental glomerulosclerosis in pediatric renal biopsies. PloS one, 14(6), e0217569.

de Vrieze, E., van de Wiel, S. M., Zethof, J., Flik, G., Klaren, P. H., & Arjona, F. J. (2014). Knockdown of monocarboxylate transporter 8 (mct8) disturbs brain development and locomotion in zebrafish. Endocrinology, 155(6), 2320-2330.

Ding, Y. J., & Chen, Y. H. (2012). Developmental nephrotoxicity of aristolochic acid in a zebrafish model. Toxicology and applied pharmacology, 261(1), 59-65.

Freire, C., Lopez-Espinosa, M. J., Fernández, M., Molina-Molina, J. M., Prada, R., & Olea, N. (2011). Prenatal exposure to organochlorine pesticides and TSH status in newborns from Southern Spain. Science of the total environment, 409(18), 3281-3287.

Freire, C. (2016). Pesticides and Thyroid Function. *Thyroid Toxicity*, 123.

Fukuyo, Y., Nakamura, T., Bubenshchikova, E., Powell, R., Tsuji, T., Janknecht, R., & Obara, T. (2014). Nephrin and Podocin functions are highly conserved between the zebrafish pronephros and mammalian metanephros. Molecular medicine reports, 9(2), 457-465.

Ghosh, R., Siddarth, M., Singh, N., Tyagi, V., Kare, P. K., Banerjee, B. D., ... & Tripathi, A. K. (2017). Organochlorine pesticide level in patients with chronic kidney disease of unknown etiology and its association with renal function. Environmental health and preventive medicine, 22(1), 49.

Giordani, S., & d'Amora, M. (2018). The utility of Zebrafish as a model for screening developmental neurotoxicity. Frontiers in neuroscience, 12, 976.

Guo, H., Jin, Y., Cheng, Y., Leaderer, B., Lin, S., Holford, T. R., ... & Niu, J. (2014). Prenatal exposure to organochlorine pesticides and infant birth weight in China. Chemosphere, 110, 1-7.

Guo, Y., Chen, L., Wu, J., Hua, J., Yang, L., Wang, Q., ... & Zhou, B. (2019). Parental co-exposure to bisphenol A and nano-TiO2 causes thyroid endocrine disruption and developmental neurotoxicity in zebrafish offspring. *Science of the Total Environment*, *650*, 557-565.

Ichimura, K., Fukuyo, Y., Nakamura, T., Powell, R., Sakai, T., Janknecht, R., & Obara, T. (2013). Developmental localization of nephrin in zebrafish and medaka pronephric glomerulus. Journal of Histochemistry & Cytochemistry, 61(4), 313-324.

Iglesias, P., Bajo, M. A., Selgas, R., & Díez, J. J. (2017). Thyroid dysfunction and kidney disease: an update. Reviews in Endocrine and Metabolic Disorders, 18(1), 131-144.

Jayasinghe, S., Lind, L., Salihovic, S., Larsson, A., & Lind, P. M. (2018). High serum

levels of p,p'-DDE are associated with an accelerated decline in GFR during 10 years follow-up. Science of the total environment, 644, 371-374.

Jia, P. P., Ma, Y. B., Lu, C. J., Mirza, Z., Zhang, W., Jia, Y. F., ... & Pei, D. S. (2016). The effects of disturbance on Hypothalamus-Pituitary-Thyroid (HPT) axis in zebrafish larvae after exposure to DEHP. PloS one, 11(5), e0155762.

Kang, H., Lee, J., Lee, J. P., & Choi, K. (2019). Urinary metabolites of organophosphate esters (OPEs) are associated with chronic kidney disease in the general US population, NHANES 2013–2014. Environment international, 131, 105034.

Kim, S., Park, J., Kim, H. J., Lee, J. J., Choi, G., Choi, S., ... & Choi, K. (2015). Association between several persistent organic pollutants and thyroid hormone levels in cord blood serum and bloodspot of the newborn infants of Korea. PLoS One, 10(5), e0125213.

Kotb, A. M., Simon, O., Blumenthal, A., Vogelgesang, S., Dombrowski, F., Amann, K., ... & Endlich, N. (2016). Knockdown of ApoL1 in Zebrafish Larvae Affects the Glomerular Filtration Barrier and the Expression of Nephrin PLoS One, 11(5),

Lee, J. Y., Kim, M. J., Deliyanti, D., Azari, M. F., Rossello, F., Costin, A., ... & Petratos, S. (2017). Overcoming monocarboxylate transporter 8 (MCT8)-deficiency to promote human oligodendrocyte differentiation and myelination. EBioMedicine, 25, 122-135.

Li, X., Chuang, P. Y., D'Agati, V. D., Dai, Y., Yacoub, R., Fu, J., ... & He, J. C. (2015). Nephrin preserves podocyte viability and glomerular structure and function in adult kidneys. Journal of the American Society of Nephrology, 26(10), 2361-2377.

Liu, C., Shi, Y., Li, H., Wang, Y., & Yang, K. (2011). p,p′-DDE disturbs the homeostasis of thyroid hormones via thyroid hormone receptors, transthyretin, and hepatic enzymes. Hormone and metabolic research, 43(06), 391-396.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods (San Diego, Calif.) 25, 402-408.

Lopez-Espinosa, M. J., Vizcaino, E., Murcia, M., Llop, S., Espada, M., Seco, V., ... & Ballester, F. (2009). Association between thyroid hormone levels and 4, 4′-DDE concentrations in pregnant women (Valencia, Spain). Environmental research, 109(4), 479-485.

Meeker, J. D., Altshul, L., & Hauser, R. (2007). Serum PCBs, p, p′-DDE and HCB

predict thyroid hormone levels in men. Environmental research, 104(2), 296-304.

Miceli R, Kroeger PT, Jr, Wingert RA. Molecular mechanisms of podocyte development revealed by zebrafish kidney research. Cell Dev Biol. 2014

Mortensen, A. S., & Arukwe, A. (2006). The persistent DDT metabolite, 1, 1-dichloro-2, 2-bis (p-chlorophenyl) ethylene, alters thyroid hormone-dependent genes, hepatic cytochrome P4503A, and pregnane× receptor gene expressions in atlantic salmon (Salmo salar) parr. Environmental Toxicology and Chemistry: An International Journal, 25(6), 1607-1615.

Nash, S. B., Poulsen, A. H., Kawaguchi, S., Vetter, W., & Schlabach, M. (2008). Persistent organohalogen contaminant burdens in Antarctic krill (Euphausia superba) from the eastern Antarctic sector: a baseline study. Science of the Total Environment, 407(1), 304-314.

O'Connor, J. C., Frame, S. R., Davis, L. G., & Cook, J. C. (1999). Detection of thyroid toxicants in a tier I screening battery and alterations in thyroid endpoints over 28 days of exposure. Toxicological sciences: an official journal of the Society of Toxicology, 51(1), 54-70.

Porazzi, P., Calebiro, D., Benato, F., Tiso, N., & Persani, L. (2009). Thyroid gland development and function in the zebrafish model. Molecular and cellular endocrinology, 312(1-2), 14-23.

Poulsen, A. H., Kawaguchi, S., King, C. K., King, R. A., & Nash, S. M. B. (2012). Behavioural sensitivity of a key Southern Ocean species (Antarctic krill, Euphausia superba) to p, p′ -DDE exposure. Ecotoxicology and environmental safety, 75, 163-170.

Rohr, K. B., & Concha, M. L. (2000). Expression of nk2. 1a during early development of the thyroid gland in zebrafish. Mechanisms of development, 95(1-2), 267-270.

Rylander, L., Wallin, E., Jönssson, B. A., Stridsberg, M., Erfurth, E. M., & Hagmar, L. (2006). Associations between CB-153 and p, p′ -DDE and hormone levels in serum in middle-aged and elderly men. Chemosphere, 65(3), 375-381.

Saadati, N., Abdullah, M. P., Zakaria, Z., Rezayi, M., & Hosseinizare, N. (2012). Distribution and fate of HCH isomers and DDT metabolites in a tropical environment–case study Cameron Highlands–Malaysia. Chemistry Central Journal, 6(1), 130.

Samoh, A. N. H., & Ibrahim, M. S. (2008). Organochlorine pesticide residues in the major

rivers of Southern Thailand. *Malaysian Journal of Analytical Sciences*, *12*(2), 280-284.

Siddharth, M., Datta, S. K., Bansal, S., Mustafa, M., Banerjee, B. D., Kalra, O. P., & Tripathi, A. K. (2012). Study on organochlorine pesticide levels in chronic kidney disease patients: association with estimated glomerular filtration rate and oxidative stress. Journal of biochemical and molecular toxicology, 26(6), 241-247.

Singh, R., Nielsen, A. L., Johansen, M. G., & Jørgensen, A. L. (2003). Genetic polymorphism and sequence evolution of an alternatively spliced exon of the glial fibrillary acidic protein gene, GFAP. Genomics, 82(2), 185-193.

Sohn, R. L., Huang, P., Kawahara, G., Mitchell, M., Guyon, J., Kalluri, R., ... & Gussoni, E. (2009). A role for nephrin, a renal protein, in vertebrate skeletal muscle cell fusion. Proceedings of the National Academy of Sciences, 106(23), 9274-9279.

Stanifer, J. W., Stapleton, H. M., Souma, T., Wittmer, A., Zhao, X., & Boulware, L. E. (2018). Perfluorinated chemicals as emerging environmental threats to kidney health: A scoping review. Clinical Journal of the American Society of Nephrology, 13(10), 1479-1492.

Sun, H. J., Li, H. B., Xiang, P., Zhang, X., & Ma, L. Q. (2015). Short-term exposure of arsenite disrupted thyroid endocrine system and altered gene transcription in the HPT axis in zebrafish. Environmental pollution, 205, 145-152.

Torres, P., Miglioranza, K. S. B., Uhart, M. M., Gonzalez, M., & Commendatore, M. (2015). Organochlorine pesticides and PCBs in southern right whales (Eubalaena australis) breeding at Península Valdés, Argentina. Science of the Total Environment, 518, 605-615.

Tiedeken, J. A., & Ramsdell, J. S. (2009). Zebrafish seizure model identifies p, p′ -DDE as the dominant contaminant of fetal California sea lions that accounts for synergistic activity with domoic acid. Environmental health perspectives, 118(4), 545-551.

U.S. Department of Health and Human Services, Agency for Toxic Substances and Disease Registry. (2002). Toxicological Profile for DDT, DDE, and DDD.

Verner, M. A., Chevrier, J., Ngueta, G., Rauch, S., Bornman, R., & Eskenazi, B. (2018). Early-life exposure to p, p′ -DDT and p, p′ -DDE in South African children participating in the VHEMBE study: An assessment using repeated serum measurements and pharmacokinetic modeling. Environment international, 119, 478-484.

Wang, X., Liu, K. C., Sun, G. J., Han, L. W., Wang, R. C., Peng, W. B., ... & Hou, H. R. (2016). Evaluation of nephrotoxic effects of aristolochic acid on zebrafish (Danio rerio) larvae. Human & experimental toxicology, 35(9), 974-982.

Wang, Y., Zhang, S., Cui, W., Meng, X., & Tang, X. (2018). Polycyclic aromatic hydrocarbons and organochlorine pesticides in surface water from the Yongding River basin, China: Seasonal distribution, source apportionment, and potential risk assessment. Science of the Total Environment, 618, 419-429.

Wolff, M. S., Zeleniuch-Jacquotte, A., Dubin, N., & Toniolo, P. (2000). Risk of breast cancer and organochlorine exposure. Cancer Epidemiology and Prevention Biomarkers, 9(3), 271-277.

Wu, L., Ru, H., Ni, Z., Zhang, X., Xie, H., Yao, F., ... & Zhong, L. (2019). Comparative thyroid disruption by o, p'-DDT and p, p'-DDE in zebrafish embryos/larvae. Aquatic Toxicology, 216, 105280.

Xu, C., Niu, L., Liu, J., Sun, X., Zhang, C., Ye, J., & Liu, W. (2019b). Maternal exposure to fipronil results in sulfone metabolite enrichment and transgenerational toxicity in zebrafish offspring: Indication for an overlooked risk in maternal transfer?. Environmental pollution, 246, 876-884.

Xu, C., Sun, X., Niu, L., Yang, W., Tu, W., Lu, L., ... & Liu, W. (2019a). Enantioselective thyroid disruption in zebrafish embryo-larvae via exposure to environmental concentrations of the chloroacetamide herbicide acetochlor. Science of The Total Environment, 653, 1140-1148.

Yin, W., Naini, S. M., Chen, G., Hentschel, D. M., Humphreys, B. D., & Bonventre, J. V. (2016). Mammalian target of rapamycin mediates kidney injury molecule 1-dependent tubule injury in a surrogate model. Journal of the American Society of Nephrology, 27(7), 1943-1957.

Yoshida, M., & Macklin, W. B. (2005). Oligodendrocyte development and myelination in GFP-transgenic zebrafish. Journal of neuroscience research, 81(1), 1-8.

Yu, L., Lam, J. C., Guo, Y., Wu, R. S., Lam, P. K., & Zhou, B. (2011). Parental transfer of polybrominated diphenyl ethers (PBDEs) and thyroid endocrine disruption in zebrafish. Environmental science & technology, 45(24), 10652-10659.

Zennaro, C., Mariotti, M., Carraro, M., Pasqualetti, S., Corbelli, A., Armelloni, S., ... & Messa, P. (2014). Podocyte developmental defects caused by adriamycin in zebrafish embryos and larvae: a novel model of glomerular damage. PLoS One, 9(5), e98131

Zhai, W., Huang, Z., Chen, L., Feng, C., Li, B., & Li, T. (2014). Thyroid endocrine disruption in zebrafish larvae after exposure to mono-(2-ethylhexyl) phthalate (MEHP). PloS one, 9(3), e92465.

국문 초록

제브라피시 배아와 성어에서 *p,p'*−DDE의 갑상선호르몬 교란과 신경 및 신장 영향 연구

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DDT (dichloro-diphenyl-trichloroethane)는 제 2차 세계 대전 이후 가장 널리 사용된 유기염소계 살충제이나 큰 독성으로 인해 사용이 금지되었다. 그러나 질병 통제와 같은 특수한 용도로 여전히 사용되고 있고, 그 대사체인 *p,p'*-DDE는 잔류성이 높아 여전히 환경과 체내에서 검출되고 있다.

p,p'-DDE는 갑상선 교란 가능성이 있음이 여러 연구를 통해 밝혀졌다. 또한 실험연구와 인구 관찰 연구에서 *p,p'*-DDE 노출에 따른 신경계 이상 및 신장 기능 손상도 보고되고 있다. 갑상선호르몬의 변화와 신경 및 신장 기능 간의 연관성이 보고된 바 있으나, 분자적인 수준에서 이를 함께 보는 연구는 제한적인 실정이다.

따라서 본 연구에서는 제브라피시 배아/자어와 성어를 이용하여 갑상선호르몬 교란 기전과 신경 및 신장 독성 가능성을 확인해보고자 하였다. 배아/자어에서는 갑상선호르몬 교란 기전을 확인하기 위해, 수정 후 2시간 이내의 배아를 0, 0.01, 0.03, 0.1 μM(0.1 % v/v DMSO)에 6일간 노출시켰다. 신경 독성을 확인하기 위해, 수정 후 2시간 이내의 배아를 0, 0.1, 0.3, 1, 3 μM(0.1 % v/v DMSO)에 5일간 노출시키고 관련 유전자 변화를 관찰하였다. 또 신장 독성을 확인하기 위해, 수정 후 2시간 이내의 배아를 0, 0.01, 0.03, 0.1, 0.3, 1, 3, 10 μM(0.1 % v/v DMSO)에 4일간 노출시키고 관련 유전자 변화를 관찰하였다. 수컷 성어에서는 갑상선호르몬 교란 기전과 신경 및 신장 독성을 확인하기위해, 0, 0.03, 0.1, 0.3, 1 μM(0.01 % v/v DMSO)에 21일간 노출시키고 관련 유전자와 T3 호르몬 변화를 관찰하였다.

배아/자어에서는 갑상선호르몬 생합성 및 대사에 관여하는 *crh, tshβ, nkx2.1*, *tg, dio1*, *dio2* 유전자 발현이 증가하였다. 신경계에서는 수초(myelination) 및 시냅스 형성에 관여하는 *mbp*, *syn2a*, *gfap* 유전자 발현이 유의하게 감소하였다. 사구체 여과에 관여하는 *nephrin* 유전자 발현은 유의한 수준으로 감소하였으나, 전신 발달에 관여하는 *wt1a*는

차이를 보이지 않았다.

성어에서는 갑상성호르몬 조절과 관련된 *crh, tshr, mct8, nis, tg, dio1, dio2* 유전자 발현이 증가하는 경향을 보였고, T3 호르몬도 통계적으로 유의한 수준으로 증가하였다(p<0.05). 유전자와 호르몬 모두 *p,p'*-DDE 노출에 따라 갑상선호르몬의 합성을 증가시키는 방향으로 나타났다. 자어와는 달리, 신경계와 관련된 유전자 중 *gfap*는 유의한 수준으로 증가했고, *mbp*와 *c-fos*는 유의하지는 않으나 증가하는 패턴을 보였다. 또 신장에서는 사구체 여과에 관여하는 *podocin*과 손상 지표로 알려진 *kim-1*이 통계적으로 유의한 수준으로 증가했다(p<0.05).

본 연구는 제브라피시 배아/자어와 성어에서 *p,p'*-DDE 노출에 따른 갑상선호르몬 교란 기전과 신경 및 신장 발달 저해 영향을 유전자와 호르몬 변화를 통해 확인하였다. 갑상선 호르몬은 신경 및 신장 발달에 핵심적인 역할을 수행하므로, 갑상선계 교란이 신경 및 신장 영향을 일으키는 기전 연구가 필요하다.

주요어: *p,p'*-DDE, 제브라피시, 갑상선호르몬, 내분비계 교란, 신장 독성, 신경계, 스크리닝
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